Activation of Liver X Receptor Alleviates Ocular Inflammation in Experimental Autoimmune Uveitis

Hongxia Yang,¹ Shijie Zheng,¹ Yiguo Qiu,¹ Yan Yang,¹ Chaokui Wang,¹ Peizeng Yang,¹ Qihong Li,² and Bo Lei¹

¹Department of Ophthalmology, the First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, Chongqing Eye Institute, Chongqing, China

²Department of Ophthalmology, College of Medicine, University of Florida, Gainesville, Florida, United States

Correspondence: Bo Lei, Department of Ophthalmology, 1 You Yi Road, Yu Zhong District, Chongqing 400016, China; bolei99@126.com.

Submitted: September 24, 2013

Accepted: March 26, 2014

Citation: Yang H, Zheng S, Qiu Y, et al. Activation of liver X receptor alleviates ocular inflammation in experimental autoimmune uveitis. *Invest Ophthalmol Vis Sci*. 2014;55:2795–2804. DOI:10.1167/iov.13-13323

PURPOSE. To investigate whether a synthetic LXR agonist TO901317 (TO90) ameliorates ocular inflammation in a mouse model of experimental autoimmune uveitis (EAU) and to explore its underlying mechanism.

METHODS. EAU was induced with subcutaneous injection of IRBP_{161–180} peptide (SGIPYII SYLHPGNTILHVD) in B10.RIII mice. TO90 (50 mg/kg/d) or vehicle was administered orally for successive 16 days or 8 days as prevention or effector phase, respectively. The severity of EAU was evaluated with clinical and histological scores. The levels of LXRs, NF-κB subunit p65, and an LXR target gene ABCA1 in the retina were detected with real-time PCR and Western blotting. The expressions of proinflammatory genes, including TNF-α, IL-1β, IL-6, MCP-1, IFN-γ, and IL-17, were detected by real-time PCR. IRBP-specific lymphocyte proliferation was detected by MTT. Intracellular IFN-γ and IL-17 in CD4⁺ T cells were measured by flow cytometry.

RESULTS. We found both LXRα and LXRβ were expressed in mouse retina. After administering TO90 orally to B10.RIII mice, the expression of LXRα but not LXRβ was upregulated in the naïve mice. Compared with naïve mice, LXRα expression was increased in vehicle and TO90-treated EAU mice, but the LXRβ expression was unchanged. The protein level of ABCA1 was enhanced in TO90-treated naïve and EAU mice but was unchanged in vehicle-treated EAU mice, suggesting activation of LXRα by TO90 is ligand dependent. TO90-mediated activation of LXRα improved the clinical and morphological scores in EAU mice. Meanwhile, activation of LXRα decreased the expressions of proinflammatory cytokines, including TNF-α, IL-1β, IL-6, MCP-1, IFN-γ, and IL-17 in the retina. TO90 treatment inhibited IRBP-specific immune responses. The proportions of Th1 and Th17 expressing IFN-γ and IL-17 were reduced in TO90-treated EAU mice in both prevention and effector phases. Furthermore, TO90 significantly downregulated the expressions of an NF-κB subunit p65 at the protein and mRNA levels.

CONCLUSIONS. TO90 activates LXRα and potently attenuates ocular inflammation in EAU. Alleviation of ocular inflammation could partially result from inhibition of the NF-κB signaling pathway. TO90 reduces IFN-γ and IL-17 expression in both prevention and treatment scenarios. Our data suggest that the LXR agonist may become a novel class of therapeutic agent for autoimmune uveitis.

Keywords: LXR, uveitis, retina, EAU, NF-κB

As a major cause of vision loss worldwide,¹ uveitis is a group of vision-threatening intraocular inflammatory diseases² caused by autoimmune disorders or by infection.³ The conventional treatment approach entails corticosteroids and immunosuppressive agents, which may cause serious systemic side effects. Thus, it is desirable to search novel therapeutics for this devastating condition.

Experimental autoimmune uveitis (EAU) is an animal model of human autoimmune posterior uveitis, and it is well established that CD4⁺ T cells mediated the pathology of EAU.⁴ A body of evidence implicated IFN-γ, produced by interphotoreceptor retinoid-binding protein (IRBP)-specific Th1 effector cells, as playing the major role in EAU. Recently it has been demonstrated that IL-17 plays a critical role in

experimental and human uveitis.⁵ The latest studies suggesting that Th1 and Th17 each represent a fully competent pathogenic effector phenotype, and either Th17 or Th1 effector response can drive ocular autoimmunity.⁶ There is evidence that liver X receptors (LXRs) mediated inhibition of Th1- and Th17-cell differentiation and autoimmunity.^{7,8} Increasing interest has been shown in LXRs as regulators of inflammatory and immune response.^{9,10} LXRs are ligand-activated transcription factors that belong to the nuclear receptor superfamily. LXRs have two isoforms: LXRα and LXRβ. LXRα is expressed in liver, intestine, and adipose tissue, whereas LXRβ is generally expressed in many tissues.¹¹ LXRs are also expressed in immune cells, such as T cells, dendritic cells, and monocytes/macrophages,^{12,13} implying that these lipid-regulated nuclear receptors have a

