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Original Article

Retinal AAV8-*RS1* Gene Therapy for X-Linked Retinoschisis: Initial Findings from a Phase I/IIa Trial by Intravitreal Delivery

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This study evaluated the safety and tolerability of ocular *RS1* adeno-associated virus (AAV8-*RS1*) gene augmentation therapy to the retina of participants with X-linked retinoschisis (XLRS). XLRS is a monogenic trait affecting only males, caused by mutations in the *RS1* gene. Retinoschisin protein is secreted principally in the outer retina, and its absence results in retinal cavities, synaptic dysfunction, reduced visual acuity, and susceptibility to retinal detachment. This phase I/IIa single-center, prospective, open-label, three-dose-escalation clinical trial administered vector to nine participants with pathogenic *RS1* mutations. The eye of each participant with worse acuity ($\leq$63 letters; Snellen 20/63) received the AAV8-*RS1* gene vector by intravitreal injection. Three participants were assigned to each of three dosage groups: 1e9 vector genomes (vg)/eye, 1e10 vg/eye, and 1e11 vg/eye. The investigational product was generally well tolerated in all but one individual. Ocular events included dose-related inflammation that resolved with topical and oral corticosteroids. Systemic antibodies against AAV8 increased in a dose-related fashion, but no antibodies against RS1 were observed. Retinal cavities closed transiently in one participant. Additional doses and immunosuppressive regimens are being explored to pursue evidence of safety and efficacy (ClinicalTrials.gov: NCT02317887).

INTRODUCTION

X-linked retinoschisis (XLRS) is a monogenic X-linked disease affecting approximately 12,000 males in the United States.[1–4] Two characteristic clinical hallmarks of this disease are (1) structural retinal lamellar separations (i.e., schisis cavities), particularly involving the central macular region of the retina from early age; and (2) a characteristic electroretinogram (ERG) waveform with the b-wave amplitude (from bipolar cell activity) reduced disproportionately to the photoreceptor a-wave, often referred to as an electronegative ERG response. The abnormal ERG implicates the photoreceptor synapse, and recent work demonstrates a deficiency of signaling protein localization and function of the photoreceptor post-synaptic output to the rod bipolar cells.[5]

Retinoschisis affects visual acuity from an early age, and men with this condition typically have visual limitations in their daily function, including difficulty with reading, and often they fail to meet vision requirements for a driver's license. Nearly all retina shows progressive structural changes and often progresses to macular atrophy by middle age.[6] In the peripheral retina, schisis cavities predispose to more extensive retinal separations, and on occasion retinal detachment occurs even at younger age. This frequently has a disastrous outcome, as the fragile retina makes surgical repair difficult.[7] The retinal fragility in XLRS disease has implications for gene-based therapeutic approaches, as these currently involve sub-retinal vector administration requiring surgical manipulation of the retina.

XLRS manifests haplosufficiency, as female carriers have no evidence of the disease and demonstrate preservation of visual function and retinal structure across their lifespan.[8] This gives biologic plausibility to using gene transfer as a therapeutic intervention, as partial restoration of RS1 expression would be expected to stabilize or even improve retinal structure and visual function. An *Rs1*-knockout mouse disease model recapitulates the structural and functional manifestations of human XLRS disease, and it affords an opportunity to test the efficacy of gene transfer therapy.[9] Studies in the *Rs1*-knockout mouse model provided proof of principle that the *RS1* adeno-associated virus (AAV8-*RS1*) vector can enter the retina after intravitreal delivery, lead to closure of the schisis cavity, and restore the retinal architecture as well as give functional improvement to synaptic visual signaling.[10] Expression is not achieved in wild-type retina after intravitreal injection, thus implicating XLRS disease pathology changes the internal limiting membrane (ILM) barrier.[11] The human trial builds on this possibility that, in XLRS participants, the viral vector will penetrate

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the retina, due to a compromised ILM, and transduce the targeted cells, as in the *Rs1*-knockout mouse model. Structural and functional rescue are achievable even in older XLRS mice, signifying the possibility of rescue in a mature human retina. Furthermore, a single intravitreal administration of a self-complementary AAV8-*RS1* vector provided sustained therapeutic effect and RS1 expression for at least 9 months post-injection, the longest time point evaluated in the study.[10]

However, critical questions remain on whether the preclinical findings in the XLRS mouse eye will translate effectively to humans. Several questions arise as to (1) whether intravitreal vector application in human can yield retinal expression, (2) how the human immune system will respond to intravitreal vector delivery, and (3) how doses effective in the mouse eye should be scaled to the much larger human eye.

Here we report on a phase I/IIa dose-escalation trial of AAV8-*RS1* retinal gene transfer by intravitreal injection. This is the first clinical study to explore treating a monogenic retinal degeneration by intravitreal application of an AAV8 vector serotype. The outcome measures focused on safety parameters and assessed the evidence of biologic activity.

RESULTS

Participants

The first participant was dosed in February 2015. Table 1 provides the participants' age, mutation, and demographics. Eight participants were Caucasian and one was Hispanic. The study and fellow eyes of each participant had similar features of cavity degree and extent. The study participants' median baseline visual acuity score was 52 letters (20/100) for study eyes and 57 letters (20/80) for fellow eyes.

Safety Profile of AAV8-*RS1*

Intravitreal injections of AAV8-*RS1* were generally well tolerated at all doses through 18 months after application. None of the systemic adverse events (AEs) were considered related to the investigational product, but some systemic AEs, such as increased blood sugar and the white blood count (WBC) shift, were likely related to the treatment with oral prednisone (Table S1). As anticipated for any intravitreal injection, some participants had minimal peri-injection discomfort and subconjunctival hemorrhage.

Ocular adverse events considered related to the AAV8-*RS1* consisted primarily of instances of ocular inflammation. There were no cases of endophthalmitis or retinal detachment. Four participants (one at 1e10 vg and three at 1e11 vg) had transient intraocular inflammation that was managed with oral and topical corticosteroid treatment (Figure 1). All cases resolved and did not recur after the cessation of corticosteroids. In the 1e10-vg group, participant 5 had a mild vitritis at 1 month post-injection and was treated with a short course of oral prednisone, starting at 60 mg and tapered to off over 2 weeks.

All three participants (7–9) at 1e11 vg showed anterior chamber inflammation of +1 to +3 cells,[12] which manifested at weeks 2–4 post-injection, and all three at 1e11 vg had vitritis by week 4, which resolved within 2–6 weeks on treatment with oral and ocular topical corticosteroids. Participant 7 had +2 anterior chamber inflammation at week 2, followed within days by mild vitritis (1+ cell). Participant 8 had +1 anterior chamber inflammation at week 4, followed by a vitritis (1+ cell).

