Effective Arrestin–Specific Immunotherapy of Experimental Autoimmune Uveitis with RTL: A Prospect for Treatment of Human Uveitis

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Introduction

Autoimmune uveitis is a group of human leukocyte antigen (HLA) associated, noninfectious inflammatory diseases of the eye that shows different clinical manifestations often leading to blindness.1,2 Uveitis is prevalent worldwide and affects an estimated 2.3 million Americans. Association between the presence or absence of certain HLA molecules and susceptibility to develop particular autoimmune disorders has been documented.3,4 However, the ocular diseases associated with class I or class II alleles may depend

Purpose: To evaluate the immunotherapeutic efficacy of recombinant T cell receptor ligands (RTLs) specific for arrestin immunity in treatment of experimental autoimmune uveitis (EAU) in humanized leukocyte antigen (HLA-DR3) transgenic (Tg) mice.

Methods: We generated de novo recombinant human DR3-derived RTLs bearing covalently tethered arrestin peptides 291–310 (RTL351) or 305–324 (RTL352). EAU was induced by immunization of HLA-DR3 mice with arrestin or arrestin peptides and treated with RTLs by subcutaneous delivery. T cell proliferation and cytokine expression was measured in RTL-treated and control mice.

Results: RTL351 prevented the migration of cells outside of the spleen and the recruitment of inflammatory cells into the eye, and provided full protection against inflammation from EAU induced with arrestin or arrestin peptides. RTL351 significantly inhibited T cell proliferation and secretion of inflammatory cytokines interleukin 2 (IL-2), interferon γ (IFN-γ), IL-6, and IL-17 and chemokines (macrophage inflammatory proteins [MIP-1α] and regulated and normal T cell expressed and secreted [RANTES]), which is in agreement with the suppression of intraocular inflammation. RTL350 (“empty,” no peptide) and RTL352 were not effective.

Conclusions: Immunotherapy with a single RTL351 successfully prevented and treated arrestin-induced EAU in HLA-DR3 mice and provided proof of concept for therapy of autoimmune uveitis in human patients. The beneficial effects of RTL351 should be attributed to a significant decrease in Th1/Th17 mediated inflammation.

Translational Relevance: Successful therapies for autoimmune uveitis must specifically inhibit pathogenic inflammation without inducing generalized immunosuppression. RTLs can offer such an option. The single retina-specific RTLs may have a value as potential immunotherapeutic drug for human autoimmune uveitis because they effectively prevent disease induced by multiple T cell specificities.
on the ethnic origin of the population studied.\textsuperscript{5,6} Vogt-Koyanagi-Harada (VKH) disease, sympathetic ophthalmitis, and birdshot retinochoroidopathy are uveitic conditions that demonstrate a significant HLA association with both HLA class II and class I molecules, indicating an underlying autoimmune process for disease. Among the class II-associated uveitic diseases, the \textit{HLA-DRB1*0405} allele, encoding a variant of the HLA-DR4 antigen, was found to be significantly increased in a Japanese population of VKH patients.\textsuperscript{7,8} Clinically similar to the VKH syndrome, sympathetic ophthalmitis is also associated with HLA-DR4 subtypes in Japanese, British, and Irish populations, and is associated with HLA-DRB1*04 and DQA1*03 genotypes in Caucasian patients, similar to Japanese patients.\textsuperscript{9,10} Pars planitis, a form of intermediate uveitis, was found to be frequently associated with the HLA-DR2 suballele (DR15, HLA-DR51, and HLA-DR17), implying an immunogenic predisposition.\textsuperscript{1,11,12} The strongest association of pars planitis with HLA-DR2 was in some patients with multiple sclerosis (MS).\textsuperscript{11,12} However, intermediate uveitis that was not related to MS was reported to be associated with HLA-DR3.\textsuperscript{2} A high prevalence of DR3 alleles was also detected in children with chronic uveitis and juvenile rheumatoid arthritis who had antinuclear antibodies (ANA).\textsuperscript{13} In all cases of panuveitis, DR4 was found to be a statistically significant factor, and the absence of DR1 in patients with posterior uveitis may be a protecting factor.\textsuperscript{2} There are many reports showing the association of HLA-DR3 in ocular sarcoidosis.\textsuperscript{14–16} In general, HLA-DR3 can be an important gene for uveitis susceptibility alone, or in association with other autoimmune disease, because a clustering of multiple autoimmune diseases has been observed in families.\textsuperscript{4,17,18}

