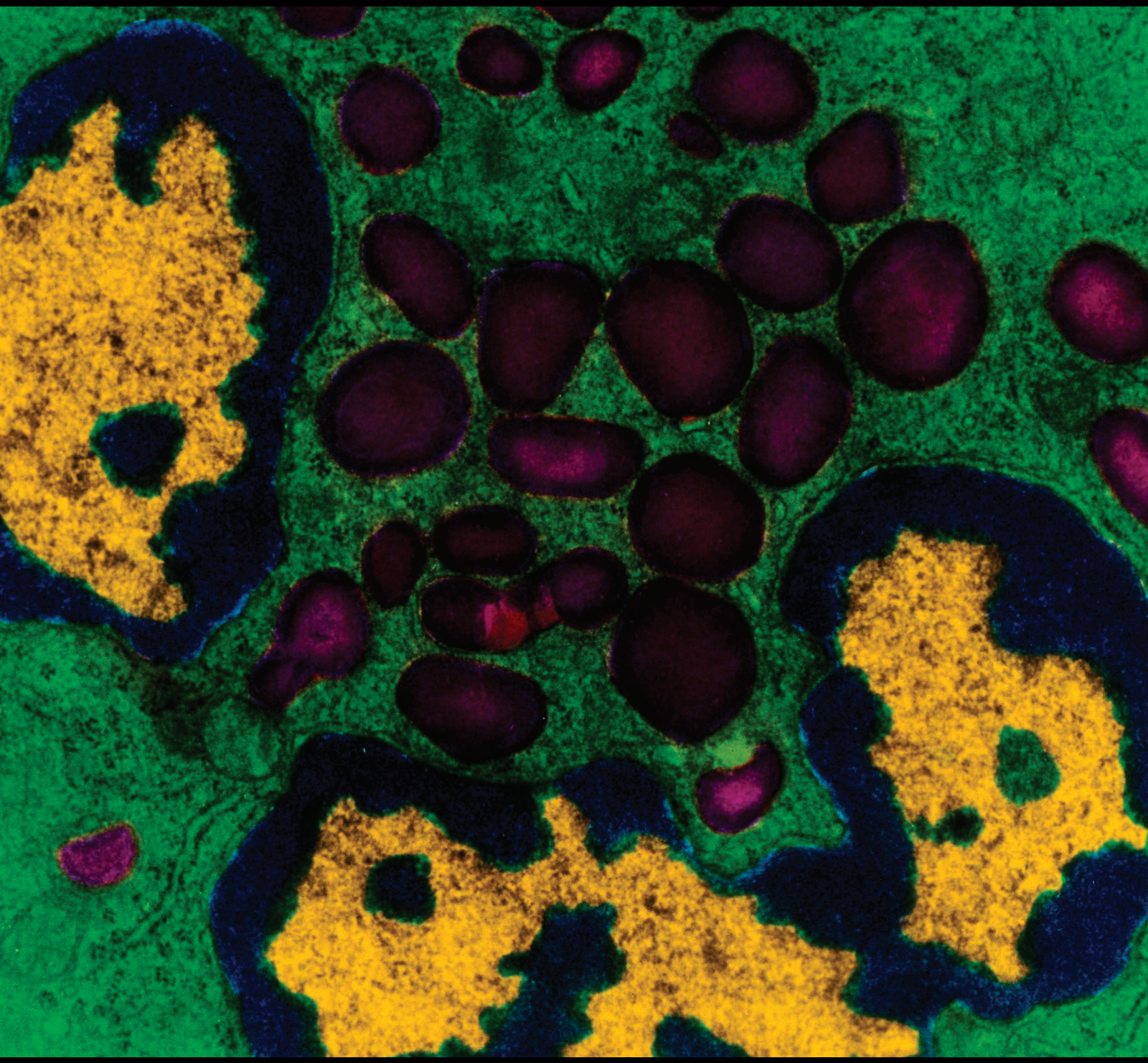
Mediators of Inflammation

Ocular Inflammation

Guest Editors: Valentín Huerva, Francisco J. Ascaso,
and Andrzej Grzybowski



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Editorial Board

Contents

Ocular Inflammation, Valentín Huerva, Francisco J. Ascaso, and Andrzej Grzybowski
Volume 2015, Article ID 398076, 2 pages

Proteasome Inhibitor Bortezomib Suppresses Nuclear Factor-Kappa B Activation and Ameliorates Eye Inflammation in Experimental Autoimmune Uveitis, Sheng-Min Hsu, Chang-Hao Yang, Fang-Hsiu Shen, Shun-Hua Chen, Chia-Jhen Lin, and Chi-Chang Shieh
Volume 2015, Article ID 847373, 10 pages

Disturbances of Modulating Molecules (FOXP3, CTLA-4/CD28/B7, and CD40/CD40L) mRNA Expressions in the Orbital Tissue from Patients with Severe Graves' Ophthalmopathy, Przemyslaw Pawlowski, Natalia Wawrusiewicz-Kurylonek, Anja Eckstein, Joanna Reszec, Wlodzimierz Luczynski, Kristian Johnson, Adam Kretowski, Alina Bakunowicz-Lazarczyk, Maria Gorska, Jacek Szamatowicz, Lech Chyczewski, and Janusz Mysliwiec
Volume 2015, Article ID 340934, 9 pages

Markers of Inflammation and Fibrosis in the Orbital Fat/Connective Tissue of Patients with Graves' Orbitopathy: Clinical Implications, Przemyslaw Pawlowski, Joanna Reszec, Anja Eckstein, Kristian Johnson, Andrzej Grzybowski, Lech Chyczewski, and Janusz Mysliwiec
Volume 2014, Article ID 412158, 9 pages

Chitosan Oligosaccharides Attenuate Ocular Inflammation in Rats with Experimental Autoimmune Anterior Uveitis, I-Mo Fang, Chang-Hao Yang, and Chung-May Yang
Volume 2014, Article ID 827847, 15 pages

The Role of Inflammation in the Pathogenesis of Macular Edema Secondary to Retinal Vascular Diseases, Francisco J. Ascaso, Valentín Huerva, and Andrzej Grzybowski
Volume 2014, Article ID 432685, 6 pages

Intraperitoneal Infusion of Mesenchymal Stem/Stromal Cells Prevents Experimental Autoimmune Uveitis in Mice, Joo Youn Oh, Tae Wan Kim, Hyun Jeong Jeong, Hyun Ju Lee, Jin Suk Ryu, Won Ryang Wee, Jang Won Heo, and Mee Kum Kim
Volume 2014, Article ID 624640, 9 pages

Immune Recovery Uveitis: Pathogenesis, Clinical Symptoms, and Treatment, Beata Urban, Alina Bakunowicz-Łazarczyk, and Marta Michalczuk
Volume 2014, Article ID 971417, 10 pages

Decreased Expression of the Aryl Hydrocarbon Receptor in Ocular Behcet's Disease, Chaokui Wang, Zi Ye, Aize Kijlstra, Yan Zhou, and Peizeng Yang
Volume 2014, Article ID 195094, 11 pages

Effect of the Toll-Like Receptor 4 Antagonist Eritoran on Retinochoroidal Inflammatory Damage in a Rat Model of Endotoxin-Induced Inflammation, Feyzahan Ekici, Emine Esra Karaca, Şafak Korkmaz, Osman Yüksel, Özlem Gülbahar, Murat Alper, Sevim Ercan, and Meral Or
Volume 2014, Article ID 643525, 9 pages

Proinflammatory Cytokines and C-Reactive Protein in Uveitis Associated with Behçet's Disease, Marina Mesquida, Blanca Molins, Victor Llorenç, Maite Sainz de la Maza, María Victoria Hernandez, Gerard Espinosa, and Alfredo Adán
Volume 2014, Article ID 396204, 8 pages

Regulation of Toll-Like Receptor Expression in Human Conjunctival Epithelial Cells, Jing Li, Melina Setiawan, Hong Wu, Roger W. Beuerman, and Peiquan Zhao
Volume 2014, Article ID 493596, 9 pages

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Editorial

Ocular Inflammation

Valentín Huerva,[1,2] Francisco J. Ascaso,[3,4] and Andrzej Grzybowski[5]

[1]*Department of Ophthalmology, Universitary Hospital Arnau de Vilanova, 25198 Lleida, Spain*
[2]*IRB Lleida, 25198 Lleida, Spain*
[3]*Department of Ophthalmology, "Lozano Blesa" University Clinic Hospital, Zaragoza, Spain*
[4]*Instituto Aragonés de Ciencias de la Salud, Zaragoza, Spain*
[5]*Department of Ophthalmology, Poznań City Hospital, Poznań, Poland*

Correspondence should be addressed to Valentín Huerva; vhuerva@gmail.com

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Ocular inflammation has become a hot topic in ophthalmology, involving also several areas of medicine: internal medicine, surgery, basic research, physiology, pharmacology, microbiology, immunology, rheumatology, pharmacology, or laboratory. There are many ocular inflammatory diseases in different locations, including orbit, ocular adnexa, ocular surface, conjunctiva, cornea, sclera, uvea, retinal vessels, and optic nerve. Management of ocular inflammation presents diagnostic and therapeutic challenges, ranging the etiology and prognosis from benign, self-limited conditions to organ-threatening disorders. Ocular inflammation is a component of eye surgery, sometimes leading to its complications, such as macular cystoid edema, and often being targeted by pharmacologic therapy or less invasive surgical procedures. Recently, it has become clear that some assumed to be noninflammatory disorders, like age-related macular degeneration [1] and macular edema secondary to diabetic retinopathy [2] or retinal vein occlusion, are dependent on some inflammatory mediators and thus should be treated, at least partially, as inflammatory disorders.

Ocular inflammation is also often present in patients with systemic inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, sarcoidosis, Wegener's granulomatosis, Sjögren's syndrome, polyarteritis nodosa, primary antiphospholipid syndrome, Behçet's syndrome, Kawasaki disease, Cogan's syndrome, and relapsing polychondritis. Eye involvement in these conditions can not only be the first symptom of the disease but also serve as a biomarker of the severity of systemic inflammation. Furthermore, immunological therapy is being developed to assist the return of healthy ocular immune responses and immune privilege in the eye.

This special issue reports some pathogenetic, diagnostic, and therapeutic aspects of ocular inflammation. We believe that papers on markers or receptors which play an important role in the pathogenesis of the ocular inflammation deserve special attention. P. Pawlowski et al. showed that macrophages and CD4 T lymphocytes are both engaged in the active, severe, and long stage of inflammation in the orbital tissue of patients with Graves' orbitopathy (GO) in "*Markers of inflammation and fibrosis in the orbital fat/connective tissue of patients with Graves' orbitopathy: clinical implications*." F. Ekici et al. reported that Eritoran treatment resulted in less inflammatory damage in terms of serum and retinochoroidal tissue parameters in "*Effect of the toll-like receptor 4 antagonist Eritoran on retinochoroidal inflammatory damage in a rat model of endotoxin-induced inflammation*."

M. Mesquida et al. showed that increased serum levels of IFN-, TNF-, and IL-17A and hsCRP were associated with active uveitis associated with Behçet's disease (BD) and might serve as markers of disease activity in "*Proinflammatory cytokines and C-reactive protein in uveitis associated with Behçet's disease*." F. J. Ascaso et al. reviewed the role of inflammation in the pathogenesis of macular edema (ME) secondary to retinal vascular diseases. ME is a nonspecific sign of numerous retinal vascular diseases. The role of inflammatory processes in the genesis of both diabetic macular edema (DME) and ME secondary to retinal vein occlusion (RVO) is discussed. The paper discusses the inflammatory

mediators which are implicated, the effect of the different intravitreal therapies, the recruitment of leukocytes mediated by adhesion molecules, and the role of retinal Müller glial (RMG) cells.

Finally, another report concludes that, in patients with CMV in the HAART era, immune recovery may be associated with a greater number of inflammatory complications, including ME and epiretinal membrane formation. Given the range of ocular manifestations of HIV, routine ocular examinations and screening for visual loss are recommended in patients with CD4 counts <50 cells/μL in "*Immune recovery uveitis: pathogenesis, clinical symptoms, and treatment*."

We hope that the readers of this special issue will find accurate data and updated reviews on the mechanisms of the different spectrum of ocular inflammatory disorders. Also, important questions may be resolved, such as inflammatory responses in DME, inflammatory cells in orbital tissues of GO patients, and markers of disease activity in BD cases among others.

Valentín Huerva
Francisco J. Ascaso
Andrzej Grzybowski

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Research Article

Proteasome Inhibitor Bortezomib Suppresses Nuclear Factor-Kappa B Activation and Ameliorates Eye Inflammation in Experimental Autoimmune Uveitis

Sheng-Min Hsu,[1,2] Chang-Hao Yang,[3] Fang-Hsiu Shen,[4] Shun-Hua Chen,[4] Chia-Jhen Lin,[2] and Chi-Chang Shieh[1,5]

[1] *Institute of Clinical Medicine, College of Medicine, National Cheng-Kung University, No. 35, Siao-Dong Road, Tainan 70403, Taiwan*
[2] *Department of Ophthalmology, National Cheng-Kung University Hospital, Tainan 70403, Taiwan*
[3] *Department of Ophthalmology, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei 10051, Taiwan*
[4] *Department of Microbiology and Immunology, College of Medicine, National Cheng-Kung University, Tainan 70101, Taiwan*
[5] *Department of Pediatrics, National Cheng-Kung University Hospital, Tainan 70403, Taiwan*

Correspondence should be addressed to Chi-Chang Shieh; cshieh@mail.ncku.edu.tw

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Bortezomib is a proteasome inhibitor used for hematologic cancer treatment. Since it can suppress NF-κB activation, which is critical for the inflammatory process, bortezomib has been found to possess anti-inflammatory activity. In this study, we evaluated the effect of bortezomib on experimental autoimmune uveitis (EAU) in mice and investigated the potential mechanisms related to NF-κB inactivation. High-dose bortezomib (0.75 mg/kg), low-dose bortezomib (0.15 mg/kg), or phosphate buffered saline was given after EAU induction. We found that the EAU is ameliorated by high-dose bortezomib treatment when compared with low-dose bortezomib or PBS treatment. The DNA-binding activity of NF-κB was suppressed and expression of several key inflammatory mediators including TNF-α, IL-1α, IL-1β, IL-12, IL-17, and MCP-1 was lowered in the high-dose bortezomib-treated group. These results suggest that proteasome inhibition is a promising treatment strategy for autoimmune uveitis.

1. Introduction

Uveitis is among the most important causes of blindness and severe visual impairment worldwide. About 15 to 30% of uveitis occurs in the choroid and adjacent retina and hence is classified as posterior uveitis or uveoretinitis [1]. Posterior uveitis tends to damage the photoreceptor cells and lead to permanent blindness. This severe intraocular inflammatory disease is often associated with autoimmune responses to unique retinal proteins [2]. Current therapies for uveitis are based largely on immunosuppressive treatment including corticosteroids, antimetabolites, and alkylating agents. Due to the nonspecific nature and the dose-limiting side effects of these drugs, the results of current treatment for autoimmune-mediated uveitis remain unsatisfactory [3]. Novel approaches to control the inflammatory process in uveitis hence are being keenly developed both in humans and in animal models [4].

Experimental autoimmune uveitis (EAU), in which eye inflammation is induced by active immunization with retinal antigens, is the most often used rodent model for the study of autoimmune uveitis [5]. The typical histological appearance of EAU resembles that of human posterior uveitis, with inflammatory cells infiltrating the vitreous cavity, retina, and choroid and causing damage to the photoreceptor cell layer [3]. Nuclear factor-kappa B (NF-κB) has a pivotal role in inducing inflammation. Several previous studies have shown that there is an increased NF-κB activation in EAU, and the inhibition of NF-κB can ameliorate inflammation [6, 7]. Several NF-κB-regulated inflammatory mediators, including interleukin- (IL-) 1, IL-6, tumor necrosis factor- (TNF-)

α, interferon- (IFN-) γ, monocyte chemoattractant protein- (MCP-) 1, and inducible nitric oxide synthase (iNOS), were found to increase in animals with EAU and may be modulated by treatment targeting NF-κB [3].

The degradation of ubiquitinated IκB by the proteasome is important for the activation of NF-κB [8, 9]. Meanwhile, inhibition of NF-κB activation has been shown to be beneficial in animal models of experimental autoimmune disease, such as myasthenia gravis, psoriasis, arthritis, and autoimmune encephalomyelitis [10–13]. However, little is known about the effectiveness of proteasome inhibition in treating autoimmune uveitis. Here, we showed the effectiveness of bortezomib, a 26S proteasome inhibitor, in inhibiting IRBP-induced EAU.

2. Materials and Methods

2.1. Mice. Female C57BL/6J (B6) mice (8- to 12-weeks-old) were obtained from the Laboratory Animal Center at the National Cheng-Kung University and used for all experiments. All experiments were performed in compliance with a protocol approved by the Institutional Animal Care and Use Committee of the National Cheng-Kung University and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Induction and Treatment of EAU. EAU was induced as previously described with modifications [14]. Briefly, mice were immunized with 100 μL of an emulsion of phosphate buffered saline (PBS) containing 200 μg of human IRBP peptide 1–20 ($hIRBP_{1-20}$) (GPTHLFQPSLVLDMAKVLLD) and complete Freund's adjuvant (CFA) containing 500 μg of inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA). Mice received the emulsion at two sites on the lower back, followed by an intraperitoneal (i.p.) injection of 1.5 μg pertussis toxin (PTX) as an additional adjuvant. Mice were treated with PBS, bortezomib (Millennium Pharmaceuticals, Cambridge, MA) at the doses of 0.75 or 0.15 mg/kg (Velcade (H) and (L) groups, resp.), or etanercept (Enbrel, Wyeth Pharmaceuticals, Hampshire, UK) at the dose of 5 mg/kg in 0.1 mL by i.p. injection twice a week starting on the day of EAU induction.

2.3. Clinical Scoring of EAU. Ocular fundus of the mouse eyes was examined by slit lamp twice a week from the 7th day after induction until the end of experiments for clinical signs of EAU. Pupils were dilated using tropicamide and phenylephrine hydrochloride ophthalmic solutions. The severity of inflammation was clinically graded on a scale of 1–5 as described previously [15]. Briefly, 0 = no inflammation; 1 = focal vasculitis $\leq$ 5 spots or soft exudates $\leq$ 5; 2 = linear vasculitis or spotted exudates < 50% of the retina; 3 = linear vasculitis or spotted exudates $\geq$ 50% of the retina; 4 = retinal hemorrhage or severe exudates and vasculitis; 5 = exudative retinal detachment or subretinal (or vitreous) hemorrhage. A mouse was considered to have uveitis if at least one of its eyes had a score of two or more. The severity of uveitis is represented as the highest clinical score achieved by either eye in a mouse.

