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 CD4+CD44+CD62L− T cells and IL-7Rα expression were analyzed by flow cytometry. The activated CD4+CD44+ 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 CD4+CD44+CD62L− lymphocyte number, it upregulated IL-7Rα 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 CD4+CD44+ lymphocytes suggests that the activation of OX40 promotes the generation or expansion of uveitogenic memory T cells. (Invest Ophthalmol Vis Sci. 2011;52:8520–8526) DOI: 10.1167/iovs.11-7664

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, CD4+ 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 CD4+CD44+CD62L− T-cell population, it increases IL-7Rα 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 female 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) 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 0 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.13 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 OX40.4

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.13

Immunohistochemistry of OX40 Staining

Paraffin-embedded sections of human eye globes were dehydrated and then steam in pressure cooker for 20 minutes in 1× EDTA/Tri 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 mouse-specific primer pairs (IFN-γ sense 5'-TCA AGT GGC CAC GCA GGT-3' and antisense 5'-ATG CCA ACA CAG TGC TGT CT-3'; IL-17A sense 5'-TCA AGT GGC CAC GCA GGT-3' and antisense 5'-ATG CCA ACA CAG TGC TGT CT-3'; Blimp-1 sense 5'-GAC AAT CGA GGC CAC GCA GGT-3' and antisense 5'-GAC AAT CGA GGC CAC GCA GGT-3'; Bcl-6 sense 5'-TCA GAG TAT GCT GAT TCT AGC GA-3' and antisense 5'-ATG TGC CGG GGC CAC GCA GGT-3'; Bcl-6 sense 5'-GAC AAT CGA GGC CAC GCA GGT-3' and antisense 5'-GAC AAT CGA GGC CAC GCA GGT-3'; Bcl-6 sense 5'-GAC AAT CGA GGC CAC GCA GGT-3'; Bcl-6 sense 5'-GAC AAT CGA GGC CAC GCA GGT-3'; Bcl-6 sense 5'-GAC AAT CGA GGC CAC GCA GGT-3'; Bcl-6 sense 5'-GAC AAT CGA GGC CAC GCA GGT-3'). Real-time PCR was performed using a master mix (RT² Real-Time PCR Master Mix; SA Biosciences, Frederick, MD), running for 40 cycles at 95°C for 15 seconds and at 55°C for 40 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 research 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. 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 g OX40-activating antibody (OX86) by tail vein injection on days 0 and 4 of IRBP 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 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

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933458/)

**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](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933458/)

**Figure 2.** OX40-activating antibody treatment prolongs and exacerbates EAU in B10.RIII mice with IRBP161–180 peptide. OX40-activating antibody (100 µg/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.

**Table 1.** Patient Demographic Information

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stage resulted in exacerbated and protracted EAU (Fig. 3A). In either OX40-activating antibody-treated group, the mice consistently exhibited more severe retinal destruction, vasculitis, and marked retro-retinal hemorrhage/separation (Figs. 3B, 3C). In addition, more severe chorioid inflammation was seen in the mice treated with OX40-activating antibody (Fig. 3D).

The fact that treatment with OX40-activating antibody at disease onset augmented EAU suggested that stimulation of OX40 also enhances effector T-cell function. Recent studies have shown that Th1 and Th17 T cells are both capable of inducing EAU. To further characterize OX40-enhanced uveitis, we examined the impact of OX40 activation on ocular IFN-γ and IL-17A transcript expression. Total RNA from whole eye was isolated on day 25 after EAU induction. Real time-PCR revealed a marked increase of IFN-γ and IL-17A transcripts in the group that received OX40-activating antibody during IRBP161–180 sensitization or uveitis onset compared with the control group with EAU (Fig. 4).

