rAAV.sFlt-1 Gene Therapy Achieves Lasting Reversal of Retinal Neovascularization in the Absence of a Strong Immune Response to the Viral Vector

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Purpose. To determine the efficacy of rAAV.sFlt-1–mediated gene therapy in a transgenic mouse model of retinal neovascularization (trVEGF029) and to assess whether rAAV.sFlt-1 administration generated any deleterious, long-lasting immune response that could affect efficacy.

Methods. trVEGF029 mice were injected subretinally with rAAV.sFlt-1 or phosphate-buffered saline. Fluorescein angiography and electroretinography were used to compare the extent of fluorescein leakage from retinal vessels and retinal function, respectively. A group of eyes was enucleated, and the retinal vasculature and morphology were studied by confocal and light microscopy. Cells were isolated from the posterior eyecups and spleens of a further group, and immune cell subset populations were investigated by flow cytometry. sFlt-1 protein levels in the eyes were evaluated by ELISA.

Results. After a single rAAV.sFlt-1 injection, sFlt-1 protein levels were upregulated, and there was a reduction in fluorescein leakage from the retinal vessels and an improvement in retinal function. Confocal microscopy of isolectin-IB4-labeled retinal wholemounts showed more normal-appearing capillary beds in rAAV.sFlt-1–injected than in PBS-injected trVEGF029 mouse eyes. Light microscopy demonstrated retinal morphology preservation, with fewer aberrant vessels invading the outer nuclear layer of rAAV.sFlt-1–injected eyes. Furthermore, the immune response to subretinal injection of rAAV.sFlt-1 was limited to a transient increase in $CD45^+$ leukocytes that disappeared by 4 weeks after injection. This transient increase was localized to the eye and did not affect long-term therapeutic efficacy.

Conclusions. The data support the notion that rAAV.sFlt-1 gene therapy is safe and effective for the long-term inhibition of deleterious blood vessel growth in the eye. (*Invest Ophthalmol Vis Sci.* 2009;50:4279–4287) DOI:10.1167/iovs.08-3253

In the past decade, gene therapy development strategies and advances in viral vectorology have come to the forefront of novel therapeutics. However, recent gene therapy trials on human subjects revealed two major obstacles: (1) activation of innate immunity which resulted in a systemic inflammatory response leading to the death of a human subject after hepatic infusion of adenovirus carrying the ornithine-transcarboxylase gene,[1] and (2) insertional mutagenesis that resulted in the development of leukemia-like disease in some young human subjects with X-linked severe combined immunodeficiency after retroviral gene therapy.[2,3]

The recombinant adenoassociated virus (rAAV) vectors used in clinical trials have not been directly linked to any serious adverse effects.[4–7] However, a participant in a current phase 1/2 dose-escalation study for safety assessment of an rAAV2 vector in adults with inflammatory arthritis died after treatment,[8] but the death was not directly linked to the rAAV vector.[9] Three ongoing Leber's congenital amaurosis clinical trials have also not reported any serious adverse events associated with subretinal injection of the rAAV vector.[10–12] rAAV is still one of the most promising gene delivery systems for broad-spectrum applications. It is a nonpathogenic parvovirus and requires a helper virus to replicate.[13,14] rAAV contains no viral genes, thus minimizing the probability of eliciting an undesirable host cellular response. In addition, rAAV transduces both dividing and nondividing cells, allowing targeting of a broad array of cells and tissues.[15–21]

In animal models, rAAV mediates long-term expression of the transgene in many tissue types,[11,16,19,22–31] but this has not been observed in human subjects.[5] In one clinical study, the short-lived rAAV-mediated F.IX expression in patients with severe hemophilia B was attributed to cell-mediated immunity targeting the rAAV capsid antigens.[5] This outcome contradicted previous reports that rAAV is nonimmunogenic. Other studies have reported humoral immune responses to rAAV capsid proteins.[28,32,33] A recent study found high levels of T cells specific to vector capsids based on rAAV2 and a related rAAV variant after intramuscular injection.[34] In addition, elicitation of immune responses due to delivery route,[28,32,33,35,36] or nature of transgene expressed, has been observed.[20,36–38] In some instances, the ability of a particular transgene to induce an immune response is dependent on the type of viral vector[32] or promoter used.[39] Altogether, these findings suggest that several factors, singly or in combination, may lead to an immune response that could limit the duration of transgene expression

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and/or the effectiveness and safety of rAAV readministration.[40–42]

The eyes, being small and isolated paired organs readily accessible for drug delivery, are ideal candidates for gene therapy. However, a cell-mediated immune response against the vector has the potential to damage the retina, resulting in visual impairment or loss. Previously, we demonstrated long-term regression of neovascular vessels after injection of rAAV.sFlt-1 (a rAAV vector carrying the soluble human vascular endothelial growth factor [VEGF] receptor 1 [sFlt-1]) into the eyes of the trVEGF029 mouse, a mouse model for VEGF-driven retinal neovascularization.[43–45] We reported long-term sFlt-1 expression and regression of pathologic and neovascular changes, with no clinically or histologically detectable signs of toxicity.[19] In this study, we further characterized the efficacy of the rAAV.sFlt-1 vector in trVEGF029 mice and determined whether it elicited a cell-mediated immune response that might negatively affect the outcome of our gene therapy regimens.

