Effects of Systemic and Intravitreal TNF-α Inhibition in Experimental Autoimmune Uveoretinitis

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PURPOSE. To investigate the effect of systemic or local TNF-α inhibition with etanercept on experimental autoimmune uveoretinitis (EAU).

METHODS. EAU was induced by immunizing B10.RIII mice with IRBPp161-180 or by adoptively transferring uveitogenic splenocytes. Mice received systemic or local treatment with etanercept in the afferent or efferent phase. For systemic treatment, mice were injected intraperitoneally. For local treatment, etanercept was injected intravitreally or subconjunctivally. Control mice received PBS. EAU scores were determined histologically. Splenic cells were assessed for [3H]thymidine incorporation. ELISA was performed to measure levels of cytokines produced by splenocytes. Vitreous cavity-associated immune deviation (VCAID) was induced by intravitreally injecting ovalbumin and evaluated by measuring DTH reaction.

RESULTS. After systemic treatment with etanercept in the afferent phase, EAU disease scores, IRBP-specific cell proliferation, and production of Th1, Th2, and Th17 cytokines were reduced. EAU also improved after intravitreal etanercept treatment in the afferent phase, with unaltered IRBP-specific proliferation, reduced IFN-γ, but increased IL6 and IL-10 secretion. VCAID induction was impaired after intravitreal etanercept treatment. No amelioration of EAU or reduction in IRBP-specific cell response was found after systemic or intravitreal treatment in the efferent phase or after subconjunctival treatment. After adoptive transfer, etanercept- and PBS-treated recipients showed similar disease severity and antigen-specific proliferation of splenocytes.

CONCLUSIONS. It can be concluded that TNF-α participates mainly in the immunopathology in the induction phase of EAU. The mechanism of action underlying EAU improvement may be different for local and systemic etanercept treatment. (Invest Ophthalmol Vis Sci. 2013;54:39–46) DOI:10.1167/iows.12-10138

Experimental autoimmune uveoretinitis (EAU) is an animal model for human posterior uveitis that can be induced by immunizing mice with the retinal antigen interphotoreceptor retinoid-binding protein (IRBP) or its peptides. It is a T cell-mediated disease, which predominantly affects the posterior segment of the eye and closely resembles the histopathological characteristics and clinical manifestations of human posterior uveitis,1–3 which is a common cause of visual loss.

Numerous investigations have indicated a significant role of TNF-α during the course of EAU. TNF-α is produced by several types of immune and inflammatory cells such as monocytes, macrophages, neutrophils, mast cells, NK-cells, or T cells. In EAU, TNF-α acts as an important inflammatory mediator and plays a key role in initiating and maintaining the inflammatory processes by orchestrating leukocyte infiltration, dendritic cell maturation, and macrophage activation and driving Th1 cell responses.5–6 It has been shown that intraocular levels and expression of TNF-α are elevated during the course of EAU.7,8 Moreover, not only is TNF-α expression increased in inflammatory cell infiltrates, but also in resident retinal cells such as microglia, Muller cells, and RPE cells.9–11 Thus, the extent of TNF-α production by resident retinal cells could influence susceptibility to EAU.10

IRBP-immunized mice that were receiving injections of recombinant human TNF showed marked increases in incidence and severity of EAU and immune responses to IRBP12. Neutralizing TNF-α systemically had a suppressive effect on the severity and incidence of EAU13–16 and delayed the onset of inflammation.15,16 Consequently, TNF-α represents a promising target for intraocular immune intervention.

Etanercept is a p75 TNF receptor/Fc fusion protein and has already been approved in the United States and Europe for the treatment of several autoimmune diseases (e.g., rheumatoid arthritis).17,18 Etanercept has also been shown to be moderately effective in treating patients with uveitis.19,20 In experimental studies, etanercept binds mouse TNF with an affinity comparable to that of human TNF,17 and systemic administration of etanercept impaired the course of various diseases in mice.21–25

However, adverse effects of systemic TNF-α inhibition may develop, such as infections or even malignant diseases.24–27 To avoid such side effects, local treatment of the eye may be beneficial.

