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BASIC SCIENCE

# Inhibitory role of transforming growth factor β2 in experimental autoimmune anterior uveitis

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## Abstract

**Purpose** Experimental autoimmune anterior uveitis (EAAU) is a clinically relevant animal model for human idiopathic anterior uveitis (IAU). The role of the immunomodulator transforming growth factor β2 (TGF-β2) in EAAU pathology is unknown. In this study, we investigated the regulatory role of TGF-β2 in EAAU.

**Methods** EAAU was induced in male Lewis rats by footpad injection of melanin-associated antigen (MAA). TGF-β2 was administered intravenously (iv) in MAA-sensitized rats during the induction of EAAU, or after the clinical onset of uveitis. MAA-sensitized rats injected similarly with an equal volume of PBS served as control. Animals were examined daily between days 7 and 30 post-injection for the clinical signs of uveitis using slit lamp biomicroscopy. Animals were sacrificed at various time points and eyes were harvested for histological analysis to assess the course and severity of inflammation. For histopathological analysis, paraffin sections of harvested eyes were stained with hematoxylin and eosin. Popliteal lymph nodes (LNs) were used for $CD4^+CD25^+FoxP3^+$ T regulatory (Tregs) population analysis and for $CD4^+$ T cell proliferation assay.

**Results** Administration of recombinant TGF-β2 during the early stages of EAAU prevented the induction of uveitis. Compared to PBS, the presence of TGF-β2 in the cell culture significantly ($p < 0.05$) inhibited the proliferation of $CD4^+$ T cells in response to MAA. In MAA-sensitized Lewis rats, iv treatment with recombinant TGF-β2 resulted in significantly ($p < 0.05$) increased percentage of Tregs compared to animals treated similarly with PBS. Thus, TGF-β2 inhibited the induction of EAAU by inhibiting $CD4^+$ T cell proliferation and increasing the number of Tregs. Injection of TGF-β2 in rats with active EAAU resulted in diminished disease activity. Unfortunately, this treatment did not lead to the early resolution of EAAU.

**Conclusions** TGF-β2 plays a critical role in regulation of intraocular inflammation in EAAU. Findings reported in this study improve our understanding of immunopathology of IAU and suggest that recombinant TGF-β2 may be a promising therapeutic agent for human IAU.

**Keywords** Anterior uveitis · Inflammation · Transforming growth factor β2 · T regulatory cells · $CD4^+$ T cells · Experimental autoimmune anterior uveitis

## Introduction

Idiopathic anterior uveitis (IAU) is the most prevalent intraocular inflammatory disease in humans and is characterized by the inflammation of the iris and/or the CB. Visual complications associated with recurrent and/or untreated IAU lead to the permanent loss of vision [1–3]. Human IAU has been considered an autoimmune disease where an immune response affects only the eye [1–3]. Evidence from animal models and limited human studies support a crucial role of T cells in the development of uveitis [4–19].

Experimental autoimmune anterior uveitis (EAAU) is an autoimmune ocular inflammatory disease and it serves as a clinically relevant animal model for idiopathic human anterior uveitis. This animal model has been extensively used by us and other investigators to understand the etiopathogenesis of IAU [6–8, 11–21]. EAAU is a $CD4^+$ T cell-mediated autoimmune disease that can be induced in Lewis rats by immunization with melanin-associated antigen (MAA) in the hind footpad. The

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cardinal features of EAAU include lymphocytic infiltration in the iris, the CB, and the anterior chamber of the eye [14–21].

We have published that the induction of EAAU can be prevented by inducing immune tolerance against MAA [18, 19]. We further reported that the levels of transforming growth factor β2 (TGF-β2) increased in the popliteal lymph nodes (LNs) of tolerized animals [18, 19]. However, the specific role of TGF-β2 in the pathogenesis of EAAU remains unclear. In this study, we investigated the ability of TGF-β2 to modulate inflammatory responses in EAAU.

## Materials and methods

### Animals

Pathogen-free male Lewis rats (5–6 weeks old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). This study was approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences, Little Rock, AR.

