In Vitro–Generated Autoimmune Regulatory T Cells Enhance Intravitreous Allogeneic Retinal Graft Survival

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Purpose. The authors demonstrated that in vitro-generated α-melanocyte stimulated hormone (MSH)-induced Treg cells specific to ocular autoantigen suppress ocular autoimmune disease in vivo when adoptively transferred. They examined the possibility of using these ocular autoantigen-specific Treg cells to promote the survival of a retinal allograft placed in the mouse vitreous.

Methods. Enhanced green fluorescent protein (eGFP)-C57BL/6 neonatal retinal microaggregates were injected into the vitreous of B10-RIII mice before the adoptive transfer of interphotoreceptor retinoid-binding protein (IRBP; an ocular antigen) or ovalbumin (OVA)-specific α-MSH-induced Treg cells. GFP transplants were imaged in vivo on days 7 and 12. In addition, on day 12, the eyes were cryosectioned and immunostained with a panel of neuronal and immune cell markers.

Results. GFP allografts underwent no detectable changes in size on days 7 and 12 in the B10-RIII mice injected with IRBP-specific Treg cells; however, mice that received OVA-specific Treg cells or no Treg cells experienced remarkable reductions in graft size on day 12. Only one quarter of the original size was seen. Using neuronal-specific markers, immunohistochemistry showed that the architecture of the retinal allografts in the IRBP Treg cell-injected group had intact rosettes and neuronal cells on the outermost layer, whereas the allografts in the OVA Treg cell-injected mice were disorganized. Immune cell-specific markers demonstrated that Treg cells and activated microglial cells were found in the retinal allografts of the mice injected with IRBP Treg cells, but not in the retinal allografts of the OVA Treg-injected mice.

Conclusions. These results demonstrate that adoptive transfer of α-MSH-generated IRBP-specific Treg cells promotes retinal allograft survival and development. (*Invest Ophthalmol Vis Sci.* 2007;48:5112–5117) DOI:10.1167/iovs.07-0175

Since the early 1970s, different organs, such as cornea, kidney, and liver, have been transplanted into humans with reasonable rates of success; however, formidable barriers remain to accomplish this type of transplantation success for neuronal tissues such as retina.[1–3] It is unknown whether transplanted neural cells can survive for extended periods of time in recipients and can form proper connections with host cells. It is also unknown how these transplants are recognized by the host immune system and whether immunity can affect the integrity of the transplanted retinal tissue.[4]

Retinal transplantation is a promising approach for restoring sight to patients with visual impairment caused by disease and age-related damage of the retina.[5–9] In mouse models of retinal damage, transplanted retinal pigment epithelial (RPE) cells delay age-related changes in retinal layers and offer a possible approach to deal with cell death in aging retinas.[10] Retinal transplants between major histocompatibility complex (MHC)-incompatible animals survive well when placed into the anterior chamber, suggesting a mechanism of immunosuppression in the eye that promotes retinal graft survival but may not be active in the subretinal space.[11] The survival of the retinal graft in the anterior chamber of the eye has been attributed to the immune-privileged nature of the ocular microenvironment.[12] Part of ocular immune privilege is systemic induction of the regulatory immunity associated with the phenomenon of anterior chamber-associated immune deviation (ACAID).[13] When retinal transplants are placed in the anterior chamber, they produce systemic suppression in immune responses to retinal transplantation antigens and induce retinal antigen-specific regulatory immunity.[14–16] In contrast, a retinal allograft placed in the subretinal space (the area between the photoreceptors and the RPE monolayer) induces an alloantigen-specific delayed hypersensitivity (DH) response as the graft is rejected.[11] In addition, subretinal transplants of allogeneic embryonic retinal cells in mice do not organize or survive when they express MHC class I or class II.[17] $CD4^+$ and $CD8^+$ T cells are activated by the allogeneic retinal transplant and by the recipient $CD4^+$ T cells recognizing donor MHC class II by direct alloantigen recognition.[18] Tolerance to retinal autoantigens remains intact, and the host retina remains in good condition after the allogeneic retinal graft is rejected.[19] These results suggest that the placement of the retinal graft in the posterior segment of the eye either fails to induce tolerance or does not benefit from the immune privilege that protects the recipient retina from autoimmunity.

