Article

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

Madison Kyger¹, Aneta Worley¹, Jianya Huan², Hugh McDowell³, W. Clay Smith³, Gregory G. Burrows^{2,4,5}, Mary J. Mattapallil⁵, Rachel R. Caspi⁶, and Grazyna Adamus¹ ✉

¹Ocular Immunology Laboratory, Casey Eye Institute, Department of Ophthalmology, School of Medicine, Oregon Health and Science University, Portland, OR

²Department of Neurology, School of Medicine, Oregon Health and Science University, Portland, OR

³Department of Ophthalmology, School of Medicine, University of Florida, Gainesville, FL

⁴Center for Hematologic Malignancies, Division of Hematology and Medical Oncology, Knight Cancer Institute, Oregon Health and Science University, Portland, OR

⁵Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, OR

⁶Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD

Correspondence: Grazyna Adamus, Ocular Immunology Laboratory, Casey Eye Institute, BRB, Oregon Health and Science University, 3181 SW Sam Jackson Pk Rd, Portland, OR 97239, USA, e-mail: adamusg@ohsu.edu

Received: 29 August 2012

Accepted: 1 January 2013

Published: 5 February 2013

Keywords: experimental autoimmune uveitis; immunotherapy; inflammation

Citation: Kyger M, Worley A, Huan J, et al. Effective arrestin–specific immunotherapy of experimental autoimmune uveitis with RTL: a prospect for treatment of human uveitis. *Trans Vis Sci Tech.* 2013;2(2):1, <http://tvstjournal.org/doi/full/10.1167/tvst.2.2.1>, doi:10.1167/tvst.2.2.1

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 peptide 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-1a] 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.

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

on the ethnic origin of the population studied.^{5,6} Vogt-Koyanagi-Harada (VKH) disease, sympathetic ophthalmia, 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 *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.^{7,8} Clinically similar to the VKH syndrome, sympathetic ophthalmia 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.^{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.^{1,11,12} The strongest association of pars planitis with HLA-DR2 was in some patients with multiple sclerosis (MS).^{11,12} However, intermediate uveitis that was not related to MS was reported to be associated with HLA-DR3.² A high prevalence of DR3 alleles was also detected in children with chronic uveitis and juvenile rheumatoid arthritis who had antinuclear antibodies (ANA).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.² There are many reports showing the association of HLA-DR3 in ocular sarcoidosis.^{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.^{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).^{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.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.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.^{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).^{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.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.^{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.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.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.^{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.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.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 (Arr_{291–310}) and RTL352 bearing arrestin residues 305–324 (Arr_{305–324}) overlapping at amino acid residues 305–310. The arrestin peptide sequences correspond to previously reported immunodominant uveitopathogenic epitopes.^{33–35} 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 281–302.³³ 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 HLA-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-A β 0 background (A β 0.DR3) and express the human DR3 antigen as their only MHC class II molecules.^{32,36} The mice originally were obtained by Rachel Caspi (National Eye Institute) from Chella David (Mayo Clinic), who generated transgenic the HLA-DR mice on B10 H-2Ab⁰ KO (negative for mouse MHC class II) as described by Kong et al.³⁶ Once in the National Eye Institute (NEI), HLA-DR3-positive males and females were immunized to develop uveitis. Animals that got the highest disease scores by fundus examination were selected as parents for future generations. These breeder pairs could be homozygous or heterozygous for the transgene. These mice were not backcrossed to any wild type (WT) B10 strain since WT B10 mice are not susceptible to arrestin-induced EAU. Before their use in experiments, all mice were screened for the presence or absence of HLA-DR and mouse I-A/I-E to assure that the mice express the correct genes but they were

not screen for the DR homozygosity. For this project, the mice were maintained and bred at the Animal Department of Oregon Health and Science University (OHSU). 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.³⁷ For phenotype screening, approximately 50 μ L 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 A β 0 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.³² The human arrestin peptides 291–310 (Arr_{291–310}) and 305–324 (Arr_{305–324}) 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 β 1 α 1 RTL350 molecule was constructed based on the human primary sequence of DR3 (DRA*0101/DRB1*0301) and then two RTLs bearing the covalently tethered Arr_{291–310} or Arr_{305–324} peptides were made (Table). De novo synthesis of the human DR3-derived RTL gene encoding 210 amino acids that contain the α 1 and β 1 domains of the human HLA DR3 (DRA*0101/DRB1*0301) with and without (“empty” RTL) sequences encoding arrestin peptides followed our previously described protocol³⁸ 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