2.4. Histopathological Evaluation. Whole eyes were collected at the peak of the clinical response (21 days after induction of EAU), immersed in 10% formaldehyde, and then stored until being processed. Fixed and dehydrated tissues were embedded in paraffin and 3 μm sections were cut through the cornea-optic nerve plane and then stained with hematoxylin and eosin (H&E). Presence or absence of disease was evaluated in a blinded fashion by examining six sections cut at different levels for each eye. The severity of inflammation was histologically graded on a scale of 1–4 as described previously [16]. Briefly, 0 means no change; 1 means mild cell infiltration and focal retinal folds; 2 means moderate cell infiltration and retinal folds; 3 means moderate to heavy cell infiltration and extensive retinal folding with detachments; 4 means heavy cell infiltration with diffuse retinal detachment. Therefore, leukocytes infiltration into the vitreous cavity and retinal folding were considered as posterior uveitis.

2.5. Preparation of Retinal Lysate for Luminex Analysis. The eyes were enucleated from euthanized mice. The eyeballs were cut at the equator around the ora serrata, and the posterior pole of the eyes was separated from the anterior pole and lens. From the posterior pole, the neurosensory retina was extracted from retinal pigment epithelial layer. The extract from six retinas was placed in 300 μL of 0.5% NP-40 (Abcam) on ice (one minute) and briefly sonicated five times for 10 seconds at probe intensity of 7 (MicrosonTM XL2000 Ultrasonic liquid processor, Qsonica, LLC, Newton, CT). After removal of the insoluble material by centrifugation (200 ×g for 5 min), the protein concentration of the retinal extract was measured at 280 nm on ND-1000 Spectrophotometer. Then, the retinal lysate was used for Luminex analysis as below.

2.6. Analysis of Inflammatory Mediators in Retinas by Luminex. Quantification of TNF-α, IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-12, IL-17, and MCP-1 in retinal tissues was carried out using murine multiplexing bead immunoassays (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Briefly, 25 μL of retinal samples in PBS was incubated with antibody-coupled beads. After series of washes, a biotinylated detection antibody was added to the beads, and the reaction mixture was detected by the addition of streptavidin-phycoerythrin. The bead set was analyzed using a flow-based Luminex 200 suspension array system (Luminex Corporation, Austin, TX, USA).

2.7. Measurement of Proteasome Activity in the Retina. The chymotrypsin-like and trypsin-like activity of the proteasome of the retinas in the bortezomib or PBS-treated mice which were sacrificed 21 days after EAU induction was determined using commercial proteasome assay kits (Proteasome-Glo assay systems; Promega) according to the manufacturer's instructions. Briefly, the Suc-LLVY-Glo substrate (for chymotrypsin-like activity) or Z-LRR-Glo substrate (for trypsin-like activity) was added to the mixture of

the Proteasome-Glo buffer and the luciferin detection reagent and incubated at room temperature for 1 hour. The retinal tissue was minced in 100 μL of ice-cold PBS containing 5 mM EDTA followed by centrifugation at 12,000 g at 4°C for 10 minutes. A 50 μL of retinal sample was added by equal volume of reagent mixture and incubated for 90 minutes. Finally the luminescence of retinal sample was detected by a microplate luminometer (Promega).

2.8. Nuclear Protein Extract and Electrophoretic Mobility Shift Assay (EMSA) of NF-κB. Nuclear protein extracts were obtained as described previously [17]. Briefly, the retinas were minced in 0.5 mL of lysis buffer (10 mM HEPES, 1.5 mM KCl, 10 mM $MgCl_2$, 1.0 mM DTT, and 1.0 mM PMSF). The tissue was homogenized, followed by centrifugation at 5,000 g at 4°C for 10 minutes. The sediment was suspended in 200 μL of extraction buffer (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and 4 μM leupeptin), and the suspension was incubated on ice for 30 minutes. The sample was then centrifuged at 12,000 g at 4°C for 30 minutes. The supernatant containing the nuclear proteins was collected and stored at −70°C until use. The protein concentration was determined with a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). The EMSA was performed with an NF-κB DNA-binding protein detection system (Pierce Biotechnology) according to the manufacturer's instructions. A 10 μg nuclear protein was incubated with a biotin-labeled NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) for 30 minutes in binding buffer. The specificity of the DNA protein binding was determined by adding a 100-fold molar excess of unlabeled NF-κB oligonucleotide for competitive binding 10 minutes before adding the biotin-labeled probe.

2.9. Statistical Analysis. Values are shown as the mean ± SD. For statistical comparison, data were analyzed by the Wilcoxon signed-rank test, Student's *t*-test, or Chi-square test using Prism 5.0 software. In all tests, *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. A High Dose of Bortezomib Significantly Decreased Uveoretinitis in EAU Mice. EAU was induced in mice by injecting 200 μg of $IRBP_{1-20}$ emulsified with CFA subcutaneously and 1.5 μg of pertussis toxin (PTX) intraperitoneally as described in Section 2. At the same time, high- (0.75 mg/kg) and low-dose (0.15 mg/kg) bortezomib were injected intraperitoneally into mice and then twice a week until the end of the experiments. A group of mice which received PBS instead of bortezomib served as controls. In PBS-treated group, the disease showed sign of inflammation 9–15 days later and developed over the following 4-5 days when it reached the peak. Mice that received $IBRP_{1-20}$ immunization plus treatment with high-dose bortezomib exhibited a significant delay in disease onset and a significantly lower peak EAU score over time (Figure 1(a), Table 1). While both the saline-treated and low-dose bortezomib-treated mice had higher incidence of disease (14 of 19 and 10 of 17, resp.), we observed that the high-dose bortezomib-treated mice had a significantly lower incidence of EAU (3 of 19, $P < 0.05$) (Table 1). The mice that received saline treatment had a mean clinical severity score of 2.16 ± 0.25 while the mice that received high-dose bortezomib treatment had a mean clinical severity score of 0.58 ± 0.18 ($P < 0.05$) (Table 1). The mice that received low-dose bortezomib treatment showed a slightly lower mean clinical severity score of 2.00 ± 0.23, which is not significantly different from that of the saline-treated control group. The fact that the majority of mice given high-dose bortezomib had peak scores of 1 or lower (i.e., mild or no disease) indicated a suppressive activity of bortezomib on EAU. In addition, examination of H&E stained paraffin fixed slides revealed that retinal sections of eyes from EAU mice that received high-dose bortezomib had a reduced cell infiltration into the vitreous cavity and their retinal layer structures lacked the retinal folds observed in the saline-treated mice (Figures 1(b)–1(e)). There was no mortality or extraocular morbidity associated with the bortezomib treatment in the experimental animals. The body weight and the level of hemoglobin of the mice did not differ significantly between the saline- and bortezomib-treated groups at the end of experiment (data not shown). There was also no tumor growth or infection after bortezomib treatment in our study.

Table 1: Effect of high-dose versus low-dose bortezomib (Velcade) on EAU[a].

Treatment	Incidence	Mean peak disease score
Saline	14/19	2.16 ± 0.25
Vel (L)[b]	10/17	2.00 ± 0.23
Vel (H)[c]	3/19[*]	0.58 ± 0.18[#,+]

[a]Data are compiled from three experiments in which similar results were obtained.
[b]Low-dose bortezomib 0.15 mg/kg ip treatment.
[c]High-dose bortezomib 0.75 mg/kg ip treatment.
[*]$P < 0.05$, via the Chi-square test, between saline and Vel (H) groups.
[#]$P < 0.05$, via the Wilcoxon signed-rank test, between saline and Vel (H) groups.
[+]$P < 0.05$, via the Wilcoxon signed-rank test, between Vel (L) and Vel (H) groups.

3.2. Bortezomib Treatment Suppressed EAU More than TNF-Alpha Antagonist Treatment. Previous studies showed that TNF-α antagonist could also suppress uveitis in human and mice [18, 19]. We hence compared the effect of suppression of EAU by bortezomib or TNF-α antagonist etanercept. Bortezomib (0.75 mg/kg) or etanercept (5 mg/kg) was injected into EAU mice twice a week from the day of EAU induction. A group of mice that received PBS (0.1 mL/mouse) served as controls. Mice that received $IRBP_{1-20}$ immunization plus treatment with bortezomib exhibited a significant delay in disease onset and a significantly lower peak EAU score over time (Figure 1(f), Table 2). The mice that received treatment with etanercept also had lower incidence and mean peak disease score. However, the differences between saline- and etanercept-treated groups did not reach statistical significance (Table 2, $P = 0.06$). Therefore, treatment with

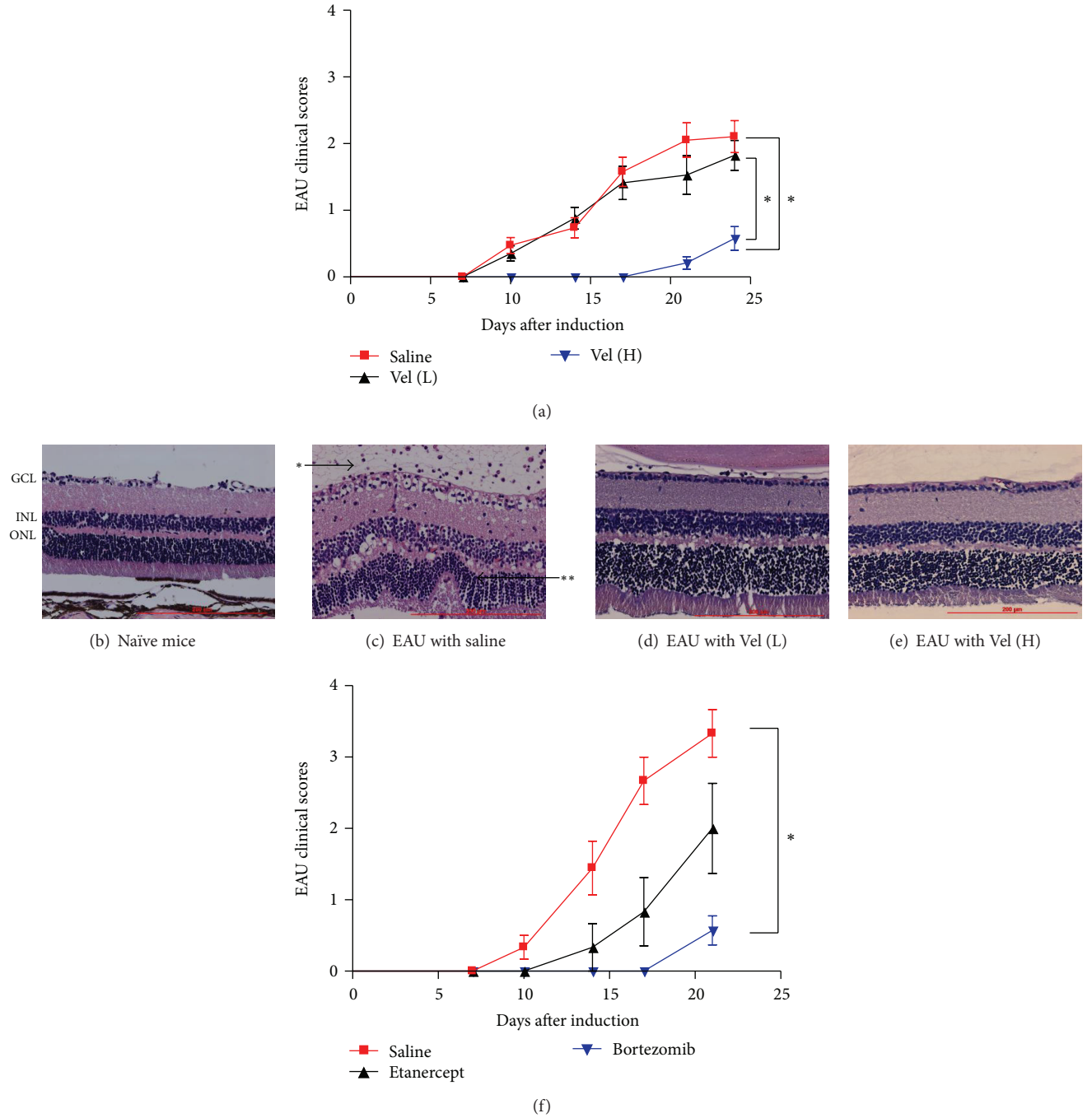

Figure 1: Effect of bortezomib on clinical course of EAU induced with IRBP. (a) Comparison of clinical scores of EAU mice treated with high-dose (0.75 mg/kg) bortezomib (red line, $n = 19$), low-dose (0.15 mg/kg) bortezomib (black line, $n = 17$), or PBS (blue line, $n = 19$) in 0.1 mL. Data shown are the mean clinical score (ordinate) of each experiment group over time (abscissa) and the sum of three independent experiments. Comparison of (the course of the clinical symptoms) high-dose bortezomib-treated EAU mice (blue line) with saline-treated mice (red line) shows a significant difference and is indicated as (*). Comparison of high-dose bortezomib-treated (blue line) with low-dose bortezomib-treated (black line) EAU mice also shows a significant difference and is indicated as (*). $^*P < 0.05$, via the Wilcoxon signed-rank test. (b), (c), (d), and (e): photomicrographs of H&E stained retinal tissue. Representative photomicrographs of paraffin-fixed H&E stained slides of the retina of (b): naïve C57BL/6 mice without EAU induction, (c): EAU mice that received 0.1 mL PBS treatment (* indicates leukocytes in vitreous cavity; ** indicates retinal folds), (d): EAU mice that received low-dose (0.15 mg/kg) bortezomib treatment, and (e): EAU mice that received high-dose (0.75 mg/kg) bortezomib treatment. The experiment was repeated three times with similar results. GCL: ganglion cell layer. INL: inner nuclear layer. ONL: outer nuclear layer. (f) Average clinical score over time of EAU in mice with high-dose (0.75 mg/kg) bortezomib (blue line, $n = 9$), etanercept (5 mg/kg) (black line, $n = 6$), or saline (0.1 mL/mouse) treatment (red line, $n = 7$). Data shown are the mean clinical score (ordinate) of each experiment group over time (abscissa) and the sum of two independent experiments. $^*P < 0.05$, via the Wilcoxon signed-rank test.

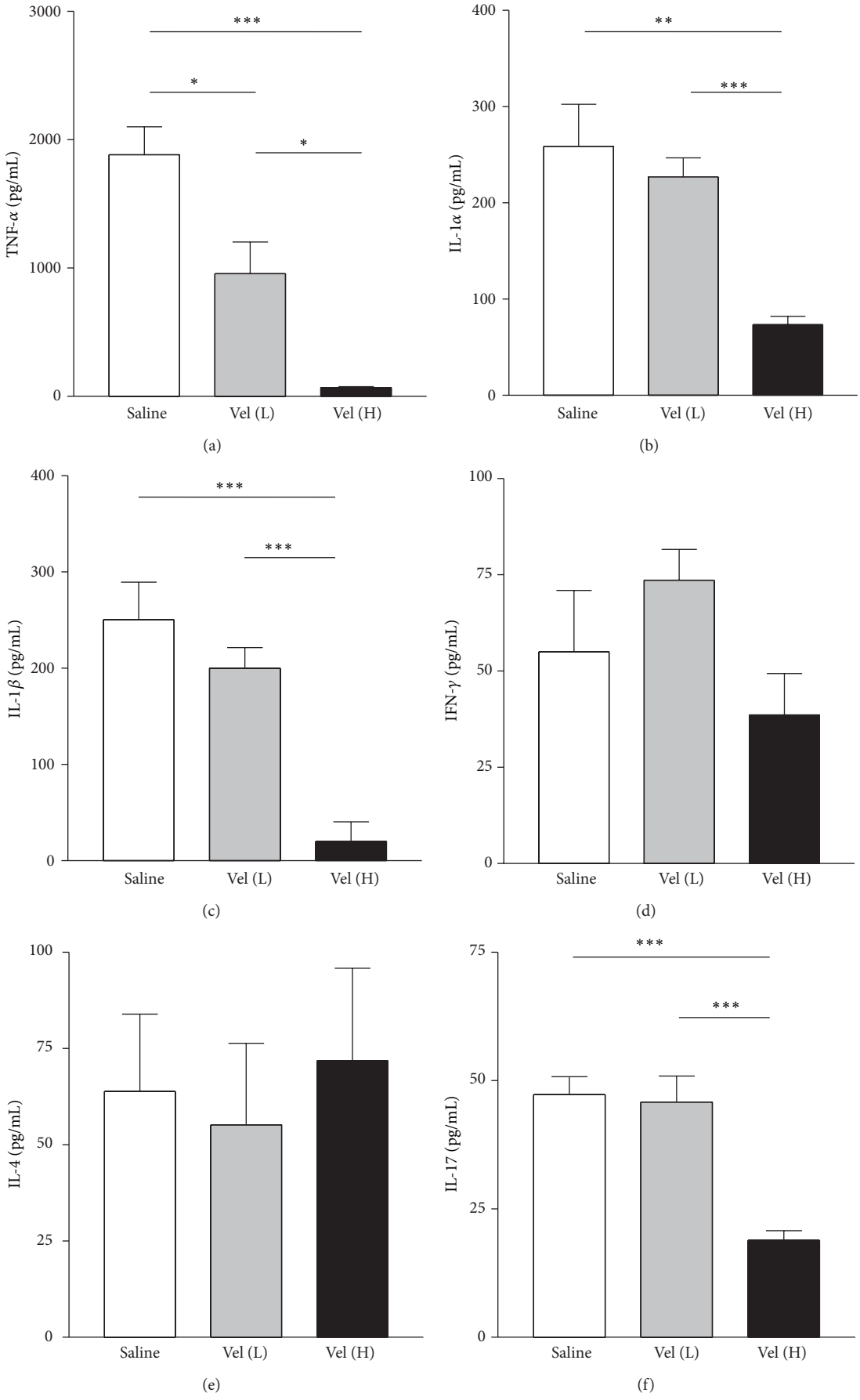

Figure 2: Continued.