Another part of ocular immune privilege is the regional suppression of immune activity mediated by neuropeptides and cytokines produced within the ocular microenvironment.[20] These immunomodulating neuropeptides and cytokines prevent the activation of inflammation mediated by innate and adaptive immune cells. Moreover, they induce immune cells to further regulate immunity itself and function as suppressor immune cells. One of the mediators of this regional immunosuppression is the neuropeptide α-melanocyte-stimulating hormone (α-MSH), which is constitutively present in healthy aqueous humor.[21] One of the mechanisms of α-MSH-mediated immunosuppression is its ability to promote the induction of antigen-specific $CD25^+CD4^+$ T regulatory (Treg) cells.[22] Another immunosuppressive factor in aqueous humor, transforming growth factor (TGF)-β2, enhances α-MSH induction of Treg cells.[23] The induction of Treg cells by α-MSH requires that the T cells already be primed and that they be exposed to α-MSH when they are restimulated through their T-cell receptor by antigen-presenting cells presenting the priming antigen. For these Treg cells to mediate suppression in vivo, their specific antigen must be expressed in the targeted tissue, and they suppress the activation of other T cells responding to the same or different antigen in the same tissue

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site. The ability of α-MSH-induced Treg cells to suppress immunity was demonstrated when α-MSH-induced Treg cells specific for an ocular autoantigen were used in adoptive transfer experiments to subdue experimental autoimmune uveoretinitis (EAU), a mouse model of human endogenous uveitis.[24]

Given that we can generate retinal autoantigen-specific Treg cells with α-MSH and that these Treg cells can suppress neighboring T cells in retinal tissues, we sought to determine whether adoptive transfer of ocular autoantigen-specific, α-MSH-induced Treg cells can promote the survival of a retinal allograft placed in the vitreous.

Materials and Methods

Animals and Reagents

Donor retinal tissues were from neonatal eGFP-C57BL/6 mice. Adult C57BL/6 ($H2^b$) and B10.RIII ($H2^r$) mice served as syngeneic and allogeneic graft recipients. Neonatal eGFP-B6 mice were bred in the Schepens Eye Research Institute animal facility. The other adult mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All experimental procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Schepens Institutional Animal Care and Use Committee. The immunodominant peptide of human IRBP, corresponding to amino acids 161 to 180 (IRBPp), was synthesized for us by Invitrogen Life Technologies (Carlsbad, CA) and was used to induce autoimmune uveoretinitis in B10.RIII mice. Complete Freund adjuvant and incomplete Freund adjuvant were purchased from Difco Laboratories (Detroit, MI).

Generation and Adoptive Transfer of α-MSH-Induced Treg Cells

To prepare α-MSH/TGF-β2-induced antigen-specific Treg cells, we followed our previously published methods.[23,24] The B10.RIII mice were immunized subcutaneously with 50 μg IRBPp emulsified in complete Freund adjuvant. Draining lymph nodes were collected 7 days after the injection of antigen, passed through a 70-μm strainer (BD Flacon; VWR, West Chester, PA), and resuspended in HBSS without Ca^{2+} or Mg^{2+} (Invitrogen Life Technologies, Carlsbad, CA). Red blood cells were removed from the lymph node cell suspension with red blood cell-lysing buffer (Sigma Chemical, St. Louis, MO). Lymph node cells were resuspended in serum-free medium (RPMI 1640) supplemented with 10 μg/mL gentamicin (Sigma Chemical), 0.01 M HEPES, 1× nonessential amino acid (NEAA) mixture (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), 1 mg/mL bovine serum albumin, and 1/500 dilution of ITS+ culture supplement (Sigma Chemical). The resuspended lymph node cells were added to the wells (1×10^6 cells/well) of a 96-well plate. To the lymph node cell cultures were added 5 μg/mL IRBPp and 30 pg/mL α-MSH. Four hours later, 5 ng/mL TGF-β2 was added to the cultures. After 24 hours of incubation, 1×10^6 αMSH/TGF-β2 treated lymphoid cells (α-MSH-induced, IRBP-specific Treg) were injected intravenously into naive, syngeneic mice on the same day the mice received intravitreal injections of allogeneic neural retinal microaggregates. To generate Treg cells specific to OVA, we used a method similar to generating IRBP antigen-specific Treg cells, except the lymph node cells were from the draining lymph nodes of mice immunized with OVA (200 μg/mL) and the isolated lymph node cells were stimulated in vitro with 100 μg/mL OVA added to the OVA-primed lymph node cell cultures.[24]