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

RTL Name	Tethered Peptide	RTL Amino Acid Sequences
RTL350	“Empty” (no peptide)	MGDTRPRFLEYSTSECHFFNGTERTVRYLDRYFHNQEE NVRFDSVGEFRAVTELGRPDAEYWNSQKDLLEQKR GRVDNYCRHNYGVVESFTVQRRV ^a IKEEHVIIQAEFYLNPDQSGEFMFDFDGDGEIFHVDMAKKETVWRLEEFGRFASFEAQGALANIAVDKANLEIMTKRSNYTPITN
RTL351	Human arrestin 291–310	MGNRERRGIALDGKIKHEDTNLDTRPRFLEYSTSEC HFFNGTERTVRYLDRYFHNQEENVRFDSVGEFRAVTELGRPDAEYWNSQKDLLEQKRGRVDNYCRHNYGVVESFTVQRRV ^a IKEEHVIIQAEFYLNPDQSGEFMFDFDGDGEIFHVDMAKKETVWRLEEFGRFASFEAQGALANIAVDKANLEIMTKRSNYTPITN
RTL352	Human arrestin 305–324	MGHEDTNLASSTIIKEGIDRTVDTRPRFLEYSTSECHFFNGTERTVRYLDRYFHNQEENVRFDSVGEFRAVTELGRPDAEYWNSQKDLLEQKRGRVDNYCRHNYGVVESFTVQRRV ^a IKEEHVIIQAEFYLNPDQSGEFMFDFDGDGEIFHVDMAKKETVWRLEEFGRFASFEAQGALANIAVDKANLEIMTKRSNYTPITN

Primary amino acid sequence of the $\beta 1\alpha 1$ DR3-derived RTL constructs used in this study, and covalently-tethered arrestin peptide antigens in bold underlined text.

^a indicates $\beta 1\alpha 1$ junction

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,³⁹ circular dichroism to monitor secondary structure of the molecules,^{38,39} and testing with a panel of MHC class II-specific antibodies for validation of MHC class II epitope integrity.³⁹ 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 μ g bovine arrestin or 200 μ 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 μ g/mouse *Bordetella pertussis* toxin (Sigma Chemical Co.) intraperitoneal-

ly. 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 μ 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.

Lymphocyte Proliferation Assay (LPA)

The assay was performed in 96-well tissue culture plates in triplicate using RPMI medium containing 10% fetal bovine serum (FBS), 5×10^{-5} M 2-mercaptoethanol, and 50 $\mu\text{g/mL}$ gentamycin. Splenocytes were seeded at a density of 2×10^5 per well and incubated with RPMI medium only, 1 μg concanavalin A, 5 μg arrestin, Arr_{291–305}, or Arr_{305–324} at 37°C in 5% CO₂ for 72 hours and then pulsed with 1 μCi tritiated thymidine/well for an additional 18 hours. The cells were harvested onto a glass fiber filter and the thymidine uptake was assessed by the liquid scintillation counting in a Betaplate counter (model 1250; Wallac Pharmacia, Espoo, Finland). The data were expressed as a stimulation index (SI), which was calculated by dividing the proliferation (cpm incorporated) measured in the presence of antigen by the proliferation measured with medium alone.