potential to affect the function of the immune system as well.^{14,15}

Ligand-activated LXRs induce genes involved in cholesterol efflux in macrophages, including the ATP-binding cassette (ABC) transporters, ABCA1, ABCG1, and apolipoprotein E (ApoE),¹⁶ and inhibit the expression of inflammatory mediators, cytokines, and matrix metalloproteinase 9 in lipopolysaccharide-treated mouse macrophages.¹⁷ The anti-inflammatory effect of LXRs has been attributed to nuclear inhibition of transcription factor- κ B (NF- κ B) signaling.^{18,19} NF- κ B forms a heterodimer or a homodimer of the subunit members. In the cytoplasm of unstimulated cells, NF- κ B binds to I κ B, which is natural inhibitor of NF- κ B and prevents it from entering the nucleus. The most common activated form of NF- κ B in inflammatory cells consists of a p65 subunit and a p50 or p52 subunit.^{20,21} Previous study has demonstrated that NF- κ B p65 is distributed in the retina of EAU mice but not in control mice. The translocation of NF- κ B p65 in the retinal Müller's cells of EAU mice suggests that NF- κ B is involved in EAU.²²

The endogenous ligand for LXRs is oxysterol.¹⁶ Several synthetic ligands, including TO901317 (TO90) and GW3965, have shown greater potency and efficacy compared with oxysterol, and offer pharmacological tools in exploring the biologic mechanisms of the LXR family.^{23,24} In this study, we investigated whether LXRs are associated with the pathogenesis of EAU and the possibility of using an LXR agonist as a therapeutic agent in uveitis. We tested the hypothesis that activation of LXRs by an exogenous ligand TO90 could inhibit ocular inflammation in EAU through suppressing the NF- κ B signaling pathway. In addition, we examined whether TO90 inhibited IRBP-specific immune response and studied whether TO90 suppressed Th1 and Th17 cells in the prevention and the effector phases.

MATERIALS AND METHODS

Ethics Statement

This study was carried out according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. Every effort was made to minimize animal discomfort and stress.

Animals and Reagents

B10.RIII mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions. Human IRBP peptide-spanning amino acid residues 161 to 180 (IRBP₁₆₁₋₁₈₀, SGIPYIISYLHPGNTILHVD) was synthesized by Shanghai Sangon Biological Engineering Technology & Services Ltd. Co. (Shanghai, China). Complete Freund's adjuvant (CFA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). TO90 (Cayman, Ann Arbor, MI, USA) was dissolved in 100% dimethylsulfoxide (DMSO) at 100 mg/mL and was stored in aliquots at -20°C . Before administration to B10.RIII mice, the DMSO was diluted with PBS to a final DMSO concentration of 2%, at which no direct effect of DMSO was observed on NF- κ B activity.²⁵

Mice aged 8 to 12 weeks were randomly divided into four groups: TO90-treated EAU group, vehicle-treated EAU group (2% DMSO in PBS), naïve mouse group, and naïve mouse treated with TO90 group. Starting from 2 days before to 14 days (-2 to 14 d) after immunization, mice were treated with TO90 (orally 50 mg/kg/d) for 16 successive days. For effector-phase treatment, TO90 or vehicle was administered from day 8 to day 14 after immunization. Control mice received the same volume of vehicle.

Induction and Clinical Assessment of EAU

EAU was made following a previous protocol.^{26,27} Briefly, mice were immunized subcutaneously at the base of the tail and both thighs with 50 μg human IRBP₁₆₁₋₁₈₀ peptide in 100 mL PBS, emulsified 1:1 vol/vol in CFA (Sigma-Aldrich) supplemented with 1.0 mg/mL mycobacterium tuberculosis strain. A total 200- μL emulsion was given in one mouse. Clinical signs of EAU were examined by slit-lamp microscopy from day 7 to day 21 after immunization. The clinical severity of ocular inflammation was assessed by two independent observers in a masked manner, and scored on a scale of 0 to 5.²⁷

Histopathology

Eyeballs were enucleated at day 14 after IRBP peptide immunization and were fixed in 4% buffered paraformaldehyde for 1 hour at room temperature. Tissues were embedded in paraffin. Serial 4- to 6- μm sections were collected through the pupillary-optic nerve axis and stained with hematoxylin and eosin. At least four sections of each eye were evaluated histologically. The severity of EAU was graded in a masked fashion on a scale of 0 to 4, as described earlier.²⁷

Western Blotting Analysis

Western blotting was performed as described previously.²⁸ Retinas were dissected from enucleated eyeballs at day 14 after immunization. Protein was extracted by radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), including 1% proteases inhibitor (Beyotime). Equal amounts of protein (50 μg) were separated by SDS-PAGE electrophoresis, using 10% polyacrylamide gels. The resolved proteins were electroblotted onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat milk and incubated with specific primary antibodies against LXR α or LXR β (1:500; ABCAM, Cambridge, UK), p65 (1:1200; ABCAM), and ABCA1 (1:500; ABCAM) overnight at 4°C . Blots were washed and incubated with a secondary antibody for 1 hour at 37°C . Bands were analyzed using Image J software, version 1.43 (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Analysis was normalized against a housekeeping protein β -actin. The measurements were repeated three times in each experiment.

