Activation of OX40 Prolongs and Exacerbates Autoimmune Experimental Uveitis

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PURPOSE. T cells are essential for the development of autoimmune uveitis. Although the costimulatory molecule OX40 promotes T-cell function and expansion, it is unclear whether OX40 is implicated in ocular inflammation. The purpose of this study was to examine the role of OX40 in uveitis.

METHODS. Experimental autoimmune uveitis (EAU) was induced in B10.RIII mice by subcutaneous injection of interphotoreceptor retinoid-binding protein peptide 161–180 (IRBP161–180). Some mice received an intravenous administration of OX40-activating antibody on days 0 and 4 after IRBP161–180 sensitization or on days 10 and 14 of uveitis onset. The severity of EAU was evaluated by histology at different time points. In addition, ocular inflammatory cytokine expression was determined by real time-PCR, and peripheral activated CD45CD44CD62L− T cells and IL-7Ra expression were analyzed by flow cytometry. The activated CD45CD44+ lymphocytes were rechallenged with IRBP161–180 in vitro to assess their antigen recall response.

RESULTS. The authors demonstrated a marked OX40 expression by infiltrating lymphocytes in enucleated human eyes with end-stage inflammation. In addition, the administration of OX40-activating antibody prolonged and exacerbated the disease course of EAU. Moreover, activation of OX40 not only increased CD45CD44CD62L− lymphocyte number, it upregulated IL-7Ra expression in the activated T-cell population. Lastly, these cells exhibited a stronger interferon-γ response to IRBP161–180 restimulation in vitro.

CONCLUSIONS. The results reveal a pathogenic role of OX40 in uveitis. Furthermore, the upregulation of IL-7R in CD45CD44+ lymphocytes suggests that the activation of OX40 promotes the generation or expansion of uveitogenic memory T cells.

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Uveitis is a serious ophthalmologic disorder characterized by intraocular inflammation. It is commonly associated with many systemic immune-mediated diseases (e.g., sarcoidosis, ankylosing spondylitis, inflammatory bowel disease). Uveitis has a high prevalence (115.3/100,000) in the United States and is comparable to diabetes as a major cause of visual loss.1,2 Although the etiology of uveitis is multifactorial, CD45+ T lymphocytes play an important role in the pathogenesis of uveitis by recognizing uveitogenic antigen and orchestrating the immune response.3

During T-cell activation, costimulatory molecules provide a pivotal signal to the T-cell response. OX40 (CD134) is a well-recognized costimulatory molecule in the TNF receptor superfamily. It is induced in activated T cells.4,5 By interacting with OX40L on antigen-presenting cells, OX40 triggers the phospho-inositol 3-kinase (PI3K)-AKT signaling pathway, leading to NF-κB translocation.6 Unlike constitutively expressed CD28, which is responsible for the initial T-cell activation, OX40 provides a second wave of costimulation to enhance T-cell effector response, proliferation, and survival.7,8

Many forms of uveitis and autoimmune diseases display a chronic and relapsing clinical course. Both effector and memory T cells contribute to the recurrent inflammatory response in these disorders. After antigen encounter and T-cell receptor activation, T lymphocytes differentiate into subsets with phenotypic and functional distinction. Short-lived effector T cells orchestrate and maximize the immune response, whereas some antigen-experienced T cells become long-lasting memory cells that are responsible for the antigen recall response. Many studies have shown that OX40 promotes the development of effector and memory T cells.9,10 Although OX40 has been involved in a number of clinically common and important autoimmune diseases,10,11 little is known of the role of OX40 in uveitis. Recently, we reported12 that blocking OX40 signaling using anti-OX40 ligand antibody attenuated inflammatory cell infiltration in mouse uveitis models. In addition, the activation of OX40 augmented the effector function of T cells in acute ocular inflammation.12 However, it remains to be further defined whether OX40 is implicated in the pathology of human uveitis and other more completely characterized models, such as experimental autoimmune uveitis (EAU).