ELISA

ELISA polystyrene plates were coated with 1 μg of arrestin or MHC $\alpha 1\beta 1$ 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-thiazoline-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

Splenocytes from treated and untreated mice were incubated in stimulation medium with arrestin, or either of the arrestin peptides Arr_{291–305} or Arr_{305–324} for 48 hours as follows: 1×10^6 cells/well were cultured with arrestin (20 $\mu\text{g/mL}$) or with synthetic peptides (25 $\mu\text{g/mL}$) 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 $\pm$ SEM). Statistical analysis was performed with a GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) using a two-tailed Student's *t* test or the significance between the controls and treatment groups was determined by one-way analysis of variance (ANOVA). Differences with a *P* value less than 0.05 were considered significant and are denoted by an asterisk.

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 $\alpha 1$ and $\beta 1$ domains of human HLA-DR3 without a covalently-tethered peptide) that served as a control, RTL351 bearing the covalently-tethered Arr_{291–310} peptide, and RTL352 bearing the covalently-tethered Arr_{305–324} 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

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 μ g arrestin in CFA in HLA-DR3⁻ (A β 0.DR3⁻) and HLA-DR3⁺ Tg mice. (B) Effectiveness of RTL351 in inhibition of EAU induced with arrestin after subcutaneous delivery of RTL351 therapy (100 μ g/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 μ g RTL350 (empty RTL without peptide tethered) and 100 μ g 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 μ g 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 $\pm$ SEM) of three experiments. * $P < 0.01$, ** $P < 0.0001$ when the response is compared with vehicle controls ($n = 14$, one way ANOVA $P = 0.0004$).

(one eye positive for 1 day post immunization (PI)-treatment and two eyes positive for 14 days PI, treatment with EAU score 1).

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 μ g 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 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 $\times 4$). (C–F) Cross-section of retinas from different RTL therapies of arrestin-induced 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 $\times 40$).

was also effective in suppression of EAU induced with peptide Arr_{291–305} (not shown). Peptide Arr_{305–324} did not induce EAU, and RTL352 tethered to Arr_{305–324} was not effective in suppressing arrestin-induced EAU, suggesting that effector T cells specific for Arr_{305–324} 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 Arr_{291–305}-specific CD4 T cells also induced tolerogenic effects, leading to the suppression of EAU induced by the whole arrestin molecule that contains

Figure 3. RTL351 prevents the migration of cells from the spleen. Splenocytes were counted in spleens of naïve 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 naïve 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 $\pm$ SD of six mice/group (naïve mice group had three mice). Statistical differences between control and treated groups were calculated using one-way ANOVA ($P > 0.00001$).

multiple epitopes in these mice³¹ and also in humans.^{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 μ g RTL351. The dose of 100 μ g 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 μ g RTL351 using the same treatment regimen suppressed the development of intraocular inflammation in 62% mice. Doses over 50 μ g 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 DR3 mice with EAU and treated with RTL351, these spleens retained almost 2 times more cells than the spleens of mice immunized arrestin. As is shown in Figure 3, the spleen cell number remained significantly increased compared to naïve, vehicle-treated, RTL350, and RTL352-treated mice ($n = 6/\text{group}$, $P < 0.0001$, one-way ANOVA) when the counts were taken on day 35 at the end of experiments. RTL351 given to naïve mice as well as an empty RTL had no effect on the spleen (Fig. 3). The reduced number of cells in arrestin-immunized mice might indicate their migration from the spleen towards the eye to cause inflammation. The increased number of splenocytes was revealed in RTL351 treatments started at 14 days PI or 21 days PI, suggesting that T cells were trapped in the spleen of treated mice and that RTL351 prevented their trafficking outside the spleen to the eye during the priming phase of the inflammatory responses.

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.^{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.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