Participant 9 had the greatest inflammatory response, beginning about day 13 with +3 anterior chamber cellular inflammation and +1 vitritis, accompanied by increased intraocular pressure. The vitritis peaked at day 21 with 2+ cell and 2+ haze, both of which resolved over the following weeks on topical and oral prednisone. Coincident with the vitritis, the retina showed a few dot hemorrhages in the inferior peripheral retina, and mild retinal venous leakage was seen on fluorescein angiogram (FA) on day 13, indicating vasculitis, which progressively resolved on FA at month 3 and was no longer observed on an FA at month 10. He also developed a post-inflammatory posterior vitreous detachment in the study eye complicated by a small peripheral retinal tear requiring laser retinopexy at 2 months and subsequent vitreous hemorrhage at 9 months requiring vitrectomy. His visual acuity returned to baseline and was maintained through 18 months. By 18 months after vector application, these ocular parameters had returned to baseline for all nine participants.

Systemic Immune Responses

None of the participants demonstrated a humoral antibody response to the RS1 protein at any time point following vector dosing (Table 2). The three participants in the low-dose group 1 (1e9 vg) had no or minimal increase in serum-neutralizing AAV8 antibody titers, including participant 3 who had quite low but detectable antibody titers of 1:10 at baseline (Table 2; Figure 1). Participant 4 in group 2 (1e10 vg) had a detectable pre-existing neutralizing antibody (NAb) titer of 1:160 to the AAV capsid at baseline, which increased steadily throughout the study and remained high after 18 months in the absence of intraocular inflammation at any time point. All 3 participants in group 3 (1e11 vg) had a rise in antibody titers prior to manifesting anterior inflammation and vitritis.

The appearance of anterior chamber inflammation coincided with antibody titers rising to $\geq$1:160. In general, antibody titers in group 3 peaked by week 2 and then showed modest declines for all 3 participants, but they remained elevated >1:160 to month 18.

We also looked at whether *in vivo* administration of AAV-*RS1* vector led to an expansion of AAV capsid-specific T cell responses, using an enzyme-linked immunosorbent spot (ELISPOT) assay (see Figure S2). A peptide pool containing overlapping sequences of AAV8 capsid protein was used to stimulate peripheral blood mononuclear cells (PBMCs) collected from participants before (day 0) and after vector administration (days 14 and 60). Only participant 9 exhibited a significant increase in interferon gamma (IFNγ)-producing T cells against AAV capsid at day 14, which coincided with the onset of ocular

Table 1. Demographics and Ocular Baseline Findings

Group and Participant	Dose (vg/eye)	Study Eye	Age (Years)	Acuity (Letters BL1/BL2) Study Eye	Acuity (Letters BL1/BL2) Fellow Eye	OCT Findings (OU)	ERG b-Wave:a-Wave Ratio Study Eye	ERG b-Wave:a-Wave Ratio Fellow Eye	Genotype	Resulting RS1 Protein
Group 1										
1	1e9	OS	72	34/39	47/46	macular atrophy	0.73	0.7	exon 4 c.304C > T; p.Arg102Trp	misfolded RS1[39]
2	1e9	OD	52	52/54	54/52	macular atrophy	0.98	1.08	exon 5 c.354delCinsGGTGTGCC TGGCTCTCCA; p.Asp118GluX14	premature termination, null phenotype[40]
3	1e9	OD	52	43/40	57/55	macular atrophy	0.46	0.54	exon 5 c.422G > A; p.Arg141His	secreted[41]
Group 2										
4	1e10	OD	56	49/52	56/54	macular atrophy	0.75	0.87	exon 6 c.589C > T; p.Arg197Cys	missense (misfolded?)
5	1e10	OS	34	27/28	57/55	macular atrophy	0.61	0.6	exon 6 c.535A > G; p.Asn179Asp	misfolded RS1[39]
6	1e10	OD	53	60/59	66/69	few central cavities	1.12	0.96	exon 6 c.574C > T; p.Pro192Ser	misfolded RS1[39]
Group 3										
7	1e11	OS	47	53/53	61/62	central schisis	0.72	0.66	exon 6 c.631G > A; p.Ala211Thr	missense (misfolded?)
8	1e11	OD	23	37/41	71/67	central schisis	0.76	0.83	exon 4 c.306_308dupGCT; p.Leu103dup	RS1 protein with one extra amino acid
9	1e11	OD	39	54/54	66/70	central schisis	0.65	0.68	exon 4 c.208G > A; p.Gly70Ser	misfolded RS1[41]

The exon 5 mutation found in participant 2 leads to premature termination in the coding sequence and gives a null phenotype. Interestingly, this phenotype leads to a particularly severe and progressive form of XLRS.[40]

inflammation. His T cell response tapered off by day 60. Two other participants (2 and 7) showed increased baseline IFNγ production relative to the six non-responders, and, by days 14 and 60, responses in both participants returned to levels not different from controls. The increased baseline pro-inflammatory cytokine production might be due to non-specific activation of both innate and adaptive immune cells.

Retinal Function Evaluation

Visual Acuity

Visual acuities in the study eye of all participants at month 18 were the same as baseline, within the variability of repeat testing for XLRS as determined previously (Figure 2).[13] Throughout the 18-month follow-up, best corrected visual acuity (BCVA) for the study eyes varied less than ±10 letters from baseline measurements (Figure 2), with the exception of participant 9, who had decreased visual acuity (−12 letters) in the study eye at week 2 due to inflammation and a large decrease in acuity at month 9 due to vitreous hemorrhage; acuity subsequently returned to baseline after vitrectomy (Figure 2).

Retinal Sensitivity on MP1 Testing

Retinal sensitivity was monitored over the course of the study. Results generally fell within 2-decibel (dB) change with some exceptions. Participant 7 (1e11 vg) showed a steady decline in mean sensitivity of extra-scotomatous points in the study eye that reached a plateau at 12 months. A similar trend was observed for mean sensitivity of responding points (Figure 3, upper panel). Participants 1 (1e9 vg) and 8 (1e11 vg) had declines in mean sensitivity of extra-scotomatous points in the study eye over the 3–9 months following treatment. In both cases, extra-scotomatous sensitivity returned essentially to the lower limit of variability at subsequent times (Figure 3, dashed line). Participant 9 experienced a transient dip in sensitivity of extra-scotomatous points at month 3 but, subsequently, returned to or above baseline. None of the fellow eyes had systematic changes in mean retinal sensitivity from any of the regions analyzed (Figure 3, lower panel). Participant 5 had a transient reduction in fixation stability within the injected eye, but the variation in the number of responding points was within the limit of repeatability for both eyes for all participants (Figures S3–S5). Our global impression is that no significant effect was observed in either gain or loss of retinal sensitivity during the study.