Preparation of Donor Neonatal Neural Retinal Microaggregates

Eyes were enucleated from humanely killed neonatal eGFP-C57BL/6 mice and were placed in 4°C calcium/magnesium-free Hanks balanced salt solution (HBSS). The anterior segment and lens were dissected, and the neuronal retina was pealed from the pigmented epithelial layer and placed in HBSS. Dissected retinas were minced into small fragments with a #10 scalpel. Neonatal neural retinal (NNR) fragments were further separated by gentle trituration using a fine glass pipette with an internal diameter of 1 mm. The retinal tissue suspension, a mixture of microaggregates and individual cells, was allowed to settle for 30 minutes at 4°C, the supernatant was removed, and the NNR microaggregates were used in the grafting procedures.[25]

Grafting Procedures

The eye of the recipient was gently projected from the eye socket using a #8 suture to form a small loop loosely tied around the back of the eyeball. The pupil was dilated by an application of tropicamide (Akorn, Buffalo Grove, IL), and the eye was anesthetized with an application of 0.5% proparacaine hydrochloride (Akorn). The mouse was placed on the stage of a dissecting microscope, and an O-ring was placed around the eye after to an application of Gonak (Akorn). This allowed the fundus of the retina to be clearly visible through the microscope. A penetrating wound was made in the posterior portion of the eye wall using a 30-gauge needle with a 45° bevel. The NNR microaggregates (equal to one fourth of a neonatal retina) were injected through the wound into the vitreous. Once the injections were completed, gentamicin ophthalmic topical ointment was applied to the injection site to guard against infection.

In Vivo Observation and Immunohistochemistry

The grafts were examined over a period of 3 to 12 days. Under anesthesia, the eye fundus of the mice was examined using a dissecting microscope attached to a fluorescence setup. To view the grafts located in the posterior portion of the eye, the pupil was dilated and the eye was covered with Gonak and a cover slide. The live images of the GFP fluorescence graft were recorded using a digital camera (SPOT, Diagnostic Instruments, Sterling Heights, MI) attached to the dissecting scope. Image J (National Institutes of Health, Rockville, MD) software was used to measure the size of the GFP grafts. The areas of the GFP grafts were compared by ANOVA (GraphPad Instat, San Diego, CA).

On day 12, the eyes were enucleated from the humanely killed mice and cryoprotected in 30% sucrose solution. The eyes were embedded in OCT medium, and 8-μm cryosections were prepared for standard immunohistochemistry methods. To evaluate the immunobiology of the graft, a panel of antibodies to detect immune cell markers, we used GS-lectin (microglial cells; Sigma, St. Louis, MO), CD3 (T cells; BD Bioscience-PharMingen, San Diego, CA), expression of transplantation antigen IA^b (BD Bioscience-PharMingen), and Treg cell marker FoxP3 (eBioscience, San Diego, CA). The retinal structure was probed using antibodies for neurons (Neurofilament-H; Sigma, St. Louis, MO), GFAP (astrocytes; Sigma), and rhodopsin (photoreceptors; Millipore-Chemicon, Billerica, MA). The secondary antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA) were tagged with Cy3 to contrast with the GFP emission of the graft.

Results

Alloretinal Graft Survival in Mice Injected with Treg Cells

We injected into the vitreous of naive B10.RIII mouse eyes eGFP-C57BL/6 NNR microaggregates. On the same day the mice were injected intravenously with α-MSH/TGF-β2–generated Treg cells that were specific to IRBP or OVA. To follow the fate of the eGFP-C57BL/6 NNR microaggregate grafts under syngeneic conditions, we injected the microaggregates into the vitreous of naive C57BL/6 mouse eyes. These mice were not injected with Treg cells. On days 7 and 12 after the microaggregate injections, we visualized the GFP fluorescence from the NNR microaggregates using an epifluorescence microscope connected to a digital camera. Fluorescent fundus images were recorded, and the size of the transplant (area of GFP