METHODS

Generation of rAAV.sFlt-1

The rAAV.sFlt-1 construct described previously[31] was produced in human embryonic kidney (HEK-293) cells using three-plasmid cotransfection and purified by heparin affinity chromatography by the Virus Vector Core Facility (University of North Carolina, Chapel Hill, NC). The genomic titer of the virus was 8×10^{12} particles/mL.

Mice

trVEGF029 mice and wild-type control littermates were housed in cages at a constant temperature of 22°C, with a 12/12 hour light/dark cycle. Food and water were available ad libitum. All procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and according to the guidelines of the Animal Ethics Committees of The University of Western Australia, University of Queensland, and the National Health and Medical Research Council of Australia.

Anesthesia and Subretinal Injection in Mice

Anesthetization of mice, pupil dilation, and subretinal injection were performed as described previously.[46] Briefly, 1 μL of rAAV.sFlt-1 (8×10^9 particles) or vehicle (sterile phosphate-buffered saline [PBS]) was injected into the subretinal space. Only successfully injected mice (appearance of a partial retinal detachment by indirect ophthalmoscopy) were included in the study.

Clinical Photography and Grading of Neovascularization

After anesthesia and pupil dilation, color fundus photography (CFP) and fluorescein angiography were performed and the extent, intensity, and stage of neovascularization were graded as described previously.[19] Data generated was analyzed using the Mann-Whitney U test.

Electroretinography

Full-field (Ganzfeld), scotopic flash electroretinography (ERG) was performed simultaneously on both eyes of anesthetized mice at 3 months after injection, as described previously.[47] Flash stimuli were presented over an intensity range of -4 to $+3$ log cd · s/m^2. Photoreceptoral (PIII) responses were obtained by fitting the leading edge of the a-wave with the model of Hood and Birch[48] to determine the maximum saturated PIII amplitude (Rm_{P3}).

Histology and Immunohistochemistry

For specific labeling and visualization of retinal vasculature and immune cell infiltration, the eyes were enucleated and fixed as described previously.[45] In brief, deparaffinized sections, and retinal wholemounts were incubated with biotinylated isolectin-IB4. Bound lectin was detected through incubation with fluorescein isothiocyanate (FITC)-labeled peroxidase (ExtrAvidin; Sigma-Aldrich, St. Louis, MO). Sections were counterstained with DAPI nuclear stain (1:1000 dilution) for 15 minutes, followed by three washes in PBS. Isolectin-IB4/DAPI-stained sections and detailed structure of the vascular beds and lesions in isolectin-IB4-stained wholemounts were analyzed by confocal microscopy (MRC1000; Bio-Rad, Hercules, CA), as described previously.[45]

Measurement of sFlt-1, Mouse VEGF, and Human VEGF Levels

rAAV.sFlt-1 and PBS-injected eyes, enucleated at 4 weeks after injection, and age-matched C56Bl/6 mouse eyes were homogenized in 200 μL PBS and centrifuged. The supernatant was collected and exogenous human sFlt-1, mouse VEGF (mVEGF), and human VEGF (hVEGF) protein levels were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The minimum detectable dose for human sFlt-1, mVEGF, and hVEGF for the assays are <3.5 pg/mL, <3 pg/mL, and <5.0 pg/mL, respectively. Data were analyzed with Student's *t*-test.

Flow Cytometry

Spleens were harvested at 1, 2, and 4 weeks after injection. Single-cell suspensions were prepared in cold PBS/1% BSA by mechanical disaggregation and filtration through a 30-μm pore size mesh (Sefar Filter Specialists Pty Ltd., Blacktown, NSW, Australia). At the same time points, eyes were enucleated and dissected at the limbus. The posterior eyecup comprising the sclera, choroid, and retina was digested by collagenase (type IV; Invitrogen, Carlsbad, CA) for 30 minutes at 37°C and washed in cold PBS/1% BSA, and then a single-cell suspension was prepared by grinding the tissues between frosted glass slides.

Antibody labeling was performed for 60 minutes at 4°C with the following fluorochrome-labeled antibodies: CD4 APC (Clone RM4-5), CD8 APC-Cy7 (clone 53-6.7), CD11b FITC (clone M1/70), CD19 PE (clone 1D3), and CD45 PE-Cy7 (clone 30F11) (BD Biosciences, San Diego, CA). For each sample, 1×10^6 events were collected (FACS Canto; BD Biosciences). Appropriately stained controls were used to check compensation settings and nonviable cells were excluded from analysis with 7AAD (Sigma). Sample files were analyzed with FlowJo software (Tree Star, San Carlos, CA) and statistical analysis was performed with the Mann-Whitney *U* test and one-way ANOVA (Tukey's Multiple Comparison Test).

Intracellular Cytokine Staining

Splenocytes or ocular cells were resuspended in RPMI-1640 (10% fetal bovine serum [FBS]) containing brefeldin A (GolgiPlug; BD Biosciences) at 10 mg/mL. The cells were incubated at 37°C 5% CO_2 for 5 hours at 2×10^6/200 μL/well in 96-well round bottomed plates containing phorbol 12-myristate 13-acetate (PMA), plate-immobilized αCD3 (50 mg/mL overnight at 4°C), or 2 ng antigen specific peptide (SNYNKSVN from AAV2 H-2b restricted epitope[34]; Proteomics International Pty. Ltd., East Perth, WA, Australia). Cells were washed and then stained at 4°C with CD8-APC Cy7, CD4-PE, CD45-PECy7, and TCR-FITC, as described earlier. After they were washed, the cells were fixed overnight (Cytofix/Cytoperm; BD Biosciences) at 4°C. They were then washed and stained (Perm-Wash; BD Biosciences) with APC-conjugated IFNγ (XmG1-2; Biolegend, San Diego, CA) antibody or isotype control for 30 minutes on ice. The cells were washed and flow cytometry and analysis performed, as described earlier.