ERG

No clinically significant change in ERG amplitudes was observed for study eyes in either the a-waves or b-waves over the 18 months following AAV8-*RS1* application. Changes in dark-adapted ERG

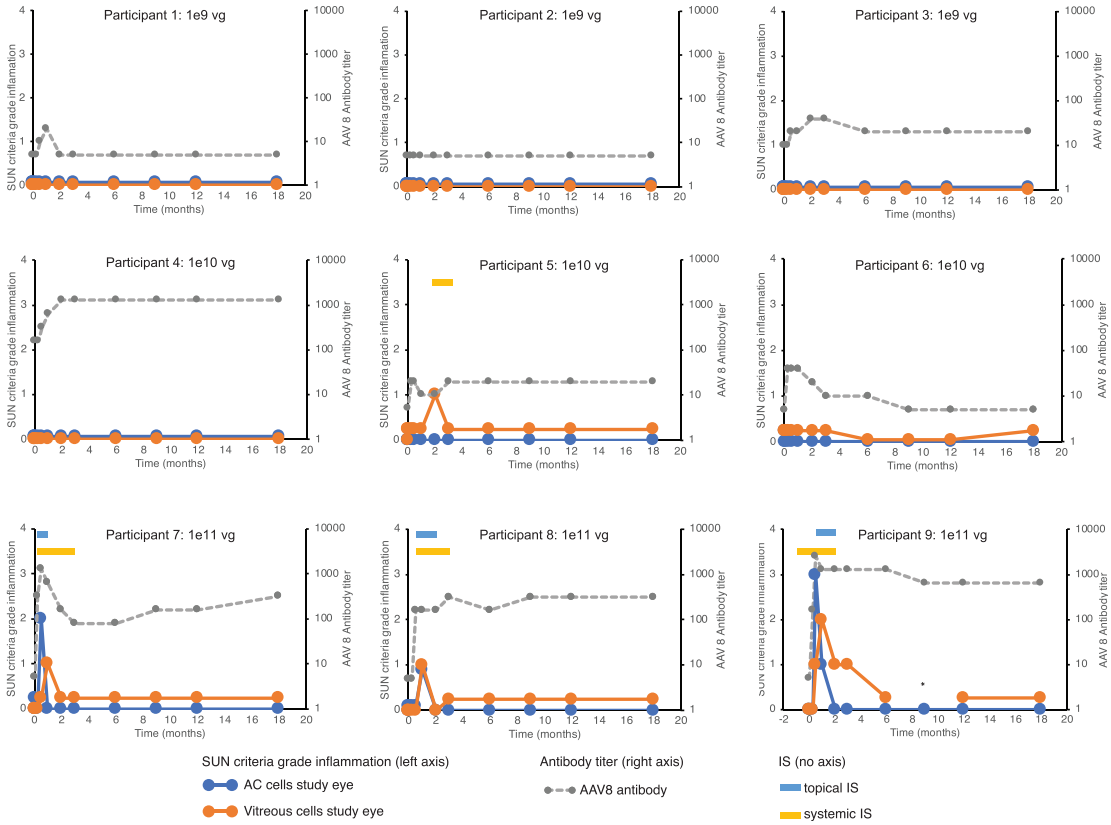

Figure 1. Ocular Inflammation and AAV-Neutralizing Antibodies over 18 Months

Figure shows level of inflammatory cells within the anterior chamber (AC, blue) or vitreous (orange) for each participant from baseline (time = 0) to 18 months. Cells were determined per the Standardization of Uveitis Nomenclature (SUN) criteria system. Neutralizing antibody titers to AAV8 are shown in gray. Only participant 4 had pre-existing NAb titer (1:160), whereas the remaining were <1:10. Bars at top show use and timing of systemic (yellow) or topical (light blue) immunosuppression (IS) for each participant. Asterisk indicates unable to take a reading from participant 9 at this time due to vitreous hemorrhage.

a-wave amplitude to moderately bright flashes (3 cd/m^2) from individual eyes (Figure 4) were within the range of the coefficient of repeatability that we previously defined for XLRS participants.[13] ERG a-wave amplitudes to a scotopic saturating flash (30 cd/m^2), to the light-adapted single ERG flash, and to the 30-Hz response amplitudes were all within the coefficient of repeatability for both study and fellow eyes (Figure S6, upper panel). These results collectively indicate no statistically significant ERG changes over the 18 months following injection.

We gave close attention to the ratio of b-wave:a-wave for possible indication of treatment outcome (Figure S6, lower panel), as this would be influenced by synaptic integrity. For participant 9, the dark-adapted ERG for moderate (3 cd/m^2) and bright (30 cd/m^2) stimuli revealed a decrease in a-wave amplitude during inflammation 1 month after vector application, which thereby increased the b-wave:a-wave ratio for the injected study eye in the absence of a significant change in the b-wave. The changes of a-wave amplitude and b-wave:a-wave ratio of participant 9 are within the limits of repeatability at month 1 (Figure 4) and remained so over the 18-month follow-up.

OCT Changes

Group 1 (1e9-vg) participants were older participants who had macular thinning at enrollment. Their optical coherence tomography (OCT) central macular thickness (CMT) measurements reflected the thinned retinas, which were maintained throughout the study with negligible variability (maximum study visit change, 6.5%). Group 2 (1e10-vg) and 3 (1e11-vg) participants showed some variability in CMT measurements during the study (Figure 5). At weeks 2 and 4, some study eyes demonstrated a reduction of cystic changes and CMT, but similar changes were generally also observed in corresponding fellow eyes.

Participant 9 in the high-dose group (1e11 vg) had shallow schisis cavities across the macula of both eyes at baseline. 2 weeks after vector application, the cystic cavities in the study eye closed completely as visualized on OCT imaging (Figure 6), with minimal change in the fellow eye. Concurrent with the cavity closure, the study eye manifested moderate anterior segment inflammation and an acute rise of intraocular pressure. By 1 month post-treatment, the cystic spaces reopened in the study eye and were comparable to the schisis at baseline

Table 2. Dose-Dependent Ocular Inflammation and Humoral Responses

Group and Participant	Dose (vg/Eye)	Inflammation Onset (Days Post-injection and Maximum)	Baseline Anti-AAV8 Titer	Highest Anti-AAV8 Titer	18-Month Anti-AAV8 Titer	Time (Weeks Post-injection) of Presentation of Clinical Inflammation	Time of Anti-AAV8 Rise to 160 or above	Time When Inflammation Resolved	Highest Anti-RS1 Antibody Levels at Any Time Point
Group 1									
P1	1e9	none	<10	20	<10	none	NR	NA	ND
P2	1e9	none	<10	<10	<10	none	NR	NA	ND
P3	1e9	none	10	40	20	NA	NR	NA	ND
Group 2									
P4	1e10	none	160	1,280	1,280	none	from baseline	NA	ND
P5	1e10	day 42; Vit 1 + cell	<10	20	20	4 weeks	NR	3 months	ND
P6	1e10	none	<10	40	<10	None	NR	N/A	ND
Group 3									
P7	1e11	day 14; AC 2+, Vit 1+ cell	<10	1,280	320	2 weeks	1 week	2 months	ND
P8	1e11	day 30; AC 1+, Vit 1+ cell	<10	320	320	4 weeks	2 weeks	2 months	ND
P9	1e11	day 13; AC 3+, Vit 2++ cell	<10	2,560	640	2 weeks	1 week	6 months	ND

P, participant; NA, not applicable; NR, not reached; ND, no anti-RS antibody detected.