### Induction and evaluation of EAAU

MAA was isolated from bovine iris and CB as previously described by us [14, 17]. Male Lewis rats were immunized with 100 μl of stable emulsion containing 75 μg of MAA emulsified (1:1) in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) using a single-dose induction protocol in the hind footpad as previously described by us [14, 16–19]. Pertussis toxin was used as an additional adjuvant. Animals were examined daily between days 7 and 30 post-injection for the clinical signs of uveitis using slit lamp biomicroscopy. EAAU was scored by an observer unaware of the experimental design. Intensity of uveitis was scored in a masked fashion on the arbitrary scale of 0 to 4, as follows: 0, normal; 1, dilated iris vessels and thickened iris and ciliary body; exudates in the anterior chamber with protein, a few scattered inflammatory cells, or both; 2, moderate infiltration of inflammatory cells in the iris, ciliary body, or both; moderate number of inflammatory cells within the anterior chamber; 3, heavy infiltration of inflammatory cells within the iris and ciliary body and within the anterior chamber; 4, heavy exudation of cells with dense protein aggregation in the anterior chamber; inflammatory cell deposits on the corneal endothelium. Eyes were also harvested at various time points for histological analysis to assess the course and severity of inflammation using the criteria previously reported [14, 16–19].

### TGF-β2 administration

Recombinant human TGF-β2 (PeproTech, Rocky Hill, NJ) was used for in vivo injections. Human TGF-β2 has high degree of homology (> 90%) with rat TGF-β2 at the amino acid level and it cross-reacts with its rat counterpart (PeproTech). TGF-β2 was administered by two different routes: intravenous (iv) and intraperitoneal (ip) in pilot experiments. Since anterior chamber (AC) injections are not feasible after the onset of EAAU because eyes are inflamed, TGF-β2 was not delivered into the AC of the eye in the current study. In our pilot experiments, a therapeutic effect on EAAU was observed when TGF-β2 was administered intravenously and not intraperitoneally. Therefore, administration of TGF-β2 via iv route was used in the following experiments.

MAA-sensitized Lewis rats received a single iv injection of recombinant TGF-β2 1 day before immunization (day − 1), on the day of immunization (day 0), and 1 day after immunization (day + 1). Control animals were treated similarly with equal volume of sterile phosphate buffered saline (PBS). To perform TGF-β2 dose titration, two different doses—500 ng/injection and 1 μg/injection of recombinant TGF-β2—were used separately in our initial experiments. Since iv injection of 500 ng and 1 μg of TGF-β2 gave same results, 500 ng of TGF-β2 was used in subsequent experiments.

In another set of experiments, Lewis rats immunized with MAA received a single iv injection of recombinant TGF-β2 after the onset of EAAU on days 12 and 14 post-immunization. MAA-sensitized rats treated similarly with equal volume of sterile PBS served as control. Clinical and histopathological examination (described above) was used to determine the onset, severity, and duration of EAAU.

### Histology

For histology [18, 19], freshly enucleated rat eyes were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24 h at room temperature, dehydrated in ethanol through ascending series of ethanol concentrations and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin (H&E) purchased from Fisher (Fair Lawn, NJ). Sections were examined using a light microscope (Carl Zeiss Meditec, Inc., Thornwood, NY).

### Cell preparation

Popliteal LNs were harvested on day 5 for Treg population analysis and on day 12 for $CD4^+$ T cell proliferation assay. A single-cell suspension was prepared as previously described [18, 19]. Briefly, the tissue was mashed between the frosted slides and cells were filtered through cell strainer and washed once with Dulbecco's phosphate buffered saline (DPBS). Total lymphocytes were purified using Histopaque gradient (Sigma Aldrich) according to manufacturer's protocol. The cells were suspended in complete RPMI 1640 culture medium containing L-glutamine (Mediatech, Herndon, VA), 1% (*v*/*v*) MEM essential vitamin mixture (Life Technologies, Rockville, MD), NEAA (BioWhittekar, Allendale, NJ),

mixture of antibiotics (penicillin 100 U/ml, streptomycin 100 U/ml and amphotericin B 0.25 μg/ml) purchased from BioWhittekar, Allendale, NJ, and 10% (*v*/*v*) FCS (Mediatech, Herndon, VA).