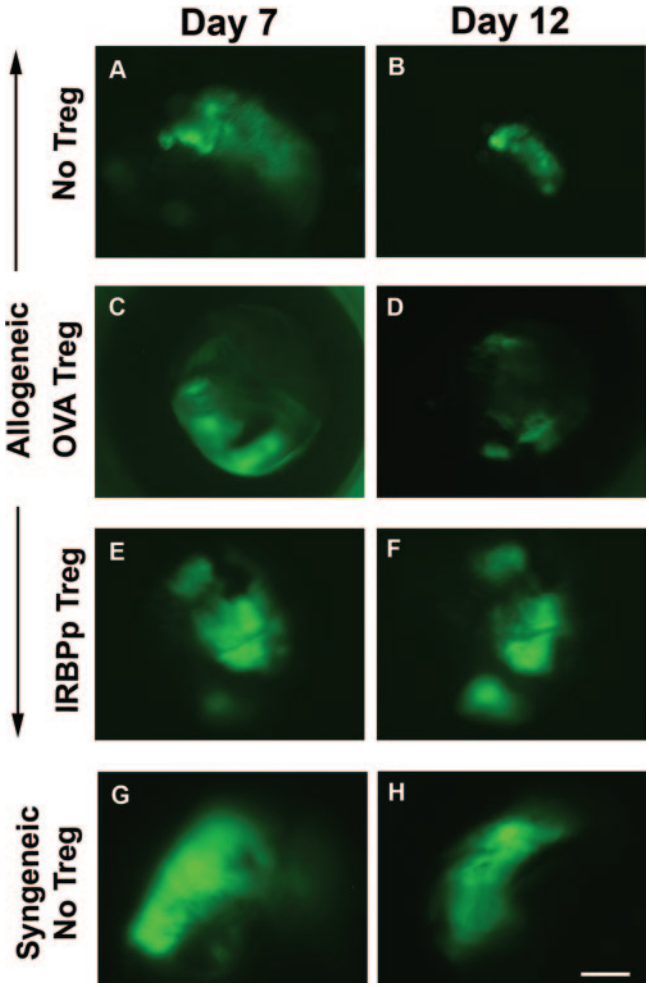

FIGURE 1. Photomicrographs of the in vivo images of eGFP-G57BL/6 NNR microaggregate transplants in the vitreous of allogeneic and syngeneic mouse eyes. *Left*: images taken on day 7. *Right*: images taken on day 12. The images are florescent fundus photomicrographs of eyes from allogeneic B10.RIII mice that received no treatment (**A**, **B**), allogeneic B10.RIII mice that were injected with OVA Treg cells (**C**, **D**), allogeneic B10.RIII recipients injected with IRBPp Treg cells (**E**, **F**), and syngeneic C57BL/6 recipients with no Treg cell injection (**G**, **H**). Each image is representative of the average response on day 7 and day 12 in each treatment group (see Fig. 2). Scale bar, 500 μm.

illumination) was measured. In C57BL/6 mice that received syngeneic eGFP-C57BL/6 NNR microaggregates, the detectable area of the GFP NNR grafts from day 7 to day 12 (Fig. 1) was reduced by 6% (Fig. 2), possibly because of compaction of the NNR microaggregate graft. In the B10.RIII mice that received allogeneic GFP NNR microaggregates and no Treg cells, the detectable area of their allogeneic GFP NNR microaggregate graft was diminished significantly from day 7 to day 12 (Fig. 1) by 72% (Fig. 2). A similar reduction in the allogeneic GFP graft area was seen in B10.RIII mice that received injections of Treg cells specific to OVA (Fig. 1); the area of the allogeneic GFP graft was reduced by 52% (Fig. 2). In contrast, the detectable area of the GFP allograft did not change in B10.RIII mouse eyes when the mice were injected with Treg cells specific to IRBP (Figs. 1, 2). On day 12, the detectable areas of the GFP-NNR microaggregates grafts in recipient of IRBP Treg cells were significantly greater than the detectable areas of the grafts in mice that received no Treg cells or Treg cells specific to a nonocular antigen ($P < 0.01$; Fig. 2). Therefore, the injection of Treg cells specific to an ocular autoantigen contributes to the promotion of retinal allograft survival.