(Figure 5). By 6 weeks, the cavity spaces had increased substantially by nominally 100% over baseline; but, by 4 months, they had returned again to near pre-treatment measurements. The fellow eye for participant 9 showed minimal change in the macular schisis cavities throughout this time, and the CMT by OCT varied less than 28% across the 18-month follow-up.

Participant 6 (group 2, 1e10 vg) had a 100% increase of CMT on OCT measurements in the study eye, followed soon afterward by a similar 100% increase of CMT in the fellow eye at 6 months post-injection. The similarity of study and fellow eyes for participant 6 was distinguished from the unilateral complete cavity closure for participant 9. Neither participant 6 nor 9 showed any correlation of functional acuity change with the OCT anatomic changes.

DISCUSSION

There are currently no approved treatments for XLRS. Gene therapy has gained significant attention over the past decade, particularly for genetic ophthalmic diseases. XLRS is particularly attractive as a target for gene therapy because mutations are in a single gene, *RS1*, and the preclinical studies in *Rs1*-knockout (KO) mouse show rapid treatment benefit to both retinal structure and function. Functional evaluation of the target tissue can be monitored non-invasively in human trials by measurements of visual acuity and ERG testing, and structural evaluation of retinal cavities can be monitored with OCT imaging. This clinical configuration makes XLRS a favorable disease target for exploring ocular gene therapy.

We found that a single intravitreal administration of the AAV8-*RS1* vector of up to 1e11 vg/eye was generally well tolerated. Ocular events included dose-related inflammation that resolved with topical and oral corticosteroids, though in participant 9 inflammation was complicated by a posterior vitreous detachment, retinal tear, and vitreous hemorrhage, which were successfully treated with laser and vitrectomy. Evidence for successful transduction of the vector in the human retina was evaluated indirectly by observations of structural or functional change that are beyond the natural history of the disease. Functionally, we saw no significant gain or loss of visual acuity, with the exception of participant 9 (1e11 vg) with acuity changes attributable to transient ocular media clouding. This participant had an approximately 40% reduction in ERG amplitudes in the study eye 1–3 months following injection, which coincided with intraocular inflammation, and later he had a transient drop in acuity in the study eye during the period of a vitreous hemorrhage. As the inflammation resolved, the ERG amplitudes subsequently improved, and after vitrectomy the visual acuity returned to baseline. For all nine participants, regardless of whether they experienced intraocular inflammation, visual acuity at 18 months post-injection was similar to baseline values.

Macular visual sensitivity on MP1 testing was quite variable and did not provide a clear indication of gain or loss. While some individuals, including participants 7 and 8 (both at 1e11 vg), had sensitivity reductions for at least one time point that exceeded the limits of variability that we previously determined,[13] this occurred for both eyes and

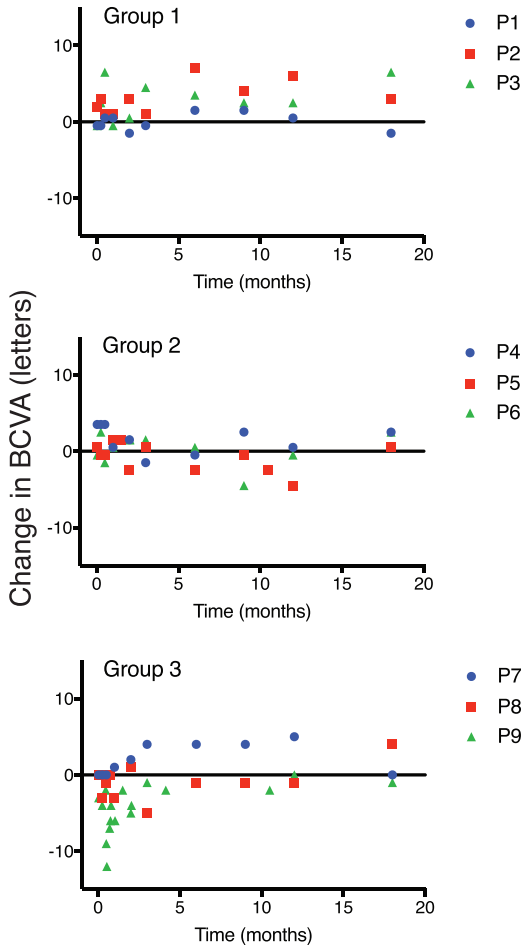

Figure 2. Change in Study Eye Acuity

The change in best corrected visual acuity from the baseline value is shown for each participant over 18 months from baseline (time = 0). Participant 9 had decreased visual acuity at days 14 and 15 with onset of inflammation and subsequent recovery by months 3–6; just prior to the month 9 visit, his visual acuity dropped to −54 letters (data not shown on chart) due to vitreous hemorrhage, with subsequent recovery to baseline at 12 months.

likely represents measurement variability beyond what we previously documented with a small dataset.

Retinal structural OCT imaging yielded the interesting observation of cavity closure in the study eye of participant 9 (1e11 vg) 2 weeks after vector application. Cavity closure is observed in high frequency in *Rs1*-KO mice treated with this clinical vector.[14] The essentially total closure of cavities across the macula of the study eye of participant 9 was not observed in the fellow eye, and it could putatively be consequent to RS1 protein expression. The macular cystic cavities subsequently reopened in the study eye during the course of ocular inflammation. No change in visual function occurred when the cavities were closed, but this was a period of concurrent inflammation. Other interpretations are possible, but cavity closure is unlikely to be a response to the corticosteroids, as closure was not observed in any fellow eyes or in individuals 7 and 8 who received the 1e11 vg/eye and were treated with oral and/or topical steroids once ocular inflammation occurred.

Other factors beyond NAbs likely affect successful retinal expression of the transgene, including vector tropism and the ILM barrier at the retinal surface. AAV8 targets both mouse and nonhuman primate (NHP) photoreceptors,[15,16] but the ILM is a barrier to retinal entry of AAV vectors following intravitreal administration both in mouse and NHP,[11,16] irrespective of capsid serotype. However, based on our studies in the *Rs1*-KO mouse model, AAV8 penetrates and expresses RS1 in amounts that result in structural and functional recovery in the XLRS mouse eye.[10] This implicates that the XLRS retina is compromised in some fashion and permits AAV to enter the retina and lead to RS1 protein synthesis and subsequent structural and functional improvement of the synaptic visual signaling pathway. The human trial builds on this possibility that in XLRS participants the viral vector will penetrate the retina, due a compromised ILM and other retinal changes associated with the disease, and transduce the targeted cells as in the *Rs1*-knockout mouse model. Currently, there is not a large animal disease model for XLRS.