### Staining for Tregs

The Fc receptor was blocked by incubating the cells with anti-rat CD32 antibody (BD Biosciences, San Jose, CA) for 30 min. The cells were then surface stained using anti-rat CD4-APC and anti-rat CD25-PE antibodies from Biolegend (San Diego, CA). After treating with antibodies for 40 min, cells were washed and stained with FITC labeled FoxP3 antibody (eBiosciences, San Diego, CA) using FoxP3 staining kit according to manufacturer's instructions (ebiosciences). The stained cells were analyzed using FACS Calibur (BD Biosciences). The percentage of $CD4^+CD25^+FoxP3^+$ Tregs was calculated using WinMDI [18, 19].

### In vitro cell proliferation assay

In vitro T cell proliferation was performed as previously described by the authors [18, 19]. Total lymphocytes were harvested from lymph nodes of MAA-sensitized animals on day 12 post-immunization. The cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using Cell Trace CFSE Cell Proliferation Kit according to manufacturer's protocol (Molecular Probes, Invitrogen, Carlsbad, CA) and were cultured with MAA (20 μg/ml) for 5 days in the absence and presence of TGF-β2 (10 ng/ml). On day 6, non-adherent cells were collected and labeled with anti-rat CD4-APC antibody. The stained cells were then analyzed using FACS Calibur. The percentage of proliferating cells was calculated using WinMDI.

### Statistical analysis

Data were analyzed and compared using Student's *t* test or Mann-Whitney *U* test using Statistica Program (Statsoft, Inc., Tulsa, OK) and differences were considered statistically significant with $p < 0.05$.

## Results

### Effect of recombinant TGF-β2 on the induction of EAAU

To study the effect of TGF-β2 on the induction of EAAU, MAA-sensitized Lewis rats received a total of three iv injections (one injection/time point) of recombinant TGF-β2 (500 ng/injection) at the following time points: 1 day before immunization (day − 1), on the day of immunization (day 0), and 1 day after immunization (day + 1). Another set of MAA-sensitized animals treated similarly with an equal volume of PBS served as control. Treatment with TGF-β2 resulted in complete inhibition of induction of EAAU as determined by clinical (Fig. 1a) and histological (Fig. 1c) examination. In these rats, no infiltration of the inflammatory cells was noted in the iris, the CB, and the anterior chamber of the eye. Conversely, similar treatment with PBS did not alter the course or the severity of EAAU (Fig. 1a, b). The iris, the CB, and the anterior chamber of the PBS-treated rat exhibited heavy exudation of inflammatory cells (Fig. 1b).

### Mechanism of TGF-β2-mediated prevention of induction of EAAU

We next investigated the underlying mechanisms responsible for inhibition of EAAU induction by TGF-β2 treatment. To address this, we investigated the proliferation of $CD4^+$ T cells in the presence of TGF-β2 (in vitro) as well as determined the effect of TGF-β2 on generation of Tregs (in vivo) using popliteal LNs that drain the site of MAA injection.

#### Proliferation of $CD4^+$ cells (in vitro)

Since EAAU is a $CD4^+$ T cell-mediated autoimmune disease [16–19], experiments were carried out to assess the effects of TGF-β2 on the proliferation of $CD4^+$ T cells in response to MAA. Proliferation of $CD4^+$ T cells in the presence and absence of recombinant TGF-β2 was compared using an in vitro cells proliferation assay. Total lymphocytes purified from popliteal LNs of MAA-sensitized animals sacrificed at day 12 post-immunization were labeled with CFSE and were cultured with MAA (20 μg/ml) for 5 days in the presence of TGF-β2 (10 ng/ml) or equal volume of PBS. On day 6, these in vitro cultured CFSE-labeled lymphocytes were labeled with anti-rat CD4-APC and analyzed using flow cytometry to investigate the effect of TGF-β2 on proliferation of $CD4^+$ T cells. Our data shows that compared to PBS (Fig. 2a, c), the presence of TGF-β2 in the cell culture significantly inhibited the proliferation (in vitro) of $CD4^+$ T cells in response to MAA (Fig. 2b, c).