The aim of the present experimental study was to investigate whether local TNF-α inhibition with etanercept improves EAU and whether the treatment also induces systemic immune modulation.

MATERIALS AND METHODS

All experimental procedures are in line with the ARVO statement on the use of animals in ophthalmology and vision research.

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**Animals and Immunization**

B10.RIII mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were immunized subcutaneously with 100 μg human IRBP peptide 161-180 (IRBPp161-180) (EMC Microcollections, Tuebingen, Germany) in emulsion with complete Freund’s adjuvant (1:1 vol/vol) (total volume 200 μL). In addition, mice received 0.4 to 0.5 μg pertussis toxin (both Sigma-Aldrich, Taufkirchen, Germany) intraperitoneally.

**Treatment of Mice with Etanercept**

Mice were injected intraperitoneally (systemic treatment) with 200 μg of etanercept (Wyeth Pharmaceuticals, Hampshire, UK). For local treatment, mice were injected unilaterally. Here, 50 μg/2 μL etanercept were injected intravitreally (intraocular treatment) into the left eye using a 10-μL syringe (Hamilton, Bonaduz, Switzerland) and a 33-G needle (Hamilton). For subconjunctival treatment of the right eye, 200 μg/30 μL etanercept was injected using a 100-μL syringe (Hamilton) and a 30-G needle. Systemic and local treatments were performed in the afferent or efferent phase (Table 1). Controls received equivalent volumes of PBS.

**Histology**

Sections from paraffin-embedded eyes (7 μm) were stained with hematoxylin-eosin, and the histopathological score was evaluated according to the number, type, and extent of lesions and the infiltration of inflammatory cells on a scale from 0 to 4.1,28

**Cell Culture, Proliferation Assay, and Cytokine ELISA**

Single-cell preparations from spleens were harvested on day 21 postimmunization (p.i.). After mechanical disruption, erythrocytes were removed by lysis, and cells were suspended in RPMI 1640 medium (Biochrom, Berlin, Germany) with 10% fetal calf serum (PAA, Pasching, Austria), 2% HEPES/mercaptoethanol, and 1% ciprofloxacin (Fresenius, Bad Homburg, Germany). Cells were stimulated with 10 μg/mL IRBPp161-180 or incubated with medium only at 37°C and 5% CO₂. For the proliferation assay, 1.2 × 10⁵ cells were seeded on a 96-well plate at a final volume of 180 μL/well and incubated for 72 hours. For another 18 hours, cells were pulsed with 1 μCi/well of [³H]-thymidine. [³H]-thymidine uptake was measured by liquid scintillation counting.

To determine the concentration of IFN-γ, IL-2, IL-6 (Becton Dickinson, Heidelberg, Germany), and IL-17 (R&D Systems, Wiesbaden, Germany) in cell culture supernatants (collected after 24 hours) by using ELISA, 2.5 × 10⁵ cells were incubated on a 24-well plate at a final volume of 500 μL. ELISA procedures were performed according to the manufacturer’s instructions.

To assess the antigen-specific response of splenocytes in the present study, baseline proliferation and cytokine production were subtracted respectively.

**EAU Induction by Adaptive Transfer**

Donor mice were immunized as described above. On day 14 after immunization, single-cell suspensions of spleens and lymph nodes were stimulated with 20 μg/mL IRBPp161-180 and cultured as described by Agarwal and Gaspi29 for 72 hours. After purifying cells by Ficoll density gradient centrifugation, 2 × 10⁷/200 μL cells were injected intraperitoneally into recipient mice.29 Recipient mice were then treated systemically by injecting 200 μg etanercept intraperitoneally on days −1, 1, 3, and 5 after adoptive transfer. On day 15 after adoptive transfer, eyes and spleens were collected to evaluate EAU severity or antigen-specific proliferation, respectively.