The macular structural schisis configuration of participant 9 may have been optimal for observing biological effect of cavity closure, as the retina lacked the high dome-shaped central schisis elevation frequently seen in XLRS-affected individuals. His schisis was a low-elevation honeycomb lamellar configuration limited to and uniform across the macula entirely surrounding the fovea. This roughly matches the peri-macular ring of retinal entry seen for AAV vectors following vitreal injection in NHP with anatomically normal retinae. One can postulate that the shallow honeycomb schisis may close more readily than a highly elevated central balloon of schisis.

Gene therapy experiments in mice, NHPs, and humans have shown that recombinant AAV (rAAV) delivery can trigger immune responses to AAV capsid or the transgene. Humoral immunity to rAAV capsid represents the first barrier in the form of antibody-mediated vector neutralization. We measured serum NAb titers directed against AAV8 capsid and also separately against the transgene product RS1 protein to better understand the immune response.

None of the participants demonstrated antibodies against the RS1 protein transgene product at any time point. However, several participants exhibited a rise in systemic AAV8 antibodies following vector application. Overall, we observed a dose- and time-dependent increase in neutralizing AAV8 antibody titer in the serum of all the participants except participants 1 and 2 who received the lowest viral dose (1e9 vg). Participant 3, at the 1e9-vg dose, and participants 5 and 6, at the 1e10-vg dose, showed only modest increases in AAV8 titer from <10 to 1:40; these increases peaked by 2 weeks post-administration and then stabilized. These antibody levels in participants in our group 2 (1e10 vg) are roughly similar to the neutralizing anti-AAV antibody levels reported by earlier sub-retinal gene therapy trials.[17,18] They are also similar to the few reported intravitreal gene

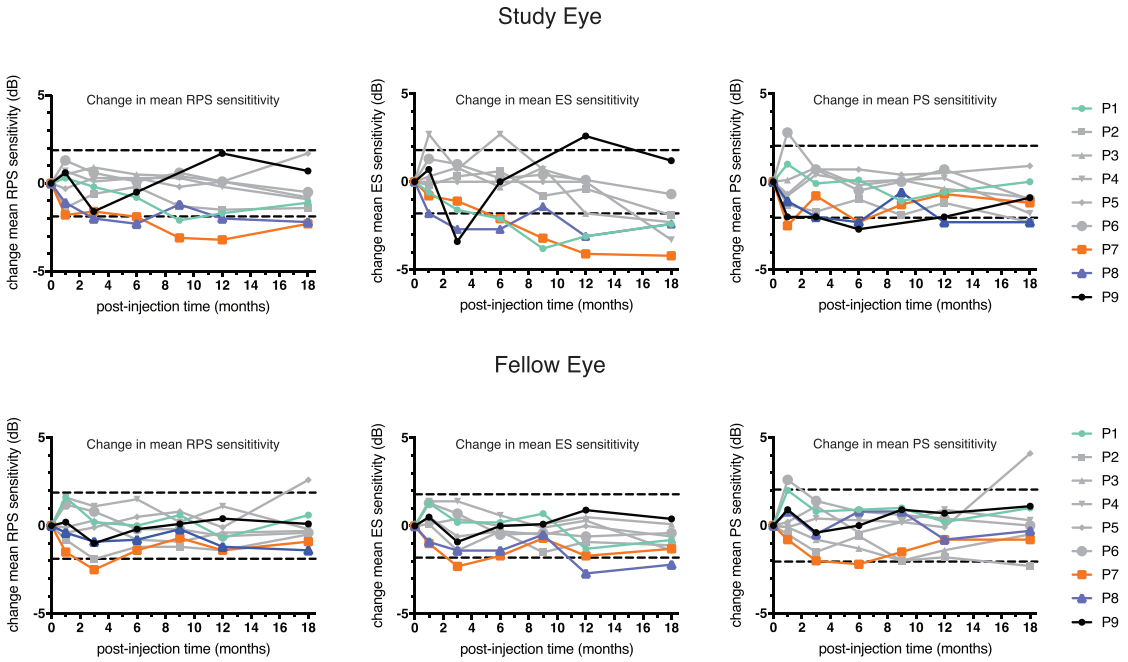

Figure 3. Mean Retinal Sensitivities

The change in mean sensitivities of MP1 testing, for the responding points (RPs) and the extra-scotomatous (ES) and para-scotomatous (PS) points from study eyes (upper panels) and fellow eyes (lower panels), are plotted as a function of time relative to baseline 2 (time = 0). For reference in interpreting the data, the dashed lines indicate the calculated limits of repeatability expected for XLRS subjects (see the Materials and Methods). The 2-dB change is cautionary for being a meaningful deviation from normal, based on our data (Jeffrey et al.[13]) and previous papers (Chen et al.,[42] Dimopoulos et al.,[43] Palkovits et al.,[44] Wong et al.,[45] Wong et al.,[46] and Wu et al.,[47]) Raw data for all five MP1 sensitivity parameters are shown for both eyes of all participants in Figures S3–S5. See also Figure S1, which shows the variability of mean sensitivity of RPs relative to both baseline values.

therapy trials in which many patients without pre-existing NAb show no or only mild elevation in titer when given 1–3 e10 vg/eye.[19–21]

We had selected AAV8 because of the reported low seroprevalence of AAV8 NAb of about 17% in the human population.[22] In our cohort of nine participants, only one had significant AAV8 antibodies at baseline (participant 4, 1:160). Participant 4 mounted a considerable increase in anti-AAV8 antibodies (increased titer of >30- to 120-fold) between 2 weeks and 2 months post-application. His higher antibody titers persisted through 18 months of follow-up, yet he never manifested clinical ocular inflammation. It has previously been reported that individuals with prior exposure to an antigen mount considerable immune response upon re-exposure.[23] In previous human AAV trials[19–21] and NHP studies,[24] subjects that had preexisting NAb generally had higher NAb levels post-injection compared to subjects that did not. Heier et al. demonstrated in their preclinical data and clinical trial results that subjects with high baseline titers of NAb exhibit reduced transgene expression, and they concluded that such patients may not be good candidates for gene therapy by intravitreal administration.[19]

All three participants in the 1e11-vg group developed substantial NAb titers against AAV8 (1:320–1:2,560), although they had nondetectable levels pre-dosing. The titer increase occurred within 2 weeks of vector application for two participants, and the higher titers persisted during the 18-month follow-up, highlighting the persistence of the NAb response to AAV8. These are considerably higher NAb titers than for comparable sub-retinal delivery where participants showed no or only low induction of anti-AAV antibodies. Interestingly, participant 9 (1e11-vg dose), who was pre-treated with oral steroid prior to dosing, did not demonstrate a qualitative difference in the timing or level of titer rise compared to the 2 other participants in this dose cohort. These data support the working premise that the intravitreal space is less immune privileged than the sub-retinal space.