In this study, we demonstrated a robust infiltration of OX40+ cells in the human eye with end-stage inflammation. In addition, OX40-activating antibody treatment augments EAU. Furthermore, enhanced OX40 activation in EAU not only expands the CD45CD44CD62L+ T-cell population, it increases IL-7Ra and Bcl-6 expression. Thus, these findings suggest that OX40 may play an instrumental role in the upregulation of activated/memory T cells during the course of ocular inflammation.
METHODS

Mice
Six-week-old B10.RIII mice (Jackson Laboratory, Bar Harbor, ME) were used for the experiments. The animal experimental protocols were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by our institutional animal care and use committee.

Experimental Autoimmune Uveitis
EAU was induced in B10.RIII mice by subcutaneous immunization (near the base of the tail) with 40 μg interphotoreceptor retinoid-binding protein peptide 161–180 (IRBP161–180) (Ser-Gly-Ile-Pro-Tyr-Ile-Ile-Ser-Tyr-Leu-His-Pro-Gly-Asn-Thr-Ile-Leu-His-Val-Asp) (AnaSpec, Fremont, CA) in 200 μL complete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO) with Mycobacterium tuberculosis strain H37RA. The eyes were harvested for histology at different time points during the experiment.

Activation of OX40
Some B10.RIII mice were also treated with OX40-activating antibody (Clone OX86; 100 μg/mouse) by tail vein injection on days 0 and 4 or days 10 and 14 after IRBP161–180 immunization. The OX40-activating antibody was produced in the laboratory of one of the authors (AW) from hybridomas and was affinity purified on protein G columns. This monoclonal antibody is a rat IgG1 that specifically interacts with mouse OX40, leading to the enhancement of T-cell activation and function. Furthermore, this antibody promotes a T-cell response in wild-type mice but not in OX40 knockout animals, suggesting that this agonistic antibody specifically activates OX40.

Cell Culture, Isolation, and Stimulation
After B10.RIII mice were euthanized, their submandibular draining lymph nodes and spleens were removed. Single-cell suspensions were prepared by passing the tissue through a 70-μm cell strainer (BD Biosciences, Mountain View, CA). Splenic red blood cells (RBCs) were lysed with 1× RBC lysis buffer (Sigma-Aldrich, St. Louis, MO) at room temperature for 5 minutes. The cell suspension was washed twice with RPMI 1640 and then cultured in RPMI 1640 with 10% fetal bovine serum (FBS) in an atmosphere of 95% air and 5% CO2 at 37°C with 4 μg/mL IRBP161–180 peptide for 72 hours.

Histology
For histologic evaluation, the eyes were fixed in 3% paraformaldehyde. Then the tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Ocular inflammation was assessed by light microscopy, and the severity of EAU was graded on a four-point scale based on inflammatory cell infiltration, retinal folding, and destruction.

Immunohistochemistry of OX40 Staining
Paraffin-embedded sections of human eye globes were rehydrated and then processed in a pressure cooker for 20 minutes in 1× EDTA/Trip buffer (pH 9.0). After incubation in 3% H2O2 in methanol quench solution for 10 minutes, these slides were stained with 1:100 dilution of isotype IgG or anti-human OX40 antibody (PharMingen, San Diego, CA) for 1 hour at room temperature. Next, the slides were rinsed with Tris-buffered saline with Tween, followed by peroxidase-conjugated anti-mouse IgG (ImmPress Reagent; Vector Laboratories, Burlingame, CA). Finally, diaminobenzidine was added to detect OX40 staining, and the slides were counterstained with hematoxylin.

Flow Cytometry
B10.RIII splenocytes were suspended in phosphate-buffered saline (PBS) containing 2% FBS. Anti-CD4, anti-CD44, anti-CD62L and anti-IL-7R antibodies conjugated with different fluorescent colors (eBioscience, San Diego, CA) were used to label these cell surface markers for 60 minutes on ice. After PBS wash, the cells were fixed with 1× fixation solution at 4°C.

For intracellular staining of IFN-γ, the lymphocytes of submandibular draining lymph nodes and spleen were harvested from IRBP161–180-immunized mice and further cultured with 4 μg/mL IRBP161–180 in vitro for 24 hours. Then these cells were stimulated with phorbol myristate acetate (PMA; 50 ng/mL) and ionomycin (1 μg/mL) for 5 hours. Brefeldin A (1:1000) was added for 2 hours. The cells were collected and stained with fluorescein isothiocyanate-labeled anti-mouse CD4 antibody for 30 minutes. After PBS wash, the cells were fixed and permeabilized overnight with 1× fixation/permeabilization solution (eBioscience) at 4°C. Then they were stained intracellularly with phycoerythrin (PE)-labeled monoclonal antibody against IFN-γ (eBioscience) for 1 hour at 4°C. Data acquisition was performed on a flow cytometer, and data were analyzed using acquisition and analysis software.