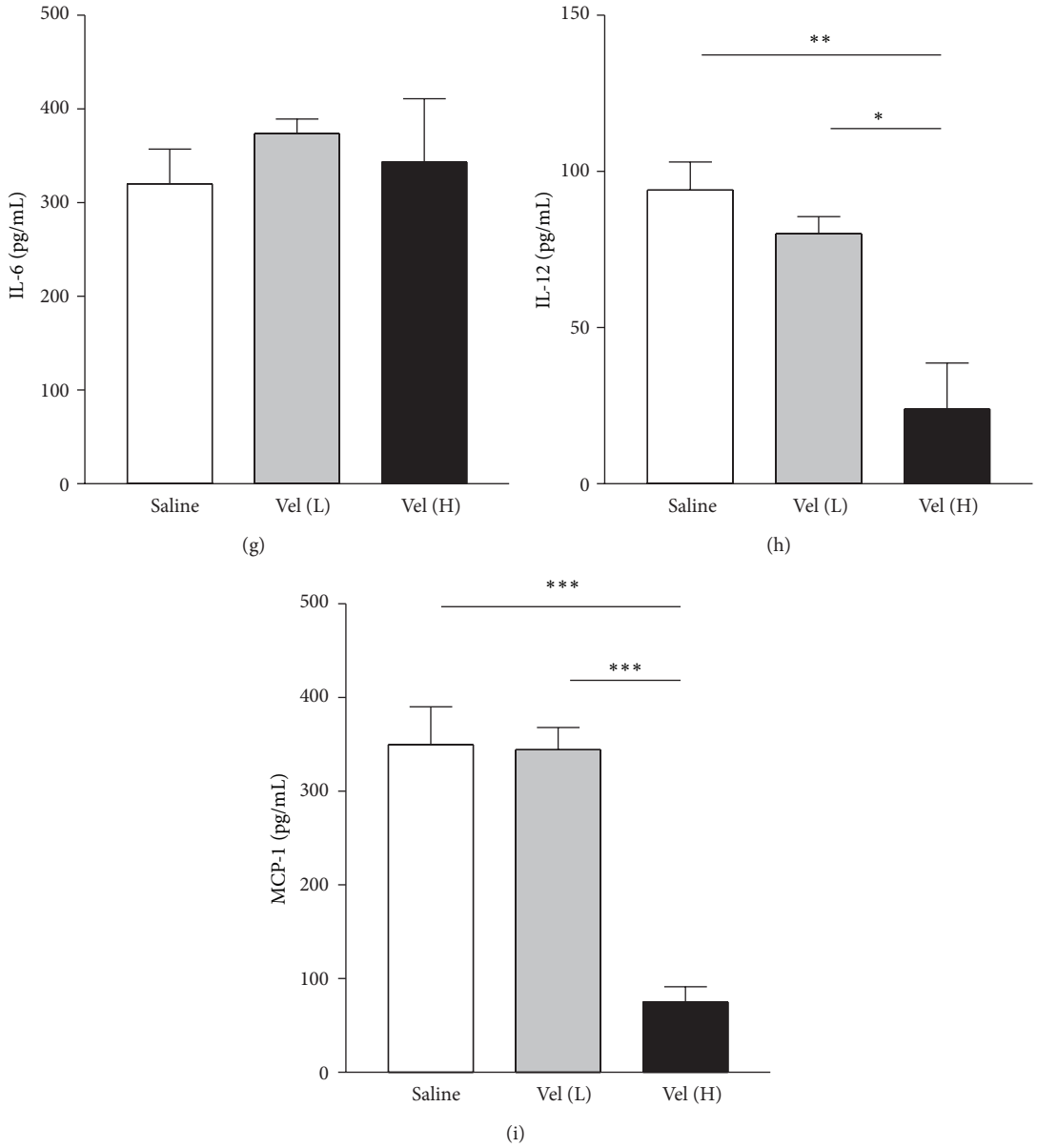

Figure 2: The evaluation of protein expression of inflammatory mediators in retinas of EAU mice in Luminex analysis. Decreased expression of TNF-α (a), IL-1α (b), IL-1β (c), IL-17 (f), IL-12 (h), and MCP-1 (i) relative to the expression in the saline-treated group was noted in the high-dose bortezomib (Vel [H]) group but not in the low-dose bortezomib (Vel [L]) group except for TNF-α. In addition, there was no significant difference on the expression of IFN-γ (d), IL-4 (e), and IL-6 (g) in retinas between bortezomib and saline-treated mice. Data are expressed as the mean SD of three independent experiments (bar graph). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, via Student's t-test.

bortezomib suppressed the development and severity of EAU more effectively than the TNF-α antagonist etanercept. There was no mortality, morbidity, tumor growth, or infection associated with the bortezomib or etanercept treatment in the EAU mice at the end of experiment.

3.3. The Influence of Bortezomib on the Levels of Inflammatory Mediators in Retina of EAU Mice. We then measured the cytokine levels in retinas in EAU mice with different treatment. When comparing the saline-treated group with low-dose bortezomib group, we found that TNF-α level was lower in the low-dose bortezomib-treated group, while the levels of other cytokines were not significantly different (Figures 2(a)–2(i)). Meanwhile, the levels of TNF-α, IL-1α, IL-1β, IL-12, IL-17, and MCP-1 in retina were significantly lower in the EAU mice treated with high-dose bortezomib when compared with saline-treated mice ($P < 0.05$ in all paired comparisons) (Figures 2(a), 2(b), 2(c), 2(f), 2(h), and 2(i)). However, there was no significant difference noted in IFN-γ, IL-4, and IL-6 between high-dose bortezomib-treated and saline-treated EAU mice (Figures 2(d), 2(e), and 2(g)). When comparing the groups treated with high-dose and low-dose bortezomib, we found that the levels of TNF-α, IL-1α, IL-1β, IL-12, IL-17,

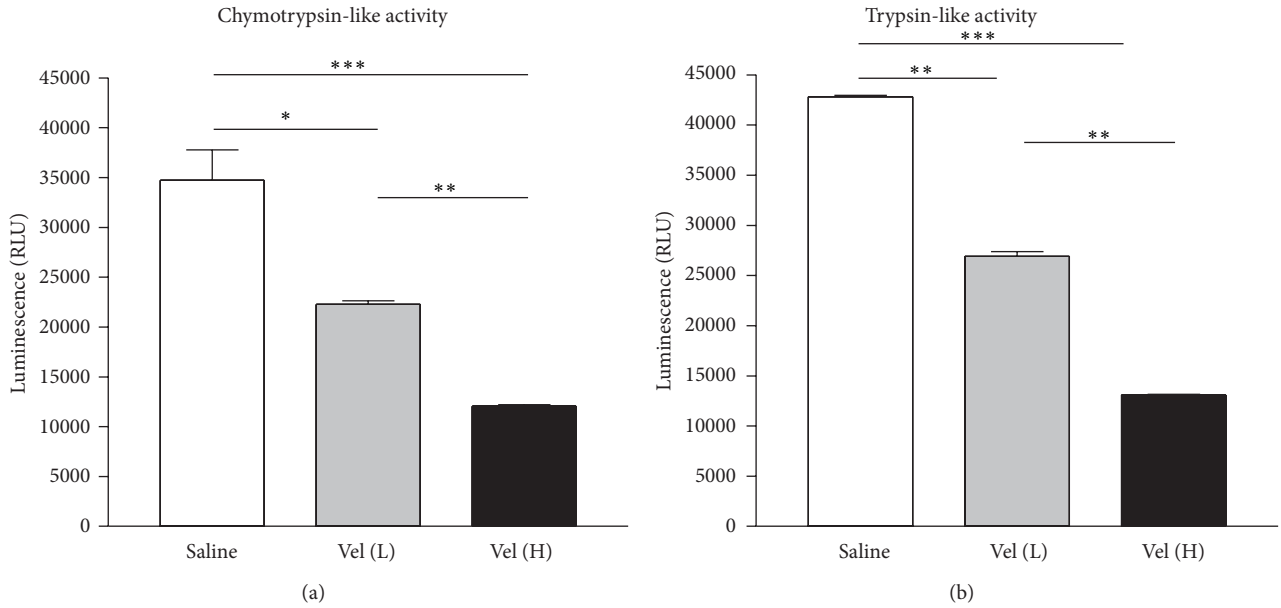

Figure 3: Evaluation of chymotrypsin-like and trypsin-like activity of the proteasome. Compared to the saline-treated EAU group, there was significantly decreased activity of chymotrypsin-like (a) and trypsin-like activity (b) in the low-dose [Vel (L)] and high-dose bortezomib [Vel (H)] groups. The activity was also markedly lowered in the high-dose bortezomib-treated group compared with the low-dose bortezomib-treated group. The data are expressed as the mean ± SD of the mean in 5 mice for each group (bar graph). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, via Student's t-test. The experiment was repeated three times with similar results.

Table 2: Effect of etanercept versus bortezomib (Velcade) on EAU.

Treatment	Incidence	Mean peak disease score
Saline	8/9	3.33 ± 0.33
Etanercept[a]	3/6	2.00 ± 0.63
Velcade[b]	0/7[*]	0.57 ± 0.20[#]

[a]Etanercept 5 mg/kg ip treatment.
[b]Bortezomib (Velcade) 0.75 mg/kg ip treatment.
[*]$P < 0.05$, via the Chi-square test, between saline and bortezomib group.
[#]$P < 0.05$, via the Wilcoxon signed-rank test, between saline and bortezomib group.

and MCP-1 were lower in the high-dose bortezomib group in comparison with the low-dose bortezomib group ($P < 0.05$ in all paired comparisons) (Figures 2(a), 2(b), 2(c), 2(f), 2(h), and 2(i)).

3.4. Bortezomib Treatment Significantly Reduced the Proteasome Activity of EAU Mice. We then performed proteasome protease activity assays to evaluate the suppressive effect of bortezomib treatment in retinal tissue. The signal of luminescence indicates chymotrypsin-like or trypsin-like activity in the retinal tissues of EAU mice. The signals were significantly lower in the high-dose bortezomib-treated group when compared with the saline or low-dose bortezomib-treated group ($P < 0.05$) (Figures 3(a) and 3(b)). There was also a significant difference in the signal of luminescence between the low-dose bortezomib and saline-treated groups (Figures 3(a) and 3(b)).

3.5. The Increased Binding of NF-κB and DNA in EAU Mice Was Inhibited by Bortezomib Treatment. The involvement of NF-κB pathway during bortezomib treatment in EAU was analyzed with EMSA. Compared to the naïve group, the NF-κB DNA binding increased after EAU induction significantly (Figure 4, Shift of Naïve and Saline). The increased activity of NF-κB DNA binding after EAU induction was markedly inhibited by treatment with low-dose bortezomib. High-dose bortezomib treatment further suppressed the NF-κB DNA binding (Figure 4, Shift of Vel (L) and Vel (H)). Adding a 100-fold molar excess of unlabeled NF-κB probe completely blocked the binding of the labeled probe to the NF-κB DNA complex (Figure 4, Shift of 100X). Therefore, bortezomib reduced the binding of NF-κB DNA in a dose-responsive manner. The results from protease inhibition (Figure 3) and NF-κB DNA binding implicated that the activation of NF-κB was effectively suppressed by proteasome inhibition.

4. Discussion

Our study demonstrated that bortezomib, a 26S proteasome inhibitor, is active in suppressing NF-κB activation and is effective in inhibiting ocular inflammation and reducing the production of inflammatory mediators in EAU. Our results indicate that inhibition of proteasome may be a promising approach to treating autoimmune uveitis.

Recent evidence indicated that NF-κB has a pivotal role in EAU and that the inhibition of NF-κB activation can reduce the levels of tissue inflammation by lowering the inflammatory mediators and cell infiltration into the uvea [6, 7].

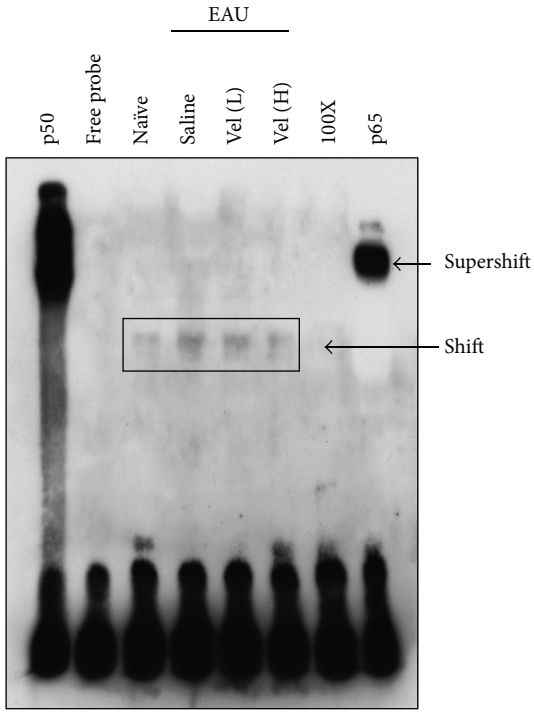

Figure 4: EMSA for the evaluation of the NF-κB DNA-binding activity in naïve mice and different groups of EAU mice. *Lane 1*: p50 subunit of NF-κB. *Lane 2*: free probe (FP). *Lane 3*: naïve C57BL/6 mice. *Lane 4*: EAU mice with saline treatment. *Lane 5*: EAU mice treated with low-dose bortezomib (Vel [L]). *Lane 6*: EAU mice treated with high-dose bortezomib (Vel [H]). *Lane 7*: 100-fold molar excess of unlabeled NF-κB probe. *Lane 8*: anti-p65 subunit of NF-κB. The sample was pooled from both eyes of five mice in each group. Data are representative of results in three independent experiments.

Since proteasomal degradation of the inhibitory factor IκB is important for NF-κB activation, the inhibition of proteasome maintains NF-κB in the inactive state in the cytosol and prevents its nuclear translocation. Proteasome inhibition has been found to be effective in treating several animal models of autoimmune disease such as myasthenia gravis, psoriasis, arthritis, and autoimmune encephalomyelitis [10–13]. Moreover, Chen et al. have shown the anti-inflammatory effect of proteasome inhibitor on endotoxin-induced uveitis in rats [17]. The proteasome inhibitors bortezomib, due to their activity to suppress nonlysosomal protein degradation, has been used in the treatment of hematologic cancers in clinical settings [20]. In this study, we chose bortezomib based on its high efficacy at minimal concentrations and tolerable and manageable adverse effects in treating human hematologic diseases [21]. To our knowledge, our study is the first to demonstrate its anti-inflammatory effect in EAU.

Being an autoantigen-induced autoimmune condition, EAU has an inflammation dominated by acute inflammatory cytokine response [3]. In our study, we found that levels of inflammatory mediators including TNF-α, IL-1α, IL-1β, IL-12, IL-17, and MCP-1 increased significantly in saline-treated EAU mice when compared with those in naïve mice (Figure 2). TNF-α is a major proinflammatory cytokine and plays a central role in autoimmune uveitis [3]. TNF-α antagonists have been used clinically to treat ocular inflammatory disorders successfully [19, 22]. Therefore, in this study we compared the anti-inflammatory effect between proteasome inhibitor bortezomib and the TNF-α antagonist, etanercept, in EAU. Etanercept, a chimeric protein of human TNF-α receptor and Fc portion of immunoglobulin G heavy chain, can neutralize TNF-α and inhibit its proinflammatory activity in both humans and mice [18, 23, 24]. We found that bortezomib treatment in EAU mice could suppress not only TNF-α but also many other inflammatory mediators such as IL-1α, IL-1β, IL-12, IL-17, and MCP-1 in retinas so the autoimmune uveitis could be more effectively suppressed with bortezomib than with TNF-α antagonist etanercept (Figure 1(f)). Bortezomib, which may suppress multiple inflammatory cytokines through inhibiting NF-κB activation, hence appears to be a better anti-inflammatory agent in treatment of autoimmune uveitis than TNF-α antagonists.

The mechanisms of the anti-inflammatory effects of bortezomib, however, may be more complex than the inhibition of NF-κB activation. As the induction of EAU apparently involves the antigen presentation of the immunogenic antigen by major histocompatibility complex class I and class II molecules, the inhibition of proteasome, which is a critical component of the antigen processing, may also affect the autoimmune recognition process in sensitization and stimulation phases [25–29]. In addition, proteasome inhibitors have been demonstrated to trigger the apoptosis of leukocytic cells, which may contribute to their immunosuppressive and antitumor effect [30–33]. Proteasome inhibition hence may suppress several key steps necessary for activating the autoimmune responses in EAU.

In our study, the mice were treated with bortezomib from the same day when EAU was induced to ensure the onset of the drug's effect during early stage of the disease development. We have not evaluated the drug's efficacy when it is applied after EAU is full-blown, as usually is the case in clinic settings. At the end of experiment after bortezomib or etanercept treatment, there was no mortality, tumor growth, or severe infection noted. However, since the proteasome and its ubiquitous distribution regulate the wide range of biological functions, the systemic adverse effects associated with proteasome inhibitors deserve meticulous consideration.

In summary, we demonstrated that bortezomib ameliorated experimental autoimmune uveitis in mice in a dose-dependent manner. Reduced intraocular inflammation was associated with the inhibition of NF-κB activation and decreased expression of many inflammatory mediators. Our encouraging results indicate that drugs targeting the proteasome may be an effective treatment strategy for autoimmune uveitis in the future.