Real-Time PCR Analysis

Total RNA was isolated from mouse retinas using the TRIZOL reagent (Ambion, Carlsbad, CA, USA). For quantitative PCR, total RNA was reverse transcribed using RT Primer Mix and oligo dT primers (Takara, Dalian, China). The cDNA was quantified by real-time PCR on a Bio-Rad c-1000 (Bio-Rad Laboratories, Hercules, CA, USA) using primers specific for mice. Mouse LXR α , Primer ID (Mm-QRP-20309); mouse LXR β , Primer ID (Mm-QRP-20331); mouse p65, Primer ID (Mm-QRP-20324); mouse TNF- α , Primer ID (Mm-QRP-20147); mouse IL-6, Primer ID (Mm-QRP-20026); mouse IL-1 β , Primer ID (Mm-QRP-20148); mouse monocyte chemoattractant protein-1 (MCP-1), Primer ID (Mm-QRP-20287); mouse IFN- γ , Primer ID (Mm-QRP-20021); mouse IL-17, Primer ID (Mm-QRP-20606); and standardized glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primer (Mm-QRP-20043) (GeneCopoeia, Inc., Rockville, MD, USA) were used. The specificity of all the primers was verified by GeneCopoeia, Inc. PCR amplification was performed in a volume of 20 μL , using all-in-one quantitative PCR mix (GeneCopoeia, Inc.). The conditions were 95°C for 10 minutes, followed by 40 cycles of 10 seconds at 95°C , 20 seconds at 60°C , and 15 seconds at 72°C .

FIGURE 1. Expression of LXR α and LXR β in mouse retina was detected by Western blotting and real-time PCR. Mice were treated with either TO90 or vehicle. Treatment was initiated 2 days before an IRBP peptide immunization and continued daily to day 14. Retinal protein and mRNA were measured at day 14. (A) LXR α and LXR β expressions in normal naïve retina with and without TO90. Both protein and mRNA levels of LXR α were lower than those of LXR β in normal naïve retina, and were increased by TO90 treatment, whereas LXR β levels were not changed by TO90 treatment. (B) LXR α and LXR β expression in EAU retina with and without TO90. The expression of LXR α but not LXR β was increased in EAU mice in contrast to naïve mice. Both LXR α and LXR β levels were not further altered in TO90-treated EAU mice. The relative expressions of mRNA and protein were normalized to GAPDH and β -actin, respectively. Data are mean $\pm$ SEM and are representative of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001. Six mice were used in each group.

Fluorescence data were acquired at 72 to 95°C to decrease the nonspecific signal, and amplification of specific transcripts was confirmed by melting curve profiles at the end of each PCR. Measurements were masked to group assignment.

Lymphocyte Proliferation and Cytokine Expression

The spleens and draining lymph nodes were removed from immunized mice on day 14. Cell suspension was prepared by mechanical disruption and followed by a passage through a sterile stainless steel screen. For proliferation and cytokine assay, cells (2×10^5 in 100 μ L) were cultured in triplicate with RPMI 1640 medium (Gibco, Grand Island, NY, USA) and 10% fetal bovine serum in the presence of 10 μ g/mL IRBP₁₆₁₋₁₈₀ peptide, 1 μ g/mL Concanavalin A (Con A; Sigma-Aldrich) or medium alone respectively for 72 hours. Proliferation was detected by a modified MTT assay using a cell-counting kit (Cell Counting Kit-8; Sigma-Aldrich) as described previously.²⁷ The expressions of IFN- γ and IL-17 in the lymphocytes were measured by real-time PCR.

Flow Cytometry Analysis

For intracellular cytokine evaluation, the lymphocytes (2×10^5 in 100 μ L) were pretreated with 100 ng/mL phorbol-12-myristate-13-acetate and 1 μ g/mL ionomycin for 1 hour at 37°C, 10 μ g/mL brefeldin A (Sigma-Aldrich) for another 4 hours, then washed, fixed, and permeabilized using the Cytofix/Cytoperm kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The cells were stained intracellularly with fluorescent antibodies, including anti-mouse CD4-APC, anti-mouse IFN- γ -PE-Cyanine 7, and anti-mouse IL-17A-PE (eBioscience) for 30 minutes.

Statistical Analysis

Data were presented as mean $\pm$ SEM. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). A Mann-Whitney *U* test was used to compare the EAU score. Continuous variables of band intensity and relative mRNA expression experiments were analyzed with the unpaired Student's *t*-test. One-way ANOVA followed by a Bonferroni correction were applied for multiple comparisons. A *P* value less than 0.05 was considered statistically significant.

RESULTS

LXR Expression in Normal and EAU Mouse Retina

We examined the expressions of LXRs in the retinas of normal mice and EAU mice with and without administration of TO90. Western blotting analysis and real-time PCR showed that both LXR α and LXR β were expressed in retina of naïve mice, but the level of LXR α was significantly lower than that of LXR β . After TO90 treatment, the LXR α level was increased but the LXR β level was unchanged (Fig. 1A). There was a significant increase in LXR α expression in the retina of EAU mice in contrast to naïve mice at day 14 after immunization, whereas LXR β expression remained unchanged. Both the LXR α and LXR β were unaltered in the EAU mice after TO90 treatment compared with vehicle treatment at the protein and mRNA levels (Fig. 1B).