ELISA
Fifty microliters of culture media of isolated B10.RIII splenocytes stimulated with IRBP161–180 for 72 hours from various experimental groups were collected for ELISA to measure IFN-γ and IL-17A levels according to the manufacturer’s protocols (BioLegend, San Diego, CA).

Real-Time PCR
Total RNA from cultured CD4+ cells was isolated with a purification kit (RNeasy Mini Kit; Qiagen, Valencia, CA). First-strand cDNA synthesis was accomplished with an oligo (dT)-primed reverse transcriptase kit (Omniscript; Qiagen). Gene-specific cDNA was amplified by PCR using mouse-specific primer pairs (IFN-γ: sense 5′-TCA AGT GCCGATA GAT GTG GAA GAA-3′ and antisense 5′-TGG CTC TGC AGG ATT TTC ATG-3′; IL-17A: sense 5′-TGTC GGG GCT ACA GTG AAG GCA-3′ and antisense 5′-GAC AAT CGA GCC CAG CCA GGT-3′; Bcl-6: sense 5′-TCA GAG TAT TGCT GAT TCT AGC TGG CTA-3′ and antisense 5′-TGG ATG CAG TGG TTG CTG CTG-3′; Il10: sense 5′-ACA GAG GCC GAG TTT GAA GAG A-3′ and antisense 5′-AGG AGT GCC TGG GTC GTA A-3′; B-cell lymphoma 6; sense 5′-ATG CCA ACA CAG TGC TGT CT-3′ and antisense 5′-AAG CAC TTG GGG TGC AG-3′). Real-time PCR was performed using a master mix (RT2 Real-Time PCR Master Mix; SABiosciences, Frederick, MD), running for 40 cycles at 95°C for 15 seconds and at 55°C for 30 seconds. The mRNA levels of investigated genes in each sample were normalized to β-actin mRNA and quantified using the following formula: 2 [(Ct/β-actin – Ct/gene of testing gene)]. The result was expressed as the fold difference in the groups stimulated with OX40-activating antibody compared with the group without additional OX40 activation.

Statistical Analysis
Data are expressed as the average ± SD. For EAU scoring, the median difference between control and experimental groups was compared using the Mann-Whitney U test. Other statistical probabilities were evaluated by Student’s t-test or ANOVA, with a value of \( P < 0.05 \) considered significant.

RESULTS

Expression of OX40 in Human Nonseeing Eyes with Chronic End-Stage Inflammation
One reason OX40 has not been extensively studied in uveitis is likely because of the limited availability of human eye tissue with inflammation. To investigate whether OX40 is involved in human ocular inflammation, we recently acquired four surgical specimens of human nonseeing eyes with chronic end-stage inflammation. The demographic information (age and sex) and diagnoses of these patients are summarized in Table 1. After
confirming marked lymphocytic inflammation in the anterior and posterior segments by histology, we performed immunohistochemistry staining to examine OX40 expression in these eye specimens. As illustrated in representative tissue (Fig. 1A), intense infiltration of lymphocytes was present in the ciliary body region, and a large percentage of these cells strongly expressed OX40. In addition, clusters of OX40+ lymphocytes were observed in the choroid in these human eye specimens. The expression of OX40 within the diseased human eye was prominent, consistent with the potential clinical importance and relevance of studying OX40 in ocular inflammation.

Exacerbation of EAU by OX40 Activating Antibody

In light of this finding, we used the B10.RIII EAU model to further characterize the role of OX40 in uveitis. Recent research14,15 has linked OX40L polymorphism to susceptibility to systemic lupus erythematosus and atherosclerosis. We postulated that enhanced OX40 function by aberrant OX40L engagement or stimulation contributes to inflammation in the eye. OX40-activating antibody has been widely used in OX40 research.16,17 This approach is especially helpful to mimic the gain-in-function change of OX40 signaling in many pathologic conditions. Therefore, we asked whether enhancement of OX40 activation would exacerbate ocular inflammation primarily by augmenting antigen sensitization or amplifying effector lymphocyte function.