Architecture of Surviving Allogeneic Retinal Transplants

Because there was a significant difference in allograft survival 12 days after transplantation, we examined the architecture of the surviving allogeneic retinal transplants 12 days after their insertion into the vitreous. To determine whether the presence of Treg cells specific to an ocular autoantigen contributed to promoting retinal allograft survival, we immunostained NNR microaggregate grafts with antibodies to NFh, GFAP, and rhodopsin to detect the distribution of retinal neurons, astrocytes, and photoreceptors, especially rods, which contain the targeted autoantigen IRBP. The NNR microaggregates in eyes of B10.RIII mice that received intravenous injections of α-MSH/TGF-β2–induced Treg cells specific to the IRBP formed organized retinal structures (Figs. 3A, 3C, 3E). NFh staining showed that most retinal neurons gathered on the outermost layer (Fig. 3A), as in the host retina. GFAP staining showed that the astrocytes were arranged in a layer adjacent to the outermost layer (Fig. 3C). Rhodopsin staining showed that photoreceptors filled most of the structure of the retinal graft (Fig. 3E). In the eyes of B10.RIII that received intravenous injections of Treg cells specific to OVA, the grafted microaggregates that could be found did not form an organized structure (Figs. 3B, 3D, 3F). NFh staining showed retinal neurons scattered throughout the retinal grafts (Fig. 3B). Similar observations were found when the grafts were stained for GFAP astrocytes (Fig. 3D). Rhodopsin staining in these grafts showed that only some photoreceptors were found and that they were distributed randomly in the graft including the outermost layers (Fig. 3F). These observations suggest that the presence of Treg cells specific to ocular autoantigens help promote healthy development and structural ordering of the neonatal retinal tissue allografts.

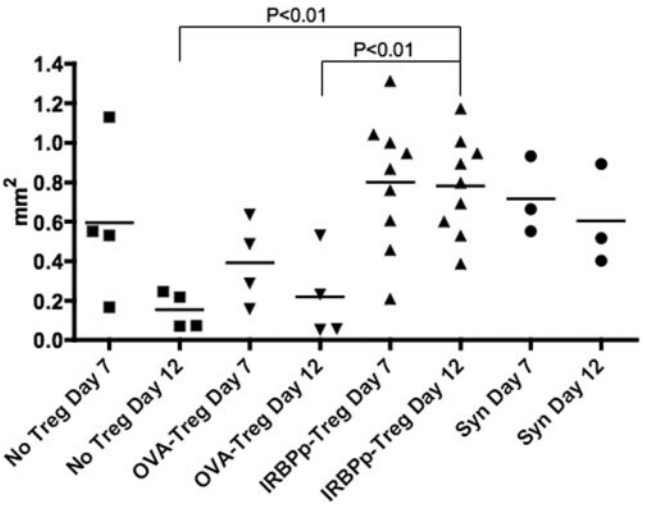

FIGURE 2. Scatter graph of the square area of GFP fluorescence of the retinal transplants on day 7 and day 12 per mouse in each treatment group; allogeneic B10.RIII mice that received no treatment (No Treg), allogeneic B10.RIII mice that were injected with OVA Treg cells, allogeneic B10.RIII recipients injected with IRBPp Treg cells, and syngeneic C57BL/6 recipients with no Treg cell injection (Syn). The average square area is indicated by the *horizontal bar* in each group. Significance as indicated was calculated by ANOVA analysis and Tukey multiple comparison test, with $P \leq 0.05$ considered significant.