Human noninfectious autoimmune uveitis is heterogeneous in terms of clinical presentation, but patients often recognize the same retinal antigens, including two major photoreceptor-specific autoantigens that induce experimental autoimmune uveitis (EAU), interphotoreceptor retinoid-binding protein (IRBP), and retinal arrestin (known as S-antigen).\textsuperscript{19,20} Heterogeneity in specific antigenic recognition can be related to a variety of diagnoses, different stages of disease activity, and their duration. Also, two patterns of T cell responses have been observed, one of which appears to be disease-specific, and the other specific to individual patients.\textsuperscript{21} Retinal antigens, including arrestin seem to play a role in the pathogenesis of idiopathic uveitis, and different pathogenic epitopes of this protein may be involved in different subsets of patients.\textsuperscript{22} Earlier studies evaluating the role of T cells in uveitis patients showed that peptides of human arrestin stimulated the cellular response in blood lymphocytes.\textsuperscript{21,23–25} A significant number of patients responded to two arrestin peptides called peptides “M” (amino acid positions 303–322), and “N” (amino acid positions 287–306).\textsuperscript{23,26} The knowledge on epitopes in antigenic proteins involved in pathogenic processes is essential in development of treatment strategies using these protein molecules and their fragments as possible targets.

EAU is an organ-specific, T cell–mediated autoimmune disease, which serves as a model for several human autoimmune intraocular inflammations. EAU targets retinal antigens, causing irreversible destruction of photoreceptor cells and visual loss, that is driven by Th1 and Th17 effector T cells.\textsuperscript{27} Previously, we showed that novel immunotherapy using recombinant T cell receptor ligand (RTL) drugs, which represent the minimal T cell receptor (TCR) ligand for uveitogenic T cells, suppressed the entry of T cells into the eye of Lewis rats, stopping inflammation, and protecting the animals from EAU.\textsuperscript{28,29} In those studies, IRBP-specific RTL220 was very effective in treatment of active and recurrent EAU when delivered at onset of clinical signs. Moreover, RTL220 was also effective in treatment of retinal degeneration and vascular pathology in Royal College of Surgeons (RCS) rats.\textsuperscript{30} Recently, “humanized” mice have served as powerful tools not only in understanding the role of antigens and HLA class II molecules in predisposition and onset of human diseases, but also in developing immunotherapies, including therapy for uveitis.\textsuperscript{4} A humanized HLA transgenic (Tg) mouse model of EAU for human class II molecules and deleted for mouse major histocompatibility complex (MHC) class II showed that mice expressing HLA-DRB1*0301 were highly susceptible to uveitis caused by arrestin and IRBP, in contrast to their parental wild-type strains.\textsuperscript{31,32} Pathogenic epitopes important in the induction of EAU that bind and present in the context of human MHC class II molecules have also been identified.\textsuperscript{31} HLA-DR3 tetramers loaded with a pathogenic epitope of arrestin could detect antigen (AG)-specific autoreactive T cells in draining lymph node cells of mice with EAU, and sorted tetramer-labeled cells could transfer EAU to naive mice, providing strong evidence for pathogenicity of arrestin.\textsuperscript{31}

In our current studies, we focused on RTL immunotherapy for autoimmune uveitis, targeting
immunopathogenic peptides of arrestin in the context of human class II in HLA-DR3 transgenic mice. We constructed new RTLs bearing either of two covalently-tethered arrestin peptides, RTL351 bearing arrestin residues 291–310 (Arr291–310) and RTL352 bearing arrestin residues 305–324 (Arr305–324) overlapping at amino acid residues 305–310. The arrestin peptide sequences correspond to previously reported immunodominant uveitopathogenic epitopes. These peptides were found to elicit in vitro T cell responses from patients affected by different uveitic diseases. The immunodominant arrestin epitope for the humanized DR3 Tg mice was identified within amino acid sequences 291–310 that overlaps with the previously characterized arrestin peptide “N” residues 281–302. The goal of the studies was to examine whether the arrestin-specific RTLs could efficiently stop EAU in humanized mice. Our findings demonstrate that immunotherapy with a single RTL351 successfully prevented and treated arrestin-induced EAU in DR3 mice and provide proof of concept for therapy of autoimmune uveitis in human patients.