Increase of CD4+CD44+IL-7Rα+ T Cells by OX40 Activating Antibody

Previously, we showed that the activation of OX40 enhanced effector T-cell function in the ovalbumin-induced acute uveitis model. In this study, we investigated whether the stimulation of OX40 could expand activated T cells while exacerbating EAU severity and augmenting ocular inflammatory cytokine expression. Three weeks after IRBP161–180 priming, T-cell activation markers CD44 and CD62L of splenic CD4+ T lymphocytes were analyzed by flow cytometry in the mice treated with and without OX40-activating antibody. As illustrated in Figure 5, the activation of OX40 during IRBP161–180 sensitization significantly increased the CD4+CD44+CD62L+ population.

Given that uveitis often displays a chronic and recurrent clinical course, we asked whether OX40 also promotes memory T-cell development in EAU. IL-7 is essential to the long-term survival of naive and memory CD4+ T cells, and the cellular response to IL-7 is significantly influenced by IL-7R expression. 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. The expression of IL-7R enhances memory T-cell survival. 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).

In the present study, the mean fluorescence intensity (MFI) of IL-7R in EAU with OX40-activating antibody treatment was higher than in the control group (Fig. 6C). It has been shown that the surface IL-7R is increased in activated T cells. The increase in IL-7R expression in these cells suggests that OX40-activating antibody treatment enhances the activation of T cells, which could contribute to the exacerbation of uveitis.
**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, Bcl-6, 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.\textsuperscript{5,6,9,10} In the B10.RIII EAU model, we found that activation of OX40 during the IRBP\textsubscript{161–180} priming phase or at disease onset markedly augments ocular inflammation. This suggests that OX40 not only boosts the antigen priming process but also amplifies the pathologic T-cell response. It has been shown that both activated effector T cells and Treg express OX40.\textsuperscript{25} In contrast to our observations, Weinberg et al.\textsuperscript{17} recently reported that OX40-activating antibody ameliorates experimental autoimmune encephalomyelitis 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\textsubscript{161–180} 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\textsuperscript{+} 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.\textsuperscript{18} 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.\textsuperscript{26,27} We have recently reported\textsuperscript{12} 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-\gamma 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,\textsuperscript{28,29} we found that OX40 agonistic antibody treatment significantly expands CD4\textsuperscript{+}CD62L\textsuperscript{−}CD27\textsuperscript{−} lymphocytes in the EAU model. In addition, the stimulation of OX40 increases IL-7R\alpha expression in this activated T-cell population. IL-7 is a common \( \gamma \) (\( \gamma \)c) 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.\textsuperscript{30,31} In addition, the cellular response to IL-7 is regulated by the expression of IL-7R. IL-7R consists of IL-7R\alpha and the \( \gamma \)c chain subunit. Distinct from other \( \gamma \)c 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, studies\textsuperscript{32,33} have demonstrated the dependence of memory T-cell survival on IL-7 and IL-7R. Our study has shown that OX40 primarily upregulates IL-7R\alpha in CD4\textsuperscript{+}CD44\textsuperscript{+} T cells, suggesting that these activated lymphocytes become memory T cells or memory precursors.

In addition to unique cytokine milieu, T-cell differentiation requires intrinsic signals from master transcription factors. Bcl-6 and Bclmp-1 are reciprocal transcription factors that play key roles in determining lymphocyte destiny.\textsuperscript{34} They were initially found to regulate B- and T-follicle helper cell differentiation. However, the latest studies\textsuperscript{22,23} demonstrate that Bcl-6 and Bclmp-1 ubiquitously control the development of effector and memory CD4\textsuperscript{+} T cells. Bcl-6 promotes memory T-cell development, whereas Bclmp-1 enhances effector T-cell proliferation and function. In addition, \( \gamma \)c cytokines have been shown to induce the expression of Bcl-6 and Bclmp-1.\textsuperscript{35} Here, we have shown that the activation of OX40 results in a reciprocal change of Bcl-6 and Bclmp-1 in the eyes of the mice developing EAU, thus further supporting the notion that OX40 promotes memory T-cell development in uveitis.

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**