TO90 Activated an LXR Target Gene ABCA1

To determine whether LXR was activated by TO90 and was functional, we evaluated the protein expression of an LXR

FIGURE 2. Expression of an LXR target gene ABCA1 in mouse retina. Retinal protein was isolated from TO90-treated and vehicle-treated naïve and EAU mice at day 14 after immunization. Treatment with TO90 was initiated 2 days before IRBP peptide immunization and continued daily to day 14. ABCA1 protein was robustly increased in normal and EAU mice after administration of TO90, but was unchanged in vehicle-treated EAU mice, suggesting that LXR was activated by oral administration of TO90. The relative expression was normalized to β -actin. Data are mean $\pm$ SEM and are representative of three independent experiments. **P* < 0.05, ***P* < 0.01. Six mice were used in each group.

target gene ABCA1.^{29,30} ABCA1 protein expression was unchanged in EAU animals compared with the naïve mice. However, ABCA1 level was remarkably elevated in TO90-treated naïve and EAU mice, suggesting retinal LXRs were activated by TO90 in a ligand-dependent manner (Fig. 2).

TO90 Ameliorated Clinical and Histological Scores in Both Prevention and Effector Phases of EAU Mice

Compared with vehicle-treated EAU mice, the clinical score was significantly lower at days 12, 14, and 16 in TO90-treated mice in both prevention and effector phases (Fig. 3A, *P* < 0.05). The anterior chamber showed conjunctival hyperemia, hypopyon, and posterior synechiae in the vehicle-treated EAU mice at day 14 (Fig. 3C). No inflammatory signs were seen in the TO90-treated mice (Figs. 3D, 3E).

To evaluate histological scores, eyes were collected at day 14 after immunization. Representative histology images of TO90-treated and vehicle-treated EAU mice are shown in Figures 3F to 3H. In the vehicle-treated EAU mice, retinal folds and inflammatory cells were seen at day 14 (Fig. 3F). TO90 treatment significantly reduced the histological damages (Figs. 3G, 3H). Histological severity was significantly milder in both prevention and effector phases in the TO90-treated EAU mice than in the vehicle-treated EAU mice (Fig. 3B; *P* < 0.05).

TO90 Downregulated Inflammatory Gene Expression in EAU Mice

We tested the gene expressions of proinflammatory cytokines IL-1 β , TNF- α , IL-6, MCP-1, IFN- γ , and IL-17, which are representative cytokines in EAU mice.³¹ Samples were collected

FIGURE 3. TO90 reduced the severity of clinical and histological scores in EAU mice in both prevention and effector phases. TO90 or vehicle was administered orally starting from day 2 before to day 14 after IRBP peptide immunization as the prevention phase, and from day 8 to day 14 as the effector phase. (A) Clinical scores were assessed with a slit lamp from day 7 to day 21 after immunization. The severity of clinical signs was remarkably relieved on days 12, 14, and 16 in the two phases in TO90-treated EAU mice. (Controls: $n = 6$ to 14; TO90 50 mg/kg: $n = 8$ to 14). (B) Histological scores in EAU mice were assessed on sections stained with hematoxylin and eosin obtained at day 14 after immunization. Retinal damage was ameliorated in TO90-treated EAU mice in both the prevention and effector phases ($n = 4$ –5). Representative anterior segment pictures and histological images of vehicle-treated (C, F) and TO90-treated (D, E, G, H) EAU mice at day 14 after immunization. Corneal edema, conjunctival hyperemia, hypopyon and posterior synechiae (C), inflammatory cellular infiltration, and retinal folds (F) were evident in vehicle-treated EAU mice, but not in TO90-treated groups. Data are shown as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Results are combined from two separate experiments.

at day 14 after immunization. The retinas of TO90-treated EAU mice showed significantly lower levels of IL-1 β , TNF- α , IL-6, MCP-1, IFN- γ , and IL-17 than those of the vehicle-treated EAU retinas (Fig. 4). TO90 treatment downregulated the expression of these proinflammatory cytokine genes in situ.

TO90 Downregulated NF- κ B p65 in EAU Mice

NF- κ B activation is involved in the inflammation response of EAU and NF- κ B inhibitors reduce inflammation in EAU.^{22,32} We investigated whether the effect of TO90 was associated with the NF- κ B pathway. The protein and gene expressions of NF- κ B subunit p65 were determined at day 14 after IRBP administration. Our results showed that TO90 downregulated the transcriptional activity of nuclear p65 in EAU mice (Fig. 5; $P < 0.01$).

TO90 Suppressed the Proliferation of Lymphocytes Specifically

We studied whether TO90 specifically inhibited IRBP peptide-induced immune response. Lymphocytes from spleens and draining lymph nodes were isolated and incubated for 72 hours in vitro with IRBP_{161–180} peptide, Con A (positive control), or medium alone (negative control), respectively. The proliferation of lymphocytes was assayed. We found that lymphocyte proliferation was similar in TO90- and vehicle-treated EAU mice when incubated with Con A. Lower proliferation of lymphocytes was observed in the TO90-treated group than the control group when exposed to IRBP_{161–180} peptide ($P < 0.05$) (Fig. 6A). Lymphocytes from the two groups did not show a detectable proliferation when cultured with medium alone.