Methods

Animals

Randomized transgenic HLA-DR3 (DRA*0101/DRB1*0301) mice of 8 to 10 weeks of age were used for experiments. HLA-DR3 Tg mice carry the HLA-DRA*0103 and DRB1*0301 genes on their MHC class II. These mice are negative H2-Aβ0 background (Aβ0.DR3) and express the human DR3 antigen as their only MHC class II molecules. The mice originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic). The mice were genotyped for the presence or absence of HLA-DR3 by polymerase chain reaction (PCR) according to the protocol provided by the Mayo Clinic Animal Facility to assure the expression of the correct gene. For phenotype screening, approximately 50 μL peripheral blood was obtained from the saphenous vein, and then blood cells were stained with monoclonal antibodies specific for human MHC class II DR (Sigma Chemical Co., St. Louis, MO). Stained cells were analyzed under a fluorescent microscope. Based on the genotype and phenotype results, the HLA-DR3+ mice were separated from HLA-DR3− mice. DR3− littermates do not express any HLA class II molecules, and their genotype is equivalent to that of Aβ0 mice. Both groups of mice were used in experiments as specified. The experiments were repeated at least three times to achieve statistical significance across treatment groups. The care and use of the animals complied with guidelines of the OHSU Institutional Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology.

Arrestin and Peptides

Arrestin was purified from bovine retinas using previously described methods. The human arrestin peptides 291–310 (Arr291–310) and 305–324 (Arr305–324) were synthesized commercially by Peptide2.0 Inc. (Chantilly, VA), using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. The peptide sequences for human DR3-derived RTLs are shown in the Table.

Construction of RTL

An empty β1α1 RTL530 molecule was constructed based on the human primary sequence of DR3 (DRA*0101/DRB1*0301) and then two RTLs bearing the covalently tethered Arr291–310 or Arr305–324 peptides were made (Table). De novo synthesis of the human DR3-derived RTL gene encoding 210 amino acids that contain the α1 and β1 domains of the human HLA DR3 (DRA*0101/DRB1*0301) with and without (“empty” RTL) sequences encoding arrestin peptides followed our previously described protocol and involved a four step, PCR-based gene synthesis (PGS) protocol using synthetic primers. Manufacturing of RTLs was done by fermentation of Escherichia coli bacteria harboring multiple copies of a recombinant DNA plasmid from which the RTL...
proteins were expressed. The protein was produced intracellularly and was subsequently harvested, denatured in organic solvents, purified by fast protein liquid chromatography (FPLC), and then reconstituted by refolding under dialysis with phosphate-buffered saline (PBS). Every RTL manufactured underwent extensive biochemical and biophysical characterization that included gel-shift assays to document native disulfide bond formation, circular dichroism to monitor secondary structure of the molecules, and testing with a panel of MHC class II-specific antibodies for validation of MHC class II epitope integrity. RTLs were kept frozen prior to use.

**Induction and Assessment Experimental Autoimmune Uveitis**

Both HLA-DR3\(^+\) and HLA-DR3\(^-\) gender-randomized mice were immunized to induce EAU at the age of 8 to 10 weeks. They received 100 \(\mu\)g bovine arrestin or 200 \(\mu\)g arrestin peptide emulsified 1:1 in complete Freund’s adjuvant (1:1; Sigma Chemical Co.) supplemented with *Mycobacterium tuberculosis* subcutaneously at the base of the tail. At the time of immunization mice received 0.5 \(\mu\)g/mouse *Bordetella pertussis* toxin (Sigma Chemical Co.) intraperitoneally. Eyes were collected after 35 to 36 days, fixed in 10% phosphate buffered formaldehyde overnight followed by embedding in paraffin. Five micrometer sections were prepared, stained with hematoxylin and eosin (H&E), and evaluated for inflammation by light microscopy. The presence or absence of eye disease was examined in a masked fashion. Histological severity of EAU was scored on a scale of 0 to 4 based on the degree of inflammation and damage to the retina. The minimal score was mild inflammatory cell infiltration of the ciliary body, choroid, vitreous, or retina and then progressively higher scores were assigned depending on the presence of vasculitis, granuloma formation, retinal folding and/or detachment, cellular infiltration, and photoreceptor damage.

**Systemic Treatment with RTL**

HLA-DR3\(^+\) transgenic mice with ongoing EAU were treated with RTL350, RTL351, or RTL352 in sterile PBS or vehicle at 10 to 100 \(\mu\)g per dose administered subcutaneously in the back. Mice received a total of five doses of RTL every day and then once a week for the duration of the experiments. The eyes and spleen were collected at the end of experiments on day 35 to 36 post immunization.