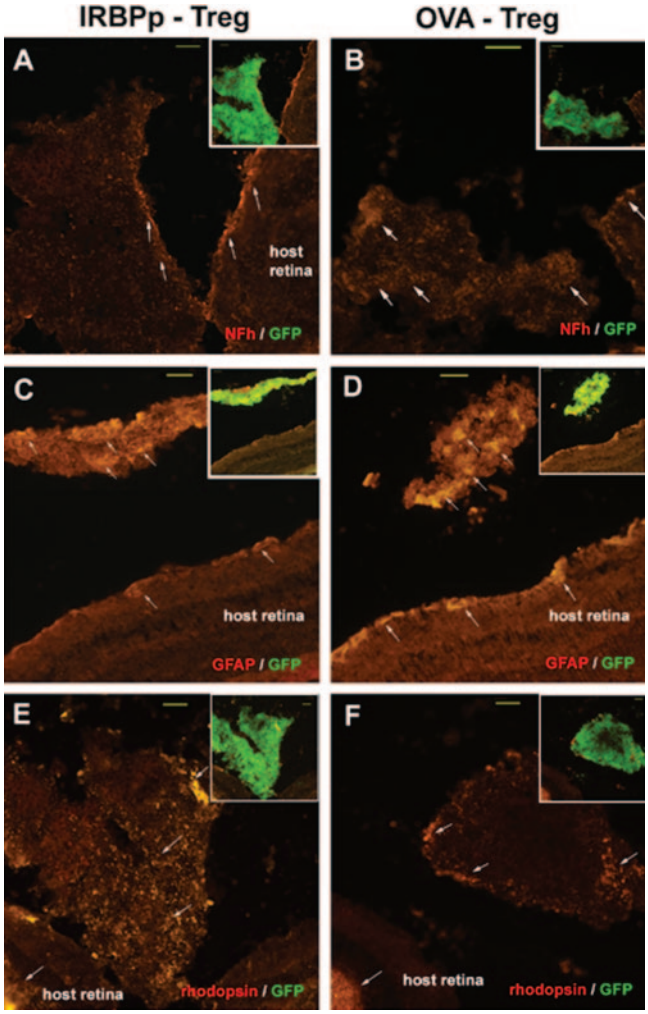

FIGURE 3. Photomicrographs of immunohistochemical staining of NNR microaggregate transplants to characterize the tissue architecture 12 days after transplantation. *Inset*: merged image of the immunohistochemical staining and the GFP fluorescence of the NNR graft. *Left*: transplants in the vitreous of B10.RIII recipients that were injected with IRBPp-Treg cells. *Right*: transplants in the vitreous of mice injected with OVA-Treg cells. Sections were stained with antibodies against NFh (**A**, **B**), GFAP (**C**, **D**), and rhodopsin (**E**, **F**). Images are representative of each treated group of mice. *Arrows*: positive labeling. Scale bar, 50 μm.

Appearance and Location of Immune Cells in Allogeneic Retinal Transplant

It has been shown that microglia act as passenger leukocytes that upregulate MHC class II after transplantation.[17] The appearance and location of microglia in the retinal graft reformed by NNR microaggregates may be important in the survival of the allogeneic grafts. We stained the surviving NNR microaggregates with GS-lectin to detect microglial cells and with antibodies against IA^b of the donor. In addition, we stained the grafts with anti–CD3 antibody to detect infiltrating T cells. We found that the NNR microaggregates in the eyes of B10.RIII mice that received injections of IRBP-specific Treg cells have microglia cells (GS-lectin stained) located in the outermost layer of the graft, and in the central lumen of the rosettes formed by the cells of the microaggregates (Fig. 4). Similar central lumen staining for IA^b was observed. Interestingly, $CD3^+$ T cells and $FoxP3^+$ Treg cells[26] were found in the grafts (Fig. 4). Grafted NNR microaggregates in the eyes of B10.RIII mice that were injected with OVA-specific Treg cells also stained for microglia in the outermost layer and inside the lumen of the rosettes; however, only the microglia that line the periphery of the central lumen of the rosettes stained for IA^b (Fig. 3). No $CD3^+$ T cells or $FoxP3^+$ Treg cells were found in these retinal transplants. These results demonstrate that Treg cells to ocular autoantigen may regulate the function and distribution of immune cells in the allograft, contributing to retinal allograft survival.

DISCUSSION

We previously demonstrated that α-MSH enhanced by TGF-β2 can be used to generate antigen-specific effector Treg cells in vitro and that we can use these generated Treg cells if they are specific to IRBPp to suppress EAU.[22] We now present experimental results that further demonstrate that the α-MSH–induced IRBPp-specific Treg cells can be used to suppress the accelerated rejection of allogeneic retinal grafts placed in the vitreous cavity and to enhance the development of an organized retinal structure by the allogeneic NNR microaggregates.

In vivo imaging demonstrated that the size of the allogeneic retinal graft in mice receiving IRBPp-Treg cells on day 12 was similar to that of syngeneic transplants in that the overall changes in the size of the microaggregates were insignificant. However, the sizes of the allogeneic retinal grafts in mice receiving OVA-Treg cells or no Treg cells shrank significantly from day 7 to day 12. Although the area measured in vivo only covered two dimensions, there was a consistent finding in the histologic sections such that by day 12 the GFP-expressing retinal grafts in the control groups (OVA-Treg cells or no Treg cells injected) were always smaller than the syngeneic grafts, sometimes were harder to find, and rarely had rosette formations. In contrast, the retinal allografts in IRBPp-Treg–injected mice were as large as the syngeneic grafts and had multiple rosettes within the graft.