To this end, we first compared the severity of EAU between the groups with and without further OX40 activation. Some B10.RIII mice received 100 \( \mu g \) OX40-activating antibody (OX86) by tail vein injection on days 0 and 4 of IRBP161–180 immunization. EAU (inflammatory cell infiltration, vasculitis, retinal folding, and destruction) was scored on days 14 and 21, respectively. As shown in Figure 2, the mice developed marked ocular inflammation in response to IRBP161–180 priming, and the uveitis receded on day 21 in the control group without further OX40 stimulation. However, the mice treated with OX40-activating antibody exhibited persistent and severe posterior uveitis on day 21 (Fig. 2). This result suggested that activation of OX40 enhances and prolongs the ocular immune response to antigen challenge.

Next, we asked whether further activation of OX40 during the onset of EAU could affect the outcome of ocular inflammation. Thus, we treated B10.RIII mice with 100 \( \mu g \) OX40-activating antibody during IRBP161–180 sensitization (days 0 and 4) or at the time of disease onset (days 10 and 14), and EAU was assessed on day 25 (10 days after the completion of OX40-activating antibody treatment during EAU onset). Compared with controls with untreated EAU, the activation of OX40 early after IRBP161–180 sensitization (days 0 and 4) or in the early disease

**Table 1. Patient Demographic Information**

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<td>Sympathetic ophthalmia</td>
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Figure 1. OX40 expression in an enucleated human eye with end-stage uveitis. Paraffin-embedded specimen was stained with isotype IgG and anti-human OX40 antibody. (A) Invasion of numerous OX40+ lymphocytes (brown) in the rim of the ciliary body. (B) Clusters of OX40+ lymphocytes (arrows) in the choroid (representative image of four patients).

Figure 2. OX40-activating antibody treatment prolongs and exacerbates EAU in B10.RIII mice with IRBP161–180 peptide. OX40-activating antibody (100 \( \mu g \)/mouse) was administered intravenously during IRBP161–180 sensitization (days 0 and 4). The mice were euthanized on days 14 and 21, respectively. Eyes were harvested at these time points for histologic EAU evaluation.
expression. Three weeks after IRBP161–180 priming, T-cell activation during IRBP161–180 sensitization significantly increased the CD4\(^+\)CD44\(^+\)IL-7R\(^+\) T cells, and the cellular response to IL-7 is significantly influenced by IL-7R expression.\(^{31}\) It has been shown that the surface level of IL-7R is downregulated when naive T cells are activated and IL-7R reappears in the lymphocytes that commit to memory lineage.\(^{29}\) The expression of IL-7R enhances memory T-cell survival.\(^{21}\) To study the effect of OX40 on memory T cells in uveitis, we examined whether OX40 activation affects IL-7R expression in naive CD4\(^+\)CD44\(^+\) and activated CD4\(^+\)CD44\(^+\) T cells. Flow cytometry showed that control EAU mice had an average of 9.28% IL-7R\(^+\) cells in splenic CD4\(^+\)CD44\(^+\) lymphocytes. The OX40-activating antibody administered on days 0 and 4 or at uveitis onset augmented IL-7R\(^+\) cells to 12.81% and 14.84%, respectively (Fig. 6A). Nevertheless, the activation of OX40 did not increase IL-7R expression in the CD4\(^+\)CD44\(^+\) population (Fig. 6A). In addition, the mean fluorescence intensity (MFI) of IL-7R expressed by the

**Figure 3.** OX40-activating antibody treatment at disease onset exacerbates EAU in B10.RIII mice with IRBP\(_{161-180}\) peptide. OX40-activating antibody (100 \(\mu\)g/mouse) was administered intravenously during IRBP\(_{161-180}\) sensitization (days 0 and 4) or at EAU onset (days 10 and 14) after IRBP\(_{161-180}\) immunization. On day 25, the mice were euthanized. Eyes were harvested for histologic EAU evaluation (A). Representative histology of OX40-activating antibody-enhanced EAU from 7 mice: (B) Severe retinal damage, retro-retinal separation, and hemorrhage (closed arrows). (C) Retinal vasculitis (open arrows). (D) Inflammatory infiltrates in the choroid (arrowheads).