**Induction of Vitreous Cavity-Associated Immune Deviation**

Vitreous cavity-associated immune deviation (VCAID)30 was induced by intravitreally injecting ovalbumin (OVA) (Sigma-Aldrich). Here, 50 μg OVA, 50 μg OVA + 50 μg etanercept or PBS (control) were injected in a 2-μL volume. Seven days later, mice were boosted by subcutaneously injecting 100 μg OVA with complete Freund’s adjuvant (1:1 vol/vol). On day 14 after intravitreal injection, delayed-type hypersensitivity (DTH) reaction was induced by subcutaneously injecting 200 μg/10 μL OVA into the right footpad; 10 μL PBS injected into the left footpad served as control. Footpad swelling was measured 24 hours later with a micrometer. DTH reaction was calculated as follows: (24-hour measurement of the right footpad - 0-hour measurement of the right footpad) - (24-hour measurement of the left footpad - 0-hour measurement of the left footpad).

**Statistical Analysis**

For normally distributed data, Student’s t-test was conducted to compare two groups and one-way ANOVA for comparing three or more groups. For comparing non-normally distributed data of three or more groups, a Kruskal-Wallis test was used. For EAU scoring data, a nonparametric Mann-Whitney U-test was performed. A value of P less than or equal to 0.05 was considered statistically significant.

**RESULTS**

**Course of EAU after Systemic Etanercept Treatment in the Afferent Phase**

EAU improved after systemic etanercept therapy in the afferent phase. Control mice showed a severe EAU score (Fig. 1C, representing mean EAU score in this group), with heavy inflammatory cell infiltration of the retina or vitreous, typical foldings of the retina, focal serous detachment of the photoreceptor layer, and focal lesions with loss of photoreceptors. In some mice of this group, EAU eyes also formed granulomata in the choroid and retina and retinal neovascularization developed; in some cases the retinal architecture was completely destroyed (not shown in the figure). In contrast, disease was less severe in mice treated with etanercept (Figs. 1A–C).
FIGURE 1. Systemic etanercept treatment in the afferent phase of EAU. Pooled data from two independent experiments. Mean ± SD. (A) Significantly reduced histological score in the etanercept group (Etanercept group: n = 17; PBS group: n = 21; P < 0.001***). (B) Representative hematoxylin-eosin-stained section of mouse eye (magnification ×100) of the etanercept group showing mild perivascular infiltration (arrow) without structural damage of the retina. (C) Representative section of mouse eye (magnification ×100) of the control group showing more severe leukocyte infiltration in the photoreceptor layer (*) and retinal folding. (D) Significantly reduced IRBP-specific proliferation of splenocytes in the etanercept group (P < 0.001). (E) Reduced IRBP-specific production of proinflammatory cytokines by splenocytes in the etanercept group (IL-2, P = 0.025; IFN-γ, P = 0.057; IL-6, P = 0.068; IL-17, P = 0.249; IL-10, P = 0.911; IL-4, P = 1).
Inflammatory cytokines was lower than in the PBS controls (Fig. 1E).

**Course of EAU with Local Application of Etanercept in the Afferent Phase**

To elucidate whether local treatment of eyes with etanercept would also improve EAU, we treated eyes in the afferent phase after IRBP immunization. While PBS-treated mice had high EAU scores at day 21 p.i., the EAU scores were significantly lower in mice that received intraocular etanercept unilaterally, but only in the injected eye. The slight reduction of EAU scores in the untreated eye were not statistically significant (Fig. 2A).

The improved EAU scores were not accompanied by a reduced antigen-specific proliferation of splenocytes (Fig. 2B). Although the splenic IL-2, IFN-\(\gamma\) (\(P = 0.009\)), and IL-17 content was reduced in the etanercept group, levels of the Th2-associated cytokines IL-6 (\(P < 0.001\)) and IL-10 were increased (Fig. 2C).