Figure 4. Lymphocyte proliferation and serum antibodies levels against arrestin and arrestin peptides in arrestin-immunized HLA-DR3 transgenic mice. Spleens and sera were collected from RTL- and vehicle-treated groups of mice 35 days post immunization. Representative recall T cell proliferation to arrestin and peptides Arr₂₉₁₋₃₁₀ and Arr₃₀₅₋₃₂₄ in mice immunized with arrestin (A) or Arr₂₉₁₋₃₁₀ (B) measured by a lymphocyte proliferation assay; mice were treated with five doses of 100 μ g RTL351 every day and then once a week or received vehicle on the same days. Data are presented as a stimulation index relative to medium controls and are representative of three separate experiments. Bars represent the mean $\pm$ SEM of three replicate of pooled splenocytes. (C–E) Anti-arrestin and anti-MHC α 1 β 1 chain of RTL specific antibody responses were measured by ELISA in sera ($n = 6$) from vehicle- and RTL-treated HLA-DR3 Tg mice collected 35 days post immunization with arrestin or peptides. Note that RTL351 suppressed T cell response (A, B), but did not lower the anti-arrestin antibody levels (C, D). Mice did not generate antibodies to MHC α 1 β 1 chain of RTL (C–E). Statistical differences between control and treated groups were calculated using a Mann-Whitney U test; * $P < 0.05$, ** $P < 0.01$.

with RTL351 significantly suppressed arrestin and Arr₂₉₁₋₃₁₀ specific-T cell proliferation in mice immunized with arrestin as well as in mice immunized with Arr₂₉₁₋₃₁₀ peptide. T cell proliferative responses in vehicle-treated mice were much stronger to arrestin and Arr₂₉₁₋₃₁₀ than to Arr₃₀₅₋₃₂₄, suggesting that effector T cells specific for Arr₃₀₅₋₃₂₄ 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 arrestin 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- α 1 β 2 of HLA) antibodies. Only background levels of anti- α 1 β 2 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 Arr₂₉₁₋₃₁₀ peptide in vitro. In particular, the major inflammatory cytokines, including IFN- γ , 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 Arr₂₉₁₋₃₁₀ 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,

→

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-1 α) 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 Arr_{291–310} 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.⁴² 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 (cyclosporine, tacrolimus), and cytokines (TNF- α , IL-2R, and IFN- α) are offered, but they also produced unwanted side effects.⁴³ In EAU experimental models, several treatments have been evaluated targeting T cells, cytokines, chemokines, dendritic cells, angiotensin receptor (AT1-R) that suppressed clinical and histological EAU.^{28,29,44–47} RTLs consist of the α 1 and β 1 domains of MHC class II molecules that are genetically

and IL-13; (C) inflammatory chemokines RANTES and MIP-1 α . Bars represent the mean cytokine concentration of three mice per group representative of two individual experiments (mean $\pm$ SEM). Statistical differences were calculated using a Mann-Whitney *U* test; **P* < 0.05, ***P* < 0.01. Black bar = vehicle treatment, gray bar = RTL351 treatment.

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.⁴⁸ 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).^{49,50} 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 RTL401.⁵¹ Also, a monospecific RTL treatment effectively suppressed EAE induced with two different peptides.⁵¹ These observations from EAE studies complement the current findings, showing the suppression of EAU induced by immunization with multideterminant 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.⁵² 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.⁵³ 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.⁵⁴ 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.^{28,29,50} In addition, peptide alone was not effective in disease suppression EAE and retinal degeneration.^{30,50}

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.

Acknowledgments

The authors thank Chella David for HLA-DR3 transgenic mice provided to Rachel Caspi. These studies were supported in part by grants from the

National Institutes of Health (EY17781, GA; EY014864, WCS; EY006225, WCS; and core grant EY021721) unrestricted grants from the Research to Prevent Blindness to Casey Eye Institute Oregon Health and Science University and Department of Ophthalmology, University of Florida, and the National Eye Institute, National Institutes of Health intramural grant (EY000184-29, RRC).

Disclosure: **M. Kyger**, None; **A. Worley**, None; **J. Huan**, None; **H. McDowell**, None; **W.C. Smith**, None; **G.G. Burrows**, Artielle Immuno Therapeutics, Inc (P); **M.J. Mattapallil**, None; **R.R. Caspi**, None; **G. Adamus**, Artielle Immuno Therapeutics, Inc (P)