Abbreviations

EAU: Experimental autoimmune uveitis
IRBP: Interphotoreceptor retinoid-binding protein
NF-κB: Nuclear factor-kappa B.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Disturbances of Modulating Molecules (FOXP3, CTLA-4/CD28/B7, and CD40/CD40L) mRNA Expressions in the Orbital Tissue from Patients with Severe Graves' Ophthalmopathy

Przemyslaw Pawlowski,[1,2] Natalia Wawrusiewicz-Kurylonek,[3] Anja Eckstein,[4] Joanna Reszec,[1] Wlodzimierz Luczynski,[5] Kristian Johnson,[6] Adam Kretowski,[3] Alina Bakunowicz-Lazarczyk,[2] Maria Gorska,[3] Jacek Szamatowicz,[7] Lech Chyczewski,[1] and Janusz Mysliwiec[8]

[1]*Department of Medical Pathomorphology, Cathedral of Biostructure, Medical University of Białystok, 13 Waszyngtona Street, 15-269 Białystok, Poland*
[2]*Department of Pediatric Ophthalmology with Strabismus Treatment Unit, Medical University of Białystok, 17 Waszyngtona Street, 15-274 Białystok, Poland*
[3]*Department of Diabetology, Endocrinology and Internal Medicine, Medical University of Białystok, 24A Skłodowskiej-Curie Street, 15-276 Białystok, Poland*
[4]*Department of Ophthalmology, Essen University Hospital, University of Duisburg-Essen, Hufeland Straße 55, 45 122 Essen, Germany*
[5]*Department of Pediatrics, Endocrinology and Diabetology with Cardiology Division, Medical University of Bialystok, 17 Waszyngtona Street, 15-274 Białystok, Poland*
[6]*Bayer HealthCare, 10 Kaiser-Wilhelm-Allee, 51373 Leverkusen, Germany*
[7]*Department of Gynaecology and Oncologic Gynaecology, Medical University of Bialystok, 24A Skłodowskiej-Curie Street, 15-276 Białystok, Poland*
[8]*Department of Nuclear Medicine, Medical University of Bialystok, 24A Skłodowskiej-Curie Street, 15-276 Białystok, Poland*

Correspondence should be addressed to Przemyslaw Pawlowski; przemyslaw.pawlowski@umb.edu.pl

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Purpose. To evaluate the relationship between the expression of orbital tissue mRNA for FOXP3, CTLA-4/CD28/CD80/CD86, and CD40/CD40 and the severity of Graves' orbitopathy (GO). *Material and Methods.* Orbital tissue was obtained from 26 patients with GO, with mild ($n = 6$) or severe GO ($n = 20$), and 7 healthy controls. The expression of mRNA of FOXP3, CTLA-4/CD28/CD80/CD86, CD40/CD40L was measured by RT-PCR. TCR and CD3 were evaluated by immunohistochemistry. *Results.* Higher mRNA for FoxP3 (relative expression: 1.4) and CD40 (1.27) and lower expression of CTLA-4 (0.61) were found in the GO tissues versus controls. In severe GO as compared to mild GO higher mRNA expression for FoxP3 (1.35) and CD40 (1.4) and lower expression for CTLA-4 (0.78), CD28 (0.62), and CD40L (0.56) were found. A positive correlation was found between FOXP3 mRNA and CD3 infiltration ($R = 0.796$, $P = 0.0000001$). *Conclusions.* The enhanced FOXP3 mRNA expression in GO samples may suggest the dysfunction of FOXP3 cells in the severe GO. The diminished mRNA expression of CTLA-4 in severe GO may indicate inadequate T regulatory function. The enhanced mRNA expression of CD40 in severe GO and negative correlation to CRP mRNA may suggest their role in the active and inactive GO.

1. Introduction

Graves' orbitopathy (GO) is an organ-specific autoimmune disease with a multifactorial etiology, involving genes and environmental triggers, with smoking as a first example, that cause autoimmune dysregulation [1–3]. It is unknown why only a small subset of patients with Graves' disease (GD) develops GO. Vulnerability to GO manifestations most likely is connected with the highly specialized function of the orbital tissue, a unique fat depot that cushions the globe [4].

Most orbital disorders are inflammatory [1] suggesting that orbital fat may be especially prone to robust inflammatory reactions. Indeed, as compared with fibroblasts from other sites, orbital fibroblasts show exaggerated inflammatory responses to various stimuli [5, 6]. Orbital fibroblasts express CD40, a costimulatory protein present on the surface of many types of cells, including macrophages, lymphocytes, and thyrocytes. CD4+ T cells expressing the CD40 ligand (CD154) directly activate orbital fibroblasts to proliferate through the formation of CD40-CD154 bridges [7–9].

As with other autoimmune diseases, the initiating event results in loss of tolerance and autoreactivity in the orbit and subsequently leads to mononuclear cell infiltration [10]. Since T cells infiltrating the retrobulbar tissues are likely to play a key role in the pathogenesis of orbital inflammation Forkhead box P3 (FOXP3), CTLA-4/CD28 and its ligands CD80/CD86 have been considered as candidate genes for GO [11, 12]. FoxP3 is a crucial regulatory factor for the development and function of regulatory T cells (T_{regs}), and deficiency of the FOXP3 suppresses the regulatory function of T_{regs} [13]. Cytotoxic T lymphocyte antigen 4 (CTLA-4), a molecule specifically and constitutively expressed on T_{regs}, plays a key role as a negative regulator of T-cell activation [14]. The CTLA-4 gene was reported to be associated with susceptibility to GO and the G allele at exon 1 CTLA-4(49) A/G polymorphism was correlated with severity of GO [15, 16]. In addition, FOXP3 and CD40 genetic association was evaluated both in human and in animal models of GO [17, 18].

CD80/CD86 (B7-1, B7-2) counter-receptors for CD28/CTLA-4, expressed mainly on the antigen-presenting cells (APC), renders, that activated T cells expressing B7 family molecules could act as APC [12, 19]. Recently, the polymorphisms of the CD86 gene were suggested as genetic markers, which could be helpful for making the diagnosis and prognosis of GO [12].

The present study was designed to investigate the expression of mRNA of the immune regulatory molecules FOXP3, CTLA-4/CD28/CD80/CD86, and CD40/CD40L in the orbital tissues from patients who underwent orbital decompression due to Graves' orbitopathy to assess their role in the inflammatory process.

2. Materials and Methods

2.1. Patients and Controls. Human orbital fat/connective tissue was derived from the orbital tissue bank at the Department of Ophthalmology and University of Essen. It was obtained from 26 patients with GO (25 females and 1 male) who underwent orbital decompression. The mean age of patients at the time of surgery was 44.5 years (range: 36–49). Normal orbital connective tissues were derived from 7 individuals (6 females and 1 male) undergoing blepharoplastic surgery with no history of GO or any orbital inflammatory disease. The clinical activity score (CAS) of GO was estimated according to Mourits (Mourits et al., 1997). The severity of the eye disease was estimated using a modified classification of no signs or symptoms; only signs, no symptoms; signs only; proptosis; eye muscle involvement; corneal involvement; and sight visual acuity reduction (NOSPECS) described by Eckstein et al. [20]. The mean CAS was 5.92 (1–10) and the mean NOSPECS was 7.46 (2–13).

The study was approved by the Medical Ethics Committee of the University of Essen, Germany and all study participants gave written informed consent.

2.1.1. Grouping of Patients. The patients were grouped according to the CAS and NOSPECS scores on the follow-up at 11–14 months after onset of GO into those with a mild or severe course of GO. The mild signs of GO were described as CAS < 4 (almost inactive GO) and NOSPECS < 5 (mild GO), whilst the severe signs were established as CAS ≥ 4 (still active disease) and/or NOSPECS ≥ 5—"severe GO." The examiner who classified the patients was blind to the thyroid status and TRAb values.

Clinical characteristics of patients are shown in Table 1.

2.2. RNA Extraction and cDNA Synthesis. Total RNA from the tissue was isolated and purified with the use of RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. One microgram of total RNA was used to prepare cDNA, and cDNA synthesis was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

2.3. RT-PCR and Data Analysis. The levels of transcripts were measured by real-time PCR using human genes QuantiTect Assays and QuantiTect Hs_GAPDH Assay (Qiagen, Valencia, CA, USA) as a normalizer. The following genes were assessed: FOXP3, CD152 (CTLA-4), CD28, CD80 (B7-1), CD86 (B7-2), CD40, CD154 (CD40L), and CRP (C-reactive protein). Real-time PCR was performed in duplicate in 20 μL, using the QuantiTect SYBR Green PCR Master Mix (Qiagen) applying the manufacturer's instructions, and conducted in the Chromo4 Real-Time PCR Detector (BIO-RAD, USA). A standard curve construction was generated by a series of four dilutions of cDNA of the control group sample in reaction to the house-keeping gene, GAPDH. On the basis of these curves, the levels of total chosen gene transcripts were calculated after their normalization to GAPDH. The value of CT was determined by the first cycle number at which fluorescence was higher than the set threshold value.

2.4. Evaluation of TCR and CD3 Expression by Immunohistochemical Methods. Following the deparaffinization and rehydration, epitope retrieval was carried out in the EnVision

Table 1: Characteristics of patients.

	Severe course of GO	Mild course of GO	Mild + severe GO
Number of patients	20	6	26
Age	42.3 (36–46)	46.2 (42–49)	44.5 years (36–49)
Gender	19F; 1M	6F	25F; 1M
Smokers	88%	50%	77%
TRAb (mean ± SD)	23 ± 11.57	2.56 ± 1.3	18.89 ± 13.59
CAS	7.1 (4–10)	2 (1–3)	5.92 (1–10)
NOSPECS	8.55 (5–13)	3.83 (2–5)	7.46 (2–13)
Proptosis right	18 (14.5–25)	16.25 (14–18)	17.5 (14–25)
Proptosis left	19.5 (15–25)	16.5 (14–21.5)	17.5 (14–25)

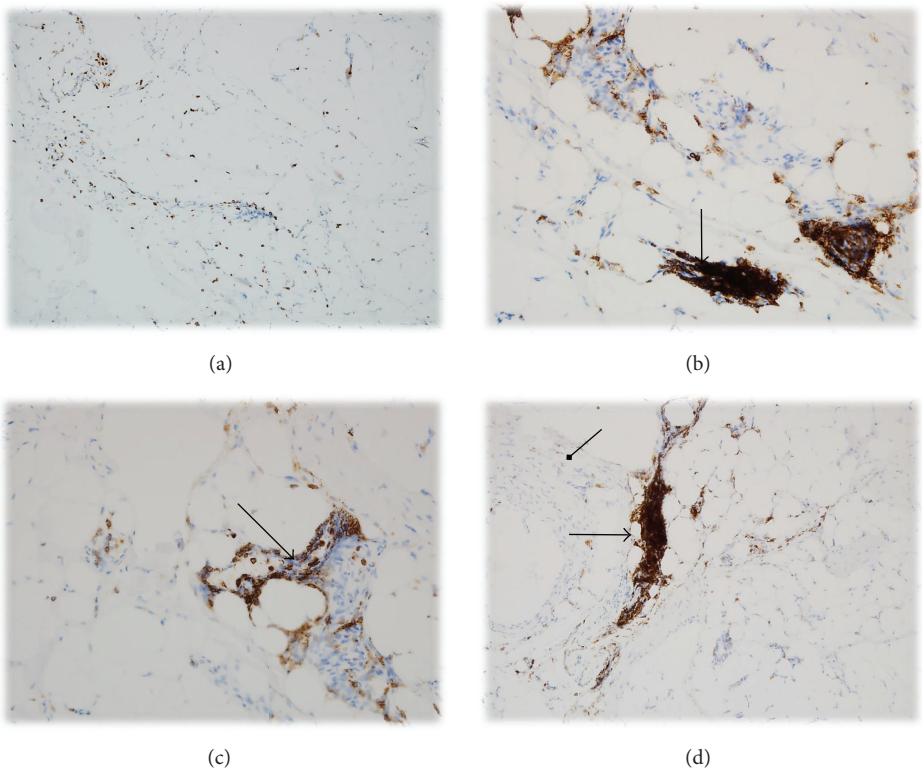

Figure 1: (a) Strong and diffuse TCR expression in lymphoid cells within the mild Graves ophthalmopathy. Magnification: 100x. (b) Focuses of the lymphoid cells aggregation (TCR expression) in the mild Graves ophthalmopathy. Magnification: 200x. ((c), (d)) Strong and diffuse TCR receptor expression in lymphocytes within severe Graves ophthalmopathy. Magnification: 200x, 100x (the arrows show T cells and fibroblasts).

Flex Target Retrieval Solution (DAKO) in high pH. Endogenous peroxidases were blocked by incubating the sections in methanol and 3% hydrogen peroxidase for 20 minutes. Next slides were incubated with mouse monoclonal antibody against TCR receptor (Novocasta) TCR alpha/beta Antibody (R73) in 1 : 100 dilution for 30 minutes in room temperature. Against CD3 receptor, were used rabbit polyclonal antibody (Anti-CD3 antibody (ab5690)) in 1 : 75 dilution for 30 minutes in room temperature. Visualization reagent EnVision (DAKO) was applied for 30 minutes followed by DAB solution for 10 minutes. The slides were then counterstained with hematoxylin and examined under the light microscope. The intensity of immunostaining was evaluated in random 10 fields under 20x magnification. The results were expressed as the percentage of cells with a strong positive staining as follows: ≤10% positive cells: negative (−), between 11% and 50% (+), and >50% positive cells (++) (see Table 2).

3. Statistical Analysis

In order to calculate mRNA expression, a comparative CT method was used for relative quantification, that is, $2^{-\Delta\Delta CT}$, following Livak and Schmittgen [21]. The results were analyzed in Statistica 9.0 for Windows (StatSoft, Poland). Owing to asymmetric data distribution, nonparametric tests

Table 2: Immunohistochemical evaluation grading score for TCR and CD3 of each specimen.

Number	Group	TCR	CD3
1	Control	0	0
2	Control	0	0
3	Control	0	0
4	Control	0	0
5	Control	0	0
6	Control	0	0
7	Control	0	0
8	Mild GO	0	0
9	Mild GO	1	1
10	Mild GO	1	1
11	Mild GO	1	1
12	Mild GO	1	1
13	Mild GO	0	0
14	Severe GO	1	1
15	Severe GO	1	1
16	Severe GO	1	1
17	Severe GO	1	1
18	Severe GO	2	1
19	Severe GO	1	1
20	Severe GO	1	1
21	Severe GO	2	1
22	Severe GO	2	1
23	Severe GO	2	2
24	Severe GO	1	1
25	Severe GO	2	1
26	Severe GO	2	1
27	Severe GO	1	1
28	Severe GO	1	1
29	Severe GO	1	1
30	Severe GO	1	1
31	Severe GO	1	1
32	Severe GO	2	1
33	Severe GO	1	1

Legend for Table 2: Immunohistochemistry scores are as follows:
0: less than 10% positive in 10 representative high power fields (HPF),
1: 11%–50% positive cells in 10 HPF,
2: more than 50% positive cells in 10 HPF.
Immunohistochemistry was done using DAB chromogen (brown staining).

were used. Significance levels were calculated in accordance with Mann-Whitney U test (differences between the control and examined group). The correlations between the clinical parameters and RT-PCR results were assessed with Spearman's rank correlation test. The level of $P < 0.05$ was regarded as significant. Due to the small amount of samples, the relative values (a comparison between the control and examined samples) below 0.75 were regarded as a decreased gene expression, between 0.75 and 1 as a comparable expression, and above 1 as an increased expression [22]. The data are presented as the relative expression of genes in examined samples compared to control samples with the example of individual data in the case of FoxP3 gene. The graphs were prepared in GraphPad Prism 5.0.

4. Results

4.1. CD3 and TCR Expression in Lymphoid Cells In Situ Immunohistochemical Evaluation

4.1.1. TCR Expression in Mild GO and Severe GO. Focal lymphoid cells aggregation with TCR expression in the mild Graves ophthalmopathy was seen in (4/6 samples) 66.66 % with mild GO and similarly in 65% with severe GO (13/20 samples). However, in 7/10 (35%) with severe GO, the expression of TCR in the lymphoid cell was evaluated as (++) (Figures 1 and 5).

In addition, no expression of CD3 or TCR was revealed in the control tissue specimens (Table 3).

4.1.2. CD3 Expression in Mild GO and Severe GO. 66.66% with mild GO (4/6) and 95% with severe GO (19/20) had focal CD3 lymphoid cells infiltrations in the orbital tissue. In addition, in one patient with severe GO (5%), the infiltration was strong and diffused (Figures 1, 2, 3, 4, and 5 and Table 3)

4.2. The mRNA Expression in GO Tissues and Control Samples. The mRNA for FoxP3, CRP, CD40, and CD86 were present in all the samples ($N = 33$, 100.0%). The mRNA for CTLA-4 was detected in 3/7, that is, 42.8% of controls, and 19/26, that is, 73.0% of GO tissue samples. The mRNA for CD28 molecule was noted in 2/7, that is, 28.5% of controls, and 10/26, that is, 38.4% of GO tissue samples. The mRNA for CD40L molecule was present only in 1/7, that is, 14.2% of controls, and 8/26, that is, 30.7% of GO tissue samples. The mRNA for CD80 was not detected in controls (0/7) and only in 8/26, that is, 30.7%, of GO tissue samples. No statistically significant differences were noted between control and examined samples concerning the expression of assessed genes. However, according to our further analysis based on quantitative RT-PCR validation of the mRNA expression changes (as stated in statistical analysis), higher amounts of mRNA for FoxP3 (relative expression: 1.4), CD28 (1.06), CD40 (1.27), CD40L (1.17), and CD86 (1.17) and lower expression of CTLA-4 (0.61) and CRP (0.62) were found in GO as compared to control tissues (see Figure 3).

Further, the expression of mRNA between tissues from patients with severe to mild GO was compared. No significant differences were noted in accordance with Mann-Whitney U test. However, in the analysis of relative amounts of mRNA, we found higher expression of mRNA for FoxP3 (relative expression: 1.35) and CD40 (1.4) and lower expression of mRNA for CTLA-4 (0.78), CD28 (0.62), and CD40L (0.56) in severe GO as compared to mild GO tissue samples. The relative expression of mRNA for assessed molecules is presented in Figure 4.

We found no correlation between the clinical data and results from RT-PCR. However, CRP mRNA correlated

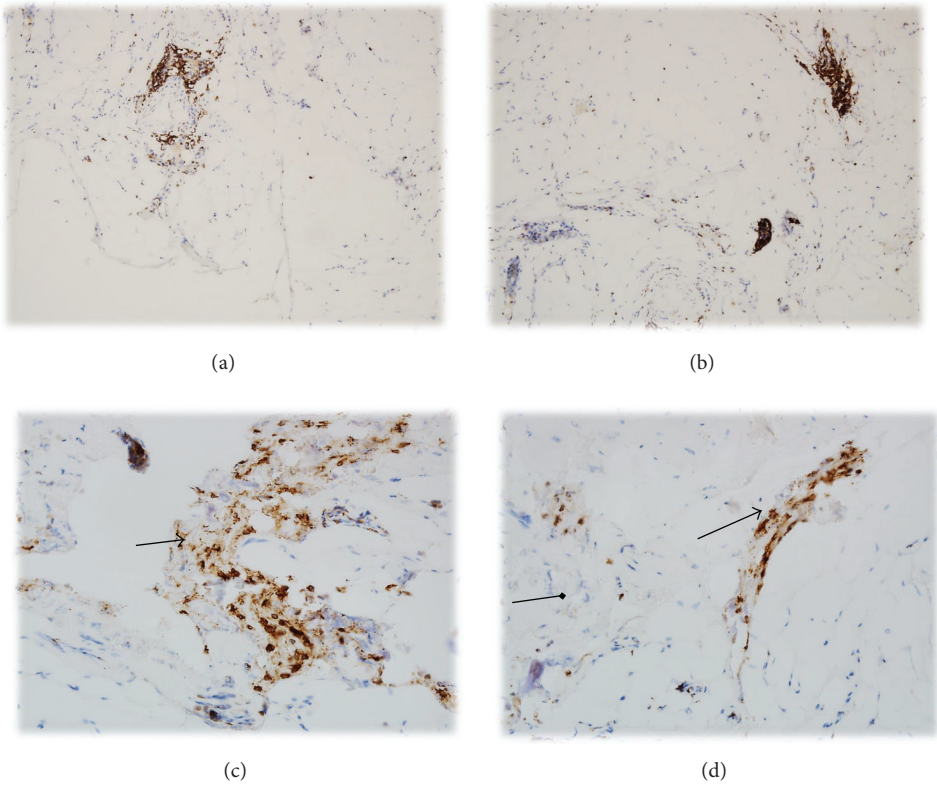

Figure 2: ((a), (b)) CD3 expression in T cells within the mild Graves ophthalmopathy. Magnification: 100x. ((c), (d)) CD3 strong expression in T cells within the severe Graves ophthalmopathy. Magnification: 200x (the arrows show T cells and fibroblasts).