It remains unclear what level of titers would block transduction or if there is a correlation between systemic and intraocular AAV8 antibody levels. Studies in mice[25] and NHPs[26] indicated that pre-existing serum titers as low as 1:5 can block vector transduction following intravascular administration targeted to the liver for Factor IX expression, and NAbs >1:100 were thought likely to persist long enough to suppress transgene expression. In the eye, following intravitreal injection of 2e10 vg AAV2-sFLT01, transgene expression monitored in the aqueous humor occurred only in patients with no detectable NAbs in the serum (4 of 5) at baseline or a titer of <100.[19] However, some participants without pre-existing serum Nab who received the highest dose of vector did not demonstrate expression, indicating that

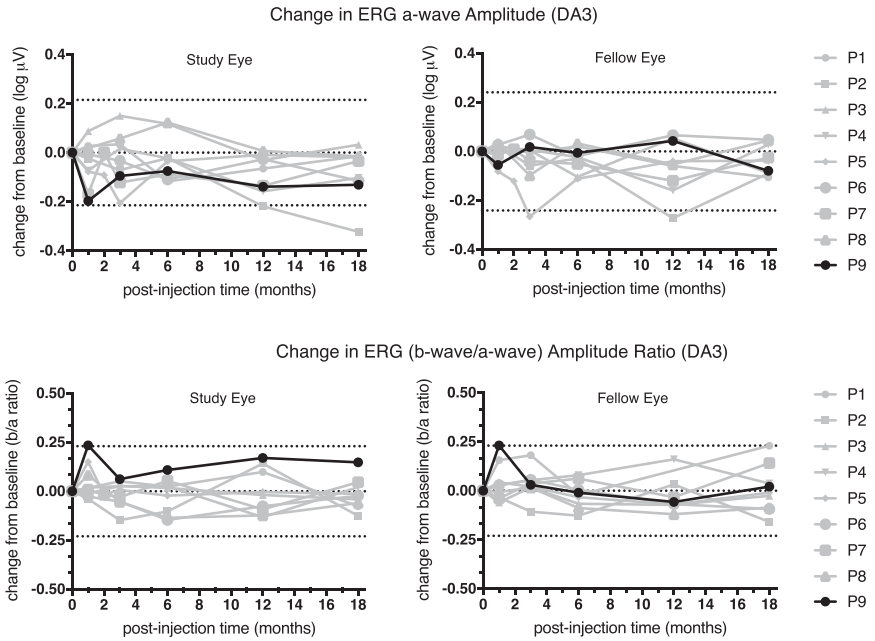

Figure 4. ERGs over 18 Months for Participants

Dark-adapted scotopic ERG amplitudes. Plots of changes in log ERG amplitudes over 18 months relative to the mean of the two baseline values (time = 0) for the study and the fellow eyes. Scotopic a-wave amplitudes were measured from the a-wave trough to b-wave peak for flash stimulus of 3 cd/m^2 (DA3). The dashed lines indicate the calculated limits of repeatability expected for XLRS subjects (see the Materials and Methods). The solid black line represents participant 9 ERG amplitudes. Scotopic ERG (b-wave:a-wave) amplitude ratios. Plots show change in ERG (b-wave:a-wave) ratio relative to the mean of two baseline (time = 0) values. ERG b-wave amplitude was measured from the a-wave trough to the response amplitude at 47 ms (see the Materials and Methods). The solid black line represents participant 9 ERG (b-wave:a-wave) ratios.

antibody titers and vector dose are not the only determinants of expression. Deciphering the importance and significance of pre-existing serum antibodies will depend on whether the serum NAb level corresponds to that in the vitreous. Kotterman et al. showed a strong correlation between serum and vitreal NAb levels in NHPs.[24] But the question is not resolved, as others indicated that serum levels are not a reliable measure of levels in the eye.[21,27]

Intravitreal delivery is desirable in affording an easy and safe route for administration and the opportunity to reach a larger expanse of retina compared to sub-retinal application. As with sub-retinal delivery for genetic ocular diseases, intravitreal delivery will require the consolidation of appropriate vector, transgene, promoter, delivery technique, patient population, and selected primary clinical outcomes to discern which entities are truly therapeutic. It is encouraging that the intravitreal approach is being advanced in several ocular gene therapy trials for posterior segment eye diseases. AAV2 vector has been administered by intravitreal injection for a mitochondrial genetic optic neuropathy[18,25,26] and also used for gene transfer to treat advanced neovascular age-related macular degeneration (AMD).[16]

This study is the first reported use of an AAV8 vector for ocular gene delivery by intravitreal application. In summary, this study showed the feasibility of this approach for delivery of an AAV8 vector into the eye. As found in other trials, we also observed a dose-dependent increase in ocular inflammation that resolved with topical and systemic steroids. Cavity closure in one participant provided the first signal of possible efficacy. Based on these promising findings, we are continuing this study and will explore additional dose levels and strategies for immune suppression to control inflammatory sequela elicited by the vector.

MATERIALS AND METHODS

Protocol Design

This prospective, nonrandomized, open-label, single-center dose-escalation phase I/IIa study was initiated and conducted at the National Eye Institute (NEI) in the Clinical Center of the NIH in Bethesda, MD. The study is registered with ClinicalTrials.gov (ClinicalTrials.gov: NCT02317887). The study adheres to the tenets of the Declarations of Helsinki. The protocol was reviewed and approved by the NIH Neurosciences Institutional Review Board and the Recombinant DNA Advisory Committee. Active oversight was provided by an independent Data and Safety Monitoring Committee. Each participant provided informed consent prior to enrollment. The eye with less acuity of each participant received a single intravitreal injection of a self-complementary AAV8 vector carrying the human *RS1* promoter and the human retinoschisin-coding sequence.[28]

Eligibility Criteria

Participants were males 18 years of age or older with an *RS1* gene mutation identified by genotyping and with at least one eye that met study eye criteria. Inclusion criteria for person-based characteristics included the following: understanding and signing the informed consent form; medically able to comply with study treatment, study testing and procedures, and follow-up visits; and agreement to use an effective barrier method of contraception for 1 year after gene transfer. Exclusion criteria included the following: actively receiving another study medication or investigational product, enrolled in another gene therapy trial, taking a systemic carbonic anhydrase inhibitor in the 3 months prior to enrollment, any condition that significantly increased the risk of needing systemic corticosteroids or systemic corticosteroid-sparing immunomodulatory agents, a separate underlying illness that could impair regular follow-up, a diagnosis or treatment of a malignancy (excluding non-melanoma skin cancer) within the previous 5 years, pre-existing ocular tumors, a known allergy to fluorescein dye or other contraindications to obtaining a fluorescein angiogram, taking a