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Table. Amino Acid Sequences for DR3 (DRA*0101/DRB1*0301)-Derived RTLs

<table>
<thead>
<tr>
<th>RTL Name</th>
<th>Tethered Peptide</th>
<th>RTL Amino Acid Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTL350</td>
<td>“Empty” (no peptide)</td>
<td>MGDTRPRFLEYSTSECHFFNGTERVRYLDRYFHNQEE NVRFDSDVGEFRAVTE LGRPDAEYWNSQKDILLEQKR GRVDNYCVRHNYGVVESFTQVQRV(^*)IIEHHVIQAEFYLE NPDAQGEFMDFDGDGEIFHVMACKETWRLFEEFGR FASFEAQGALANIAVDKANLEIMTKRSNTYPITN</td>
</tr>
<tr>
<td>RTL351</td>
<td>Human arrestin 291–310</td>
<td>MGNNRERRGIALDGIKHEDTNLDTRPRFLEYSTSEC HFFNGTERVRYLDRYFHNQEEENVRFDSDVGEFRAVTE LGRPDAEYWNSQKDILLEQKRGRVDNYCVRHNYGVVES SFTQVQRV(^*)IIEHHVIQAEFYLENPDAQGEFMDFDGDGEIFHVMACKETWRLFEEFGRFASFEAQGALANIAVDKANLEIMTKRSNTYPITN</td>
</tr>
<tr>
<td>RTL352</td>
<td>Human arrestin 305–324</td>
<td>MGHDNTLNASSTIKEGIDRTVDTTRPRFLEYSTSEC HFNGTERVRYLDRYFHNQEEENVRFDSDVGEFRAVTE LGRPDAEYWNSQKDILLEQKRGRVDNYCVRHNYGVVES FTVQVRRV(^*)IIEHHVIQAEFYLENPDAQGEFMDFDGDGEIFHVMACKETWRLFEEFGRFASFEAQGALANIAVDKANLEIMTKRSNTYPITN</td>
</tr>
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Primary amino acid sequence of the \(\beta1\times1\) DR3-derived RTL constructs used in this study, and covalently-tethered arrestin peptide antigens in bold underlined text.

\(^*\) indicates \(\beta1\times1\) junction

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Immunoglobulin G (IgG) incubated with 1:2000 diluted biotinylated anti-mouse IgG serum at 1:100 was added to each well and allowed to incubate for 1 hour. After washing, the wells were blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour, diluted in coating buffer (0.1 M Tris–HCl buffer, pH 9.0) overnight at room temperature. After washing with coating buffer, pH 4.5, containing 3% H2O2 and immediately developed for 30 minutes by incubation with peroxidase substrate (2,20-azino-bis-[3-ethylbenz-thiazo-line-6-sulfonic acid]).

2.3 Cytokines

Cytokine production was assessed by an ELISA method. ELISA polystyrene plates were coated with 1 µg of arrestin or MHC α1β1 chain of RTL per well in the coating buffer (0.1 M Tris–HCl buffer, pH 9.0) overnight at room temperature. After washing with the coating buffer and blocking with 1% bovine serum albumin (BSA) in PBS for 1 hour, diluted serum at 1:100 was added to each well and allowed to incubate for 1 hour. After washing, the wells were incubated with 1:2000 diluted biotinylated anti-mouse Immunoglobulin G (IgG) (H+L chain; Invitrogen, Grand Island, NY) antibodies for 1 hour followed by 30 minutes incubation with streptavidin conjugated to peroxidase (1:5000; Invitrogen). Color reaction was developed for 30 minutes by incubation with peroxidase substrate (2,20-azino-bis-[3-ethylbenz-thiazo-line-6-sulfonic acid]) in 0.1 M citrate–phosphate buffer, pH 4.5, containing 3% H2O2 and immediately measured at 415 nm using a Bio-Rad Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

Cytokines from treated and untreated mice were incubated in stimulation medium with arrestin, or either of the arrestin peptides Arr291−305 or Arr305−324 for 48 hours as follows: 1 × 10⁶ cells/well were cultured with arrestin (20 µg/mL) or with synthetic peptides (25 µg/mL) in 200 µL RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 1% normal mouse serum, 2-mercaptoethanol, antibiotics, glutamine, and nonessential amino acids. Then supernatants were collected and immediately frozen. A 50 µL aliquot of supernatant sample was used in triplicate in a 96-well plate. The assay was performed using a multiplex ELISA kit (Bio-Rad), including a 23-plex cytokine and chemokine panel using a manufacturer’s protocol. The experiments for multiple samples were repeated two times. Data from reactions were acquired using Luminex system and analyzed using Bio-Plex Manager software (Bio-Rad), and are presented as bar graphs (mean ± SEM).