References

- Oruc S, Duffy BF, Mohanakumar T, Kaplan HJ. The association of HLA class II with pars planitis. *Am J Ophthalmol*. 2001;131:657–659.
- Cuccia Belvedere M, Martinetti M, de Paoli F, et al. Genetic heterogeneity in uveitis. *Dis Markers*. 1986;4:243–246.
- de Kozak Y, Camelo S, Pla M. Pathological aspects of spontaneous uveitis and retinopathy in HLA-A29 transgenic mice and in animal models of retinal autoimmunity: relevance to human pathologies. *Ophthalmol Res*. 2008;40:175–180.
- Mangalam AK, Rajagopalan G, Taneja V, David CS. *HLA Class II Transgenic Mice Mimic Human Inflammatory Diseases*. In: Frederick WA, ed. *Advances in Immunology*. Waltham, MA: Academic Press; 2008:65–147.
- Martin TM, Rosenbaum JT. Genetics in uveitis. *Int Ophthalmol Clin*. 2005;45:15–30.
- Davey MP, Rosenbaum JT. The human leukocyte antigen complex and chronic ocular inflammatory disorders. *Am J Ophthalmol*. 2000;129:235–243.
- Shindo Y, Inoko H, Yamamoto T, Ohno S. HLA-DRB1 typing of Vogt-Koyanagi-Harada's disease by PCR-RFLP and the strong association with DRB1*0405 and DRB1*0410. *Br J Ophthalmol*. 1994;78:223–226.
- Alaez C, del Pilar Mora M, Arellanes L, et al. Strong association of HLA class II sequences in Mexicans with Vogt-Koyanagi-Harada's disease. *Human Immunol*. 1999;60:875–882.
- Kilmartin DJ, Wilson D, Liversidge J, et al. Immunogenetics and clinical phenotype of sympathetic ophthalmia in British and Irish patients. *Br J Ophthalmol*. 2001;85:281–286.
- Shindo Y, Ohno S, Usui M, et al. Immunogenetic study of sympathetic ophthalmia. *Tiss Antigens*. 1997;49:111–115.
- Malinowski SM, Pulido JS, Goeken NE, Brown CK, Folk JC. The association of HLA-B8, B51, DR2, and multiple sclerosis in pars planitis. *Ophthalmol*. 1993;100:1199–1205.
- Raja SC, Jabs DA, Dunn JP, et al. Pars planitis: clinical features and class II HLA associations. *Ophthalmol*. 1999;106:594–599.
- Alsaied KM, Haider MZ, al-Awadhi AM, Srivastva BS, Ayoub EM. Role of human leukocyte antigen DRB1*0307 and DRB1*0308 in susceptibility to juvenile rheumatoid arthritis. *Clin Exp Rheumatol*. 2003;21:399–402.
- Suzuki H, Ota M, Meguro A, et al. Genetic characterization and susceptibility for sarcoidosis in Japanese patients: risk factors of BTNL2 gene polymorphisms and HLA class II alleles. *Invest Ophthalmol Vis Sci*. 2012;53:7109–7115.
- Sato H, Woodhead FA, Ahmad T, et al. Sarcoidosis HLA class II genotyping distinguishes differences of clinical phenotype across ethnic groups. *Hum Mol Gen*. 2010;19:4100–4111.
- Jakob E, Reuland JE, Mackensen F, et al. Uveitis subtypes in a German interdisciplinary uveitis center—analysis of 1916 patients. *J Rheumatol*. 2009;36:127–136.
- Zenewicz LA, Abraham C, Flavell RA, Cho JH. Unraveling the genetics of autoimmunity. *Cell*. 2010;140:791–797.
- Mackay IR. Clustering and commonalities among autoimmune diseases. *J Autoimmun*. 2009;33:170–177.
- Adamus G, Chan CC. Experimental autoimmune uveitides: multiple antigens, diverse diseases. *Int Rev Immunol*. 2002;21:209–229.
- de Smet MD, Chan CC. Regulation of ocular inflammation—what experimental and human studies have taught us. *Prog Ret Eye Res*. 2001;20:761–797.
- de Smet MD, Bitar G, Mainigi S, Nussenblatt RB. Human S-antigen determinant recognition in uveitis. *Invest Ophthalmol Vis Sci*. 2001;42(X):3233–3238.
- Rai G, Saxena S, Kumar H, Singh VK. Human retinal S-antigen: T cell epitope mapping in posterior uveitis patients. *Exp Mol Pathol*. 2001;70:140–145.
- Hirose S, Donoso LA, Shinohara T, Palestine AG, Nussenblatt RB, Gery I. Lymphocyte responses to peptide M and to retinal S-antigen in uveitis patients. *Jpn J Ophthalmol*. 1990;34:298–305.