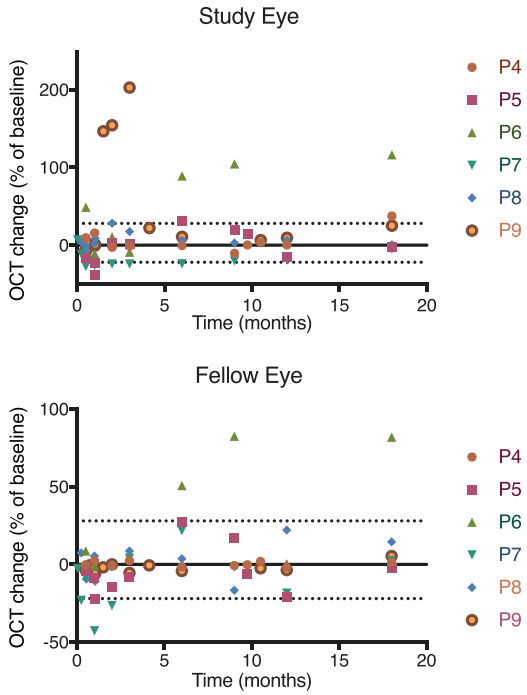

Figure 5. Change in OCT CMT Relative to Baseline

Quantitative analysis of retinal thickness was evaluated in the central macula using the ETDRS 1-mm circle, and the percent change in CMT was calculated from the mean baseline values, for examinations over 18 months from dosing, as described in the Materials and Methods. The dashed lines represent the expected repeatability for XLRS subjects (see the Materials and Methods). Data from participants 1–3 varied minimally (range 0%–6.5%) and are not depicted so as not to obscure the remaining data points.

medication that prevents the safe administration of study-related drugs, and uncontrolled hypertension.

Study eye criteria included a BCVA score of $\leq$63 Early Treatment Diabetic Retinopathy Study (ETDRS) letters (20/63 Snellen equivalent) and a reduced ERG b-wave:a-wave ratio consistent with XLRS. Study eye exclusion criteria included a history of other ocular diseases likely to contribute significantly to visual loss or likely to present special risks, presence of significant media opacities of the lens or cornea, intraocular surgery within 6 months prior to enrollment, or use of an oral or topical carbonic anhydrase inhibitor in the past 3 months.

AAV8-*RS1* Vector

AAV8-*RS1* is a replication-deficient, nonpathogenic AAV vector serotype 8 that delivers a human retinoschisin-coding sequence.[28] Gene expression is driven from a modified tissue-selective human retinoschisin promoter, thus limiting expression to ocular cells normally expressing this gene. Promoter activity is augmented by an interphotoreceptor retinoid-binding protein (IRBP) enhancer element. The vector is self-complementary, putatively giving higher expression levels and faster onset.[29,30] The good manufacturing practice (GMP) product was prepared under Dr. J. Fraser Wright at The Children's Hospital of Philadelphia (Philadelphia, PA). The vector was generated by triple transfection and purified by combined column chromatography and cesium chloride gradient ultracentrifugation to remove empty capsids.[31] The final product was diluted to appropriate concentration for delivery in 50 µL total volume into the study eye by intravitreal injection using a $^1/_2$-inch non-detachable 28G needle inserted into the anterior vitreous.

Study Design and Procedures

Nine participants were enrolled, three each at three dose levels: 1e9 vg/eye, 1e10 vg/eye, and 1e11 vg/eye. Only one eye was dosed per participant. This was a pilot safety study, and treated study eyes were neither masked nor randomized. The fellow untreated eyes were evaluated in parallel on each study visit as untreated controls. Participants underwent medical evaluations, laboratory tests, and complete ophthalmic examinations at scheduled visits, including bilateral assessment of BCVA, intraocular pressure measurement, and stereoscopic funduscopy. Baseline evaluations included medical and ocular histories; physical exam; and serology testing for syphilis, HIV, Hepatitis B and C, and tuberculosis. Structural and functional measures of the retina were made at each of two baseline visits within 1 month prior to administering the vector. Ten scheduled follow-up visits occurred at post-dosing days 1, 7, and 14 and months 1, 2, 3, 6, 9, 12, and 18. We are following participants per current FDA guidelines for gene therapy products.[32]

Participants 1–8 received no oral or topical steroid immediately prior to vector dosing. We then modified the protocol to pre-treat participant 9 with 60 mg oral prednisone beginning 2 days prior to vector administration.

Outcome Measures

The outcome was the safety of AAV8-*RS1* vector administration, and functional measures were selected to evaluate effects on retinal function. Adverse events were characterized as changes in ocular function that differed clinically from those expected in the normal course of progression of XLRS, including acute worsening of visual acuity, decreased ERG amplitude, or ocular clinical inflammation. Participants were assessed for systemic effects with serum laboratory measures and reported events.

Secondary functional and structural outcomes were also monitored. These were selected based on known effects of XLRS on retinal function, structure, and physiology. Functional measures included the following: changes in visual acuity, macular visual sensitivity, and ERG response amplitudes. Structural measures included changes of clinical retinal architecture assessed by imaging with OCT. We also monitored for circulating systemic anti-AAV8 antibodies and antibodies against RS1 protein.

The dashed lines in Figures 3, 4, and 5 are repeatability of measurements expected for XLRS participants. These values are based on our previous study of 7 XLRS subjects,[13] with four repeated measurements over 6 months, who included several participants in this

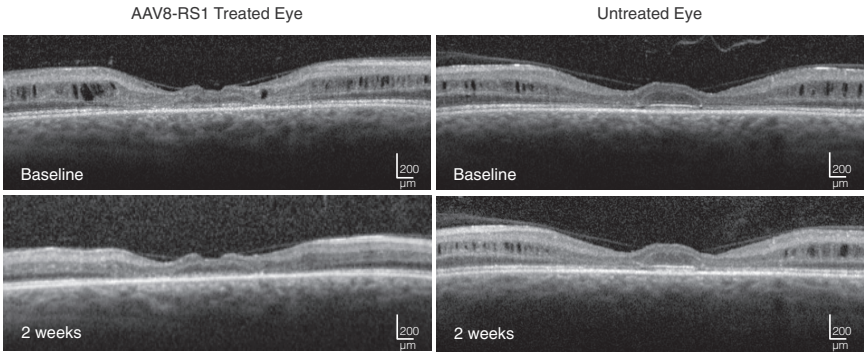

Figure 6. OCT Image of Retinal Macular Schisis Cavities in Participant 9 at Baseline and 2 Weeks after Vector Administration

Macular OCT image of participant 9 through center of fovea at pre-vector baseline shows parafoveal scarring and shallow honeycomb distribution of schisis cavities, which extended for 360 degrees across the macula and were localized to the inner nuclear layer of both eyes. 2 weeks after dosing with *AAV8-RS1* at 1e11 vg/eye, the injected eye showed complete closure of the schisis cavities, whereas the cavities persisted in the untreated fellow eye.

present AAV8-RS1 trial. These lines are provided to assist readers in interpreting fluctuations in measurement data, and they are not absolute limits, due to a limited number in the previous sample cohort.

Visual Acuity

BCVA was measured at all study visits using the JAEB Evaluation of Visual Acuity (EVA) charts[33] (electronic method to assess ETDRS letters) and recorded as the number of letters read correctly. Changes in visual acuity were measured relative to the average number of letters read at baseline 1 and baseline 2.