#### Generation of $CD4^+CD25^+FoxP3^+$ Tregs (in vivo)

We have reported that LN-derived $CD4^+CD25^+FoxP3^+$ Tregs cells play a crucial role in the induction of immune tolerance against MAA in EAAU [18, 19]. Therefore, we next determined and compared the percentage of Tregs in popliteal LNs of MAA-sensitized Lewis rats after iv injections of TGF-β2 or PBS. Lewis rats were injected intravenously with recombinant TGF-β2 (500 ng/injection) on day − 1, day 0, and day + 1 post-immunization as described above. Animals were sacrificed on day 5 post-immunization. Total lymphocytes harvested from popliteal LNs were stained for CD4, CD25,

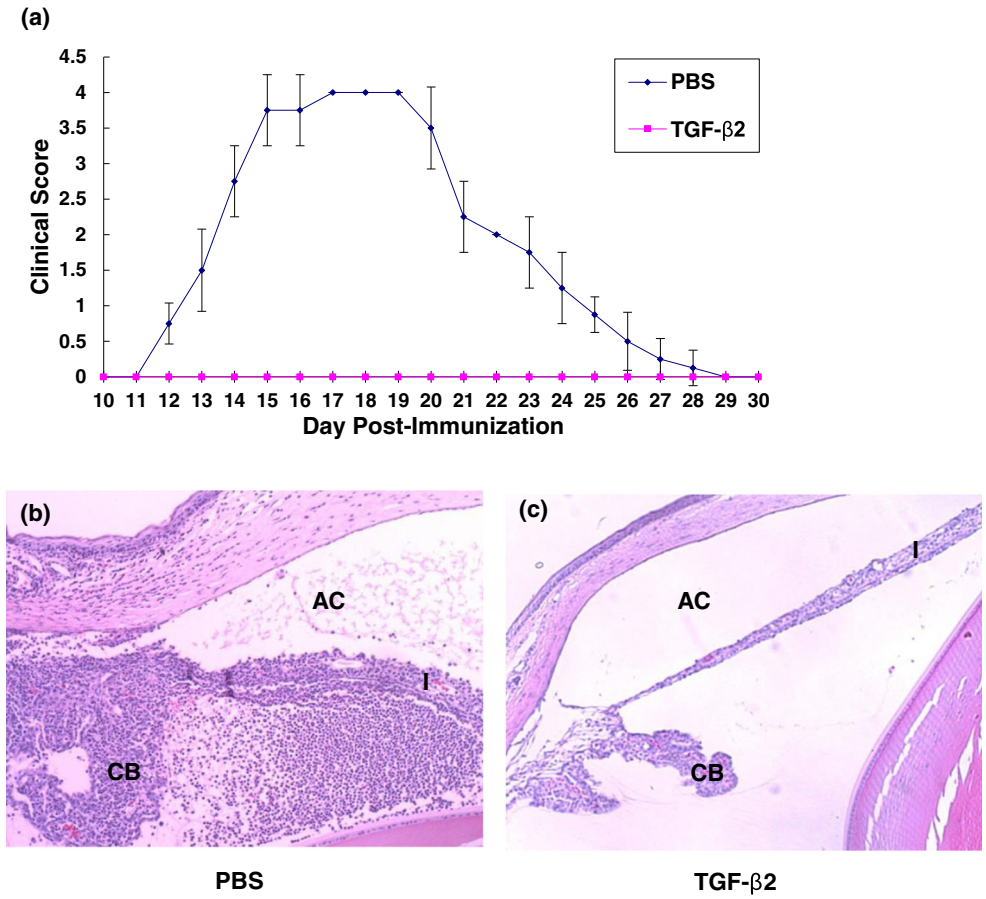


**Fig. 1** Transforming growth factor β2 (TGF-β2) inhibits the induction of experimental autoimmune anterior uveitis (EAAU). Recombinant TGF-β2 or PBS was administered intravenously 1 day before immunization, at the time of immunization, and 1 day post-immunization with melanin-associated antigen (MAA). This treatment with recombinant TGF-β2 prevented the induction of uveitis clinically (**a**). MAA-sensitized rats treated similarly with phosphate buffered saline (PBS) developed the normal clinical course of EAAU (**a**). Lewis rats injected with PBS (control) or TGF-β2 as described above were sacrificed at day 18 post-immunization. Representative H&E stained sections of harvested eyes demonstrated heavy infiltration of the inflammatory cells within the iris (I), the ciliary body (CB), and the anterior chamber (AC) of PBS-injected rats (**b**). In contrast, no inflammatory cells could be detected in the eye of Lewis rats injected iv with TGF-β2 (**c**) at this time point. The data are expressed as the mean ± SD, representative of three independent experiments. $n = 15$ rats/group. Objective magnification × 20

and FoxP3 and percentage of $CD4^+CD25^+FoxP3^+$ Tregs was determined using flow cytometry as described under "Materials and Methods." Our results (Fig. 3a–c) demonstrate that in MAA-sensitized Lewis rats, iv treatment with recombinant TGF-β2 resulted in increased percentage of Tregs (13.25 ± 0.69%) compared with MAA-sensitized animals treated similarly with PBS (8.4 ± 0.44%). These differences were statistically significant ($p < 0.05$).

## Effect of recombinant TGF-β2 on EAAU after the onset of uveitis

Experiments were carried out to investigate if active EAAU can be inhibited by administration of recombinant TGF-β2. MAA-sensitized Lewis rats received iv injection of TGF-β2 or PBS separately on two occasions after the onset of the clinical disease (on days 12 and 14 post-immunization) and the disease progression was monitored. A sharp decline in the clinical disease activity was observed in all animals at days 13–18 that received TGF-β2 via iv route (Fig. 4). However, from day 19 post-immunization onward EAAU followed the normal course of the disease in these animals. The duration of EAAU in MAA-sensitized rats that were treated with recombinant TGF-β2 after the onset of uveitis was similar to PBS-treated control rats (Fig. 4).

## Discussion

The management of patients with IAU remains a significant challenge because no specific treatment is available for this disease. IAU is treated symptomatically only using non-specific therapies such as corticosteroid and immunosuppressive agents [1–3]. Unfortunately, these treatment modalities do not address the underlying mechanisms and are associated with adverse side effects. Prolonged immunosuppression