FIGURE 4. TO90 reduced the expression of inflammatory cytokines in EAU mouse retina. Retinal mRNA was isolated from TO90- and vehicle-treated normal and EAU mice at day 14 after an IRBP peptide immunization. TO90 treatment was initiated 2 days before immunization and continued for 16 days. Real-time PCR showed decrease of inflammatory cytokines (TNF- α , IL-1 β , IL-6, MCP-1, IFN- γ , and IL-17) in TO90-treated EAU mice. The relative gene expression was normalized to GAPDH. Data are mean $\pm$ SEM and are representative of three to four independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001. Six mice were used in each group.

FIGURE 5. TO90 inhibited NF- κ B expression in EAU mouse retina. TO90 treatment was initiated 2 days before IRBP peptide immunization and continued for 16 days. Western blotting and real-time PCR showed NF- κ B subunit p65 was significantly decreased in TO90-treated mice. The relative expressions of mRNA and protein were normalized to GAPDH and β -actin, respectively. Data are mean $\pm$ SEM and are representative of three independent experiments. * P < 0.05, ** P < 0.01. Six mice were used in each group.

FIGURE 6. TO90 inhibited the systemic IRBP-specific immune response and reduced the secretion of several signature inflammatory cytokines in vitro. Lymphocytes from vehicle- and TO90-treated EAU mice were collected on day 14 postimmunization and stimulated with either IRBP peptide or Con A. The proliferative response was measured with MTT assay. TO90 treatment reduced the IRBP-specific proliferative response (A). Cytokine productions of IL-17 and IFN- γ were measured by real-time PCR. The IL-17 (B) and IFN- γ (C) productions were significantly downregulated in TO90-treated EAU mice compared with vehicle-treated animals. The relative expression was normalized to GAPDH. Data are mean $\pm$ SEM and are representative of 3 to 4 independent experiments. * $P < 0.05$. Five mice were used in each group.

TO90 specifically suppressed cellular proliferation induced by IRBP_{161–180} peptide.

TO90 Reduced the Secretion of the Signature Inflammatory Cytokines In Vitro

To examine whether TO90 suppressed immune response on different Th cell subpopulations, lymphocytes from TO90- or vehicle-treated EAU mice were collected at day 14 after immunization and measured by real-time PCR after incubation for 72 hours in vitro with IRBP_{161–180} peptide. The lymphocytes from TO90-treated EAU mice showed significantly lower levels of IL-17 and IFN- γ mRNA than those of the vehicle-treated animals (Figs. 6B, 6C). TO90 treatment suppressed the production of IFN- γ and IL-17 in vitro.

TO90 Reduced the Proportions of Cells Expressing IFN- γ and IL-17 in Lymphocytes in Both Prevention and Effector Phases

Next, we determined whether TO90 inhibited Th1 and Th17 cells in both prevention and effector phases. Lymphocytes were collected from three groups (ie, EAU, EAU plus TO90 treatment from day -2 , and EAU plus TO90 treatment from day 8) and the intracellular expressions of IFN- γ and IL-17 were examined. As shown in Figure 7, the CD4⁺ IFN- γ ⁺ and CD4⁺ IL-17⁺ Th populations were significantly reduced in TO90-treated EAU mice in both prevention and effector phases. Meanwhile,

the number of IFN- γ /IL-17 double-positive cells decreased after TO90 treatment (data not shown).

DISCUSSION

LXRs are physiological regulators of lipid and cholesterol metabolism. Emerging evidence showed that LXRs are negative regulators of inflammatory responses in various tissues including the eye.^{17,24,33,34} The potent anti-inflammatory effect of LXR agonists has been confirmed in inflammatory and autoimmune conditions, including experimental autoimmune encephalomyelitis^{7,35} and collagen-induced arthritis.^{25,36} It was reported recently that the LXR agonist GW3965 improved diabetic retinopathy (DR) and restored inflammatory genes toward nondiabetic levels in mice.³⁴ However, it is unclear whether LXRs are involved in intraocular autoimmune inflammation. Here, we demonstrated LXRs are associated with the pathogenesis of EAU. Furthermore, we found the synthetic LXR agonist TO90 suppressed inflammation in EAU efficiently in both prevention and effector phases. The suppression could be partially related to downregulation of NF- κ B p65.

To our knowledge, the present study is the first to quantitate LXR isoforms LXR α and LXR β in the retina at the protein level. In accordance with a recent immunohistochemistry and gene expression study,³⁷ LXR β is predominantly expressed in the normal mouse retina; however, retinal expressions of LXR β mRNA and protein were unchanged in EAU mice. Nevertheless, we found LXR α mRNA and protein

FIGURE 7. TO90 reduced the proportions of Th1 and Th17 cells expressing IFN- γ , IL-17 in both prevention and effector phases. Lymphocytes from vehicle- and TO90-treated EAU mice were collected on day 14 postimmunization and the frequency of IFN- γ ⁺CD4⁺ and IL-17⁺CD4⁺ T cells were tested by flow cytometry. (A, B) are representative experiments for intracellular expression of IFN- γ and IL-17, respectively. (C, D) are data of four combined independent experiments and presented as the ratio between the percentage of cells expressing IFN- γ and IL-17 among lymphocytes from the three groups. Numbers represent percentage of positive cells. Data are mean $\pm$ SEM. * P < 0.05. Five mice were used in each group.

expressions were significantly elevated in the EAU mouse retina, suggesting that LXR α signaling may be associated with response to inflammatory stimuli in this condition. We provided the first piece of evidence that LXR α expression is induced in retina after a deleterious stimulation.