TABLE 1. Mouse VEGF Concentrations in Different Control and Treatment Groups

	C57B1/6	rAAV.sFlt-1 Injected trVEGF029	PBS-Injected trVEGF029
	25.4	32.8	41.6
	28.2	27.9	40.6
	31.1	29.6	38.3
	15.6	15.4	31.9
	34.1	30.1	31.4
Mean ± SD	26.9 ± 7.1	27.2 ± 6.8	36.7 ± 4.8

Data are free mVEGF protein concentration (pg/mL).

RESULTS

Upregulation of sFlt-1 Protein Levels, Reduced Retinal Neovascularization, and Preserved Retinal Vasculature after rAAV.sFlt-1–Mediated Gene Therapy

All mice were injected 3 weeks after birth. The left eye received rAAV.sFlt-1 and the contralateral (control) eye, PBS. sFlt-1 protein levels were significantly elevated in rAAV.sflt-1–injected eyes at 4 weeks after injection compared with the contralateral eyes (1140.6 ± 407.5 pg/mL vs. 103.0 ± 9.4 pg/mL; $P = 0.029$, $n = 4$ for each group). By this age, free hVEGF protein concentrations in all the eyes tested were below the level of detection of the kit. The free mVEGF concentrations are presented in Table 1. The mean free mVEGF concentration in PBS-injected trVEGF029 mouse eyes was significantly higher than that in C57Bl/6 ($P = 0.036$) and in rAAV-sFlt-1–injected trVEGF029 mouse eyes ($P = 0.019$) but there was no significant difference ($P = 0.953$) in mean free mVEGF concentration between C57Bl/6 and rAAV.sFlt-1–injected eyes.

Fluorescein angiography of rAAV.sFlt-1–injected ($n = 28$) and PBS-injected ($n = 28$) trVEGF029 mouse eyes were blindly graded by two observers according to a scale used previously.[19] Before rAAV.sFlt-1 injection, 15 eyes had mild neovascularization, 11 eyes had moderate to severe neovascularization, and 2 eyes had cataract. At 2 months after injection, 24 eyes had mild neovascularization, and only 2 eyes showed moderate to severe neovascularization. There was no change in the number of eyes with cataract. A significant reduction in mean neovascularization scores was observed (before injection, 1.5 ± 0.58; 2 months after injection, 0.81 ± 0.57; $P = 0.0005$). In the control eyes, before PBS injection, 23 had mild neovascularization, 3 eyes had moderate to severe neovascularization, and 2 had cataract. At 2 months after injection with PBS, 9 eyes had mild and 10 eyes showed moderate to severe neovascularization. The number of eyes with cataract increased from two to nine. A significant increase in mean neovascularization scores was observed (before PBS injection: 1.08 ± 0.56, 2 months after PBS injection: 1.63 ± 1.06; $P = 0.014$).

Representative isolectin-IB4-stained sections are presented in Figures 1A–D. Isolectin-IB4 staining for endothelial cells was positive in the nerve fiber and retinal ganglion cell layers and in the area between the retinal pigment epithelium (RPE) and outer nuclear layer (ONL) in both PBS-injected (Fig. 1C) and rAAV.sFlt-1–injected (Fig. 1D) trVEGF029 mouse eyes at 2 months after injection. However, there was more isolectin-IB4-positive staining in the region between the RPE and ONL in PBS-injected trVEGF029 mouse eyes (Fig. 1C). The specificity of isolectin-IB4 staining in the sections tested was confirmed by the lack of signal in the controls (Figs. 1A, 1B) that were processed at the same time, but without primary antibody being added.

Retinal vasculature was visualized with confocal microscopy of isolectin-IB4-stained retinal wholemounts. Animal groups were: wild-type noninjected ($n = 3$) and injected ($n = 5$; left eye, rAAV.sFlt-1; right eye, PBS) and trVEGF029 mice noninjected ($n = 3$) and injected ($n = 5$; left eye, rAAV.sFlt-1; right eye, PBS). Retinal wholemounts were examined at 4 months after injection and at an equivalent age for noninjected animals.

Examination of wild-type eyes (noninjected and PBS-injected) confirmed that the injection per se did not adversely affect the retinal vasculature (Figs. 1E, 1F); furthermore, injection of rAAV.sFlt-1 itself did not induce damage (Fig. 1G). In the wild-type groups, all eyes had three distinct vascular beds comprising the superficial (nerve fiber layer/retinal ganglion