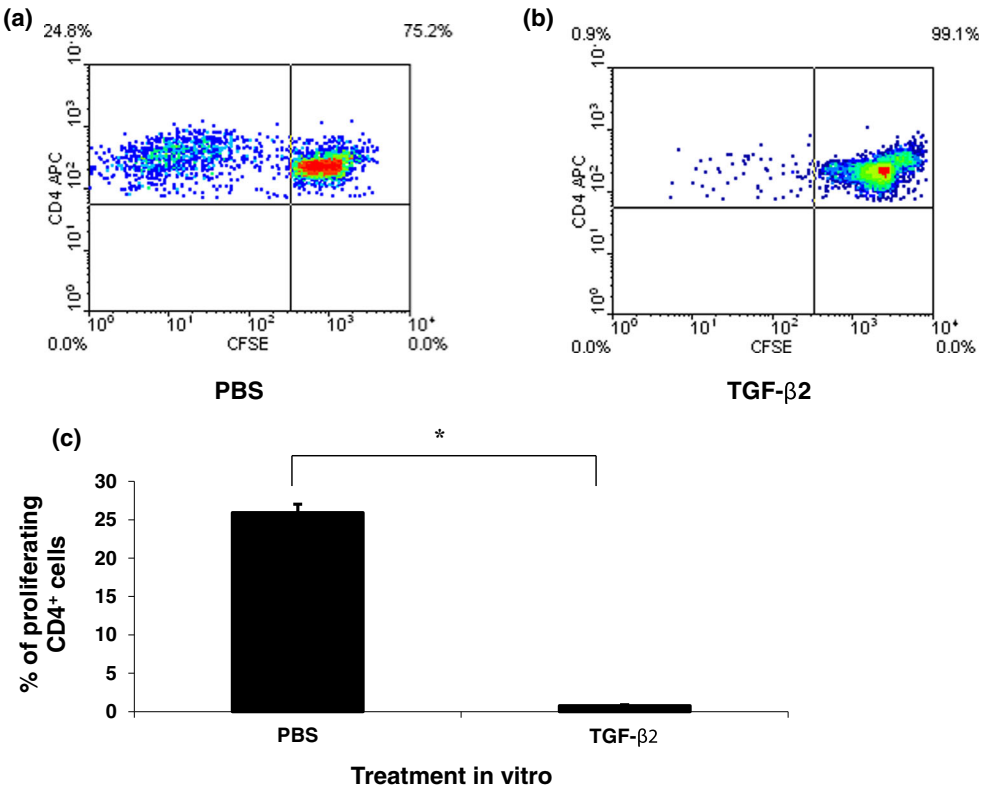


**Fig. 2** TGF-β2 inhibits the proliferation (in vitro) of $CD4^+$ T cells isolated from lymph nodes (LNs) harvested at day 12 post-immunization in response to MAA. Density plots (**a** and **b**) show representative flow cytometric data and panel **c** represents cumulative data from three separate experiments. Presence of 10 ng/ml of recombinant TGF-β2 (**b** and **c**) in culture inhibited the proliferation of $CD4^+$ T cells in response to MAA compared to the treatment with same volume of PBS (**a**, **c**). The data are expressed as the mean ± SD, representative of three independent experiments. $n = 9$ rats/group $^*p < 0.05$

results in increased morbidity. Due to the lack of safe and effective therapeutic options available to the patients with IAU, further research is required so that novel therapeutic targets can be discovered for this sight-threatening disease.

EAAU is a convenient and reliable animal model of human IAU [6–8, 11–21]. Previous reports by us, and in the literature, have shown that $CD4^+$ T cell infiltration into the iris and the CB with inflammatory cell infiltration in the anterior chamber of the