When an exogenous LXR ligand TO90 was applied to EAU mice, we found the mRNA and protein levels of LXR α and LXR β were unchanged compared with vehicle-treated EAU mice. To identify whether TO90 was functional in EAU mice, we examined the protein expression of an LXR target gene ABCA1 in the retina. We found ABCA1 protein was increased significantly in the TO90-treated EAU mice but not in the vehicle-treated EAU mice. Thus, because TO90 activated the LXR α -ABCA1 axis, the LXR α should be operational. In vehicle-treated EAU mice, although the LXR α level was increased, the ABCA1 was inactive and the proinflammatory cytokines

remained at high levels. Our data confirmed the notion that the anti-inflammatory effect of LXR α is ligand-dependent.³⁸ It was interesting that LXR α expression in EAU retina was increased; however, elevation of LXR α in EAU appeared not functional unless it was activated by its ligand TO90. We presume that increase of LXR α might be a compensatory reaction of the retina in EAU.

The anti-inflammatory action of synthetic LXR agonists can be mediated through either LXR α or LXR β .^{17,18,39} Because LXR β levels were not altered by TO90 in this study, we propose that activation of LXR α plays a major role in anti-inflammation in EAU; however, we cannot exclude the possibility that LXR β also mediated inflammation inhibition in this process. Activation of LXR β is possibly mediated by nuclear translocation and not necessarily by increasing the total LXR β protein level in the retina. Although the total LXR β protein level is not changed,

translocation of the LXR β is still possible to mediate anti-inflammation, or maybe there are other unknown factors. Clearly, further study concerning whether LXR β is functional in EAU is desirable.

Next, we examined the effect of TO90 as an anti-inflammatory agent in suppressing inflammation in EAU. We demonstrated that TO90 administered orally improved the clinical score and ameliorated the pathological manifestations of EAU. NF- κ B plays a key role in inflammatory response in EAU. Activation of the NF- κ B pathway induces various genes that contribute to the inflammatory response, including various cytokines, immune receptors, adhesion molecules, and chemokines.^{22,32,40} The secretion of cytokines and inflammatory mediators is the key characteristic of intraocular inflammatory diseases. TO90 treatment significantly reduced the expression of proinflammatory cytokines, including TNF- α , IL-1 β , IL-6, MCP-1, IFN- γ , and IL-17 in the retina of EAU mice. Moreover, it is established that ligand activation of LXR inhibits inflammatory responses via blockade of NF- κ B signaling in macrophages.^{18,41} We found that NF- κ B p65 expression was suppressed in the retina of TO90-treated EAU mice. Taken together, the suppression of ocular inflammation by TO90 treatment might result, at least in part, from NF- κ B pathway inhibition.

In EAU, the Th1- and Th17-cell response against retinal antigen led to intraocular inflammation of posterior uveitis.⁴² We focused on the T-cell response to investigate the effect of TO90 on EAU. We examined lymphocyte proliferation and cytokine production by stimulation with IRBP₁₆₁₋₁₈₀ peptide. In contrast to a nonspecific T-cell stimulation (Con A), IRBP₁₆₁₋₁₈₀ peptide antigen-mediated T-cell proliferation was significantly suppressed with TO90. The mRNA expression of IFN- γ and IL-17 in lymphocytes was decreased in vitro. Moreover, intracellular cytokine production of Th1 and Th17 after IRBP₁₆₁₋₁₈₀ peptide stimulation was suppressed in TO90-treated EAU mice. TO90 treatment in the effector phase also suppressed the production of IFN- γ and IL-17 in immune EAU mice. These results suggest that TO90 may be promising for the treatment of active (established) uveitis.

LXRs play a role in both inflammation and cholesterol metabolism. It is becoming more important to understand the crosstalk between the two processes in the retina. It is reported that the two pathways share signaling and responder molecules⁴³ and excess intracellular cholesterol may directly promote inflammation.⁴⁴ A large number of cholesterol-related genes and proteins exist in the retina.³⁷ Zheng et al³⁷ proposed that intraocular cholesterol homeostasis could be relatively independent from the rest of the body. In our study, in addition to the transcriptional suppression of NF- κ B by TO90, we showed upregulation of ABCA1, which is involved in modulating cholesterol efflux. The association between cholesterol efflux and anti-inflammation effect in uveitis remains unknown. LXR appears to be a key link between cholesterol metabolism and inflammation, and it has become a research focus in atherosclerosis and diabetes mellitus.^{44,45} Thus, in-depth investigation on LXRs may be an ideal approach to explore the crosstalk between cholesterol metabolism and inflammation.