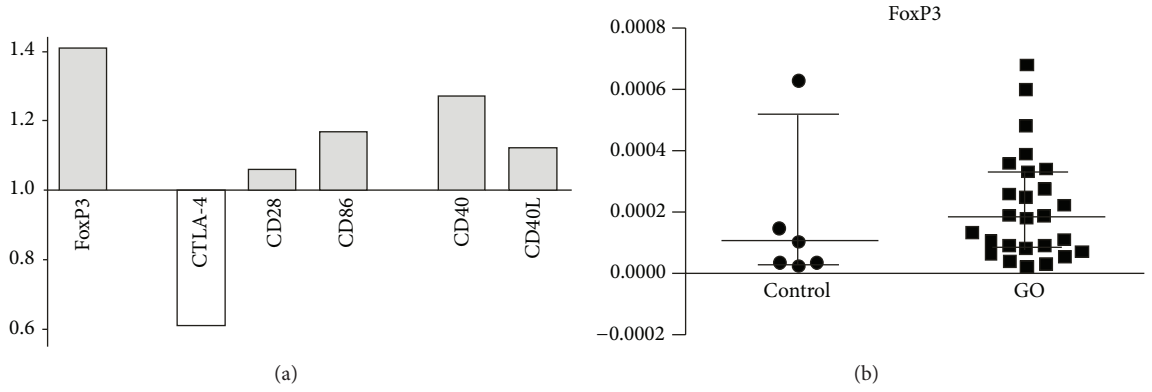

Figure 3: (a) Quantitative RT-PCR validation of the mRNA expression changes for the CD86/CD28/CTLA-4: FOXP3 and CD40/CD40L molecules in GO (mild + severe) orbital tissues compared to control tissue samples. The values above "1.0" on the y-axis show the relative higher expression in GO versus control samples and values below "1.0" indicate relatively lower expression. (b) The individual data from RT-PCR displaying the expression of mRNA for transcription factor FoxP3 in control and examined (GO) samples (medians and 25th–75th percentiles) ($P < 0.05$).

positively with FOXP3 ($R = 0.520$ and $P = 0.03$) and negatively with CD40 values ($R = -0.757$ and $P = 0.000001$). In addition, a positive correlation was found between FOXP3 mRNA and CD3 infiltration ($R = 0.358$, $P = 0.05$) and a strong correlation was found between TCR and CD3 expression ($R = 0.796$, $P = 0.0000001$) (see Table 4).

5. Discussion

The explanation why the connective tissue of the orbit and skin should be singled out for activation in GD concerns possible intrinsic differences in the residential orbital and leg cells as setting up these regions for disease involvement in GD [4].

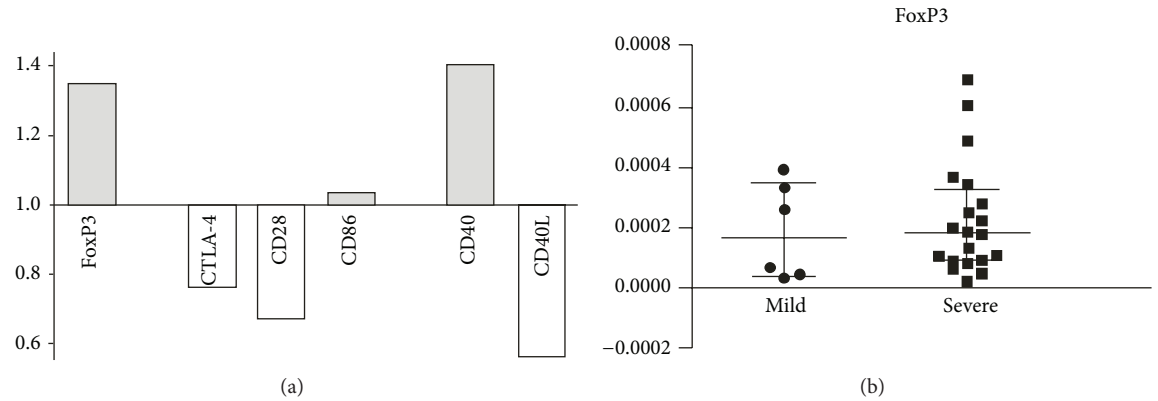

Figure 4: (a) Quantitative RT-PCR validation of the mRNA expression changes for assessed molecules in the severe GO compared to mild GO samples. The values above "1.0" on the *y*-axis show the relatively higher expression in the severe versus mild GO samples and values below "1.0" indicate relatively lower expression. (b) The individual data from RT-PCR displaying the expression of mRNA for transcription factor FoxP3 in severe GO and mild GO samples (medians and 25th–75th percentiles).

Table 3: Number of patients and the percentage of (a) TCR and (b) CD3 expression within scores 0, 1, and 2 in examined groups (see the footer of Table 2).

(a)

TCR expression	Control	Mild GO	Severe GO	Sum
≤10%	7	2	0	9
[%]	100.0%	33.34%	0.00%	
[10%–50%]	0	4	13	17
[%]	0.00%	66.66%	65.00%	
>50%	0	0	7	7
[%]	0.00%	0.00%	35.00%	

(b)

CD3 expression	Control	Mild GO	Severe GO	Sum
≤10%	7	2	0	9
[%]	100.0%	33.34%	0%	
[10%–50%]	0	4	19	24
[%]	0.0%	66.66%	95%	
>50%	0	0	1	1
[%]	0.00%	0.00%	5%	

Table 4: Spearman's correlation table with number of samples, *R*, and *P* value.

	N	*R*	*P* value
TCR expression & CD3 expression	32	0.796	**0.0000001**
CD3 expression & CTLA-4	20	−0.375	0.1
CD3 expression & Fox	**32**	**0.358**	**0.05**
CD3 expression & CD40	32	−0.124	0.5
CD3 expression & CD86	32	0.186	0.3
CRP & Fox	**32**	**0.520**	**0.003**
CRP & CD40	**32**	**−0.757**	**0.000001**

Among other immunoregulatory molecules, CTLA-4 and CD40 in GD and GO have been evaluated most extensively [15, 23]. In an early study, Vaidya et al. demonstrated that the G-carrying genotypes of the *CTLA4A/G* polymorphism are associated with an increase in risk of GO and are independent of male sex, smoking, and previous radioiodine administration [24]. Since then, many studies have evaluated the CTLA-4 polymorphism in different ethnic groups and its association with ophthalmopathy and Graves' disease [25]. For CTLA-4 function SNP C-318 T, SNP A49G (alanine/threonine polymorphism) and microsatellite- sequence in 3′UTR was determined [26]. Most recently, Borodic et al. described a case report on treatment with ipilimumab, the monoclonal antibody blocking CTLA-4 mediated T cell suppression, that has led to GO-like syndrome [27]. The decreased expression of the CTLA-4 mRNA and increased expression of its counter-receptor B7-2 (CD86) in the orbital tissue from GO may explain that patients with GD develop severe GO due to the increased autoimmune reactivity in the orbital venue. Saverino et al. found elevated soluble form of CTLA-4 (sCTLA-4) in serum from patients with autoimmune thyroid disease suggesting a possible role in immune response dysregulation [28]. Thus, the systemic elevation may be caused by increased cleavage of the surface CTLA-4 and lack of its expression in inflammation site in the orbit.

The increased CD86 mRNA expression found in GO orbital samples may suggest that CD86 costimulation promotes a direct immune response toward Th2 lymphocytes development in the orbital tissue [29]. Th2 differentiation has been shown to be involved in the humoral immune response involving B cells and antibodies production. Sigal et al. found that the CD86 costimulation was more critical for cytotoxic T lymphocytes generation [30].

Similar to CD86, CD40 and CD40L were identified as important B cell activation factors [31]. Most recently, both CD40 and CD86 were found to be elevated in the thyroid of the animal model of GD [32]. In agreement with that, we found an increased level of CD40 mRNA in the orbital tissue samples. In addition, a single nucleotide polymorphism (SNP) C/T in the untranslated region of the CD40 gene has been associated with susceptibility to Graves' disease

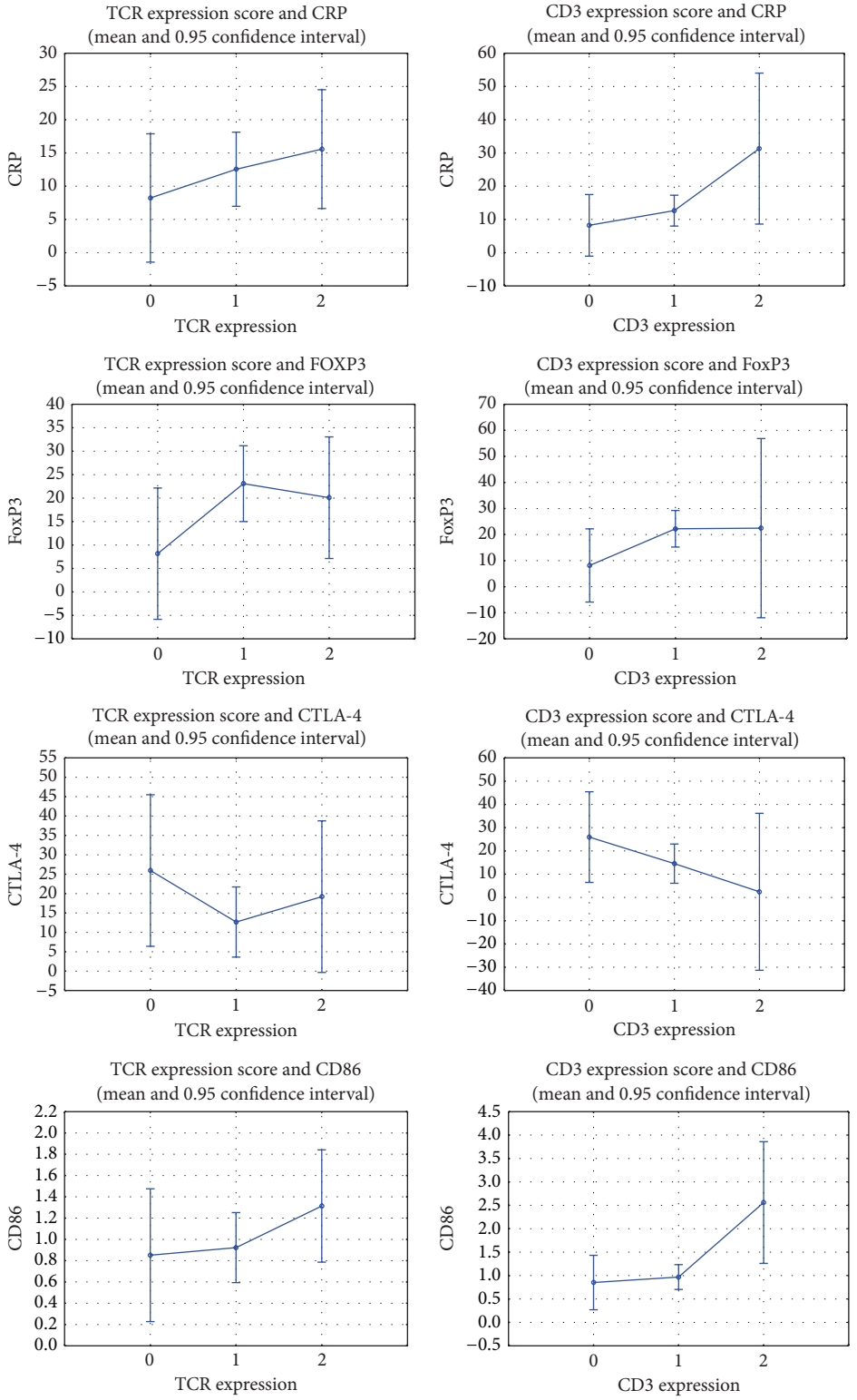

Figure 5: TCR and CD3 immunohistochemical grading score groups' (0: ≤10% positive cells; 1: 11%–50%, 2: >50% cells) mean and confidence values of CRP, FOXP3, CTLA-4, and CD86 mRNA.

in Caucasian [33] and Korean populations [2]. This SNP may alter the translational efficiency of CD40 protein [34]. Jacobson et al. showed that the T-allele makes 15.5% less CD40 than the C-allele, demonstrating that the effect of the single-nucleotide polymorphism (SNP) on CD40 expression is at the level of translation [34]. Human B cells harboring this SNP expressed 39% and 27% higher levels of surface CD40 at rest and activation, respectively, compared with controls. Moreover, this SNP has been linked with the increased production of autoantibodies [35]. However, no difference was detected in steady state CD40 mRNA expression in GD [34].

It is possible that the elevated levels of surface CD40 might amplify cell activation during tissue injury, potentially predisposing the host to GD and perhaps to GO [36]. Since ligation of CD40L on T cells by CD40 B cells or other APC was shown to be necessary for efficient activation of T cell effector functions, elevated serum concentrations of serum CD40 and CD154 could suggest the systemic activation and explain the increased CD40/CD40L mRNA level in the orbital tissue as a pathway leading to inflammatory infiltration in GO [37].

Direct evidence that any particular candidate antigen or orbital cell type is an autoimmune target in GO has been difficult to obtain due to the lack of available human orbital tissue. However, the loss of tolerance could anyway be the trigger of GO. In agreement with that, we found that the level of FOXP3 mRNA was elevated in GO patients comparing to controls. In addition, the expression of FOXP3 mRNA was also higher in patients with the severe course of GO than in patients with the mild course of GO. These disturbances could be elucidated by the fact that, by preventing the activation of autoreactive pathogenic cells, $CD4^+CD25^+FOXP3^+$ regulatory T lymphocytes (T_{regs}) have a critical role in the maintenance of self-tolerance and thus in the prevention of autoimmune disease [13]. Hence, the autoimmune response might be more enhanced in GO especially in patients with severe GO. It has been shown that the incubation with polyclonal rabbit anti-T lymphocyte (rATG) globulin increased the frequency of PBMCs of GO patients expressing T_{regs}-markers (CD25, FOXP3). Kahaly et al. have found that FOXP3/CD4 rATG-induced T_{regs} marker was more intensively expressed on GO peripheral blood leucocytes (PBLs) than on GD or normal PBLs [38]. Interestingly, FOXP3 mRNA from peripheral blood was equal in patients with active GD to that in controls [39]. It is possible that in our study the enhanced FOXP3 mRNA could be linked with a polymorphism or a dysfunction of FOXP3 that attempted to compensate the immunological response in its overexpression [18].

6. Conclusions

The enhanced FOXP3 mRNA expression in GO samples and its correlation with CD3 and CRP may suggest the involvement and perhaps dysfunction of FOXP3 lymphoid cells in the pathogenesis of severe GO. The diminished mRNA expression of CTLA-4 in severe GO may indicate inadequate T regulatory function of this molecule in severe course of GO. The enhanced mRNA expression of CD40 in severe versus mild GO and negative correlation to CRP mRNA may suggest their role not only in active but also in the late inactive phase of GO.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Markers of Inflammation and Fibrosis in the Orbital Fat/Connective Tissue of Patients with Graves' Orbitopathy: Clinical Implications

Przemyslaw Pawlowski,[1,2] Joanna Reszec,[1] Anja Eckstein,[3] Kristian Johnson,[4] Andrzej Grzybowski,[5,6] Lech Chyczewski,[1] and Janusz Mysliwiec[7]

[1] *Department of Medical Pathomorphology, Cathedral of Biostructure, Medical University of Białystok, 13 Waszyngtona Street, 15-269 Białystok, Poland*
[2] *Department of Pediatric Ophthalmology with Strabismus Treatment Unit, Medical University of Białystok, 17 Waszyngtona Street, 15-274 Białystok, Poland*
[3] *Department of Ophthalmology, University Hospital Essen and University of Duisburg-Essen, Hufeland Straße 55, 45-122 Essen, Germany*
[4] *Bayer Healtcare, Kaiser-Wilhelm-Allee 10, Leverkusen, 51-373 Nordshein-Westfalen, Germany*
[5] *Department of Ophthalmology, Poznań City Hospital, 3 Szwajcarska Street, 61-285 Poznań, Poland*
[6] *Department of Ophthalmology, University of Warmia and Mazury, 30 Warszawska Street, 10-082 Olsztyn, Poland*
[7] *Department of Nuclear Medicine, Medical University of Białystok, 24A Skłodowskiej-Curie Street, 15-276 Białystok, Poland*

Correspondence should be addressed to Przemyslaw Pawlowski; przemyslaw.pawlowski@umb.edu.pl

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Purpose. To assess FGF-β, TGF-β, and COX2 expression and immunocompetent cells in the orbital tissue of patients with severe and mild Graves' orbitopathy. *Patients and Methods.* Orbital tissue was taken from 27 patients with GO: (1) severe GO ($n = 18$), the mean clinical activity score (CAS) being 8.5 (SD 2.5); and (2) mild GO ($n = 9$), the mean CAS being 2.2 (SD 0.8), and from 10 individuals undergoing blepharoplasty. The expression of CD4+, CD8+, CD20+, and CD68 and FGF-β, TGF-β, and COX2 in the orbital tissue was evaluated by immunohistochemical methods. *Results.* We demonstrated predominant CD4+ T cells in severe GO. CD68 expression was observed in the fibrous connective area of mild GO and was robust in severe GO, while the prominent TGF-β expression was seen in all GO. Increased FGF-β expression was observed in the fibroblasts and adipocytes of severe GO. No expression of COX2 was found in patients with GO. *Conclusions.* Macrophages and CD4 T lymphocytes are both engaged in the active/severe and long stage of inflammation in the orbital tissue. FGF-β and TGF-β expression may contribute to tissue remodeling, fibrosis, and perpetuation of inflammation in the orbital tissue of GO especially in severe GO.

1. Introduction

Graves' orbitopathy (GO) is a disfiguring and sometimes blinding disease, characterized by inflammation and swelling of orbital tissues, with fibrosis and adipogenesis being predominant features [1].