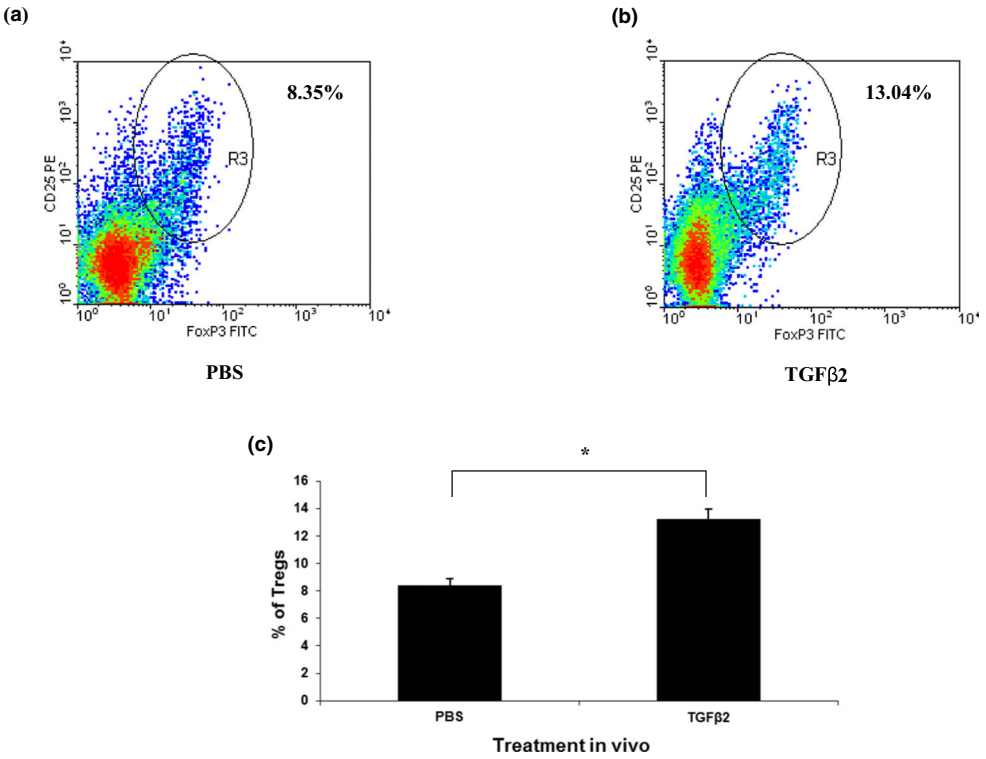


**Fig. 3** TGF-β2 induces T regulatory (Treg) cells in the popliteal LNs. $CD4^+CD25^+FoxP3^+$ Tregs in the popliteal LNs of MAA-sensitized Lewis rats after TGF-β2 administration on day − 1, day 0, and day + 1 post-immunization via iv route (**a**–**c**). Lymph nodes were collected on day 5 post-immunization and lymphocytes were stained for CD4, CD25, and FoxP3. Representative density plots show the percentage of Tregs found in animals treated with PBS (**a**) or recombinant TGF-β2 (**b**). Cumulative data from three independent experiments is shown in panel **c**. The data are expressed as the mean ± SD, representative of three independent experiments. $n = 9$ rats/group. $^*p < 0.05$

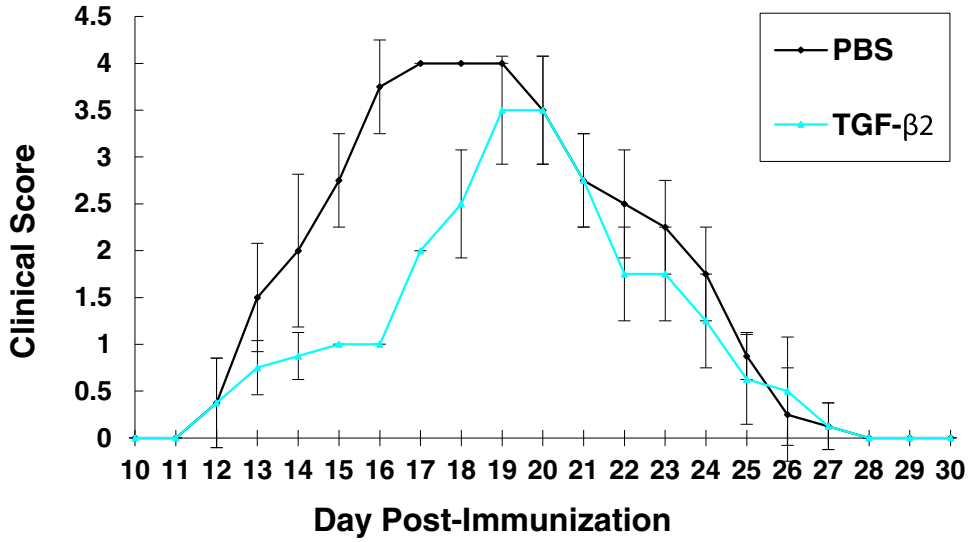


**Fig. 4** TGF-β2 suppresses active EAAU. To investigate the effect on ongoing uveitis, MAA-sensitized Lewis rats received recombinant TGF-β2 or PBS via iv route at days 12 and 14 post-immunization (after the onset of clinical disease). This treatment with TGF-β2 reduced the clinical severity of EAAU until day 18 post-immunization. However, from day 19 post-immunization, EAAU followed the normal course of the disease, similar to that observed in PBS-injected rats. On days 15, 16, 17, and 18 post-immunization, the clinical severity of EAAU in rats treated with TGF-β2 was significantly ($p < 0.05$) reduced compared to Lewis rats treated with same volume of PBS. The data are expressed as the mean ± SD, representative of three independent experiments. $n = 9$ rats/group

eye is the hallmark of EAAU pathology [6–8, 11–21]. We have previously reported that TGF-β2 plays an important role in the development of immune tolerance against MAA [18, 19]. In the current study, the immunomodulatory role for TGF-β2 in the induction and progression of EAAU was explored. TGF-β is a cytokine with multiple effects in T cell development, homeostasis, and tolerance [22–24]. Three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) are present in mammals [25, 26]. TGF-β2 is the main isoform of TGF-β in the eye and it is produced locally [25]. Importantly, TGF-β2 has been reported to play an important role in the pathogenesis of several vision-threatening ocular diseases [25–29].