LXR agonist in DR mice reduced oxidative damage, inhibited inflammation, and enhanced the repaired function of endothelial progenitor cells,³⁴ which may make it a promising new category of drug for retinal diseases. Because LXRs are involved in both metabolism and inflammation, and the LXR agonist TO90 is a nonselective ligand, it may cause systemic side effects, such as increasing triglyceride level.⁴⁶ The anti-inflammatory transrepression of LXRs is hand-in-hand with metabolic transactivation. Clinical trials using an LXR agonist LXR-623 appeared to activate LXR without causing

hepatic lipogenesis but experienced adverse neurological side effects.⁴⁷ Thus, a challenge is to develop subtype-specific, tissue-specific, and dissociating LXR ligands. Dissociating the two functions could make specific transrepression possible and avoid unwanted side effects. In fact, the first synthetic LXR compound with transrepression selectivity has been tested in vitro.⁴⁸

In summary, we have confirmed the existence of LXRs in retina and demonstrated that a synthetic LXR agonist TO90 significantly activated LXR α . TO90 administration suppressed intraocular inflammation in EAU in both prevention and effector phases. LXR α may play a major role in downregulating intraocular inflammation through partially inhibiting the NF- κ B signaling pathway. Our data suggest LXR agonists may become a novel class of therapeutic agents for uveitis.

Acknowledgments

Supported in part by the National Natural Science Foundation of China (81271033), Chongqing Key Laboratory of Ophthalmology (CSTC), National Clinical Key Department Program Fund, and the National Institutes of Health (EY021752).

Disclosure: **H. Yang**, None; **S. Zheng**, None; **Y. Qiu**, None; **Y. Yang**, None; **C. Wang**, None; **P. Yang**, None; **Q. Li**, None; **B. Lei**, None

References

1. Servat JJ, Mears KA, Black EH, Huang JJ. Biological agents for the treatment of uveitis. *Expert Opin Biol Ther*. 2012;12:311–328.
2. Yang P, Fang W, Meng Q, Ren Y, Xing L, Kijlstra A. Clinical features of Chinese patients with Behcet's disease. *Ophthalmology*. 2008;115:312–318.e4.
3. Bar-Yehuda S, Luger D, Ochaion A, et al. Inhibition of experimental auto-immune uveitis by the A3 adenosine receptor agonist CF101. *Int J Mol Med*. 2011;28:727–731.
4. Singh VK, Biswas S, Anand R, Agarwal SS. Experimental autoimmune uveitis as animal model for human posterior uveitis. *Indian J Med Res*. 1998;107:53–67.
5. Luger D, Caspi RR. New perspectives on effector mechanisms in uveitis. *Semin Immunopathol*. 2008;30:135–143.
6. Caspi RR. A look at autoimmunity and inflammation in the eye. *J Clin Invest*. 2010;120:3073–3083.
7. Cui G, Qin X, Wu L, et al. Liver X receptor (LXR) mediates negative regulation of mouse and human Th17 differentiation. *J Clin Invest*. 2011;121:658–670.
8. Solt LA, Kamenecka TM, Burris TP. LXR-mediated inhibition of CD4+ T helper cells. *PLoS One*. 2012;7:e46615.
9. Zelcer N, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. *J Clin Invest*. 2006;116:607–614.
10. Kidani Y, Bensinger SJ. Liver X receptor and peroxisome proliferator-activated receptor as integrators of lipid homeostasis and immunity. *Immunol Rev*. 2012;249:72–83.
11. Repa JJ, Liang G, Ou J, et al. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXR α and LXR β . *Genes Dev*. 2000;14:2819–2830.
12. Walcher D, Kummel A, Kehrle B, et al. LXR activation reduces proinflammatory cytokine expression in human CD4-positive lymphocytes. *Arterioscler Thromb Vasc Biol*. 2006;26:1022–1028.
13. Weichhart T, Haidinger M, Katholnig K, et al. Inhibition of mTOR blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells. *Blood*. 2011;117:4273–4283.