Results

HLA-DR3+ Tg mice were susceptible to arrestin-induced EAU at about the 70% to 80% incidence while the wild-type mice B10 were resistant to the induction of disease (no EAU). HLA-DR3+ mice with no detectable expression of DR3 were not susceptible to the induction of EAU and served as negative controls (Fig. 1A). These characteristics have been considered in the interpretation of the results. Three RTLs were constructed de novo for the studies (Table), RTL350 (“empty” RTL is a single chain construct consisting of the α1 and β1 domains of human HLA-DR3 without a covalently-tethered peptide) that served as a control, RTL351 bearing the covalently-tethered Arr291−310 peptide, and RTL352 bearing the covalently-tethered Arr305−324 peptide. To determine the therapeutic effectiveness of the RTLs, DR3+ Tg mice were immunized with the whole arrestin protein or the corresponding peptides. Development of autoimmune EAU was assessed by histopathology 35 to 36 days post immunization and treatment with RTLs. Systemic therapy consisted of five daily doses of RTL and boosts once a week over the duration of experiment administered subcutaneously. Using this protocol, we examined the most effective time of RTL delivery to suppress EAU induced with arrestin. A 100 µg RTL351 dose was initiated on day 1, 7, 14, 21 (onset), or 28 (peak) post immunization. Figure 1B demonstrates a significant inhibition of cellular inflammatory infiltration in mice receiving RTL351 (overall P < 0.0001, one-way ANOVA). EAU was almost completely inhibited when the treatment started between 1 to 14 days.
As is shown in Figure 1B, RTL351 was also effective when the treatment was administered at onset of inflammation (about 21 days PI) of arrestin-induced EAU. Remarkably, only one dose of 100 μg RTL351 delivered on day 28 post immunization when inflammation was in progress reduced cellular infiltration into the eyes in about 50% of mice. RTL351

Figure 1. Severity of EAU induced with arrestin in HLA-DR3 transgenic mice treated with five doses of RTLs every day and then once a week for the duration of experiment. (A) Mice lacking DR allele were resistant to the induction of EAU, histological scores of intraocular inflammation in eyes collected 35 days post immunization with 100 μg arrestin in CFA in HLA-DR3−/− (Aβ0.DR3−/−) and HLA-DR3+ Tg mice. (B) Effectiveness of RTL351 in inhibition of EAU induced with arrestin after subcutaneous delivery of RTL351 therapy (100 μg/dose) administered at different post immunization times starting on days 1, 7, 14, 21 (onset), and 28 (peak) post immunization; histological scores were assessed at the end of experiment on day 35 (n = 14, overall one way ANOVA P < 0.0001). (C) Lack of protection from arrestin-induced EAU when mice were treated with vehicle, 100 μg RTL350 (empty RTL without peptide tethered) and 100 μg RTL352 (tethered with Arr305–324) subcutaneously using the same treatment regimen as above. (D) Histology scores of EAU in HLA-DR3+ Tg mice immunized with arrestin and treated with 10, 50, or 100 μg per dose of RTL351; severity of eye disease was determined on day 35 or 36 PI by histological examination and the bar graphs represent average histological scores (mean ± SEM) of three experiments. *P < 0.01, **P < 0.0001 when the response is compared with vehicle controls (n = 14, one way ANOVA P = 0.0004).
was also effective in suppression of EAU induced with peptide Arr291–305 (not shown). Peptide Arr305–324 did not induce EAU, and RTL352 tethered to Arr305–324 was not effective in suppressing arrestin-induced EAU, suggesting that effector T cells specific for Arr305–324 epitope were not prevalent in this model. Also, RTL350 ("empty" DR3 domain without a bound peptide) had no effect on suppression of EAU, indicating that covalently-peptide was needed to induce tolerance (Fig. 1C). The effectiveness of the treatment with a monospecific RTL351 designed to target the Arr291–305-specific CD4 T cells also induced tolerogenic effects, leading to the suppression of EAU induced by the whole arrestin molecule that contains

Figure 2. Histopathology of posterior EAU treated with vehicle, RTL350, RTL351, or RTL352 started at 14 PI post immunization with arrestin. (A, B) Show cross-sections of humanized HLA-DR3 Tg mouse entire eye obtained after vehicle treatment (A) and RTL351 treatment (B) (magnification ×4). (C–F) Cross-section of retinas from different RTL therapies of arrestin-induce EAU as follows: (C) Vehicle, (D) RTL351, (E) RTL350, (F) RTL352. Note that treatment with RTL351 provided a complete retinal protection from inflammation when the treatment started at 14 PI (B, D). Arrows point at inflammatory cells in the retina, retinal folds, exudate in the subretinal space, and structural damage in RTL350, RTL352, and vehicle treated eyes (magnification ×40).
multiple epitopes in these mice and also in humans.\textsuperscript{23,35,40}

