Neuroprotective Effects of Recombinant T-cell Receptor Ligand in Autoimmune Optic Neuritis in HLA-DR2 Mice

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PURPOSE. Optic neuritis (ON) is a condition involving primary inflammation, demyelination, and axonal injury in the optic nerve and leads to apoptotic retinal ganglion cell (RGC) death, which contributes to the persistence of visual loss. Currently, ON has no effective treatment. The goal was to determine the effectiveness of immunotherapy with recombinant T-cell receptor ligand (RTL) in preventing ON in humanized HLA-DR2 transgenic mice.

METHODS. Experimental autoimmune encephalomyelitis (EAE) was induced with myelin oligodendrocyte glycoprotein in humanized HLA-DR2 (DRB1*1501) transgenic mice. Five consecutive doses of RTL342M were administered at the onset of ON. The development of autoimmune ON was assessed by histopathology at different time points. The levels of myelin loss, axonal loss, and RGC damage were examined by immunofluorescence.

RESULTS. HLA-DR2 mice developed chronic ON 2 days before EAE characterized by progressive neurodegeneration in both organs. RTL342M significantly suppressed inflammation in the optic nerve and spinal cord and provided protection for at least 30 days. Examination of myelin loss showed a marked suppression of demyelination and an increase in myelin recovery in the optic nerve. Moreover, RTL342M treatment revealed a neuroprotective effect on optic nerve axons and RGCs in retinas at postimmunization (PI) day 62.

CONCLUSIONS. RTL342M suppressed clinical and histologic signs of EAE/ON by preventing the recruitment of inflammatory cells into the optic nerve and showed neuroprotective effects against ON. However, to achieve full therapeutic benefit, more doses may be needed. These findings suggest a possible clinical application of this novel class of T-cell-tolerizing drugs for patients with optic neuritis. (Invest Ophthalmol Vis Sci. 2012; 53:406–412) DOI:10.1167/iovs.11-8419

Optic neuritis (ON) is a condition involving primary inflammation of the optic nerve presenting with sudden onset of painful unilateral visual loss.1 The optic nerve is a well-defined white matter tract originating from retinal ganglion cells (RGCs). ON may occur in isolation or may co-exist with multiple sclerosis (MS) or a variety of systemic autoimmune disorders.2,5 The disease typically affects young adults ranging from 18 to 45 years of age, with a mean age of 30 to 35 years and also children as young as 4 years.6 The annual incidence of ON is approximately 5 in 100,000, with a prevalence estimated to be 115 in 100,000. Acute demyelinating ON is the presenting feature in 15% to 20% of patients with MS, and it occurs at some time during the course of the disease in 50% of patients. Since there is evidence of early axonal damage in acute demyelinating ON, disease-modifying drugs are considered in patients at high risk of developing MS as a prophylaxis against permanent neurologic impairment and vision loss.3,6 Permanant vision loss from RGC death occurs in 40% to 60% of patients. Recurrences of ON after a single isolated episode are quite common. The risk of later development of clinically definite MS correlates with white matter demyelinating lesions on magnetic resonance imaging (MRI). During a 10-year follow-up, MS was diagnosed in 38% of patients with a first episode of ON who were enrolled in the Optic Neuritis Treatment Trial (ONTT).7,8 An ON episode is also a strong predictor of the 15-year risk of MS in patients with the presence of brain MRI abnormalities. The risk of developing MS increases to 72% compared with 25% in patients with no lesions on baseline brain MRI.9 Multiple factors can influence the outcome of the disease.

One important factor in susceptibility to ON is the expression of human leukocyte antigen (HLA) class II alleles that also have a strong association with MS. The subjects expressing HLA-DR2 are four times more likely to develop MS after ON. Moreover, a significant increase in frequency of HLA-DR2 was found in subjects with isolated ON (P < 0.01; RR = 2.9) compared with the normal population.10 Subjects with ON who were HLA-DR3 positive had an increased risk for the development of MS (RR = 2.8) and this risk was further enhanced when DR3 occurred in combination with DR2 (RR = 6.7). The overall increased risk that MS would develop in patients with this combination was 26 times that in the normal population.