24. Vrabec TR, Reber RN, Magargal LE, Donoso LA. S-antigen. Identification of human T-cell lymphocyte proliferation sites. *Arch Ophthalmol*. 1990;108:1470–1473.
25. de Smet MD, Yamamoto JH, Mochizuki M, et al. Cellular immune responses of patients with uveitis to retinal antigens and their fragments. *Am J Ophthalmol*. 1990;110:135–142.
26. Yamamoto JH, Minami M, Inaba G, Masuda K, Mochizuki M. Cellular autoimmunity to retinal specific antigens in patients with Behcet's disease. *Br J Ophthalmol*. 1993;77:584–589.
27. Caspi RR. A look at autoimmunity and inflammation in the eye. *J Clin Invest*. 2010;120:3073–3083.
28. Adamus G, Burrows GG, Vandenbark AA, Offner H. Treatment of autoimmune anterior uveitis with recombinant TCR ligands. *Invest Ophthalmol Vis Sci*. 2006;47:2555–2561.
29. Adamus G, Karren L, Mooney J, Burrows G. A promising therapeutic approach for treatment of posterior uveitis: recombinant T cell receptor ligand protects Lewis rats from acute and recurrent experimental autoimmune uveitis. *Ophthalmic Res*. 2010;44:24–33.
30. Adamus G, Wang S, Kyger M, Worley A, Lu B, Burrows GG. Systemic immunotherapy delays photoreceptor cell loss and prevents vascular pathology in RCS rats. *Mol Vis*. 2012;18:2323–2337.
31. Mattapallil MJ, Silver PB, Mattapallil JJ, et al. Uveitis-associated epitopes of retinal antigens are pathogenic in the humanized mouse model of uveitis and identify autoaggressive T cells. *J Immunol*. 2011;187:1977–1985.
32. Pennesi G, Mattapallil MJ, Sun SH, et al. A humanized model of experimental autoimmune uveitis in HLA class II transgenic mice. *J Clin Invest*. 2003;111:1171–1180.
33. Singh VK, Nussenblatt RB, Donoso LA, Yamaki K, Chan CC, Shinohara T. Identification of a uveitopathogenic and lymphocyte proliferation site in bovine S-antigen. *Cell Immunol*. 1988;115:413–419.
34. Donoso LA, Yamaki K, Merryman CF, Shinohara T, Yue S, Sery TW. Human S-antigen: characterization of uveitopathogenic sites. *Curr Eye Res*. 1988;7:1077–1085.
35. Rajasingh J, Singh VK, Singh V, Sharma K, Agarwal SS. Cellular immune response to retinal S-antigen & interphotoreceptor retinoid binding protein fragments in idiopathic human uveitis. *Indian J Med Res*. 1996;103:222–226.
36. Kong YC, Lomo LC, Motte RW, et al. HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice: definitive association with HLA-DRB1*0301 (DR3) gene. *J Exp Med*. 1996;184:1167–1172.
37. Knipper AJ, Hinney A, Schuch B, Enczmann J, Uhrberg M, Wernet P. Selection of unrelated bone marrow donors by PCR-SSP typing and subsequent nonradioactive sequence-based typing for HLA DRB1/3/4/5, DQB1, and DPB1 alleles. *Tiss Antigens*. 1994;44:275–284.
38. Burrows GG, Chang JW, Bachinger HP, Bourdette DN, Offner H, Vandenbark AA. Design, engineering and production of functional single-chain T cell receptor ligands. *Protein Eng*. 1999;12:771–778.
39. Huan J, Meza-Romero R, Mooney JL, Vandenbark AA, Offner H, Burrows GG. Single-chain recombinant HLA-DQ2.5/peptide molecules block alpha2-gliadin-specific pathogenic CD4+ T-cell proliferation and attenuate production of inflammatory cytokines: a potential therapy for celiac disease. *Muc Immunol*. 2011;4:112–120.
40. Zhao C, Yang P, He H, et al. Retinal S-antigen Th1 cell epitope mapping in patients with Behcet's disease. *Graefes Arch Clin Exp Ophthalmol*. 2009;247:555–560.
41. Tripathi P, Saxena S, Yadav VS, Naik S, Singh VK. Human S-antigen: peptide determinant recognition in uveitis patients. *Exp Mol Pathol*. 2004;76:122–128.
42. Offner H, Sinha S, Burrows GG, Ferro AJ, Vandenbark AA. RTL therapy for multiple sclerosis: a Phase I clinical study. *J Neuroimmunol*. 2011;231:7–14.
43. Larson T, Nussenblatt RB, Sen HN. Emerging drugs for uveitis. *Expert Opin Emerg Drugs*. 2011;16:309–322.
44. Yin H, Vistica BP, Chan CC, Strominger JL, Gery I. Inhibition of experimental autoimmune uveitis by amino acid copolymers. *J Neuroimmunol*. 2009;215:43–48.
45. Usui Y, Takeuchi M, Hattori T, et al. Suppression of experimental autoimmune uveoretinitis by regulatory dendritic cells in mice. *Arch Ophthalmol*. 2009;127:514–519.
46. Haruta H, Ohguro N, Fujimoto M, et al. Blockade of interleukin-6 signaling suppresses not only Th17 but also interphotoreceptor retinoid binding protein-specific Th1 by promoting regulatory T cells in experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci*. 2011;52:3264–3271.