Next, we attempted to determine the minimal effective dose of RTL351 in suppression of EAU in treatment experiments using from 10 to 100 μg RTL351. The dose of 100 μg RTL351 was extremely effective and completely suppressed anterior and posterior intraocular inflammation in majority of humanized mice with arrestin-induced EAU. As shown in Figure 1D the doses of 10 μg RTL351 using the same treatment regimen suppressed the development of intraocular inflammation in 62\% mice. Doses over 50 μg were more effective and prevented arrestin-EAU in a larger number of DR3+ mice. Figure 2 shows a representative retinal histopathology from DR3+ Tg mice treated with vehicle, RTL350, RTL351, and RTL352. There was a remarkable absence of inflammatory cells in the retina of the majority RTL351-treated mice, implying that RTL351 was effective in prevention and treatment of arrestin-induced EAU.

To initiate the eye inflammation in mice pathogenic T cells migrate from the periphery into the eye. When splenocytes were counted in spleens of naive mice and arrestin-immunized mice and treated with vehicle, “empty” RTL350, RTL351, and RTL352 starting at 14 PI post immunization with arrestin. RTL351 injected into naive mice serve as a control. Cell counts were determined 35 days post immunization. Splenocyte numbers increased after immunization with arrestin and migrated outside but RTL351 sequestered cells within the spleen. Bars represent the mean ± SD of six mice/group (naïve mice group had three mice). Statistical differences between control and treated groups were calculated using one-way ANOVA (P > 0.00001).

In summary, the most important finding was that systemically delivered RTL351 significantly retained increased level of cells in the spleen and prevented an influx of inflammatory cells into the eye not only in peptide-induced EAU, but more importantly, in EAU induced with the whole arrestin molecule, during which multiple T cell specificities against various pathogenic epitopes are expected to be generated after processing the antigenic protein. In fact, a number of patients have shown in vitro lymphocyte proliferation against multiple arrestin peptides.\textsuperscript{21,23,24,40,41} Our results show that RTL351 effectively inhibited T cell proliferation against pathogenic epitope Arr291–310 and also induced other tolerogenic responses to protect the mice from disease.

EAU in DR3+ Tg mice is driven by a combination of the T cell– and antibody-effector mechanisms.\textsuperscript{27} We postulated that RTL351-mediated prevention of arrestin-EAU is attributed to the inhibition of specific T cell trapped in the spleen entry into the eye due to amelioration of early inflammatory responses in the spleen. To test our hypothesis, we examined arrestin and Arr291–310-specific CD4+ T cells responses in spleens of immunized mice (Figs. 4A, 4B). HLA-DR3+ Tg mice injected with arrestin produced a strong recall T cell response to arrestin and Arr291–310, but not to Arr305–324 as measured by the lymphocyte proliferation assay. The immunization of DR3+ mice with Arr291–310 peptide-induced T cells responses to the immunizing peptide only (Fig. 4B). Treatment
with RTL351 significantly suppressed arrestin and Arr\textsubscript{291–310} specific T cell proliferation in mice immunized with arrestin as well as in mice immunized with Arr\textsubscript{291–310} peptide. T cell proliferative responses in vehicle-treated mice were much stronger to arrestin than to Arr\textsubscript{305–324}, suggesting that effector T cells specific for Arr\textsubscript{305–324} epitope are not generated in large numbers in this model.

Although RTLs were designed specifically to target pathogenic T cells, the effect of RTL351 and RTL352 on anti-arrestin antibody titers after treatment was also evaluated by ELISA. Sera were collected from mice in the end of experiment on day 35 PI. Figures 4C to 4E show that RTLs have not affected anti-arrestin antibody levels in uveitic mice immunized with arrestin or peptides, suggesting that antibodies target different epitopes than do T cells in these mice. Since the DR3 mice received multiple doses of RTLs, we determined whether the mice generated anti-HLA (anti-\(\alpha\beta\textsubscript{1}\) of HLA) antibodies. Only background levels of anti-\(\alpha\beta\textsubscript{1}\) chain antibodies were detected in all mouse sera.