Experimental autoimmune encephalomyelitis (EAE), which serves as an animal model for MS, often co-presents with ON in some animal strains. EAE can be induced by immunization with a number of myelin antigens, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG).11 Inflammatory demyelination is the pathologic hallmark of MS.12 In this study, we used a human-Affiliations:

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ized HLA-DR2 (DRβ1*1501) transgenic model, in which the animals develop MOG-induced EAE.13 As we determined in these studies, the mice also develop chronic ON. Remarkably, MOG is expressed in the outermost surface of the oligodendrocyte myelin in the optic nerve,14 which could become clinically significant in the context of suboptimal T-cell responses and T-cell-targeted treatments such as RTLS.

The goal of these studies was to determine whether recombinant T-cell receptor ligand (RTL) immunotherapy would protect HLA-DR2 mice from developing ON induced with the MOG₃₅₋₅₅ peptide. RTLS are new biologics that target T-cell-mediated inflammatory responses in an antigen-specific manner.15,16 We selected RTL342M, which is a mouse version of the human DR2-restricted RTL1000, also containing the MOG₃₅₋₅₅ peptide, because RTL1000 has been shown to be safe in MS patients in a phase 1 clinical trial.17 In these studies humanized HLA-DR2 transgenic mice that developed chronic ON associated with EAE and RTL342M suppressed inflammation, demyelination, and axonal loss in the optic nerve.

**METHODS**

**Animals**

Inbred HLA-DR2 (DRA-DRβ1*1501) transgenic mice 8 to 10 weeks old were housed at animal facilities of the VA Medical Center (Portland, OR). All experimental animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Recombinant T-Cell Receptor Ligand**

HLA-DR2 (DRA-DRβ1*1501) derived RTL covalently linked to the encephalitogenic mouse MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVHLVRNGK) was produced as previously described.18–20 RTL342M comprises the β1 and α1 domains of DR2 (DRβ1*1501; DRA*0101) covalently linked to the MOG₃₅₋₅₅ peptide.

**Induction and Assessment of ON**

To induce EAE and ON, groups of seven to 12-week-old male and female transgenic mice expressing the DR2 allele were immunized with 200 μg MOG₃₅₋₅₅ peptide in CFA containing 400 μg Mycobacterium tuberculosis H37RA subcutaneously (SC) at four sites on the flanks.13,20 In addition, the mice received 70 ng pertussis toxin on days 0 and 2 PI. At the onset of ON, the mice were given five daily 20-μg SC doses of RTL342M. Control mice received five doses of buffer vehicle (Tris buffer) at the same times. The mice were assessed daily for 100% normal goat serum in PBS containing 1% BSA and 0.2% Tween for 1 hour. After the primary antibodies were blocked in PBS with 1% BSA and 0.2% Tween, the sections were incubated for 1 hour at room temperature. The following antibodies were used: anti-MOG (1:200; Abnova, Walnut, CA), anti-CD11b (1:100; Abcam, Cambridge, MA), anti-RT7K (1:500; Millipore, Billerica, MA), anti-MBP (1:200; Abnova,)), anti-NeuN (1:100; Chemicon, Temecula, CA), and anti-GFAP (1:100; BD Pharmingen, San Diego, CA) for overnight incubation at 4°C. Next, tissue was washed and incubated in appropriate secondary antibodies in PBS with 1% BSA, 0.2% Tween for 1 hour, including anti-mouse AlexaFluor 488 (1:400), anti-rabbit AlexaFluor 594 (1:400), or anti-goat AlexaFluor 594 (1:400; all from Invitrogen, Carlsbad, CA). After the tissues was washed, DAPI (Roche, Indianapolis, IN) counterstain was applied for 1 minute, and the slides were then mounted (Fluoromount-G; Southern Biotech, Birmingham, AL) to be photographed with a confocal fluorescence microscope (Fluoview1000; Olympus, or DM5000 Leica, Deerfield, IL). Pictures were then taken at 10× and 20× magnifications and analyzed (ImagePro; Media Cybernetics, Silver Spring, MD). A negative control contained secondary antibodies only.