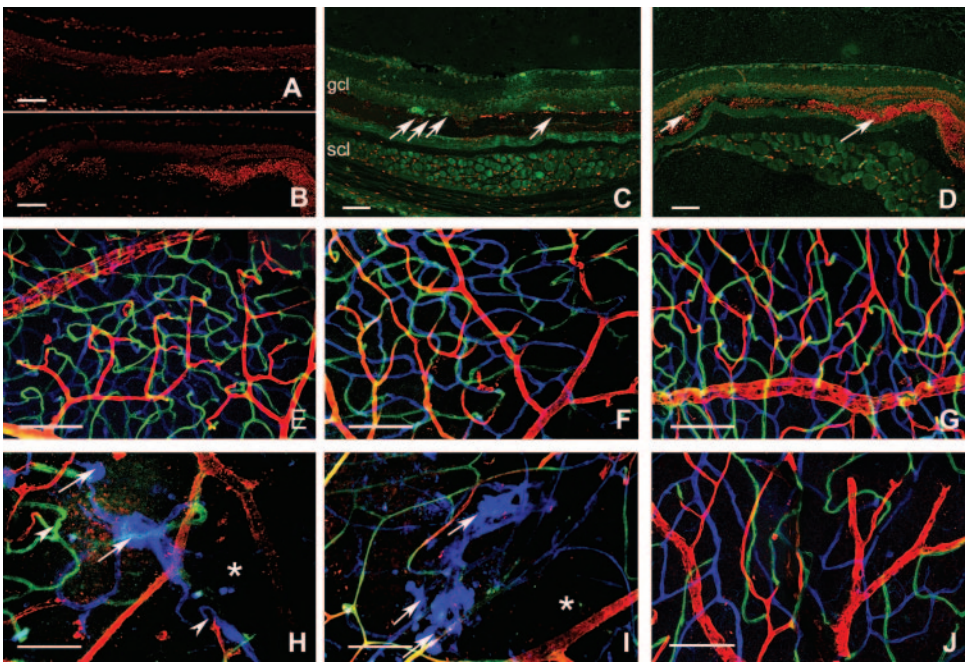

FIGURE 1. Confocal microscopy of sectioned eyes of trVEGF029 mouse (**A–D**, counterstained with DAPI) and retinal wholemounts (**E–J**) of wild-type (**E–G**) and trVEGF029 (**H–J**) mouse stained with isolectin-IB4. (**A**, **B**) No primary antibody control (**A**, PBS injected; **B**, rAAV.sFlt-1 injected) and isolectin-IB4$^+$ vessels in trVEGF029 mouse eyes at 2 months after injection (**C**, PBS-injected; **D**, rAAV.sFlt-1–injected). Superficial (*red*), mid-depth (*green*), and deeper (*blue*) vascular beds in wild-type (**E–G**) and trVEGF029 (**H–J**) mouse eyes that were not injected (**E**, **H**) or were injected with PBS (**F**, **I**), or rAAV.sFlt-1 (**G**, **J**). Vascular lesions (*arrows*), tortuous capillaries (*arrowhead*), and areas of capillary dropout (✶) were observed. Scale bar (**A–D**) 50 μm; (**E–J**) 100 μm. gcl, ganglion cell layer; scl, sclera.

cell layer, red), middle (vitread side of the inner nuclear layer, green), and deeper (sclerad side of the inner nuclear layer, blue) capillary beds which appeared as regular, nonoverlapping arrays (Figs. 1E–G).

By contrast, in both noninjected and PBS-injected trVEGF029 mouse eyes, the superficial vascular bed (red) had a very sparse capillary network, indicative of capillary dropout (Figs. 1H, 1I). Many vascular lesions comprising swollen capillaries that were twisted on themselves, as described previously,[45] were also observed (data not shown). More vascular lesions were present in the deeper capillary beds (blue; Figs. 1H, 1I). Regardless of the capillary bed in which they resided, vascular lesions predominated in the peripheral retina (data not shown). By contrast, in rAAV.sFlt-1–injected trVEGF029 mouse eyes, the superficial (red) capillary network appeared similar to normal (Fig. 1J). Furthermore, there were very few vascular lesions observed (data not shown) and the middle (green) and deeper (blue) vascular beds also appeared relatively normal (Fig. 1J).

Effect of rAAV-Mediated Ocular Gene Therapy on Visual Function and Retinal Morphology

Full-field ERGs were recorded simultaneously to allow photoreceptor-derived ERG responses to be obtained from the rAAV.sFlt-1–injected and PBS-injected contralateral eyes under identical states of anesthesia and adaptation. This was followed by retina morphology assessment by light microscopy. The ERG recordings of rAAV.sFlt-1–injected trVEGF029 mice, expressed as a percentage of the contralateral eye, and histology of rAAV.sFlt-1–injected and control eyes are presented in Figure 2. An increase in maximum saturated photoreceptor response (Rm_{P3}) values (Fig. 2G) was seen in all rAAV.sFlt-1–injected eyes ($n = 5$). Three of the five injected eyes (F080, F083, and G113) with higher Rm_{P3} values appeared to have thicker ONLs (Figs. 2B, 2D, 2F) and fewer blood vessels transgressing the ONL (Figs. 2B, 2F, arrows) when compared with the contralateral PBS-injected eyes (Figs. 2A, 2E, arrows). The intensity-response characteristics from a representative mouse (F080) showing the larger photoreceptor-generated a-wave response in the rAAV.sFlt-1–injected eye are presented in Figure 2H.