The influence of the IRBPp-Treg cells may occur locally in the vitreous. We demonstrated that on day 12, $CD3^+$ T cells were found only in the allogeneic retinal grafts of mice receiving IRBPp-Treg cells, but not in mice receiving OVA-Treg cells and rejecting the graft. It was possible that the primed T cells (both OVA-Treg and IRBPp-Treg cells) trafficked trough the eye, but only the IRBPp-Treg cells stayed in the vitreous while the OVA-Treg moved on. Such immobilized IRBPp-Treg cells would further promote immune privilege and potentially would help induce immune tolerance to the alloantigens expressed on the graft in the ocular microenvironment. In addition, these Treg cells could influence the induction of alloimmunity in regional lymph nodes. Staining for FoxP3 revealed that the surviving allografts were infiltrated with Treg cells. Because the immune mechanisms of retinal allograft rejection are still unclear, we cannot determine whether the activity of the Treg cells suppressed an allodestructive immune response or produced neurotrophic factors that promote neuronal survival and retina-mediated immune privilege. All that is known is that immunity is involved in the rejection of retinal allografts, and our results demonstrated that regulatory immunity can be used to promote retinal allograft survival.

Published studies report that GFP itself can be a transplantation antigen contributing to the rejection of grafts expressing GFP.[27,28] In addition, syngeneic transplants expressing GFP can be rejected by an anti–GFP immune response.[27] Although the loss in the syngeneic NNR microaggregate graft area was insignificant, we cannot rule out the possibility that the graft underwent a rejection process mediated by an anti–GFP immune response; however, this cannot be separated from the expected devolution of the graft because the graft does not