14. Joseph SB, Bradley MN, Castrillo A, et al. LXR-dependent gene expression is important for macrophage survival and the innate immune response. *Cell*. 2004;119:299–309.
15. Bensinger SJ, Bradley MN, Joseph SB, et al. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. *Cell*. 2008;134:97–111.
16. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*. 1996;383:728–731.
17. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med*. 2003;9:213–219.
18. Castrillo A, Joseph SB, Marathe C, Mangelsdorf DJ, Tontonoz P. Liver X receptor-dependent repression of matrix metalloproteinase-9 expression in macrophages. *J Biol Chem*. 2003;278:10443–10449.
19. Wu S, Yin R, Ernest R, et al. Liver X receptors are negative regulators of cardiac hypertrophy via suppressing NF-kappaB signalling. *Cardiovasc Res*. 2009;84:119–126.
20. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med*. 1997;336:1066–1071.
21. Firestein GS. NF-kappaB: Holy Grail for rheumatoid arthritis? *Arthritis Rheum*. 2004;50:2381–2386.
22. Kitamei H, Iwabuchi K, Namba K, et al. Amelioration of experimental autoimmune uveoretinitis (EAU) with an inhibitor of nuclear factor-kappaB (NF-kappaB), pyrrolidine dithiocarbamate. *J Leukoc Biol*. 2006;79:1193–1201.
23. Collins JL, Fivush AM, Watson MA, et al. Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. *J Med Chem*. 2002;45:1963–1966.
24. Schultz JR, Tu H, Luk A, et al. Role of LXRs in control of lipogenesis. *Genes Dev*. 2000;14:2831–2838.
25. Park MC, Kwon YJ, Chung SJ, Park YB, Lee SK. Liver X receptor agonist prevents the evolution of collagen-induced arthritis in mice. *Rheumatology (Oxford)*. 2010;49:882–890.
26. Caspi RR, Roberge FG, Chan CC, et al. A new model of autoimmune disease. Experimental autoimmune uveoretinitis induced in mice with two different retinal antigens. *J Immunol*. 1988;140:1490–1495.
27. Tian L, Lei B, Shao J, Wei L, Kijlstra A, Yang P. AAV2-mediated combined subretinal delivery of IFN-alpha and IL-4 reduces the severity of experimental autoimmune uveoretinitis. *PLoS One*. 2012;7:e37995.
28. Lin T, Qiu Y, Liu Y, Mohan R, Li Q, Lei B. Expression of adiponectin and its receptors in type 1 diabetes mellitus in human and mouse retinas. *Mol Vis*. 2013;19:1769–1778.
29. Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem*. 2000;275:28240–28245.
30. Schwartz K, Lawn RM, Wade DP. ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR. *Biochem Biophys Res Commun*. 2000;274:794–802.
31. Commodaro AG, Bueno V, Belfort R Jr, Rizzo IV. Autoimmune uveitis: the associated proinflammatory molecules and the search for immunoregulation. *Autoimmun Rev*. 2011;10:205–209.
32. Iwata D, Kitaichi N, Miyazaki A, et al. Amelioration of experimental autoimmune uveoretinitis with nuclear factor-kappaB inhibitor dehydroxy methyl epoxyquinomicin in mice. *Invest Ophthalmol Vis Sci*. 2010;51:2077–2084.
33. Jakobsson T, Treuter E, Gustafsson JA, Steffensen KR. Liver X receptor biology and pharmacology: new pathways, challenges and opportunities. *Trends Pharmacol Sci*. 2012;33:394–404.
34. Hazra S, Rasheed A, Bhatwadekar A, et al. Liver X receptor modulates diabetic retinopathy outcome in a mouse model of streptozotocin-induced diabetes. *Diabetes*. 2012;61:3270–3279.
35. Hindinger C, Hinton DR, Kirwin SJ, et al. Liver X receptor activation decreases the severity of experimental autoimmune encephalomyelitis. *J Neurosci Res*. 2006;84:1225–1234.
36. Chintalacharuvu SR, Sandusky GE, Burris TP, Burmer GC, Nagpal S. Liver X receptor is a therapeutic target in collagen-induced arthritis. *Arthritis Rheum*. 2007;56:1365–1367.
37. Zheng W, Reem RE, Omarova S, et al. Spatial distribution of the pathways of cholesterol homeostasis in human retina. *PLoS One*. 2012;7:e37926.
38. Michael DR, Ashlin TG, Buckley ML, Ramji DP. Liver x receptors, atherosclerosis and inflammation. *Curr Atheroscler Rep*. 14:284–293.
39. Zelcer N, Khanlou N, Clare R, et al. Attenuation of neuroinflammation and Alzheimer's disease pathology by liver x receptors. *Proc Natl Acad Sci U S A*. 2007;104:10601–10606.
40. Keino H. Therapeutic effect of the low molecular weight inhibitor of the NF-kappaB signaling pathway on experimental autoimmune uveoretinitis [in Japanese]. *Nihon Ganka Gakkaï Zasshi*. 2010;114:944–954.
41. Terasaka N, Hiroshima A, Ariga A, et al. Liver X receptor agonists inhibit tissue factor expression in macrophages. *FEBS J*. 2005;272:1546–1556.
42. Suzuki J, Yoshimura T, Simeonova M, et al. Aminoimidazole carboxamide ribonucleotide ameliorates experimental autoimmune uveitis. *Invest Ophthalmol Vis Sci*. 2012;53:4158–4169.
43. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. *Nat Rev Immunol*. 2008;8:923–934.
44. Im SS, Osborne TF. Liver x receptors in atherosclerosis and inflammation. *Circ Res*. 2011;108:996–1001.
45. Huang W, Glass CK. Nuclear receptors and inflammation control: molecular mechanisms and pathophysiological relevance. *Arterioscler Thromb Vasc Biol*. 2010;30:1542–1549.
46. Joseph SB, Laffitte BA, Patel PH, et al. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *J Biol Chem*. 2002;277:11019–11025.
47. Katz A, Udata C, Ott E, et al. Safety, pharmacokinetics, and pharmacodynamics of single doses of LXR-623, a novel liver X-receptor agonist, in healthy participants. *J Clin Pharmacol*. 2009;49:643–649.
48. Chao EY, Caravella JA, Watson MA, et al. Structure-guided design of N-phenyl tertiary amines as transrepression-selective liver X receptor modulators with anti-inflammatory activity. *J Med Chem*. 2008;51:5758–5765.