MP1 Retinal Sensitivity

Mesopic retinal visual sensitivity was measured following pupil dilation using a fundus-guided microperimeter (MP1, Navis Software, version 1.7.6, Nidek Technologies, Padova, Italy), as previously described.[13] Prior to testing, a single B-scan through the fovea obtained with the Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) was uploaded into the MP1 to identify retinal landmarks. Retinal sensitivity was measured at 68 points on a grid pattern covering a 20-degree field (Humphrey 10-2 pattern) centered on the fovea. At each grid point, sensitivity was measured for a 0.43-degree white stimulus (Goldmann size III) presented for 200 ms against a mesopic background (1.27 cd/m^2). After initial baseline MP1 measurements, all subsequent measurements were made using the MP1 follow-up feature. We found that baseline 2 values were consistently decreased a small amount relative to baseline 1 in both the fellow eyes (median = −1.8 dB or 34%) and the study eyes (median = −1.9 dB or 36%) for 8 of the 9 participants (Figure S1). This appeared to be a systematic shift of machine reference, which was further supported by the subsequent next measurements (at 1 month) after baseline 2, as no change in mean sensitivity of responding points was observed between baseline 2 and month 1 post-injection (study eye change = 0.3 dB; fellow eye change = −0.1 dB). Consequently, we measured change in retinal sensitivity post-injection relative to baseline 2.

The measurement values for each of the 68 data points were classified as responding (RP) or as dense scotomatous (DS) if the participant failed to respond to the highest MP1 stimulus intensity (127 cd/m^2). Based on their proximity to the scotomatous points, measured points were either para-scotomatous (PS) or extra-scotomatous (ES).[34] PS points were directly adjacent to scotomatous DS points; ES points were separated from a scotomatous point by at least one testing point. The following parameters were calculated and analyzed as a function of time after vector application: (1) number of responding points, and (2) mean sensitivities in decibels of all responding PS and ES points. Mean sensitivity of PS and ES points at follow-up was always measured over the same loci identified at baseline. Fixation stability (degree^2) was calculated from the bivariate contour ellipse area that enclosed 95% of fixation points measured during recording.[35] Repeatability coefficients (RCs) were calculated for each MP1 parameter across four visits (baseline 2, month 1, month 3, and month 6) from the fellow eye.[13]

Electroretinography

Full-field flash dark-adapted scotopic ERGs were recorded after 30 min of dark adaptation, using corneal bipolar Burian-Allen electrodes and following standards of the International Society for Clinical Electrophysiology of Vision.[36] ERG amplitudes and implicit times were analyzed.[13] Of note, as the ERG b-wave peak is often poorly defined in XLRS participants, we measured the scotopic ERG b-wave amplitude at a fixed time of 47 ms corresponding to mean ERG b-wave timing of normal participants.

OCT

Spectral domain OCT (SD-OCT) retinal imaging was performed for both eyes of each participant at each study visit except for the 1-day and 1-week examinations after vector administration visits (Cirrus HD-OCT; Carl Zeiss Meditec, Dublin, CA). The 512 × 128 scan pattern images a 6 × 6-mm segment of retina centered at the macula. Quantitative longitudinal analysis OCT scans were performed by aligning the scans spatially using functions provided within the OCT instrument software (Carl Zeiss) and then checking for accuracy. The accuracy of automated delineations of the inner and outer retinal boundaries was verified manually. OCT retinal thickness measurements in the macula were analyzed using the ETDRS-type grid position on the center of the fovea. Images were aligned to the baseline, and the percent change at each time point was calculated from the difference of the mean thickness measurements for the central subfield (central circle of diameter 1 mm) and the mean of the two baseline thickness values, divided by the mean of the two thickness

values, which was then multiplied by 100. Additional imaging was performed in some participants using a Heidelberg Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany).

Immunoassays

Serum samples were collected at baseline; days 7 and 14; and months 1, 2, 3, 6, 9, 12, and 18 for each participant, and they were evaluated for antibodies to AAV8 capsid and RS1 protein. The NAb assay and anti-*RS1* antibody levels were assessed using methods described previously.[37,38] The minimum reliable level for the NAb assay is 1:10. The minimum detectable level for the ELISA is 0.1 absorbance units.

IFNγ ELISPOT assays were performed to evaluate T cell activation using a commercial ELISPOT kit with detection antibodies and reagents per the manufacturer's instructions (MABTECH, Cincinnati, OH, USA). PepMix AAV-8 capsid protein (a mix of 182 peptides, each of which is a 15-mer with an overlap of 11 amino acids) and CEFT pool (cytomegalovirus, Epstein-Barr virus, influenza virus, and tetanus toxin, mix of 23 peptides) were obtained from JPT Peptides (JPT, Acton, MA). Peptide pool stock solutions were prepared by dissolving in DMSO to yield 0.625 μg/μL for each peptide.

Cryopreserved PBMCs were used for analyses. PBMCs were plated at 2×10^5 cells/well in plates pre-coated with an anti-human IFNγ antibody (1-D1K). Peptides in serum-free media were added to a final concentration of 1 μg/mL overnight. Wells containing media alone or media + DMSO served as negative controls, and 1 μg/mL CEFT peptides served as positive controls. The ELISPOT plate was incubated with biotinylated detector antibody (7-B6-1 biotin, 0.1 μg/mL) followed by streptavidin-alkaline phosphatase (ALP) conjugate. After incubation, the plates were developed with ALP substrate solution (5-bromo-4-chloro-3 indol phosphate/nitro blue tetrazolium [BCIP/NBT]). Cells producing IFNγ produced dark spots. The results are expressed as spot-forming cells (SFCs) per 10^6 PBMCs. The lower limit of detection is less than 3–5 IFNγ-producing T cells per 200,000 PBMCs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.05.025.

AUTHOR CONTRIBUTIONS

C.C., H.E.W., B.G.J., A.T., R.A.B., L.L.W., and P.A.S. contributed to the trial design. C.C., A.T., H.E.W., B.G.J., H.N.S., and P.A.S. were involved with the patients, the clinical aspects of the trial, and its management. C.C., H.E.W., A.T., B.G.J., Y.Z., C.V., D.M., S.H., Z.W., R.A.B., L.L.W., and P.A.S. enrolled patients, did study procedures, collected data, and conducted immunoassays. C.C., B.G.J., H.E.W., H.N.S., Z.W., C.V., R.A.B., L.L.W., and P.A.S. contributed to the data analysis. C.C., B.G.J., Z.W., C.V., R.A.B., L.L.W., and P.A.S. wrote the manuscript. C.C., B.G.J., V.J., L.L.W., and P.A.S. drafted figures and tables and supplementary information. C.C., B.G.J., C.V., D.M., T.K.P., L.Z., S.K., P.C., Z.W., R.A.B., L.L.W., and P.A.S. edited the manuscript. P.C., Z.W., and J.F.W. were responsible for the vector design and production of clinical material. All authors approved the final manuscript.

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