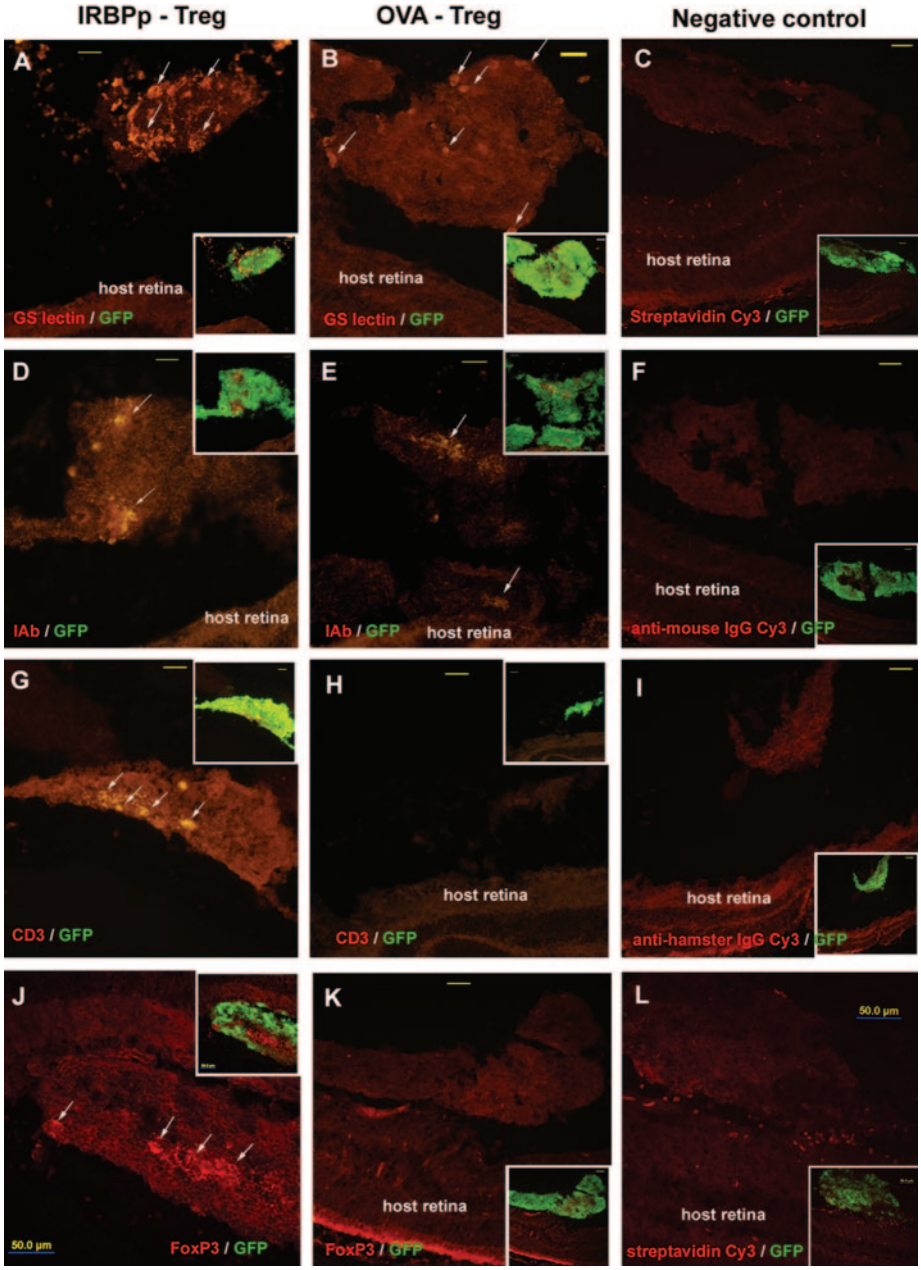

FIGURE 4. Photomicrographs of immunohistochemical staining of NNR microaggregate transplants to characterize markers of immunity 12 days after transplantation. *Insets*: merged images of the immunohistochemical staining and the GFP fluorescence of the NNR graft. *Left*: transplants in the vitreous of B10.RIII recipients injected with IRBPp-Treg cells. *Middle*: transplants in the vitreous of mice injected with OVA-Treg cells. *Right*: negative controls. Sections were stained with GS-lectin for microglial (**A**, **B**) and antibodies against MHC class II, IA^b (**D**, **E**), CD3 (**G**, **H**), and FoxP3 (**J**, **K**). Sections were also stained with corresponding secondary antibodies (negative control), including streptavidin-Cy3 (**C**), anti-mouse IgG Cy3 (**F**), anti-hamster IgG Cy3 (**I**), and anti-goat IgG Cy3 (**L**). No positive staining was found in the negative controls. Images are representative of each treated group of mice. *Arrows*: positively stained cells. Scale bar, 50 μm.

neurologically integrate itself with the host retina.[11] Interestingly, other reports using neonatal retinal tissues do not see an anti–GFP immune response in the rejection of neonatal retinal tissue grafts.[29,30] The expression of GFP in neonatal cells does not appear to be a target and may not be presented in an immunogenic manner.[31,32] Regardless of whether the NNR-microaggregated grafts express GFP as an immunogenic target, the presence of the Treg cells must also suppress any potential immunity to GFP as well as it suppresses the graft-rejecting alloimmune response.

Given that antigen-presenting cells (APCs) are central to the activation of T cells, potential APCs in the NNR microaggregate grafts are the microglia. GS lectin staining showed that the microglia were in an active state with MHC class II expression. Interestingly, these microglia occupied the lumen of the rosettes, and their primary function might have been similar to that of the retinal pigment epithelium for the phagocytosis of shed rod outer segments. Unexpectedly, fewer numbers of MHC class II–positive, GS lectin–positive microglia were found in the NNR microaggregate grafts of mice receiving OVA-Treg cells. Because microglial cells function as passenger leukocytes, they might have migrated out of the allografts and possibly had a role inducing alloimmunity. If this were the case, the IRBPp-Treg cells we injected might have prevented passenger leukocyte migration and thus further weakened the induction of alloantigen immunity. In addition, the lack of microglia in the allografts could be an indication of an unhealthy transplant.[4] The strong presence of microglia with MHC class II expression in the retinal grafts of mice injected with IRBPp-Treg cells could be the result of a strong viable graft, with class II stimulation of the Treg cells suppressing alloimmunity.

Our results demonstrate the usefulness of in vitro–derived Treg cells specific to an ocular autoantigen to promote retinal allograft survival and development. Our results further show that the aqueous humor immunosuppressive factor α-MSH can

be used with TGF-β2 to manipulate immunity from pathogenic or unfavorable immune responses to an immune response that is beneficial.

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