Assessment of Immune Responses after Subretinal Injection of rAAV.sFlt-1

The cellular immune response to rAAV.sFlt-1 therapy was assessed in the eye at 1, 2, and 4 weeks after injection using flow cytometry. Infiltrating leukocytes were identified on the basis of CD45 expression and classified as monocytes/granulocytes, B cells, $CD4^+$ T cells and $CD8^+$ T cells on the basis of CD11b, CD19, CD4, and CD8 expression, respectively. The posterior eyecup was collected from five mice in each group (rAAV.sFlt-1–injected, PBS-injected, and noninjected control) and pooled for analysis. As shown in Figure 3A, there was no difference in the total number of cells recovered from each group of mice over the course of the experiment. However, there was a significant increase in the number of $CD45^+$ cells 1 and 2 weeks after injection that disappeared by 4 weeks (Fig. 3B). Almost all of the increase seen at 1 week was attributable to an increase in $CD11b^+$ cells (Fig. 3C), because there was no difference in the number of $CD4^+$, $CD8^+$, and $CD19^+$ cells (Figs. 3D–F) at this time point.

At 2 weeks, there was a slightly higher, but not statistically significant, number of $CD11b^+$ cells present in the rAAV.sFlt-1–injected eyes ($P > 0.05$). At this time point, however, there was a significant increase ($P < 0.05$) in the number of $CD4^+$ and $CD8^+$ T cells and a possible trend toward a small increase in B cells. The number of $CD45^+$ cells (Fig. 3B) fell sharply at 4 weeks, as did that of $CD11b^+$, $CD4^+$, and $CD8^+$ cells (Figs. 3C–E). A small difference in the number of $CD4^+$ and $CD8^+$ cells between rAAV.sFlt-1– and PBS-injected or noninjected mice remained at this time point (Figs. 3D, 3E). No change in the number of $CD11b^+$, $CD4^+$, $CD8^+$, and $CD19^+$ cells was observed in the spleen during the same time course (Fig. 4).

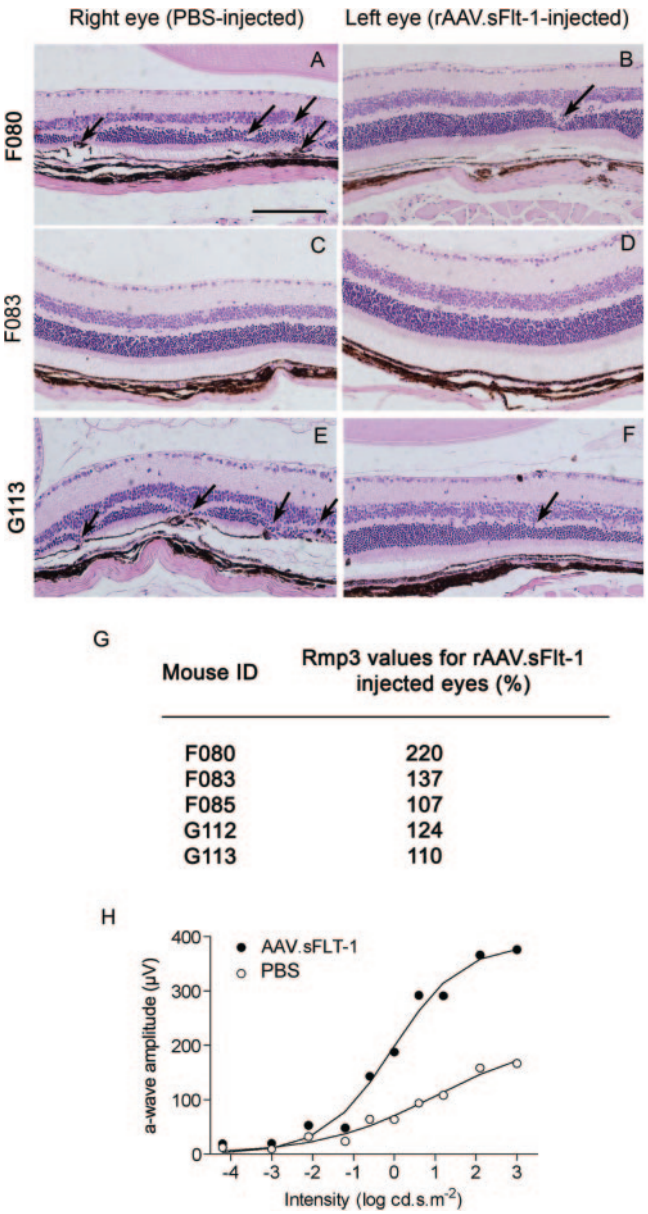

Figure 2. Retinal morphology (**A**–**F**) and ERG responses (**G**, **H**) of PBS-injected (**A**, **C**, **E**) and rAAV.sFlt-1–injected trVEGF029 (**B**, **D**, **F**) mouse eyes. Light micrographs showing the presence of more layers of photoreceptor cells and fewer vessels transgressing the outer nuclear layer (*arrows*) in rAAV.sFlt-1–injected left eyes when compared with the contralateral PBS-injected right eyes. Amplitudes of the photoreceptor-derived ERG response (Rm_{P3}) from five trVEGF029 mouse eyes expressed as a percent of the Rm_{P3} of the respective contralateral eyes (**G**) and an ERG a-wave intensity-response curve of a representative trVEGF029 mouse (**H**) at 3 months after injection. The photoreceptor response was measured over an intensity range of −4 to +3 log cd · s/m^2 at 3 months after injection of rAAV.sFlt-1. Scale bar, 165 μm.