47. Manczak M, Jiang S, Orzechowska B, Adamus G. Crucial role of CCL3/MIP-1a in the recurrence of autoimmune anterior uveitis induced with myelin basic protein in Lewis rats. *J Autoimmun.* 2002;18:259–270.
48. Ishioka GY, Adorini L, Guery JC, et al. Failure to demonstrate long-lived MHC saturation both in vitro and in vivo. Implications for therapeutic potential of MHC-blocking peptides. *J Immunol.* 1994;152:4310–4319.
49. Offner H, Sinha S, Wang C, Burrows GG, Vandenbark AA. Recombinant T cell receptor ligands: immunomodulatory, neuroprotective and neuroregenerative effects suggest application as therapy for multiple sclerosis. *Rev Neurosci.* 2008;19:327–339.
50. Huan J, Subramanian S, Jones R, et al. Monomeric recombinant TCR ligand reduces relapse rate and severity of experimental autoimmune encephalomyelitis in SJL/J mice through cytokine switch. *J Immunol.* 2004;172:4556–4566.
51. Sinha S, Subramanian S, Miller L, et al. Cytokine switch and bystander suppression of autoimmune responses to multiple antigens in experimental autoimmune encephalomyelitis by a single recombinant T-Cell receptor ligand. *J Neurosci.* 2009;29:3816–3823.
52. Sinha S, Miller LM, Subramanian S, Burrows GG, Vandenbark AA, Offner H. RTL551 treatment of EAE reduces CD226 and T-bet+ CD4 T cells in periphery and prevents infiltration of T-bet+ IL-17, IFN- γ producing T Cells into CNS. *PLoS ONE.* 2011;6:e21868.
53. Vandenbark AA, Meza-Romero R, Benedek G, et al. A novel regulatory pathway for autoimmune disease: binding of partial MHC class II constructs to monocytes reduces CD74 expression and induces both specific and bystander T-cell tolerance [published online ahead of print September 29, 2012]. *J Autoimmun.* doi:10.1016/j.jaut.2012.08.004.
54. Huan J, Kaler LJ, Mooney JL, et al. MHC class II derived recombinant T cell receptor ligands protect DBA/1LacJ mice from collagen-induced arthritis. *J Immunol.* 2008;180:1249–1257.