CD4+CD44+ T cells was compared between the control group and the group treated with OX40 antibody. Treatment with OX40-activating antibody during antigen sensitization or at uveitis onset showed an increase in IL-7Rα MFI by 14.68% ± 8.57% and 21.58% ± 9.48%, respectively. When we restimulated these lymphocytes with IRBP161–180 in vitro, the total IFN-γ production was significantly higher in the splenocytes from OX40-activating antibody-treated mice than in the control group (Fig. 6B). However, it was unclear whether the augmented cytokine expression was caused by the increase in total number of IRBP-reactive lymphocytes, cytokine production in single cells, or both. To address this question, we examined intracellular IFN-γ expression by flow cytometry. Splenocytes were harvested from EAU mice with and without OX40-activating antibody treatment during IRBP sensitization. These cells were further cultured with IRBP for an additional 36 hours, followed by PMA and ionomycin stimulation. Then intracellular IFN-γ production was analyzed in CD4+ lymphocytes. Compared with control EAU mice, we found a minimal increase of IFN-γ expression per individual CD4+ T cells in the splenocytes of the animals that received OX40-activating antibody in vivo. The MFI of intracellular IFN-γ in control and OX40-activating antibody-treated groups was 138.05 ± 2.09 and 143.73 ± 1.55, respectively. This result suggested that the augmented inflammatory cytokine expression was primarily caused by expansion of the IRBP-reactive CD4+ T-cell population. Together, these data indicate that OX40 promotes the development and expansion of uveitogenic memory lymphocytes.

Recent studies22–24 have shown that decreased Blimp-1 and increased Bcl-6 transcription regulators are instrumental in the generation of memory CD4+ precursors, respectively. To further correlate OX40 activation with the development of memory lymphocytes during uveitis, we examined the ocular transcription of IL-7Rα, Bcl-6, and Blimp-1, markers associated with memory T-cell differentiation in EAU (Fig. 7). Compared with control mice, the ocular expression of IL-7Rα and Bcl-6 tended to be enhanced in both OX40-activating antibody-treated groups regardless of the timing of OX40 activation (Fig. 7), and the increase of ocular Bcl-6 transcription became statistically significant in the mice that received OX40 activation during IRBP sensitization (P < 0.05). In contrast, a reduction of Blimp-1 transcription (41.07 ± 16.28-fold) occurred in the eyes of mice that received OX40-activating antibody during IRBP priming. Activation of OX40 at EAU onset did not suppress Blimp-1 expression (data not shown), indicating that OX40 signaling influences Blimp-1 regulation in a time-dependent manner. Thus, this result suggests that further activation of OX40 may skew the memory T-cell response in the process of uveitis development by regulating the expression of IL-7R, Bcl-6, and Blimp-1.

**FIGURE 6.** OX40-activating antibody treatment augments IL-7Rα expression of peripheral CD4+CD44+ T cells and their response to IRBP161-180 restimulation. EAU and OX40-activating antibody treatment during IRBP161-180 sensitization or at uveitis onset are described. Three weeks later, the splenocytes were harvested and labeled with anti-CD4, anti-CD44, and anti-IL-7Rα antibodies. (A) IL-7Rα expression in the CD4+CD44+ population was analyzed by flow cytometry (n = 7 mice/group). *P < 0.05. (B) Stimulation of splenocytes in vitro with IRBP161-180 (4 μg/mL) for 3 days. The IFN-γ level in the culture media was measured by ELISA (n = 7 mice/group). *P < 0.05.

**DISCUSSION**

In this study, we implicate OX40 in the severe ocular inflammation of human patients. Moreover, further activation of OX40 significantly exacerbates the severity of EAU. In addition to expanding activated T cells, OX40 can potentially exert its immunologic impact on memory T cells through the signaling of IL-7R, Bcl6, and Blimp-1.
OX40 is a key costimulatory molecule that is expressed 24 hours after T-cell activation. It has been shown to enhance effector lymphocyte function and to promote memory T-cell development.5-6,9-10 In the B10.RIII EAU model, we found that activation of OX40 during the IRBP<sub>161–180</sub> priming phase or at disease onset markedly augments ocular inflammation. This suggests that OX40 not only boosts the antigen priming process but also ameliorates the pathologic T-cell response.