The functional capacity of the T cells infiltrating the retina was examined more closely by measuring intracellular IFN-γ production after stimulation with PMA, plate-immobilized αCD3, or a CD8-restricted AAV peptide. Compared with noninjected control eyes, <5% of either $CD4^+$ or $CD8^+$ T cells

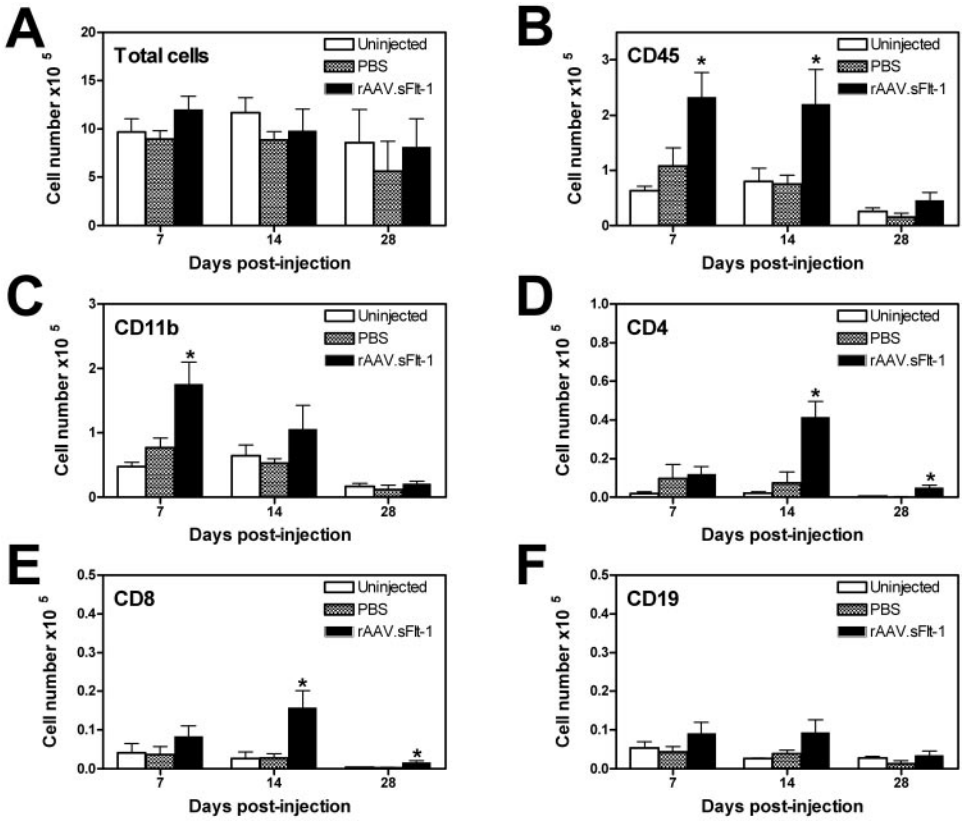

FIGURE 3. Flow cytometry results showing total number of cells (**A**), $CD45^+$ cells (**B**), $CD11b^+$ cells (**C**), $CD4^+$ cells (**D**), $CD8^+$ cells (**E**), and $CD19^+$ cells (**F**) isolated from control noninjected, PBS-injected and rAAV.sFlt-1–injected eyes at 7, 14, and 28 days after injection. Data represent the mean ± SD of three independent experiments with five mice per group per time point.