Clearly, the vulnerability to disease manifestation most likely reflects the highly specialized function of the orbital tissue, a unique fat depot that cushions the globe [2]. Regensburg et al. found that, in GO patients, 25% have orbital fat and muscle volumes within an age-specific reference range. An increase of the fat volume, characterized by proptosis, is seen in approximately 14% of GO patients [3]. However, only 3 to 5% of patients with GO have severe disease with intense pain, inflammation, and sight-threatening corneal ulceration or compressive optic neuropathy [4].

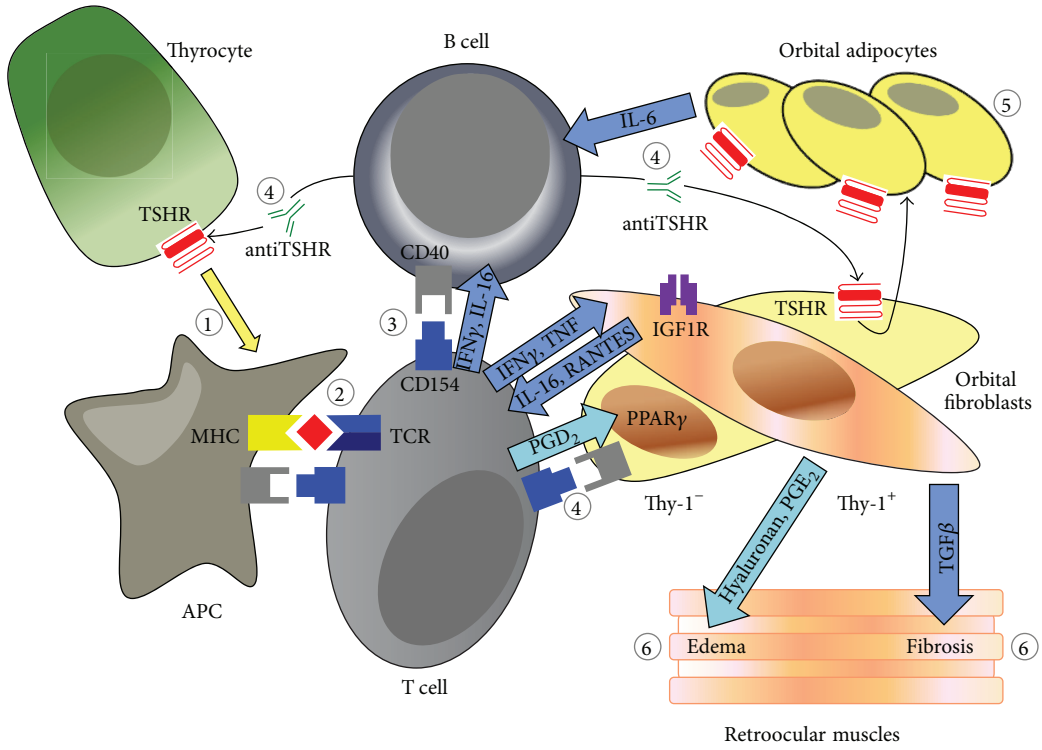

Figure 1: Immunopathogenesis of GO, highlighting the role of counterplay in the orbit between adipocytes, orbital fibroblast, and immunocompetent cells (lymphocytes T and B and macrophages) (adapted from a book chapter with the permission of Professor J. Mysliwiec).

The sparse mononuclear cell infiltrates are seen within the fatty connective tissue and the muscle endomysium [5, 6]. In the early inflammatory phase the majority of cells are T lymphocytes CD4+ and CD8+ and B lymphocytes are only occasionally seen [7, 8]. Macrophages influx is increased in the early disease and less so in late disease [9]. The inflammatory cells, T and B lymphocytes, and macrophages as well as mast cells infiltrating the orbit interact with orbital fibroblasts and amplify inflammatory/autoimmune reaction [9]. Activated human T lymphocytes by expressing cyclooxygenase-2 and producing prostaglandins drive human orbital fibroblast differentiation to adipocytes [10]. Moreover, engagement of CD40 on orbital fibroblasts triggers hyaluronan synthesis and activation of inflammatory cyclooxygenases [11].

When exposed to TGF-β, Thy-1$^+$ fibroblast differentiates into myofibroblasts with prominent cytoplasmic actin filaments that can participate in inflammation, repair, and fibrosis and are responsible for tissue remodeling in GO [12, 13] (Figure 1).

TGF-β and other inflammatory mediators (IL-16, RANTES) elaborated by resident macrophages and fibroblasts trigger T cells migration or participate directly in local inflammation [14]. The inflammatory cyclooxygenase (COX2) is usually expressed at extremely low levels under normal basal physiology conditions. COX2 is expressed at higher levels in the orbital fibroadipose tissue in GO; there seems to be a positive correlation with increasing severity of ophthalmopathy, suggesting a possible relationship with COX2 expression and orbital inflammation in GO [15, 16].

Of the cell types residing in GO tissue preadipocytes and fibroblasts are most likely target and effectors cells of the orbital immune processes [12]. Fibrogenic growth factor, oxygen free radicals, and cytokines released from inflammatory cells act upon orbital preadipocytes, in para- or autocrine manner, stimulating adipogenesis, fibroblast proliferation, and glycosaminoglycan synthesis. Expression of FGF-β in the orbital fat/connective tissue with GO and a direct relation with higher CAS suggest its important role in severity of GO [17].

Therefore we decided to perform a study aimed at comparing leucocyte infiltration pattern and inflammatory (COX2) and profibrotic (FGF-β, TGF-β) cytokine milieu in the orbital fat/connective tissue of patients with mild and severe GO taken during decompression surgery.

2. Material and Methods

2.1. Patients and Controls. Human orbital tissue was obtained from 27 patients with GO (26 females and 1 male) classified according to the European Group on Graves' Orbitopathy, who underwent orbital decompression procedures, from the orbital tissue bank at the Department of Ophthalmology and University of Essen. The mean age of patients at the time of surgery was 44.5 years (range 26–47).

Control fat/connective tissues were derived from 10 individuals (9 females and 1 male) undergoing orbital surgery for blepharoplasty with no history of GO or any orbital inflammatory disease.

Surgical specimens of orbital fat/connective tissue were obtained and immediately snap-frozen in liquid nitrogen until use.

The clinical activity score of GO (CAS) was estimated according to Mourits et al. [18]. The severity of the eye disease was estimated using NOSPECS classification (no signs or no symptoms; only signs, no symptoms; signs only; proptosis; eye muscle involvement; corneal involvement; and sight visual acuity reduction) [19].

2.1.1. Patients with Severe GO. Patients with severe GO (n = 18) (NOSPECS IV–VI) required orbital bony decompression due to optic nerve compression and limited extraocular muscle functions (22 specimens). Mean duration of thyroid disease was 2.5 (SD 1.5) years with 1.2 (SD 1.0) years for GO. Before surgery all patients had received >1 cycles of steroid regiment, and all but 2 received orbital irradiation. The mean clinical activity score was 8.5 (SD 2.5).

2.1.2. Patients with Mild GO. Patients with mild GO (n = 9) (NOSPECS III-IV) underwent orbital bony decompression to reduce proptosis or for fat resection. Mean duration of thyroid disease was 3.9 (SD 2.0) years and 3.3 (SD –1.9) for GO. Steroid regiment and received orbital irradiation were similar for the patients with severe GO. The mean clinical activity score was 2.2 (SD 0.8).

2.2. Methods. Evaluation of proteins expression was done using immunohistochemical methods. Following the deparaffinisation and rehydration, epitope retrieval was carried out in the EnVision Flex Target Retrieval Solution (DAKO) in high pH. Endogenous peroxidases were blocked by incubating the sections in methanol and 3% hydrogen peroxidase for 20 minutes. Next slides were incubated with special types of antibodies (Table 1). Visualization reagent EnVision Flex (DAKO) was applied for 30 minutes followed by DAB solution for 10 minutes. The slides were then counterstained with hematoxylin and examined under the light microscope. Immunohistochemical evaluation of each protein expression was performed by pathologist. The intensity of immunostaining was evaluated in random 10 fields under 20x magnification. The results were expressed as the percentage of cells with a strong positive staining as follows: ≤10% positive cells − negative (−), between 11% and 50% (+), and ≥51% positive cells (++) [20].

Appropriate positive and negative controls were performed.

Table 1: Antibodies used in the study for immunohistochemical staining.

Antibody	Type of antibody	Dilution
CD4	Monoclonal mouse anti-human CD4 DAKO cytomation clone 4B12	1 : 40
CD8	Monoclonal mouse anti-human CD8 DAKO cytomation clone C8/144B	1 : 50
CD20	Monoclonal mouse anti-human CD20cy DAKO cytomation clone L26	1 : 200
CD68	Monoclonal mouse anti-human CD68 DAKO cytomation clone PG-M1	1 : 100
TGFβ	Rabbit polyclonal TGFβ1 (V) antibody: sc-146 Santa Cruz Biotechnology	1 : 50
FGFβ	Rabbit polyclonal FGF-1 antibody (H-125): sc-7910 Santa Cruz Biotechnology	1 : 100
COX2	Monoclonal mouse anti-human COX-2 DAKO cytomation clone CX-294	1 : 50

3. Results

3.1. T Cells within Graves' Orbitopathy. In mild type of GO we observed T lymphocytes CD4 positive (T helpers) within the tissue in 6 out of 9 mild cases; mostly T cells were dispersed within the whole tissue and next to the small blood vessels. In 2 specimens T helpers were together with T cytotoxic cells (CD8 positive); however T CD8 were in minority. In 3 mild GO T cells were absent.

3.1.1. T Cells in Severe GO. T lymphocytes were observed in 20 out of 22 severe GO. T cells were mostly CD4 positive T helpers; only in 2 cases we also observed few CD8 positive T cytotoxic cells.

3.1.2. B Lymphocytes in GO. We did not observed presence of B lymphocytes in almost all specimens of both severe and mild GO (with only 1 exception of severe GO, where we observed a focal infiltration of B lymphocytes).

3.1.3. CD68 Expression in Mild GO. In all mild GO tissues we observed presence of CD68 positive cells—both macrophages and fibroblasts. The cells were dispersed in whole tissue, especially in more fibrotic connective tissue, rather than in fat one. In 4 out of 9 cases the cells were numerous and prominent (see Table 2).

3.1.4. CD68 Expression in Severe GO. In all severe GO specimens we observed presence of macrophages and fibroblasts; mostly the cells were numerous and were disseminated in whole tissue (in 15 cases we evaluated the staining as ++). Number of patients and the percentage of CD68 expression within score 0, 1, 2 in examined groups (see Table 3).

3.1.5. COX-2 Expression in Mild and Severe GO. We did not observe COX2 staining, in both mild and severe cases. The explanation of this may by associated with the previous steroid therapy in almost all GO patients.

Table 2: Immunohistochemical evaluation grading score for CD4/CD8, CD20, and CD68 cells and FGF-β, COX2, and TGF-β of each specimen. (a) Expression of the examined markers in the control group, (b) expression of the markers in the group of mild Graves' orbitopathy, and (c) expression of the markers in the group of severe Graves' orbitopathy.

(a)

Number	Classification	CD4/CD8	CD20	CD68	COX-2	FGF	TGF
1	Contrl.	0-0	0	1	0	0	0
2	Contrl.	0-0	0	0	0	0	0
3	Contrl.	0-0	0	0	0	0	0
4	Contrl.	0-0	0	0	0	0	0
5	Contrl	0-0	0	0	0	0	0
6	Contrl	0-0	0	1	0	1	0
7	Contrl.	0-1	1	1	0	1	0

(b)

Number	Classification	CAS score	CD4/CD8	CD20	CD68	COX-2	FGF	TGF
1	Mild GO	1	0-0	0	1	0	1	1
2	Mild GO	3	1-0	0	2	0	2	1
3	Mild GO	2	1-0	0	1	0	1	1
4	Mild GO	3	1-0	0	2	0	2	1
5	Mild GO	1	1-0	0	1	0	1	1
6	Mild GO	3	1-1	0	2	0	1	1
7	Mild GO	1	1-1	0	1	0	1	1
8	Mild GO	3	0-0	0	2	0	1	2

(c)

Number	Classification	CAS score	CD4/CD8	CD20	CD68	COX-2	FGF	TGF
1	Severe GO	10	1-0	0	2	0	2	2
2	Severe GO	10	1-0	0	2	0	2	2
3	Severe GO	9	1-0	0	2	0	2	1
4	Severe GO	8	1-0	0	2	0	2	2
5	Severe GO	7	1-0	0	1	0	1	1
6	Severe GO	10	1-0	0	2	0	2	2
7	Severe GO	10	1-0	0	2	0	2	2
8	Severe GO	9	1-0	2	2	0	2	2
9	Severe GO	10	1-1	0	2	0	2	2
10	Severe GO	10	2-1	0	2	0	2	2
11	Severe GO	5	1-0	0	1	0	1	1
12	Severe GO	10	1-0	0	2	0	2	2
13	Severe GO	10	1-0	0	2	0	2	2
14	Severe GO	6	1-0	0	2	0	1	1
15	Severe GO	5	0-0	0	1	0	1	1
16	Severe GO	5	0-0	0	1	0	1	1
17	Severe GO	5	1-0	0	1	0	1	1
18	Severe GO	8	1-0	0	1	0	2	1
19	Severe GO	10	1-0	0	2	0	2	2
20	Severe GO	7	1-0	0	2	0	1	2
21	Severe GO	5	1-0	0	1	0	2	2
22	Severe GO	10	1-0	0	2	0	2	2

Legend for Tables 2(a), 2(b), and 2(c): immunohistochemistry score:
0-less than 10% positive in 10 representative high power fields (HPF).
1-10%–50% positive cells in 10 HPF.
2-more than 50% positive cells in 10 HPF.
Immunohistochemistry was done using DAB chromogen (brown staining). Ctrl: controls.

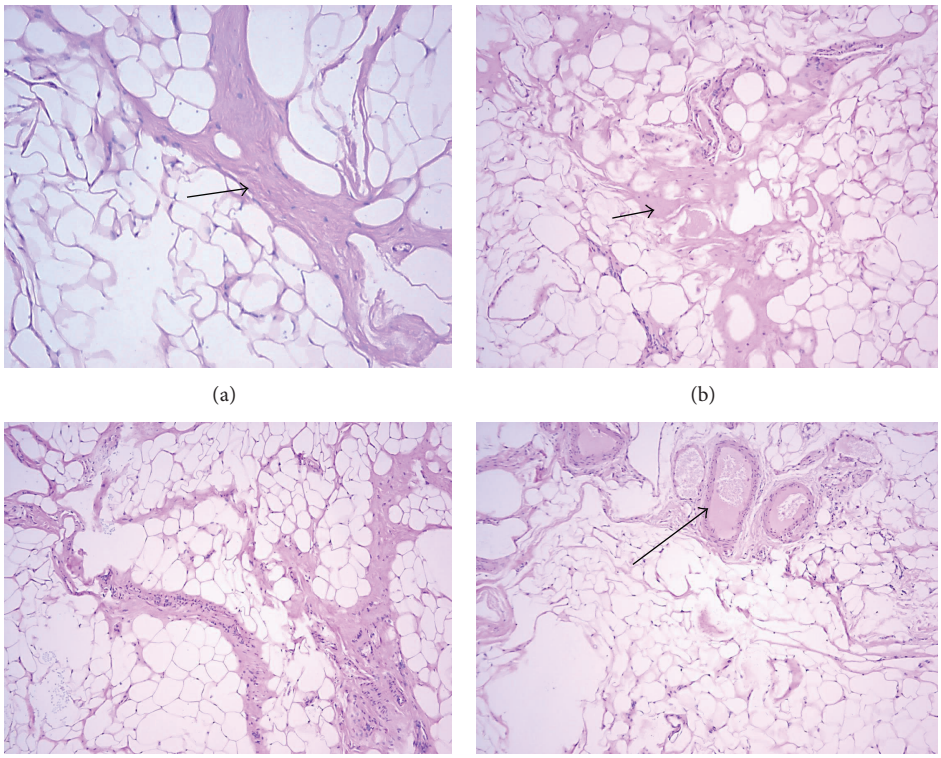

Figure 2: Hematoxylin and Eosin staining in paraffin embedded sections. (a) Mild type of GO. The arrow head shows the area of the intense fibrosis. Magn. 400x, 100x. (b) Mild type of GO. The arrow head shows the small blood vessels. Magn. 400x, 100x. (c) Severe type of GO. Significant proliferation of the fibrous connective tissue within the fat. Magn. 400x. (d) The arrow head shows the prominent blood vessels within the fatty tissue in the severe GO orbitopathy. Magn. 200x.

Table 3: Number of patients and the percentage of FGF and CD68 expression within scores 0, 1, and 2 in examined groups (see legend for Table 2).

(a)

FGF expression	Control	Mild GO	Severe GO	Sum
≤10% no.	5	0	0	5
[%]	71.43%	0.00%	0.00%	
(10%; 50%) no.	2	6	7	15
[%]	28.57%	75.00%	31.81%	
>50% no.	0	2	15	17
[%]	0.00%	25.00%	68.18%	

(b)

CD68 expression	Control	Mild GO	Severe GO	Sum
≤10% no.	4	0	0	4
[%]	57.14%	0.00%	0.00%	
(10%; 50%) no.	3	4	7	14
[%]	42.86%	50.00%	31.81%	
>50% no.	0	4	15	19
[%]	0.00%	50.00%	68.18%	

3.1.6. FGF-β Expression in Mild GO. In all mild GO tissues we observed expression of FGF-β. The staining was observed mainly in adipocytes and fibroblasts within the tissue. FGF-β was also expressed by endothelial cells of the blood vessels within the tissue. However only 2 out of 9 mild GO were evaluated as (++).

3.1.7. FGF-β Expression in Severe GO. All of the severe GO cases showed FGF-β expression within the adipose tissue as well as in the fibroblasts in the connective tissue. Also FGF-β expression was observed in numerous small blood vessels. In almost all of the examined severe GO cases (15 specimens) FGF-β expression was estimated as (++). Number of patients and the percentage of FGF-β expression within score 0, 1, 2 in examined groups (see Table 3).

3.1.8. TGF-β Expression in Mild GO. All of the examined mild GO specimens showed TGF-β expression mainly within the fibroblasts of the connective fibrous tissue; however the expression was mostly dispersed.

3.1.9. TGF-β Expression in Severe GO. Most of the examined severe GO specimens presented strong and diffused pattern of the TGF-β expression, all in the numerous fibroblasts within the connective fibrous tissue.

There was a positive correlation of CAS values with CD4 and FGF-β, TGF-β, and CD68 expression (see Table 4).