After subconjunctival etanercept treatment in the afferent phase, no significant reduction in EAU scores was detected, nor had antigen-specific proliferation of splenocytes decreased (data not shown).

**Course of EAU with Systemic or Local Etanercept Treatment in the Efferent Phase**

Treatment in the efferent phase correlated to the onset of inflammatory cell infiltration in the eyes, which arises at about day 9 after active immunization.\(^{14,31,32}\) Systemic or local (intravitreal or subconjunctival) etanercept treatment in the efferent phase did not reduce the EAU scores. Furthermore, the antigen-specific proliferation of splenocytes was not consistently decreased (Table 2).

**Course of EAU Induced by Adoptive Transfer with Systemic Etanercept Treatment**

We next investigated systemic etanercept treatment in an EAU model induced by adoptive transfer of IRBP\(_{161-180}\)-specific lymphocytes. Recipient mice developed severe EAU, exhibiting the characteristic histopathologic features of EAU induced by active immunization. There was no significant difference between etanercept-treated mice and PBS controls in terms of EAU severity (Fig. 3A) or the antigen-specific proliferation of splenocytes (Fig. 3B).

**Influence of Etanercept on VCAID Induction**

Anterior chamber-associated immune deviation (ACAID) and VCAID represent a deviant, systemic immune response induced by antigens placed in the anterior chamber or the vitreous cavity of the eye, respectively. TNF-\(\alpha\) has been suggested to play an important role in inducing ACAID or VCAID, which is mediated by regulatory T cells downregulating immunogenic inflammation.\(^{30,33,34}\) We therefore assessed the effect of intravitreal etanercept treatment on the induction of VCAID.

VCAID was induced by intravitreally injecting OVA, which was dissolved in PBS or in etanercept. Mice intravitreally injected with OVA expressed a reduced DTH response in the PBS control group (treated eyes: both groups: \(n = 17\), \(P = 0.012\); untreated eyes: etanercept: \(n = 15\); PBS: \(n = 16\), \(P = 0.052\)). (B) No difference in IRBP-specific proliferation of splenocytes between groups (\(P = 0.818\)). (C) IRBP-specific production of inflammatory cytokines by splenocytes. \(\*P < 0.05\), \(\**P < 0.01\), \(\***P < 0.001\).
comparison with PBS-treated mice. Mice receiving simultaneous intravitreal injections of etanercept and OVA showed a stronger DTH response than mice in the OVA/PBS group (Fig. 4A). In contrast, there was a trend of the OVA-specific production of IL-10 by splenocytes to be higher in the PBS/OVA group than in the etanercept/OVA group and the PBS controls, but the difference did not reach the level of significance (Fig. 4B).

DISCUSSION

TNF-α has an important role in experimental and clinical uveitis.5,6 Neutralizing TNF-α systemically delayed disease onset and reduced tissue damage in EAU.13,15,16,35 Our results of using different etanercept regimens in the EAU model suggest a primary role of TNF-α in the induction phase rather than in the efferent phase of EAU. In vitro experiments revealed enhanced antigen- and mitogen-induced proliferation of mature T cells by recombinant TNF36 and abolished the proliferative activity of responder cells by anti-TNF antibodies in a human mixed lymphocyte reaction.37 In accordance with these findings, the reduced histological EAU scores in our study were accompanied by a decreased antigen-specific proliferation of splenocytes and their production of proinflammatory cytokines. This is in line with a previous report in which the severity of EAU, antigen-specific lymphocyte proliferation, and the DTH response were reduced after systemic administration of polyclonal antibodies directed against TNF-α in the afferent phase. The authors suggested that neutralizing TNF-α inhibits the priming of antigen-specific effector T cells.13