**Axon Quantification**

Ten-micrometer cross sections of experimental and age-matched normal optic nerves were prepared every 30 μm for immunostaining with anti-RT7K antibodies. At least four sections were used from each animal. Anti-RT7K (Millipore) diluted at 1:500 were used for 1 hour followed by incubation with anti-mouse Alexa Fluor 488 diluted at 1:400 for another hour and then counterstain with DAPI (Roche) for mounting. Pictures were taken of each stained section at 40× using a confocal microscope (Fluoview1000; Olympus), and pseudocolored images were acquired for analysis. The images were then cropped to the same 220 × 130-μm area and counted in a masked fashion based on the intensity of the fluorescent green stain (ImagePro Plus; Media Cybernetics). The negative control was also stained and counted to subtract the background from all the counts.

**RGC Evaluation**

Retinas were removed from fixed left eyes, and whole mounts were made by four radial cuts representing the dorsal, ventral, temporal, and nasal sides. Cross sections (10 μm) were prepared from the right eye. The retinas were placed in a solution containing 100× diluted anti-NeuN antibodies that labeled RGCs specifically, followed by fluorescent anti-mouse Alexa488 diluted 400×. After a brief wash, the retinas were flat mounted on the slide, and retinal images were captured at 10× magnification with a fluorescence microscope (DM5000B; Leica) and photographed. RGCs were counted in a masked fashion with the imaging software.

**Myelin Quantification**

Frozen longitudinal sections of optic nerves from each treatment group were stained with Luxol fast blue without cresyl violet, to stain myelin only. Micrographs were then taken at 20× of each section with a light microscope (model BH-2; Olympus, Tokyo, Japan) with an attached camera (DP21; Olympus) and were cropped to 0.119 × 0.506 mm. The cropped images were then pixilated by the ImageJ program with the Hessian plug-in feature, as described by Grider et al.25 (Image), developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Pixilation intensity was counted from the whole assigned area in a masked fashion using Image ProPlus and graphed (Prism; GraphPad, San Diego, CA). The area was normalized to 0.1 mm².

**Immunofluorescent Staining**

Frozen long or cross sections were postfixed with 4% paraformaldehyde in PBS for 10 minutes, washed, and blocked with 10% normal goat serum in PBS containing 1% BSA and 0.2% Tween for 1 hour. After the primary antibodies were blocked in PBS with 1% BSA and 0.2% Tween, the sections were incubated for 1 hour at room temperature. The following antibodies were used: anti-MOG (1:200; Abnova, Walnut, CA), anti-CD11b (1:100; Abcam, Cambridge, MA), anti-RT7K (1:500; Millipore, Billerica, MA), anti-MBP (1:200; Abnova,)), anti-NeuN (1:100; Chemicon, Temecula, CA), and anti-GFAP (1:100; BD Pharmingen, San Diego, CA) for overnight incubation at 4°C. Next, tissue was washed and incubated in appropriate secondary antibodies in PBS with 1% BSA, 0.2% Tween for 1 hour, including anti-mouse AlexaFluor 488 (1:400), anti-rabbit AlexaFluor 594 (1:400), or anti-goat AlexaFluor 594 (1:400; all from Invitrogen, Carlsbad, CA). After the tissues was washed, DAPI (Roche, Indianapolis, IN) counterstain was applied for 1 minute, and the slides were then mounted (Fluoromount-G; Southern Biotech, Birmingham, AL) to be photographed with a confocal fluorescence microscope (Fluoview1000; Olympus, or DM5000 Leica, Deerfield, IL). Pictures were then taken at 10× and 20× magnifications and analyzed (ImagePro; Media Cybernetics, Silver Spring, MD). A negative control contained secondary antibodies only.

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Statistical Analysis

The data are expressed as the mean ± SEM. A two-tailed Student’s t-test was used to determine the statistical significance. The significance between the controls and treatment groups was determined by one-way analysis of variance (ANOVA; Prism 3.0; GraphPad). Differences with a P < 0.05 were significant.

RESULTS

Therapeutic Effect on Inflammation in Chronic ON in HLA-DR2 Mice

Our previous studies have shown that immunization of HLA-DR2 mice with MOG35-55 peptide induced chronic EAE, but, until now, it was unknown whether these mice would develop ON. In our studies, MOG immunization induced ON of a chronic nature, and the inflammatory lesions in the optic nerve correlated with the lesions in the spinal cord (not shown). Figure 1A shows that HLA-DR2 mice given MOG peptide developed chronic inflammation of the optic nerve that occurred on PI days 8 to 9 before the onset of EAE (Fig. 1B; 11–12 days), with inflammatory infiltrates in the optic nerve at onset on the long and cross sections stained with H&E. The incidence was 100%.