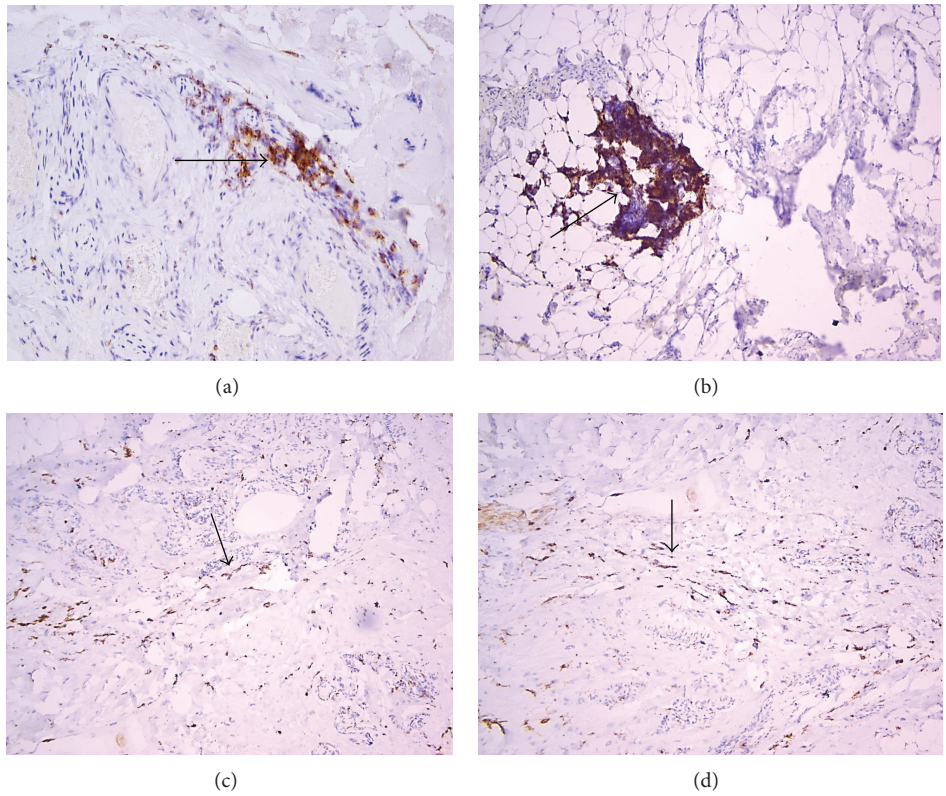

Figure 3: Immunohistochemistry with DAB staining. (a) The arrow head presents the focal cluster of T lymphocyte (CD4+) brown cytoplasmatic staining in the mild type of GO. Magn. 200x. (b) The arrow head shows the cluster of T lymphocytes (CD4+) brown staining in the severe type of GO. Magn. 100x. (c) and (d) The arrow head presents significant fibroblasts and macrophages clusters (CD68+) brown cytoplasmatic staining observed in the fibrous connective area of the adipose tissue of the mild GO (robust 3c) and severe one (d). Magn. 100x.

Table 4: Pearson's correlation of CAS values with examined parameters.

	CAS	
	R value	*P* value
CD4 expression	.4071	$P = .028$
CD8 expression	−.0632	$P = .745$
CD20 expression	.1432	$P = .459$
CD68 expression	.6017	$P = .001$
FGF expression	.7311	$P = .0001$
TGF expression	.7213	$P = .0001$

4. Discussion

Retrobulbar tissue specimens are generally not available from patients with early, active GO without prior immunomodulatory treatment (with glucocorticoids or radiotherapy).

Avunduk et al. studied 4 biopsy specimens from active GO without prior immunosuppressive treatment [21]. They demonstrated that both CD4+ and CD8+ cells were present, and a significant proportion of them were CD45RO+ cells. Infiltration of OCT (orbital connective tissue) by HLA-Dr+, CD25+, and TNF-α cells suggests that Th1-type immune reaction with the interference of proinflammatory cytokine(s) (TNF-α) may be important in the pathogenesis of disease [21].

Recently an elevation of CD4 to CD8 ratio and enhanced secretion of IL-6, IL-10, and TNFα were detected in PBMCs of GO patients compared with controls [22].

Similar to our study, previously the examined orbital tissue specimens from patients with GO who had received immunosuppressive therapy have shown predominant CD4+ infiltration and only 20–30% of infiltrating cells were CD3+CD8+ cells [23, 24].

Yang et al. examined orbital OCT-derived T cell lines from GO using immunohistochemical methods, and they reported that T cell marker CD3+ could be detected in almost 100% of cases. In addition, T cell lines consisted predominantly of CD4+ cells [24]. Similar results were found by Yang and coworkers who established 104 T cell clones from OCT biopsies of 3 patients with GO and found that approximately 70–80% were CD3+CD4+ and approximately 20–30% were CD3+CD8+ cells [24]. Nevertheless, in both published papers all examined patients had been treated either with systemic steroids and/or orbital radiotherapy. In the previous study, Eckstein et al. have shown the predominant intraorbital CD4+ T cells infiltration in GO with absence of CD8+ and CD20+ B lymphocytes [25]. These results are in agreement with our findings.

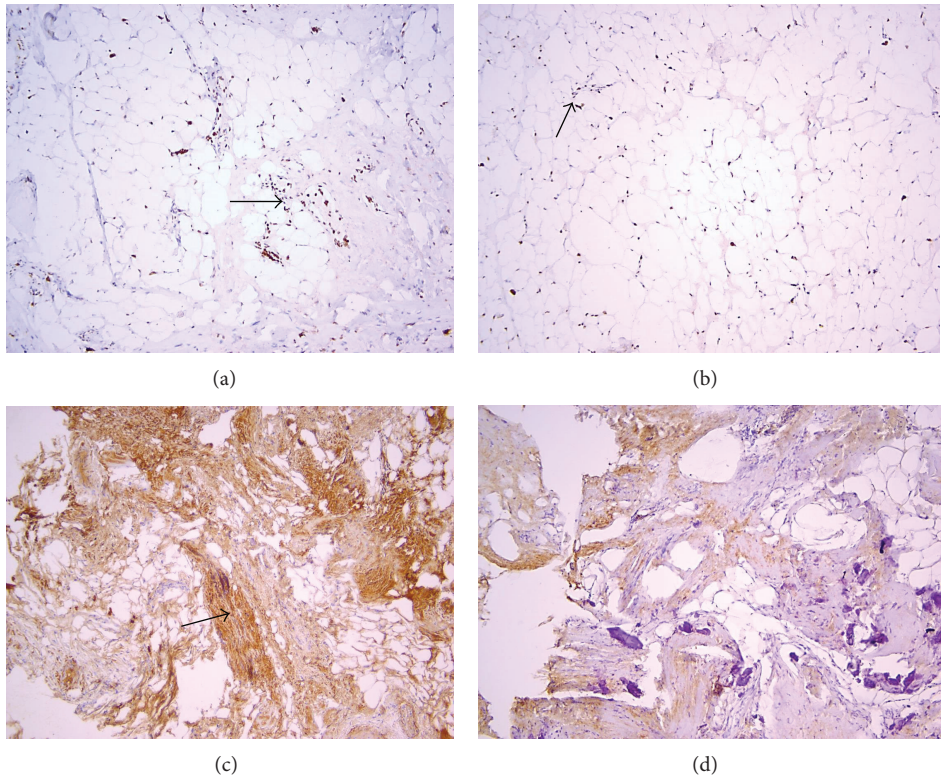

Figure 4: Immunohistochemistry with DAB staining. (a) and (b) FGF-β expression observed in the fibroblasts (a) and adipocytes (b) in the connective tissue of the severe GO. Magn. 40x. (c) and (d) A prominent TGF-β expression observed in the fibrous connective tissue of the severe (c) and mild (d) GO. Magn. 40x.

Chen et al. found that macrophage infiltration may play an important role in the pathogenesis of GO via overexpression of MCP-1 [26]. The infiltration of macrophages was located primarily around blood vessels and between mature adipocytes. Macrophage infiltration did not attenuate in GO of long duration. They also found that the expression of MCP-1 was higher in GO orbital fat than in the orbital fat of controls [26]. In our study we demonstrated CD68 macrophages' infiltration in fibrous connective and fat tissue more expressed in severe than mild GO (Figures 3(c) and 3(d)). Previously, Eckstein et al. have documented in the orbital tissue an increase of immigrant macrophages CD14 and RFD7 influx [25]. The presence of such cells in GO with strong correlation to disease activity suggests robust proinflammatory secretion on site [26]. Eckstein et al. concluded that these macrophages are recruited from freshly infiltrated monocytes, but not local resident macrophages [25]. Recently it has been found that, in GO, mast cells, monocytes, and macrophages may activate orbital fibroblasts via secretion of especially PDGF-AB and PDGF-BB [27].

Matos et al. have investigated the immunohistochemical expression of growth factors (IGF-1, PDGF-A, PDGF-B, FGF, and VEGF) in patients with Graves' ophthalmopathy [17]. IGF-1 expression was positive in 29.2% of cases. There was a direct relation with higher CAS (clinical activity score) in all of them. When CAS equal or higher than 5 was considered, the percentage of IGF-1 expression was 54.5%. FGF expression was in 5 cases (20.8%) with a direct relation in all those with higher CAS (>5), suggesting its important role in active GO (45.4%). They concluded that in all patients, except one, with positive expression of FGF, IGF-1 and VEGF showed CAS greater than 5, suggesting in this way an important role of these growth factors in the pathogenesis and severity of Graves' ophthalmopathy [17]. Significant proliferation of the fibrous connective tissue in severe GO was seen in our specimens (Figure 2(c)). In addition to our study an increased expression of FGFβ in the fibroblasts (Figure 4(a)) and adipocytes (Figure 4(b)) was observed in the connective tissue of the severe GO.

Yi and Xu observed high COX2 expression in thyroid-associated ophthalmopathy and Bloise et al. proved treatment of moderate GO with oral sodium diclofenac is a good and safe therapeutic option [28, 29]. Vondrichova et al. showed COX2 overexpression in patients in active phase compared to chronic phase of GO. Moreover, they found that diclofenac, an inhibitor of cyclooxygenases with antagonistic effects on PPAR-gamma, reduced the number of mature adipocytes by approximately 50% [16]. Recently attenuation of interleukin- (IL-) 1β-induced cyclooxygenase- (COX-) 2 and prostaglandin (PG)E2 expression in orbital fibroblasts from patients with thyroid-associated ophthalmopathy (TAO) has been proven to decrease inflammation [30]. This statement would be in agreement with our results, showing no COX-2 expression in our specimens, since most of our

studied patients received prior to orbital decompression glucocorticosteroids that may had hampered the inflammation mandated by COX2.

5. Conclusions

Macrophages and CD4 T lymphocytes are both engaged in the active/severe and long stage of inflammation in the orbital tissue. FGF-β and TGF-β expression may contribute to tissue remodeling, fibrosis, and perpetuation of inflammation in the orbital tissue of GO especially in severe GO. COX2 pathway may be hampered by systemic steroids treatment.

Abbreviations

CD:	Cluster of differentiation
CAS:	Clinical activity score
COX2:	Cyclooxygenase 2
FGF-β:	Fibroblast growth factor
GO:	Graves' orbitopathy
MCP-1:	Monocyte chemoattractant protein
RANTES:	Regulated on activation normal T cell expressed and secreted
TGF-β:	Transforming growth factor-β.

Ethical Approval

The study was approved by the Medical Ethics Committee of the University of Essen, Germany.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Chitosan Oligosaccharides Attenuate Ocular Inflammation in Rats with Experimental Autoimmune Anterior Uveitis

I-Mo Fang,[1,2] Chang-Hao Yang,[2] and Chung-May Yang[2]

[1] *Department of Ophthalmology, Taipei City Hospital, Zhongxiao Branch, Taipei, Taiwan*
[2] *Department of Ophthalmology, National Taiwan University Hospital, No. 7, Chung-Shan S. Road, Taipei 10041, Taiwan*

Correspondence should be addressed to Chang-Hao Yang; chyangoph@ntu.edu.tw

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We investigated the protective effects and mechanisms of chitosan oligosaccharides (COS) on experimental autoimmune anterior uveitis (EAAU) in rats. EAAU was induced in Lewis rats by footpad and intraperitoneal injections of melanin-associated antigen. The rats received intraperitoneal injections of low-dose (5 mg/kg) or high-dose (10 mg/kg) COS or PBS daily after the immunization. The effects of COS were evaluated by determining the clinical scores and the morphology of the iris/ciliary body (ICB). The expression of inflammatory mediators was evaluated using western blot, immunofluorescence, and ELISA. Treatment with COS significantly attenuated the clinical scores and the leukocyte infiltration in the ICB in a dose-dependent manner. COS effectively reduced the expression of inflammatory mediators (TNF-α, iNOS, MCP-1, RANTES, fractalkine, and ICAM-1). Moreover, COS decreased the IκB degradation and p65 presence in the ICB, which resulted in the inhibition of NF-κB/DNA binding activity. In an in vitro study, sensitized spleen-derived lymphocytes of the COS-treated group showed less chemotaxis toward their aqueous humor and decreased secretion of the above inflammatory mediators in the culture media. COS treated EAAU by inhibiting the activation of NF-κB and reducing the expression of inflammatory mediators. COS might be a potential treatment for acute anterior uveitis.

1. Introduction

Acute anterior uveitis (AAU) is the most common uveitis in humans. AAU can cause significant visual problems because of its recurrent nature and might result in secondary complications, such as cataract formation, cystoid macular edema, and glaucoma [1]. The exact mechanism of AAU remains unknown. Topical corticosteroids are generally the mainstay in the treatment of AAU; however, periocular injections and systemic steroids are necessary in recalcitrant cases. The long-term use of corticosteroids might produce a wide range of systemic and ocular side effects [2]. Therefore, there is increasing interest in therapies with new molecules that eliminate the side effects of corticosteroids but are as efficient in reducing ocular inflammation and preventing tissue destruction.

Experimental autoimmune anterior uveitis (EAAU), an animal model of human acute anterior uveitis, can be induced in Lewis rats by immunization with bovine melanin-associated antigen (MAA) [3–5]. The clinical course and pathology observed in EAAU are strikingly similar to the related processes in human AAU. The inflammation, which is confined exclusively to the anterior segment without retinal and choroid involvement, differs greatly from other uveitis models [6–8]. This characteristic makes EAAU the best model of human acute anterior uveitis.

Chitosan oligosaccharides (COS), the hydrolyzed product of chitosan, are a mixture of oligomers of β-1,4-linked D-glucosamine residues and are abundant in the exoskeleton of crustaceans and in the cell walls of fungi and insects [9]. COS are known to have various biological effects, including antitumor, antibacterial, anti-inflammation, antioxidative, and antiapoptotic activities [10–13]. COS are nontoxic and biodegradable and have been used as a bioactive material. In addition, COS have good solubility in water and are easily absorbed in the intestine, making them an attractive ingredient in many healthy foods or dietary supplements. We previously showed that COS exerted antioxidative effects by inhibiting NF-κB activation and attenuating oxidative-stress related retinal degeneration in rats [14]. However, the effects

of COS for the anti-inflammatory activity in a rat model of EAAU remain unknown.

In this study, we investigated the therapeutic effects and possible mechanisms of COS in EAAU rats. Furthermore, an in vitro study using sensitized spleen-derived lymphocytes was performed to verify the possible mechanisms of COS action.

2. Materials and Methods

2.1. Reagents. Chitosan oligosaccharides and paraquat were purchased from Sigma-Aldrich St. Louis, MO, USA). A green fluorescent protein (GFP) antibody was purchased from BioVision (Mountain View, CA, USA). Mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Burlingame, CA, USA). The anti-rat TNF-α, MCP-1, and RANTES antibodies were purchased form Peprotech (Rocky Hill, NJ, USA), the anti-rat fractalkine antibody was from eBioscience (San Diego, CA, USA), and the anti-p65 antibody was from Rockland (Gilbertsville, PA, USA). The MCP-1 and RANTES ELISA kits were obtained from Peprotech. The TNF-α ELISA kit was from BioLegend (San Diego, CA, USA) and the fractalkine ELISA kit was from R&D Systems (Minneapolis, MN, USA). The nitric oxide (NO) ELISA kit was purchased from Cayman (Ann Arbor, MI, USA).

2.2. Antigen Preparation and Induction of EAAU. Melanin-associated antigen (MAA) was prepared as described by Broekhuyse et al. with a modification [4]. The iris and ciliary body were carefully obtained from freshly pigmented bovine eyes. The tissue was homogenized gently and filtered through a wire mesh to remove the cellular debris and connective tissue. The homogenate was centrifuged at 1.2×10^5 g at 4°C for 15 min and washed once with PBS (pH 7.4). The resulting pellet was resuspended in 2% SDS and incubated at 70°C for 10 min. After centrifugation, the pellet was washed three times in water. The insoluble antigen was dried and stored at −20°C.

Lewis rats, 6–8-week old and weighing 125–160 g, were used for the experiment. All animals were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. To induce EAAU, the rats were given two separate injections simultaneously. The first injection was 0.05 mL MAA, suspended in PBS, emulsified (1 : 1) in complete Freund's adjuvant (Sigma-Aldrich), and injected into the left hind footpad and the second injection was 0.05 mL MAA, emulsified with 1 μg purified Bordetella pertussis toxin (List, Campbell, CA, USA) and injected intraperitoneally.

2.3. Animal Grouping and Treatment. The experimental rats were randomly divided into four groups.

Group 1: rats received footpad and intraperitoneal injections of PBS as the normal control (normal).

Group 2: rats received daily intraperitoneal injections of PBS, beginning immediately after footpad and intraperitoneal injections of MAA to induce EAAU (PBS-treated group).

Group 3: rats received daily intraperitoneal injections of 5 mg/kg COS, beginning immediately after footpad and intraperitoneal injections of MAA to induce EAAU (low-dose COS group).

Group 4: rats received daily intraperitoneal injections of 10 mg/kg COS, beginning immediately after footpad and intraperitoneal injections of MAA to induce EAAU (high-dose COS group).

In the preliminary study, we have tried 4 doses of COS (5, 10, 20, or 50 mg/kg) for intraperitoneal injections daily into the rats after immunization. However, the rats receiving 20 or 50 mg/kg COS injections became of poor appetite, decreased activity, and increased abdominal circumference after daily injections for 10 days. These rats became emaciated with distended abdomens and some rats even died after continued injections of 20 or 50 mg/kg COS. These findings indicated that intraperitoneal injections of COS at the concentrations of 20 or 50 mg/kg would cause severe toxic effects. Therefore, we chose 5 or 10 mg/kg COS to treat rats with EAAU in our study.