Two sets of in vivo experiments were performed to investigate the protective role for TGF-β2 in EAAU, one at the time of induction and another after the onset of uveitis. In the first set of experiments, recombinant TGF-β2 completely blocked the induction of EAAU when given at the time of induction of disease (days − 1, 0, and + 1 post-immunization). To determine the mechanism by which TGF-β2 inhibited the induction of EAAU, we used a combination of in vitro and in vivo experiments. We specifically analyzed the effect of TGF-β2 on the proliferation of $CD4^+$ T cells and on the generation of Tregs. In our study, the proliferative response of $CD4^+$ T cells to MAA was negligible when cultured in the presence of TGF-β2. We further observed that in MAA-sensitized Lewis rats, treatment with recombinant TGF-β2 resulted in increased percentage of Tregs in the draining popliteal LNs at day 5 post-immunization. It has been reported that TGF-β can induce $CD4^+$ T cells in the periphery to become Tregs [30]. This mechanism may be responsible for the relatively quick development of Tregs in the popliteal LNs of rats treated with TGF-β2 noted in the current study. TGF-β2 has also been implicated to suppress $CD4^+$ T cells [31, 32]. Thus, early increase in number of Tregs in the draining lymph nodes observed in the current study suggests that TGF-β2-induced Tregs suppressed the further activation of immune cells and significantly contributed to the inhibition of EAAU induction.

We previously reported that EAAU is induced in Lewis rats by a $CD4^+$ T cell response to MAA [16–19], the number of $CD4^+CD25^+FoxP3^+$ Tregs increased in Lewis rats tolerized against MAA and these Tregs were crucial for the induction of immune tolerance in this animal model [18, 19]. $CD4^+CD25^+FoxP3^+$ Tregs represent the best characterized subset of suppressive T cell population and play an important role in prevention and/or suppression of autoimmune diseases [33–35]. It is well recognized that TGF-β2, an immunosuppressive cytokine, triggers the expression of Foxp3 in $CD4^+$ T cells and subsequently converts them to $FoxP3^+$ T regulatory cells [22, 26, 30–32]. Together, our results suggest that the treatment with TGF-β2 during the early stages of EAAU completely suppressed the immune response by inhibiting $CD4^+$ T cell proliferation and by generating Tregs.

In the second set of in vivo experiments, we tried to mimic the scenario when the patients report to the clinic with active IAU. Keeping that in mind, treatment with TGF-β2 or PBS was withheld until the clinical onset of EAAU and was given on days 12 and 14 post-immunization (after the clinical onset of EAAU). Rats which received PBS developed a prototypical course of EAAU. Interestingly, animals treated with TGF-β2

developed a diminished intraocular inflammation and less severe EAAU. However, this treatment did not result in early resolution of EAAU. Failure of complete suppression of active EAAU by TGF-β2 treatment can be attributable to the fact that in these animals the immune cells are already triggered and primed to perform destined functions.

In conclusion, here, we have reported several novel observations: (i) EAAU did not develop in Lewis rats injected with TGF-β2 on the day before, on the day of, and on the day after MAA immunization; (ii) TGF-β2 mediates inhibition of EAAU induction by diminishing $CD4^+$ T cell proliferation and increasing Tregs; (iii) Lewis rats which received TGF-β2 after the onset of EAAU developed less severe anterior uveitis. Taken together, our results demonstrate that TGF-β2 plays a pivotal role in regulation of intraocular inflammation in EAAU. Disease-modifying effects of TGF-β2 observed in the present study help us in progressing our understanding of the pathogenesis of anterior uveitis and raise the possibility that recombinant TGF-β2 could be a successful therapeutic agent for treatment of human IAU. Since the effect of TGF-β2 was transient when administered systemically, continued intraocular (local) administration of the agent may induce a lasting form of immune suppression.

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## Compliance with ethical standards

This study was approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences, Little Rock, AR.

**Conflict of interest** The authors declare that they have no conflict of interest.

**Commercial relationships** None.

**Ethical statement** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.



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