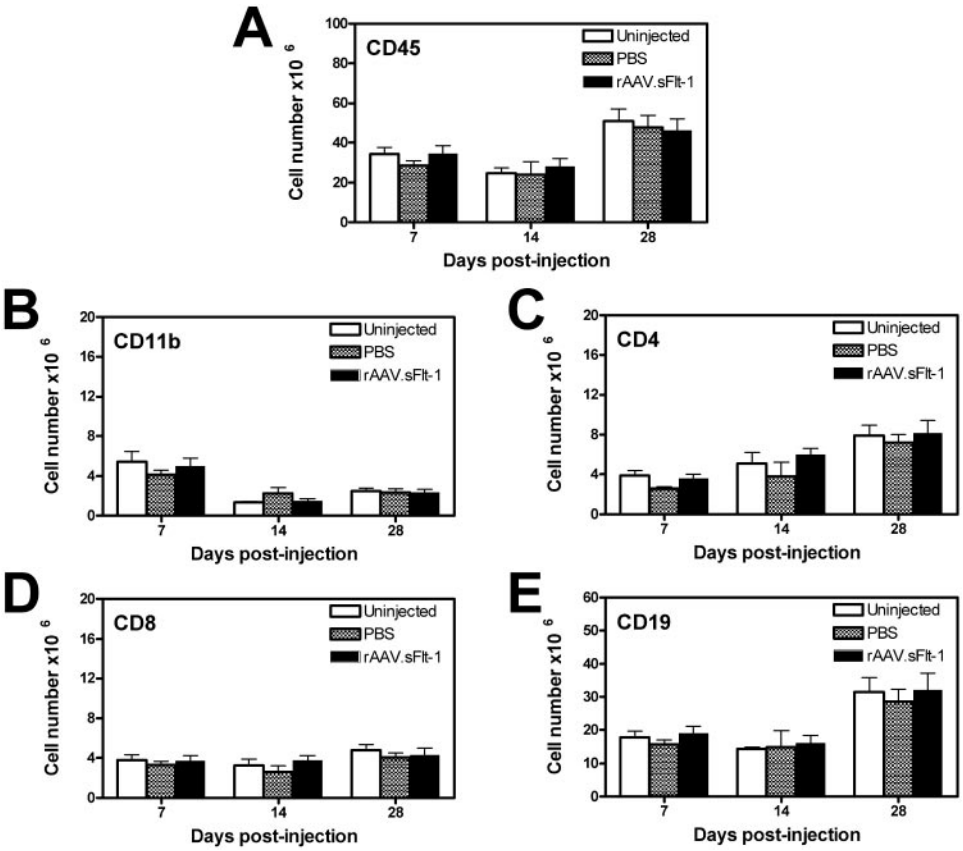

FIGURE 4. Flow cytometry analysis results showing total number of $CD45^+$ cells (**A**), $CD11b^+$ cells (**B**), $CD4^+$ cells (**C**), $CD8^+$ cells (**D**), and $CD19^+$ cells (**E**) in the spleens of control noninjected mice or mice whose eyes were injected with PBS or rAAV.sFlt-1 at 7, 14, and 28 days after injection. Data are the mean ± SD of three independent experiments with five mice per group per time point.

were found to produce IFN-γ after injection of rAAV.sFlt-1 if stimulated with either PMA or αCD3 (data not shown). The frequency of IFN-γ-producing cells did not vary significantly over time, nor were these frequencies significantly higher than those observed in T cells from noninjected eyes (data not shown). In an important finding, no significant production of IFN-γ was observed when the $CD8^+$ T cells were stimulated with a class I MHC-restricted epitope of the AAV capsid protein (data not shown). Taken together, these results provide evidence that there is no significant specific activation of T cells in the eye after injection of rAAV.sFlt-1.

Discussion

VEGF, an angiogenic and vascular permeability factor, has long been accepted as a major player in tumor metastasis, rheumatoid arthritis, and ocular neovascularization. Many antiangiogenic molecules aimed at targeting VEGF have been used successfully in experimental ocular disease and other disease models. Pegaptanib (Macugen; Eyetech, New York, NY), a 28-base ribonucleic acid anti-VEGF aptamer,[49] and ranibizumab (Lucentis; Genentech, South San Francisco, CA), a recombinant humanized monoclonal antibody fragment for hVEGF,[50] are currently used clinically for neovascular age-related macular degeneration (AMD) and ocular neovascularization, respectively. More recently, bevacizumab (Avastin; Genentech), a humanized anti-VEGF monoclonal antibody licensed as an anticancer drug for colorectal cancer, has produced promising results in inhibiting neovascularization in diabetic retinopathy[51–54] and neovascular AMD.[55–57] Although these drugs have shown very promising results, one setback is that they often need repeated delivery.[49,52,56–58]

Similar to the above therapeutic monoclonal antibodies and aptamers, sFlt-1 is a potent antiangiogenic agent that exerts its action through sequestering secreted VEGF. In addition, sFlt-1 can form a heterodimeric complex with the wild-type VEGF receptor in a dominant negative fashion, inhibiting its signal transduction.[59–61] A recent study demonstrated that administration of recombinant sFlt-1 restored avascularity in spontaneously vascularized corneas of *corn1* and *Pax6* $^{+/-}$ mice.[62] Furthermore, recombinant virus-mediated sFlt-1 expression has been used successfully in animal disease models to inhibit tumor growth[63–66] and neovascularization associated with rheumatoid arthritis[67] and ocular diseases.[19,31,68–71] We have shown through clinical photography and histologic assessment that a single rAAV.sFlt-1 injection can inhibit neovascularization of the cornea, retina, and choroid in different experimental animal models,[19,31,70] suggesting that rAAV can modulate long-term sFlt-1 expression.[19,31]

Retinal neovascularization is seen in ischemic retinopathies, such as diabetic retinopathy, central and branch retinal vein occlusion, and retinopathy of prematurity. Neovascularization is a multistep process characterized by degradation of the parent vessel basement membrane, endothelial cell migration, capillary tube formation, and endothelial cell proliferation. Although VEGF expression by photoreceptors has been described in trVEGF029 mice and neovascularization occurs toward the ONL,[43,45] the neovascular process appears similar to that in ischemic retinopathies such as diabetic retinopathy.[45] The retinal vasculature of 1-week- and 1-month old trVEGF029 mice has recently been described in detail.[45] In the present study, we observed more severe pathologic changes in older trVEGF029 mice. Capillary dropout was observed in all three vascular beds, and numerous vascular lesions were present in the middle and deeper beds. Unlike ischemic retinopathies (as trVEGF029 mice are not diabetic), capillary dropout in trVEGF029 mice is likely to be due to VEGF upregulation. Injection of VEGF into normal nondiabetic eyes was able to replicate many of the retinal vascular changes triggered by diabetes, such as intercellular adhesion molecule (ICAM)-1 upregulation, leukostasis, vascular permeability, and capillary nonperfusion or dropout.[72–75] Both retinal vascular leakage and nonperfusion seem to follow leukostasis.[75]

The inability to detect hVEGF in trVEGF029 at 7 weeks after birth is not surprising, as we have previously shown that hVEGF upregulation in trVEGF029 is transient, the level decreases with increase in age, and the severity of the pathologic changes decreases in trVEGF029 mice. We also showed that sustained hVEGF protein overexpression is not necessary for the development of vascular changes.[44] However, the presence of elevated levels of mVEGF in trVEGF029 mouse eyes may suggest that mVEGF upregulation is a consequence of events triggered by the initial upregulation of transgenic hVEGF expression. The elevated mVEGF levels which appeared to be sustained (39.8 ± 12.0 pg/mL in 12-week-old trVEGF029, $n = 12$, Lai C-M, unpublished data, 2009) could then trigger the downstream pathologic changes. Nevertheless, the human sFlt-1 protein decreased the free mVEGF protein level to that in the control mice and ameliorate the pathologic changes.