Next, we assessed the cytokine production by splenocytes collected 35 days post immunization, since the profile of systemic cytokines secretion may determine the positive outcome of uveitic diseases. The findings from the measurement of cytokines in splenocytes from arrestin-induced EAU and treated with vehicle or RTL351 are summarized in Figure 5. RTL351 caused a significant decrease in secreted inflammatory cytokines in response to the stimulation with arrestin and Arr\textsubscript{291–310} peptide in vitro. In particular, the major inflammatory cytokines, including IFN-\(\gamma\), IL-2, IL-17, and IL-6 associated with Th1...
Figure 5. Cytokine concentration in arrestin-stimulated splenocyte cultures obtained from vehicle- and RTL351-treated HLA-DR3⁺ Tg mice injected with arrestin. Splenic cells (collected 35 days PI) were stimulated with arrestin and Arr 291–310 for 48 hours and multiple cytokines were quantitated by the multiplex ELISA system. Bar graphs represent only cytokines that show significant changes in response to RTL351 treatment in mice. (A) Inflammatory cytokines IL-2, IL-17, IFN-γ, and IL-6; (B) anti-inflammatory cytokines IL-4, IL-5, IL-10, IL-13; (C) RANTES and MIP-1α.

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and Th17 cell responses known to drive EAU were significantly decreased (gray bars) as compared with the vehicle treatment (black bars; Fig. 5A). In contrast, Th2-type cytokines, including IL-4, IL-5, IL-13, and IL-10, appeared only slightly increased, if at all (Fig. 5B). Macrophage inflammatory proteins (MIP-1α) and regulated and normal T cell expressed and secreted (RANTES) inflammatory chemokines were significantly reduced after RTL351 therapy (Fig. 5C). These findings suggest that the significant decrease of systemic inflammatory cytokines is in agreement with the reduction in the arrestin-specific T cell proliferative responses after RTL351 therapy. Together, RTL351 not only inhibited T cell proliferation and secretion of inflammatory cytokines/chemokines, but also prevented trafficking of pathogenic T cells outside the spleen to the eye in mice that resulted in the suppression of inflammation in eyes of RTL351-treated mice.

**Discussion**

The current studies demonstrated the benefit of arrestin-specific RTL351 in prevention of EAU in the humanized HLA-DR3+ mouse immunized with the corresponding peptide and importantly, with the entire arrestin molecule. The key finding is that a single RTL protected DR3+ mice from EAU-induced with the whole protein, because such an immunization was shown to generate T cells specific to multiple determinants of arrestin in these mice.31 Our data provide proof of concept that immunotherapy of RTL351 carrying human epitope Arr291–310 has a potential application in treatment of human uveitis. Our findings are consistent with our previous studies showing beneficial effects of two RTLs (RTL201 and RTL220) for attenuating anterior uveitis, and acute and recurrent posterior uveitis in Lewis rats.28,29 Furthermore, no side effects from the RTL therapy were observed in the treated rats. In addition, this study nicely complements the recent findings from DR3 mice, showing the uveitogenic regions of arrestin that correspond to the determinants recognized by T cells from human patients with uveitis.31 By the use of humanized transgenic mice that present arrestin autoantigen in the context of a DR3 class II molecule we were able to identify RTL351 as a potential compound for therapy of human uveitis.

In our studies, we evaluated RTLs specific to arrestin, a 48-kDa intracellular protein also known as retinal “Soluble Antigen” (S-Ag), which is believed to be a major antigen associated with human uveitis.19 Patients showed significant blood lymphocyte proliferative responses against various arrestin peptides.21,22,35,40,41 The maximum T cell proliferation was found in response to peptides from the 231–270 amino acid region of the human arrestin sequence, and also in response to arrestin uveitogenic “N” peptide 281–302 and “M” peptide 303–320, suggesting that these epitopes play a major role in pathogenesis of a subset of human uveitis.35 Pathogenic epitopes corresponding to the previously identified peptide “N” were also defined in the humanized HLA-DR3 transgenic mouse model of arrestin-induced EAU.31 The arrestin sequence 287–306 identified as a DR3 epitope overlapped with the peptide sequence 291–310 used in our investigation. By using immunotherapeutic RTL351 treatment targeting this potentially uveitogenic epitope, we further provide evidence that anti-retinal autoimmunity is causally involved in pathogenesis of human uveitis.