In these studies, we tested whether RTL immunotherapy would suppress inflammation, demyelination, and loss of axons in the optic nerve of HLA-DR2 mice when RTL342M was administrated at the onset of ON. To evaluate the effectiveness of RTL342M therapy, the tissues were collected from treated and control mice on days 16 (peak), 30, and 62 PI. The treatment of the mice with five consecutive daily doses of RTL342M by subcutaneous administration at onset of ON (day 8) significantly suppressed development of histologic ON for the first 30 days and reduced inflammatory cell infiltrates in optic nerves (Fig. 2). The clinical scoring for EAE was performed daily and is shown in Figure 1C from a representative experiment. Figure 1D shows that the severity of ON was significantly reduced on day 16 PI with an average inflammatory score of 0.4 ± 0.24 for treated optic nerves compared with 2.8 ± 0.66 for the controls (P < 0.0094). After treatment stopped, this effect was later diminished, and the mice presented signs of ON progression. The results suggest that, although RTL342M was effective in stopping the influx of in-
Inflammatory cells, additional doses of RTL may be needed to inhibit further development of inflammation, to reach full therapeutic benefit in those mice.

Inhibition of Myelin Loss in Optic Nerves

The next experiment examined the effect of RTL342M on myelin loss in optic nerves. Figure 3 shows that demyelination of the optic nerve occurred around the time of inflammation, as determined by Luxol fast blue staining (Figs. 3A–C). The myelin loss in Figure 3 shows that myelin in normal untreated mice was nicely compact within the nerve, whereas mice with inflammation exhibited patchy loss of myelin. Moreover, the loss of myelin was profound around inflammatory lesions and was progressive in untreated mice over the 62-day observation period compared with that in normal optic nerves ($P < 0.001$).

Figure 3D shows that although the mice lost myelin due to inflammation, RTL342M protected long-term demyelination of the optic nerve compared with the control, vehicle-treated mice (days 30 and 62 PI).

Figure 4 shows representative early pathology in consecutive long sections of the optic nerve collected 16 days (peak)
Protection from Axonal Degeneration

As shown in Figures 2 and 3, inflammation and demyelination of the optic nerve were associated with progressive axonal loss. We thus analyzed RGCs and axons that comprise the optic nerve. Figure 6A shows immunofluorescent axonal immunolabeling in the optic nerves at 62 PI by RT97 antibodies, which label neurofilaments. Normal optic nerves showed densely labeled axons (Fig. 6A), but the area of axonal immunofluorescent staining decreased in vehicle-treated mice and showed a significant trend (Fig. 6B). In our experiments, some mice were fully protected by RTL342M, but other mice still showed signs of inflammation, and in those mice, there was more myelin loss and more neuronal damage. Figure 6B shows averaged results from both groups of mice and compared to the axonal immunolabeling for day 30 PI. In RTL342M-treated nerves at 62 PI, the number of labeled axons was significantly greater than in the vehicle-treated optic nerves ($P = 0.047$), layer of representative cross sections of eyes collected from vehicle- and RTL342M-treated mice. (E) Quantitative analysis of immunofluorescence-labeled RGCs with NeuN antibodies in retinal cross-sections in vehicle- and RTL342M-treated mice collected on day 62 PI. Results are expressed as mean ± SEM of fluorescent cell counts from four areas of the retina ($n = 4$) and normalized to a 0.1-mm$^2$ area.

after immunization without treatment. The inflammation was associated with the influx of inflammatory cells and the accumulation of macrophages (mac)/microglia in inflammatory lesions (arrows), as determined by immunofluorescent staining with anti-CD11b antibodies of the optic nerve (Figs. 4B). The myelin loss was apparent around inflammatory lesions, as shown using cresyl violet to counterstain the inflammatory infiltrate (Fig. 4C). In addition, early axonal damage near inflammatory cells in optic nerves (Figs. 4D–F) was revealed by immunostaining with anti-axon RT97 antibodies (Fig. 4D) and by Bielschowsky silver impregnation of the long section of the optic nerve (Figs. 4E, 4F). The cross section of the affected nerve showed axonal injury compared with healthy looking axons in normal optic nerve (Fig. 4G, 4H). Together, these results from the peak of ON indicate that inflammation had an effect on myelin and axonal loss.