Macrophages activated in the proinflammatory conditions of IFN-γ or TNF-α at the immunization site play a pivotal role in generating antigen-specific effector T cells. It has been shown that these macrophages, once programmed from the peak of disease, may overcome immunoregulatory effects of TGF-β in the posterior chamber of the eye and that nitric oxide production was not influenced by ex vivo incubation with soluble TNF-receptor/IgG fusion protein.38 From this perspective, we would not expect that the activation status of macrophages and the subsequent generation of effector T cells would be influenced by neutralizing TNF-α in the efferent phase. Thus, when the activation processes in the induction phase were completed, TNF-α blockade by etanercept was not sufficient to dampen ocular inflammatory responses in our experiments.

Clinical studies reported that, in part, uveitis patients could also be successfully treated with etanercept.19,20 In contrast to EAU in B10.RIII mice, which is characterized by a monophasic, acute course,14 periodic cycles of antigen priming can be assumed in uveitis patients, on which etanercept may act. However, other studies have shown EAU improvement after treatment in the afferent and the efferent stages of EAU in a rat and mouse model by using a 55-kDa tumor necrosis factor receptor (TNFR1)-Ig fusion protein.14–16 Data reported by Luger et al.39 also indicated a strong reducing effect of

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Figure 3. Systemic etanercept treatment on days −1, 1, 3, and 5 after adoptive transfer. Mean ± SD. (A) No difference in histological EAU score between groups (etanercept group: n = 8, 1.9 ± 1.0; PBS group: n = 8, 2.6 ± 0.8, P = 0.14). (B) No difference in IRBP-specific proliferation of splenocytes between groups (etanercept group: ΔCPM = 5505 ± 1890; PBS group: ΔCPM = 3588 ± 2255; P = 0.121).

neutralizing anti-TNF antibodies on EAU induced by adoptive transfer of uveitogenic Th1 cells.\textsuperscript{39} In contrast, our present data demonstrate that TNF-\(\alpha\) inhibition by etanercept did not ameliorate EAU in the adoptive transfer model, supporting the notion that etanercept acts more on the processes of T-cell priming and has only a minor impact on effector cells that had already been generated and exposed to the specific antigen.

Etanercept is a fusion protein of an antibody Fc fragment and the 75-kDa TNFR2. In contrast to the 55-kDa TNFR1, the TNFR2 was found to form relatively unstable complexes with soluble and transmembrane forms of TNF, which were characterized by high rates of association and dissociation.\textsuperscript{17,40,41} Moreover, in contrast to anti-TNF antibodies, etanercept is still able to induce reverse signaling and thus does not completely impede monocyte reactivity to antigens.\textsuperscript{42} This may explain the differences between our results and the findings of Dick et al.,\textsuperscript{15,16} Hankey et al.,\textsuperscript{14} and Lugner et al.\textsuperscript{39}

Several reports have suggested an important role of resident ocular cells in the pathogenesis of intraocular inflammation as regards expression of molecules related to professional antigen-presentation properties, reactivation of invading autoreactive T cells, induction of TNF-\(\alpha\) expression by these T cells, and local production of the TNF-\(\alpha\).\textsuperscript{9,10,43–45} We therefore analyzed the effect of local TNF-\(\alpha\) blockade at the site of inflammation by injecting etanercept intravitreally. Again, reduced EAU scores were observed after intravitreal etanercept treatment, but only in the afferent phase of disease. This is in line with results of a recently published study, in which local immunomodulation was achieved by the electrotransfer of a plasmid encoding a p55 TNF receptor/IgG1 fusion protein to the ciliary muscle. A shift toward a Th2-dominated effector response as deduced from the intraocular cytokine profile was suggested as a possible mode of action.\textsuperscript{46} While several previous studies have indicated a protective role of activating the Th2 response and the associated cytokines on the course of EAU,\textsuperscript{87–89} in IFN-\(\gamma\)-deficient mice such a deviant effector response in terms of Th1/Th2 balance has also been shown to be deleterious to the eye.\textsuperscript{50} After intravitreal etanercept treatment, we observed an immune response exerting Th2 associated elements (splenic IFN-\(\gamma\) decreased, while IL-6 and IL-10 [slightly] increased), but without a shift to a prototypic Th2 response as the secretion of IL-4 was low. In contrast, after systemic etanercept treatment, an overall reduction of proinflammatory cytokines was detected. These results of varying cytokine production and different splenic proliferation responses indicate that the mechanisms of action underlying EAU improvement may differ between systemic and intravitreal anti-TNF-\(\alpha\) treatment. The reduction of EAU scores after intraocular etanercept treatment does not seem to be primarily due to the backflow of etanercept into the conjunctiva after intravitreal injection, as EAU did not improve after subconjunctival treatment. An uptake study of FITC-labeled antisense oligonucleotides (ASON) (data not shown) indicated a rapid efflux of the agent to the regional draining lymph nodes (DLNs) after subconjunctival injection. Taking these observations together, a minor role of these DLNs in regulating EAU-mediated dynamics of inflammatory cells can be suggested under these experimental conditions.