RTLs are new biologic drugs that were designed to turn off attacking immune cells by altering the function of T cells that promote intraocular inflammatory responses. Importantly, a recent phase I clinical trial showed no significant side effects in patients.32 Based on our recent data, we believe that RTLs could supplement current treatments of uveitis in humans. Corticosteroids and immunosuppressive agents are frequently used to treat uveitis, however long term treatments lead to side effects. Newer immunomodulatory agents, including antimetabolites (azathioprine, methotrexate, mycophenolate mofetil), alkylating agents (cyclophosphamide, chlorambucil), T-cell inhibitors (cyclosporin, tacrolimus), and cytokines (TNF-α, IL-2R, and IFN-α) are offered, but they also produced unwanted side effects.33 In EAU experimental models, several treatments have been evaluated targeting T cells, cytokines, chemokines, dendritic cells, angiotsensin receptor (AT1-R) that suppressed clinical and histological EAU.28,29,44–47 RTLs consist of the α1 and β1 domains of MHC class II molecules that are genetically
linked into a single polypeptide chain covalently-tethered with a targeted pathogenic epitope. The mechanism of RTL therapy is not fully explained, but the MHC domains of the RTL may function as a carrier, protecting the peptide from proteolytic degradation. Early studies using RTL therapy for experimental autoimmune encephalomyelitis (EAE), and also EAU, have demonstrated that clinical and histological beneficial effects are peptide-specific. EAE induced with a given encephalitogenic determinant can only be treated with RTLS containing the same, but not a different peptide (e.g., MOG-specific RTL551 could not inhibit EAE induced with PLP–139-151, and PLP-specific RTL401 is ineffective in treatment of MOG–35-55 induced EAE). However, when the mice were immunized with syngeneic whole cord homogenate, which is composed of all relevant myelin antigens, EAE was successfully treated with a single RTL401. Also, a monospecific RTL treatment effectively suppressed EAE induced with two different peptides. These observations from EAE studies complement the current findings, showing the suppression of EAU induced by immunization with multidentate determinant arrestin protein with a monospecific RTL351, suggesting that RTL351 not only targeted the Arr291–305-specific T cells as it was designed to do, but may also induced tolerance effects. RTL351 strongly reduced the Th1 and Th17 response in periphery during EAU, which is important for subsequent disease progression and sequestered the uveitogenic cells within the spleen compared with untreated controls. There was about a 2-fold increase in the spleen cell numbers in RTL351-treated mice but the injection of RTL into a naïve mouse had no effect on the spleen. Likewise, in EAE, RTL551-treated mice retained the increased levels of CD4 T cells in the spleen throughout the disease but cells in vehicle- and “empty” RTL550–treated mice were still trafficking out of the periphery into the central nervous system as EAE progressed. Their studies also showed that as soon as one day after RTL551 injection there was a significant decreased frequency of CD4+ T cells in the blood, suggesting that RTL affected the antigen experienced T cells in the blood. Just published studies have identified a novel regulatory pathway that involves RTL binding to a receptor complex composed of MHC class II invariant chain (CD74), cell-surface histones and MHC class II itself on CD11b+ monocytes. RTLS with or without tethered antigenic peptide rapidly down regulated CD74 in a dose-dependent hierarchical manner, and blocked signaling of macrophage inhibitory factor, the inflammatory cytokine, for which CD74 serves as the primary receptor, and effectively reduced EAE severity. RTL constructs trigger both peptide-dependent and peptide-independent regulatory pathways that contribute to T-cell tolerance and EAE treatment effects. This is a key step in understanding RTL beneficial effects in the antigen-driven treatment of inflammatory diseases.

There is also a possibility that the interaction of a peptide/MHC class II complex with a cognate TCR, in the absence of costimulatory molecules results in a suboptimal signal in the T cell triggering FoxP3 expression. In collagen-induced arthritis, there was an increase in the FoxP3 gene in splenocytes of the RTL2001MII-treated mice in comparison to vehicle or “empty” RTL-treated mice. This suggests that up regulation of the Treg population in RTL therapy could mediate bystander suppression during the course of arthritis. Moreover, unlike RTLS with covalently bound peptide, “empty” RTLS have no protective effect on EAE and EAU, suggesting that “empty” RTLS bind to APCs but are not able to carry out tolerogenic signals to T cells. In addition, peptide alone was not effective in disease suppression EAE and retinal degeneration.

In summary, successful therapies for autoimmune disease, including autoimmune uveitis must specifically inhibit pathogenic inflammation without inducing generalized immunosuppression. RTLS can offer such an option. Our findings demonstrate the unique ability of systemically delivered arrestin-specific RTL351 to prevent and treat intraocular inflammation and suppress the immune responses directed against arrestin, suggesting its therapeutic potential for treatment of uveitis in humans. A single RTL351 can successfully treat ongoing EAU induced with the whole protein, which likely generates an immune response against multiple arrestin-determinants. Such treatment reduces Th1 and Th17 immune responses in periphery during EAU and reduces the eye infiltration by retaining cells within the spleen. Thus, the single RTL can effectively prevent disease induced by multiple T cell specificities under condition that the cognate T cell specificity is present. Therefore, RTLS specific for arrestin immunity and also earlier identified for IRBP immunity may have a value as potential immunotherapeutic drugs for autoimmune uveitis.

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