Quantitative analysis of Luxol fast blue-stained myelin evaluated at 30 and 62 days PI revealed that RTL342M slowed, and perhaps reversed, myelin loss compared with vehicle treatment (Fig. 3D), and so we examined whether myelin recovery occurred after the initial loss due to inflammation and damage to the oligodendrocytes in optic nerves. The long sections were immunolabeled with anti-MOG antibodies, markers for oligodendrocytes, and RT97 antibodies to label axons. Figure 5 shows an increase in immunofluorescent labeling of MOG$^+$ (white arrows) in the RTL-treated optic nerve on day 62 PI compared with vehicle-treated mice, which showed visibly fewer immunolabeled cells. Double immunofluorescent labeling with anti-CD11b and anti-MOG antibodies revealed a greater presence of mac/microglia (yellow arrows) in untreated optic nerve, whereas the RTL-treated optic nerve showed a reduction in microglia and an increase in MOG-stained cells. This lack of overlapping colors showing separated staining of microglia and MOG labeling may suggest oligodendrocyte recovery rather than an increase in microglia phagocytizing myelin from damaged cells. Also, there was a substantial increase in immunofluorescence of RT97-labeled axons at day 30 PI in contrast to those in vehicle controls. These findings strongly suggest oligodendrocyte regeneration and an increase in myelin in the optic nerve after initial degeneration (30 PI) due to inflammatory processes.

**FIGURE 6.** Immunofluorescent labeling of surviving axons and RGCs in the optic nerve from vehicle- and RTL342M-treated mice collected on day 62 PI. (A) Immunostaining of cross sections with anti-RT97 antibodies for axons in normal and treatment groups collected on day 62 PI. Note a visible reduction in RT97-labeled axons in vehicle-treated optic nerves compared with normal and RTL342M-treated optic nerves; (B) quantitative analysis of fluorescence density of RT97 axonal labeling in vehicle- and RTL342M-treated optic nerves collected on days 30 and 62 PI. Results are expressed as the mean ± SEM of fluorescence density in four sections 30 μm apart in stained optic nerves ($n = 4$). (C) Representative images of retinal whole mounts demonstrate NeuN-labeled RGCs on day 62 PI taken from RTL342M- and vehicle-treated mice. (D) Double-immunofluorescence labeling in the retina with anti-NeuN as a marker for RGCs and anti-GFAP antibodies as marker for astrocytes: anti-GFAP (red), anti-NeuN (green), nuclear labeling with DAPI (blue); the pictures show the ganglion cell immunostaining with anti-axon RT97 antibodies (Fig. 4D) and normalized to a 0.1-mm$^2$ area.
suggested neuroprotection. To further examine the axonal degeneration, we analyzed RGCs by preparing retinal whole mounts and retinal cross sections obtained from day 62 PI. RTL342M and vehicle-treated mice. The retinas were immunofluorescently labeled with anti-RGC antibodies against neuron-specific nuclear protein (NeuN). All retinal whole mounts from vehicle-treated mice showed a noticeable decline in the number of immunolabeled RGCs, compared with the count in RTL342M-treated mice (Fig. 6C), but analysis of the fluorescent cell count showed no statistical significance. An additional experiment of immunolabeling of RGCs with anti-NeuN antibodies on retinal cross sections also demonstrated a marked loss of RGCs in vehicle-treated retinas, which showed statistical significance (P = 0.0068; Fig. 6E). Immunolabeling with anti-GFAP antibodies specific for astrocytes revealed an increase in astrocyte labeling in vehicle-treated retinas in the ganglion cell layer (Fig. 6D) in contrast to RTL342M-treated retinas where more RGCs and fewer astrocytes were immunolabeled in the ganglion cell layer. These findings further suggest the protection of RGCs and their axons by the RTL treatment.