Our data demonstrated a more potent effect for systemic etanercept treatment than for intravitreal treatment in reducing EAU severity. In a clinical study, patients with active uveitis had elevated TNF-\(\alpha\) levels in sera and aqueous humor compared with control patients. The TNF-\(\alpha\) serum levels were higher than in the aqueous humor and thus the authors concluded that systemic participation is greater than local participation of TNF-\(\alpha\) in the pathogenesis of uveitis.\textsuperscript{51}

EAU in B10.RIII mice is characterized by an acute course of disease,\textsuperscript{14} which may make it difficult to detect the efficacy of a therapeutic intervention, such as with etanercept. TNFR-IgG fusion proteins and antibodies directed against TNF-\(\alpha\) operate by neutralizing TNF-\(\alpha\) posttranslationally. We therefore assessed an alternative local treatment strategy with ASON targeting the messenger RNA of TNF-\(\alpha\) and thus preventing TNF-\(\alpha\) synthesis.\textsuperscript{52} However, also local ASON injection had no ameliorating effect on EAU in both the afferent and the efferent phase (data not shown).

Several studies implicate a participation of intraocular TNF-\(\alpha\) in the maintenance of immune privilege by inducing apoptosis of inflammatory cells infiltrating the eye.\textsuperscript{53,54}
Furthermore, TNF-α contributes to the immune privilege of the eye as it is involved in the induction of ACAID. The induction of ACAID by intracameral injection of IRBP suppressed the development of IRBP-induced EAU in mice, whereas in EAU-affected inflamed eyes, immune privilege is compromised. Anti-TNF antibodies blocked the suppression of DTH when the antibody was co-injected with the antigen into the anterior chamber, suggesting that intraocular TNF-α is important in ACAID. We analyzed whether TNF-α blockade had an impact on the induction of VCAID by injecting etanercept intravitreally. Compared with mice that received OVA intravitreally, the etanercept/OVA group showed an increased DTH. The notion of such an immunomodulation was supported by the fact that IL-10 expression was in contrast slightly reduced. IL-10 represents a key cytokine in the induction of ACAID. Therefore, intraocular TNF-α possesses regulatory and inhibitory functions that may be impaired by intravitreal etanercept treatment. Correspondingly, the anti-inflammatory value of intravitreal treatment with other TNF-α inhibitors, such as infliximab or adalimumab, is controversial.

Taken together, etanercept treatment was more effective in ameliorating EAU in the afferent than in the efferent phase, when antigen-specific, uveitogenic T cells have already been generated. This suggests that TNF-α plays an important role predominantly in the induction phase of EAU, and that etanercept affects the generation and priming of uveitogenic effector T cells. Our data suggest that the mechanism of action underlying EAU improvement may be different for systemic and local etanercept treatment.

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References