We were able to demonstrate preservation of the three vascular beds after a single rAAV.sFlt-1 injection at 3 weeks postnatal age, an age at which the VEGF protein and mRNA levels have been shown to be still relatively high.[44] Preservation of retinal vasculature with more photoreceptors remaining, fewer aberrant vessels invading the ONL, and improvement in retinal function were seen in the treated trVEGF029 mice. Together, our data support previous findings that rAAV can mediate long-term transgene expression and that sFlt-1 can slow or stop progression of VEGF-driven pathologic changes. The results also highlight the fact that, in secretion gene therapy, technical difficulties associated with targeting a particular ocular cell type can be circumvented by delivering the therapeutic agents to ocular cells that are particularly permissive to rAAV transduction.

The cellular immune response to subretinal injection of rAAV-sFlt-1 appeared to comprise two waves of leukocyte infiltration of the retina. The first wave occurred 1 week after injection and involved an increase in $CD11b^+$ cells. CD11b is typically expressed on the surface of myeloid cells, such as monocytes, neutrophils, and other granulocytes, and thus, although we did not identify these cells in any more detail, it is likely that the first wave of infiltrating cells involved these cell types. A second wave of infiltrating cells was observed 2 weeks after rAAV-sFlt-1 and involved an increase in $CD4^+$ and $CD8^+$ T cell numbers. At this point, the number of $CD11b^+$ cells had returned to normal, and by 4 weeks, the number of T cells also fell, suggesting that the response had resolved.

Only a very low frequency (<5%) of infiltrating $CD4^+$ and $CD8^+$ T were found to produce IFN-γ after mitogenic stimulation and this did not increase or decrease over the course of the experiment. No significant production of IFN-γ was observed in $CD8^+$ T cells after antigen-specific stimulation with a rAAV2 peptide. These observations indicate that there was no increase in antigen-specific T-cell responses after rAAV.sFlt-1 injection. Taken together, the observations suggest that the initial insult that occurred with injection of rAAV.sFlt-1 resulted in short-lived infiltration of leukocytes into the eye, but this resolved within 4 weeks. Our current data do not allow us to formally rule out the possibility that the infiltrating cells were responding specifically to rAAV.sFlt-1, though there is no evidence in support of this hypothesis. Of importance, we did not find any evidence of tissue destruction in the retina in our extensive histologic examinations, we could not detect rAAV antibodies in the sera at any of the time points tested (data not

shown), and there was no impairment of the therapeutic production of sFlt-1 protein, which continued to be produced at very high levels 4 weeks after injection. Furthermore, our analysis of the spleen suggests that the delivery of rAAV.sFlt-1 in the eye did not induce systemic inflammation that could be detected in this organ. Thus, the therapy appears to elicit a transient influx of inflammatory cells in the eye, but this does not cause damage to the organ, nor does it impair the effectiveness of the therapy.

The results presented in this study indicate that subretinal rAAV.sFlt-1 gene therapy elicits only a limited cellular immune response with no evidence of humoral responses being generated. A recent study has found that the site of intraocular administration determines the development of neutralizing antibodies in mice.[76] Intravitreous administration of two different AAV vector systems elicited neutralizing antibodies that blocked AAV expression on readministration, even when the second treatment was administered by a subretinal route. In contrast, no neutralizing antibodies were generated after subretinal administration of AAV. Thus, if the initial administration was subretinal, the AAV vectors could be effectively readministered via either subretinal or intravitreous routes.[76] These results are consistent with our data showing that no rAAV antibodies were generated after subretinal administration of rAAV.sFlt-1. It is also worth noting that although the generation of neutralizing antibodies has important implications for AAV therapy requiring more than one dose, the goal of our rAAV.sFlt-1 gene therapy protocol is to administer the therapeutic agent as a single dose. Our previous published studies show that this is possible and that efficacy is retained long-term without the need for readministration.[19] An independent issue with the type of gene therapy proposed here is that preexisting humoral immunity to AAV can neutralize AAV expression. Thus, subjects undergoing AAV gene therapy may need to be screened for preexisting antibodies that could neutralize the therapy vector and those with such antibodies may require administration of an AAV vector from a different serotype. Of importance, the results presented in this study indicate that subretinal administration of rAAV, in the absence of existing neutralizing antibodies, will provide effective therapy without generating ongoing immune responses that may be deleterious to the physiology of the eye.

In summary, we have shown that rAAV.sFlt-1 gene therapy offers effective and long-lasting treatment for retinal neovascularization. Subretinal injection of this viral vector induced a transient and self-limiting inflammatory response in the eye that had no effect on the local ocular tissues, the host systemically, or expression of sFlt-1. Coupled with the ability of the rAAV vector genome to form a large concatamer that exists stably, or as a large episome, or that could integrate randomly into the host genome,[77,78] rAAV.sFlt-1 gene therapy could be regarded as useful in the treatment of chronic diseases, where long-term therapeutic gene expression is needed.

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