**DISCUSSION**

Our studies demonstrate that immunization of HLA-DR2 mice with MOG35-55 peptide-induced ON of a chronic nature that was associated with EAE; however, the onset of ON preceded the onset of EAE by at least 2 days. This observation is similar to human optic nerve disease in which ON is frequently the initial manifestation of MS.6,9 The RTL (RTL342M) used in these investigations demonstrated the potential to inhibit inflammation and promote neuroprotection and even the remyelination process. The RTL treatment at onset of ON (day 8) significantly reduced the development of both clinical EAE and histologic ON by preventing the recruitment of inflammatory cells into the optic nerve. RTL342M not only markedly suppressed inflammation, but also inhibited demyelination for up to 30 days. Moreover, the optic nerve from mice treated with RTL showed signs of myelin recovery. By 60 days, there was an increase in the number of MOG-labeled cells (oligodendrocytes) after an initial decline, which may be important in designing future treatment for human chronic or recurrent ON. Loss of myelin and axonal damage in optic nerves seemed to be the effect of inflammation that started in the proximity of inflammatory lesions. We do not have any direct evidence that axonal loss in optic nerves preceded inflammation, which is similar to previously reported studies using other animal models of ON.22–24 In our model, there was continuous demise of ON, as determined for the first time in these studies. MOG is an important central nerve system (CNS) antigen, which is expressed in larger quantities in the optic nerve, prominently in the outermost surface of the oligodendrocyte myelin.14 Thus, inflammatory cells targeting MOG appeared first around the pia, at the onset of ON.

Some experimental therapies have been reported recently for treatment of autoimmune ON, including corticosteroid therapy, which suppresses ON and prevents RGC loss if treatment is initiated before onset of optic nerve inflammation, but is less effective after inflammation has begun.26 The Boweman-Birk inhibitor (BBI), a soybean-derived serine protease inhibitor, significantly reduces the incidence of ON and prevents loss of RGCs.27 Intraocular delivery of antioxidant genes such as superoxide dismutase or catalase cloned into recombinant AAV provides a long-lasting suppression and showed a decreased RGC loss by 29%, optic nerve demyelination by 36%, axonal loss by 44%, and cellular infiltration by 34%, compared with the control eye with ON at 6 months PI.28 Lipocic acid, also an antioxidant, reduces inflammation and protected axonal injury when delivered on day 8 or 13 PI in the C57Bl/6 EAE model.29 RTL molecules are new biologics that consist of the immunodominant α1 and β1 domains of class II MHC molecules and contain covalently linked antigenic peptides,15,18,30 including the MOG35-55 peptide. By inhibiting autoreactive T-cell responses, RTLs have been shown to suppress clinical and histologic signs in various experimental autoimmune disease models, including uveitis,51,52 encephalomyelitis (EAE), artritis,33 and stroke.34 The application of RTL342M is a new therapy for ON in humanized mice. Our studies demonstrated that RTL342M had a therapeutic effect on both ON and EAE, and its treatment led to the suppression of inflammatory responses, myelin recovery, and some axonal protection. A recent study of human ON suggested that all optic nerve inflammatory lesions tend to remyelinate regardless of the size of the initially demyelinated zone.54 This study showed that smaller lesions get more complete remyelination than larger lesions, and the extent of the initial inflammatory demyelinating assault was probably the single most important factor determining success of remyelination. Moreover, the first 5 to 6 months after the demyelinating event may be crucial for the remyelinating process to succeed.34 Therefore, by suppressing acute inflammation in the optic nerve, RTL therapy could be regarded as a remyelination-enhancing therapy that could accelerate myelin recovery in the CNS if applied almost immediately after onset. Recent animal studies using SJL/J mice with established EAE could support this notion. A successful treatment with a different myelin-specific RTL401 (I-A1/PLP139-151 peptide) after the peak of EAE markedly reduces inflammation in the CNS associated with an increase in remyelinating axons and the presence of small, presumably regenerative axonal sprouts.35 These findings indicate that RTL therapy targeting pathogenic T cells may promote CNS neuroregenerative processes, and these findings may also have implications for clinical trials.

In summary, our study showed a successful therapy for ON with RTL342M administered at onset, resulting in markedly reduced inflammation, demyelination, and axonal loss. Such immunomodulatory therapy may prevent loss of myelin and thus RGC damage. Because the protection was not complete, we believe that additional doses over the course of the disease may be needed to increase the benefit of RTL treatment.

**References**