It has been shown that both activated effector T cells and Treg express OX40.25 In contrast to our observations, Weinberg et al.17 recently reported that OX40-activating antibody ameliorates experimental autoimmune encephalomyelopathy by expanding Treg numbers during the antigen-sensitization period before the disease onset. We also observed a potentially unique effect of OX40 in the pathogenesis of uveitis. Activation of OX40 at the time of IRBP<sub>161–180</sub> immunization markedly extended the disease course of EAU. These findings suggest that aberrant OX40 signaling in uveitis may augment the effector function and longevity of uveitogenic T cells.

Effector CD4<sup>+</sup> T cells can differentiate to Th1, Th2, and Th17 subsets on the basis of distinctive transcription factor and cytokine expression and function. These unique T-cell subsets undertake special immunologic tasks and responsibilities. Adding to the complexity of our immune system, some T cells are found to coexpress cytokines representative of more than one subset. Although we simplistically conceptualize that one distinctive T-cell subset mediates one particular disease, in reality multiple T-cell lineages are often involved in uveitis and other disease processes. Recently, Caspi et al.31 demonstrated that Th1 and Th17 cells are each capable of inducing EAU, depending on different antigen stimulation conditions. OX40 has been shown to promote Th1 and Th2 differentiation.26-27 We have recently reported20 that OX40 also augments Th17 effector function. In this study, we demonstrated that the activation of OX40 enhances the ocular expression of mRNA for IFN-γ and IL-17 in EAU, which suggests that OX40 promiscuously activates different T-cell subsets during inflammation.

After antigen encounter, some activated T cells become long-lasting memory cells that are responsible for the antigen recall response. Both effector and memory T cells contribute to the chronic and relapsing course of uveitis. Consistent with recent published studies,28-29 we found that OX40 agonistic antibody treatment significantly expands CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup> lymphocytes in the EAU model. In addition, the stimulation of OX40 increases IL-7R<sub>α</sub> expression in this activated T-cell population. IL-7 is a common γc cytokine that plays an indispensable role in memory T-cell development. IL-7 enhances antiapoptotic gene Bcl-2 expression and inhibits proapoptotic factors BAX and BAD.30-31 In addition, the cellular response to IL-7 is regulated by the expression of IL-7R. IL-7R<sub>α</sub> and the γc chain subunit. Distinct from other γc chain cytokine receptors that are upregulated in activated effector T cells, IL-7R is primarily expressed by naive and memory lymphocytes, suggesting its critical role in supporting these two T-cell populations. Indeed, studies32-33 have demonstrated that OX40 not only boosts the antigen priming process but also ameliorates the pathologic T-cell response.

In addition to unique cytokine milieus, T-cell differentiation requires intrinsic signals from master transcription factors. Bcl-6 and Blimp-1 are reciprocal transcription factors that play key roles in determining lymphocyte destiny.34 They were initially found to regulate B- and T follicle helper cell differentiation. However, the latest studies22-24 demonstrate that Bcl-6 and Blimp-1 ubiquitously control the development of effector and memory CD4<sup>+</sup> T cells. Bcl-6 promotes memory T-cell development, whereas Blimp-1 enhances effector T-cell proliferation and function. In addition, γc cytokines have been shown to induce the expression of Bcl-6 and Blimp-1.35 Here, we have shown that the activation of OX40 results in a reciprocal change of Bcl-6 and Blimp-1.35,36 Here, we have shown that the activation of OX40 results in a reciprocal change of Bcl-6 and Blimp-1. This provides a rationale to further study how OX40 regulates memory T-cell development.

In summary, this study underscores the role of OX40 in the pathogenesis of uveitis. It also implicates OX40 in the development of uveitogenic memory T cells. Although OX40 could directly upregulate IL-7R and Bcl-6 to facilitate the generation of memory lymphocytes, at this time we cannot exclude the possibility that the increase of IL-7R and Bcl-6 levels is secondary to the expansion of memory T cells that express these molecules. This provides a rationale to further study how OX40 regulates memory T-cell development. Further research in this field is important not only for understanding the molecular mechanism of T-cell regulation by OX40 but also for identifying downstream therapeutic targets of OX40 signaling to treat uveitis.

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**References**