Total numbers of animals used at definite time points in each group and the days to perform the experiments were summarized in Table 1.

2.4. Clinical Examination. The rats were clinically observed each day using slit lamp biomicroscopy for clinical signs of ocular inflammation. The disease severity was clinically assessed with a scale ranging from 0 to 4: 0 = normal; 1 = slight iris-vessel dilatation and some anterior chamber cells; 2 = iris hyperemia, with some limitation in pupil dilation, anterior chamber cells, and a slight flare; 3 = miotic, irregular, hyperemic, and slightly damaged iris, with a considerable flare and cells (especially with accumulation near the iris); 4 = a seriously damaged and hyperemic iris, a miotic pupil often filled with protein, and cloudy gel-like aqueous humor (AqH).

2.5. Histological Evaluation of the Anterior Segment and Quantification of Leukocytes in the AqH. In a separate experiment, three rats in each group were randomly selected and sacrificed at days 10, 14, 17, and 20, respectively. Aqueous humor (2 μL) was obtained from each rat using anterior chamber paracentesis with a 30-gauge needle. The sample was stained with 0.4% trypan blue and then observed under phase contrast microscopy to calculate the number of leukocytes.

The eyeballs were enucleated and immersed in 4% paraformaldehyde in 0.2 M phosphate buffer for 24 h. After fixation, the eyes were dehydrated with alcohol and embedded in paraffin. The specimens were cut into 5 μm sagittal sections near the optic nerve head and were stained with hematoxylin and eosin (H&E) to evaluate the cellular infiltration in the iris and ciliary body (ICB).

2.6. Western Blot Analysis. The rats of each group were sacrificed at day 14. The total protein was extracted from the ICB by lysing the sample in radioimmunoprecipitation assay (RIPA) buffer (0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic

Table 1: Summary of total number of animals at each time point in each group per experiment and days to perform the experiments.

Experiments	Number of animals at each time point in each group (n)	Days after treatment
Clinical scores	10	Days 3, 7, 10, 14, 17, 20, 25, and 30
Histology	3	Day 14
Leukocyte counts	3	Days 10, 14, 17, and 20
Western blot analysis		
TNF-α, iNOS, MCP-1, RANTES, fractalkine, ICAM, p65, and iκB	8	Day 14
Immunofluorescence		
TNF-α, iNOS, MCP-1, RANTES, fractalkine, and p65	3	Day 14
ELISA		
TNF-α, iNOS, MCP-1, RANTES, and fractalkine	5	Days 10, 14, 17, and 20
EMSA	5	Day 14

acid, 10% NP-40, 10 mM EDTA, and protease inhibitors). The extract and Laemmli buffer were mixed at a 1 : 1 ratio, and the mixture was boiled for 5 min. The samples (100 μg) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with anti-TNF-α, anti-iNOS, anti-MCP-1, anti-RANTES, anti-fractalkine, anti-ICAM-1, anti-IκB, anti-NF-κB p65, and anti-β-actin antibodies. The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody and visualized using chemiluminescence (GE Healthcare, Buckinghamshire, UK). The density of the blots was quantified using image-analysis software (Photoshop, version 7.0; Adobe Systems, San Jose, CA, USA). The optical densities of each band were calculated and standardized based on the density of the β-actin band.

2.7. Immunohistochemical (IHC) Studies. Three rats in each group were used for immunohistochemical studies. Immunohistochemistry was carried out by simultaneously blocking and permeabilizing sections with 0.2% Triton in PBS containing 5% goat serum for 1 hr at room temperature, incubating with TNF-α, iNOS, MCP-1, RANTES, fractalkine, ICAM-1, and NF-κB p65 primary antibodies diluted in blocking solution overnight at 4°C, and incubating with the appropriate fluorescent secondary antibodies (all diluted 1 : 1000) in blocking solution for 3 hrs at room temperature. The nuclei were counterstained with DAPI.

The following formula was used for the densitometric quantitation of TNF-α, iNOS, MCP-1, RANTES, fractalkine, ICAM-1, and NF-κB p65, as previously described [15] with modification:

$$\text{Immunostaining index} = \sum \frac{[(X - \text{threshold}) \times \text{area (pixels)}]}{\text{total cell number}}, \quad (1)$$

where X is the staining density indicated by a number between 0 and 256 in grayscale, and X is more than the threshold. Briefly, digitized color images were obtained as PICT files. PICT files were opened in grayscale mode using NIH image, version 1.61. Cell numbers were determined using the Analyze Particle command after setting a proper threshold.

The relative density of immunostaining was defined as immunostaining index of PBS-treated, low-dose, or high-dose COS groups divided by immunostaining index of normal group.

2.8. Quantification of the Levels of TNF-α, Nitric Oxide (NO), MCP-1, RANTES, and Fractalkine in the AqH. Five rats in each group were sacrificed at days 10, 14, 17, and 20, respectively. The concentrations of TNF-α, NO, MCP-1, RANTES, and fractalkine were determined using commercial ELISA kits according to the manufacturers' instructions. The AqH (5 μL) were diluted to 50 μL for testing, and the optical density was determined at A_{450} (absorbance at 450 nm) using a microplate reader (Bio-Rad).

2.9. Nuclear Protein Extract and Electrophoretic Mobility Shift Assay of NF-κB (EMSA). Five rats in each group were sacrificed at day 14. The ICB was minced in 0.5 mL of ice-cold buffer A containing 10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT), and 1.0 mM phenylmethylsulfonyl fluoride (PMSF). The tissue was homogenized, followed by centrifugation at 5000 g at 4°C for 10 min. The sediment was suspended in 200 μL of buffer B. The suspension was incubated on ice for 30 min. The sample was centrifuged at 12,000 g at 4°C for 30 min. The supernatant containing the nuclear proteins was collected. The EMSA was performed using an NF-κB DNA-binding protein-detection system (Pierce Biotechnology, Rockford, IL, USA). A 10 μg nuclear protein aliquot was incubated in binding buffer with a biotin-labeled NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAG-GC-3′) for 30 min and resolved in 6% nondenaturing polyacrylamide gel. The specificity of the DNA/protein binding was determined by adding a 100-fold molar excess of unlabeled NF-κB oligonucleotide for competitive binding 10 min

before adding the biotin-labeled probe. The protein-DNA-biotin complexes were blotted onto a nitrocellulose transfer membrane followed by UV cross-linking. The complexes were revealed with streptavidin-horseradish peroxidase conjugate and SuperSignal chemiluminescent substrate and then exposed to X-ray film. The density of the blots was quantified using image-analysis software (Photoshop). "Fold change" was defined as optic density of the PBS-treated, low-dose, or high-dose COS group divided by optic density of the normal group.

2.10. Cell Preparation of Spleen-Derived Lymphocytes. Single cell suspensions from the spleens of the four groups at day 14 were prepared by mashing the tissues with frosted slides (Fisher Scientific), followed byfiltration through a cell strainer (BD Biosciences, SanJose, CA, USA). The red blood cells were removed by treating the cells with red blood cell lysis buffer (2.07 g NH_4Cl, 0.25 g $NaHCO_3$, and 9.3 mg EDTA in 100 mL H_2O). The total lymphocytes werepurified by passing the cells through a Histopaque-1077 gradient (Sigma-Aldrich) according to the manufacturer's protocol. The cells were suspended in complete RPMI 1640 culture medium with L-glutamine containing 1% (v/v) minimum essential medium (Life-Gibco, Rockville, MD, USA), NEAA (BioWhittaker, Allendale, NJ, USA), a mixture of antibiotics (100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 μg/mL Amphotericin B), and 10% (v/v) fetal bovine serum (Life-Gibco). The cells of each group were exposed to 10 μg/mL MAA for 3 days.

2.11. Lymphocyte Chemotaxis Assay. Lymphocyte chemotaxis was measured using QCM chemotaxis 96-well plates fitted with 3 mm membrane inserts (Millipore). The lymphocytes of the four groups were placed in the upper chamber of a QCM apparatus, and the aqueous humor samples of the same groups were placed in the lower chamber of the QCM apparatus. After 24 hrs of incubation, the cells that migrated toward the chemoattractant were recovered from the lower chamber, and the unmigrated cells were recovered from the inserts. The migrated cells were stained with a green fluorescent dye (CyQuant GR dye, Millipore) and transferred to a 96-well flat-bottomed ELISA microplate; the fluorescence was measured at 485/535 nm using a plate reader (Perkin Elmer, Waltham, MA, USA). The data are reported in fluorescent units representing cells that migrated into the lower chamber toward the chemoattractant.

2.12. Evaluation of the Levels of TNF-α, MCP-1, RANTES, and Fractalkine in Culture Media of Spleen-Derived Lymphocytes. The culture media of spleen-derived lymphocytes of each group were obtained. The levels of TNF-α, MCP-1, RANTES, and fractalkine in the culture media were measured using ELISA kits as described previously.

2.13. Statistical Analysis. The results are expressed as the mean ± SD. To compare the numerical data among four groups, Kurskal-Wallis H test followed by post hoc Dunn test was used. A P value of 0.05 or less was considered significant. All of the data were analyzed using SPSS 10.0.

3. Results

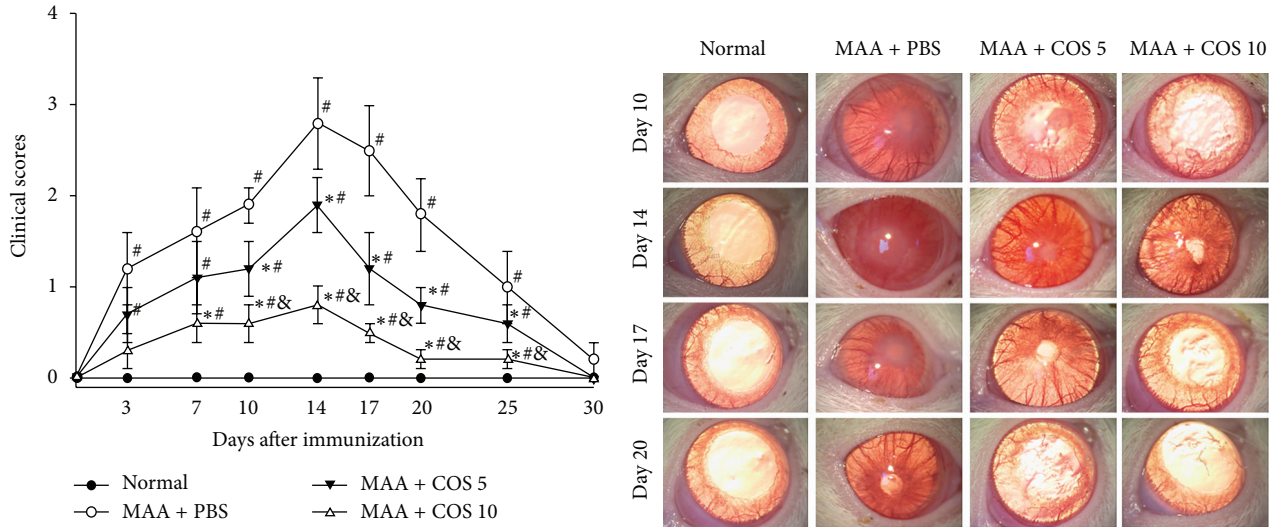
3.1. Effects of COS on Clinical Activity Scores. The rats induced with MAA began to develop signs of EAAU on day 3 after immunization. The clinical signs reached a peak at day 14 and were entirely relieved at day 30. Treatment with low-dose COS caused a significant reduction in the clinical activity scores at days 10, 14, 17, and 20 ($P < 0.05$ in all paired comparisons, $n = 10$). Moreover, the high-dose COS group demonstrated significantly decreases in the clinical activity scores throughout the clinical course, at days 7, 10, 14, 17, 20, and 25, compared with the PBS-treated group ($P < 0.05$ in all paired comparisons, $n = 10$). The clinical activity scores were significantly lower in the rats treated with high-dose COS than in the rats in the low-dose COS group at days 10, 14, 17, 20, and 25 ($P < 0.05$ in all paired comparisons, $n = 10$) (Figures 1(a) and 1(b)).

3.2. Effects of COS on Histological Changes in ICB and Leukocytes Infiltration in AqH. The histological examination revealed that the PBS-treated group had prominently increased leukocyte infiltration and tissue swelling in the ICB at day 14. Treatment with low-dose or high-dose COS resulted in a markedly decreased infiltration of leukocytes in the ICB. The effects of attenuated leukocyte infiltration and tissue swelling in the ICB were more noticeable in the high-dose COS group than in the low-dose COS group (Figure 2(a)).

In the PBS-treated group, the number of leukocytes in the AqH significantly increased at days 10, 14, 17, and 20 compared with the normal group ($P < 0.05$ in all paired comparisons, $n = 3$). The number of leukocytes was significantly lower in the rats treated with low-dose or high-dose COS compared with the rats treated with PBS at days 10, 14, 17, and 20 ($P < 0.05$ in all paired comparisons, $n = 3$). In addition, the number of leukocytes was significantly reduced in the high-dose COS group compared with the low-dose COS treatment group at days 10, 14, 17, and 20 ($P < 0.05$ in all paired comparisons, $n = 3$) (Figure 2(b)).

3.3. Western Blot for the Effects of COS on the Expression of TNF-α, iNOS, MCP-1, RANTES, Fractalkine, and ICAM-1 in the ICB. The expression levels of the TNF-α, iNOS, MCP-1, RANTES, fractalkine, and ICAM-1 proteins in the ICB were significantly higher in the PBS-treated group compared with the normal group at day 14 ($P < 0.05$ in all paired comparisons; $n = 8$). Treatment with low- or high-dose COS significantly attenuated the expression of these inflammatory mediators compared with the PBS-treated group ($P < 0.05$, low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group; $n = 8$). In addition, the levels of TNF-α, iNOS, MCP-1, RANTES, fractalkine, and ICAM-1 were more significantly reduced in the high-dose COS group than in the low-dose COS group ($P < 0.05$ in all paired comparisons; $n = 8$) (Figure 3).

3.4. IHC for the Effects of COS on the Expression of TNF-α, iNOS, MCP-1, RANTES, Fractalkine, and ICAM-1 in the ICB. The immunohistochemical studies showed increased expression of TNF-α, iNOS, MCP-1, RANTES, fractalkine, and



(a) (b)

Figure 1: (a) Effects of COS on ocular inflammation demonstrated by clinical scores. The rats in each group were clinically observed on a daily basis using slit lamp biomicroscopy for clinical signs of ocular inflammation. Disease severity was clinically assessed with a scale ranging from 0 to 4. The data are expressed as the mean ± SD ($^{*}P < 0.05$ compared with the PBS-treated group; $^{\#}P < 0.05$ compared with the normal group; $^{\&}P < 0.05$ compared with the low-dose group by Kruskal-Wallis H test with post hoc Dunn test; $n = 10$ for each group). (b) Representative clinical photographs of the four groups at days 10, 14, 17, and 20 are shown.

ICAM-1 in the ICB of the PBS-treated group at day 14. The low-dose and high-dose COS groups showed significantly decreased relative density of TNF-α, iNOS, MCP-1, RANTES, fractalkine, and ICAM-1 in the ICB, when compared with the PBS-treated group ($P < 0.05$ low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group, $n = 3$). The relative density of TNF-α, iNOS, MCP-1, RANTES, fractalkine, and ICAM-1 was more reduced in the high-dose COS group than in the low-dose COS group ($P < 0.05$ high-dose group versus low-dose group, $n = 3$) (Figure 4).

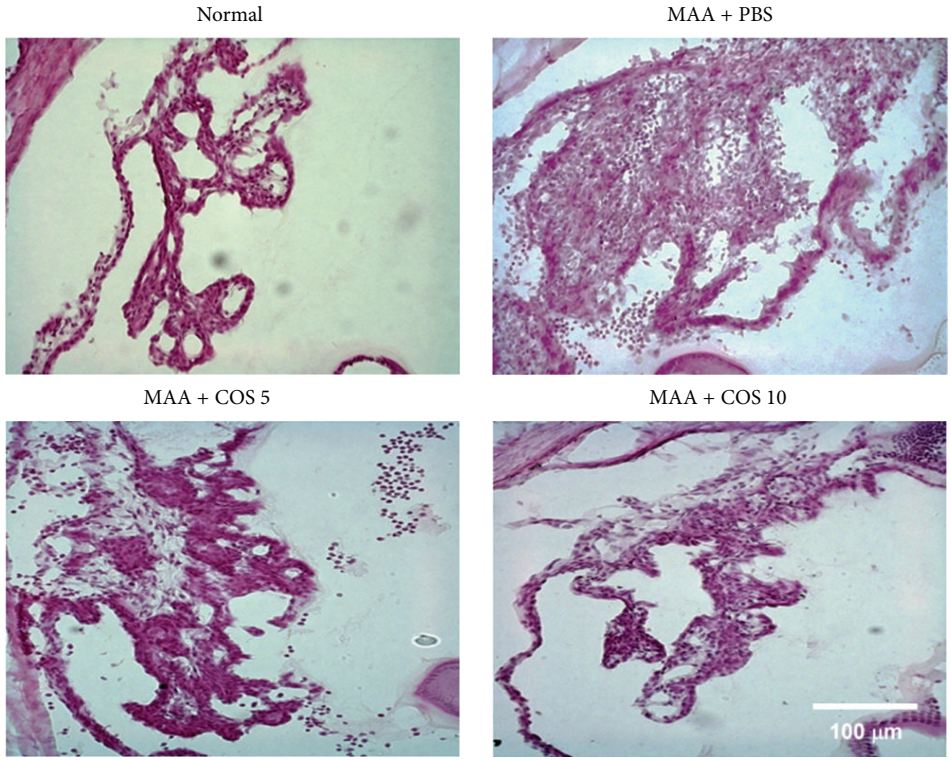
3.5. ELISA for the Effects of COS on the Levels of TNF-α, NO, MCP-1, RANTES, and Fractalkine in the AqH. In the PBS-treated group, the levels of TNF-α, NO, MCP-1, RANTES, and fractalkine in the AqH were upregulated at days 10, 14, 17, and 20. Treatment with low-dose or high-dose COS significantly reduced the levels of TNF-α, NO, MCP-1, RANTES, and fractalkine in the aqueous humor at days 10, 14, 17, and 20 compared with the PBS-treated group ($P < 0.05$ low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group, $n = 8$). In addition, the levels of TNF-α, MCP-1, fractalkine, RANTES, and NO in the aqueous humor were more reduced in the high-dose COS group than in the low-dose COS group ($P < 0.05$ in all comparisons, $n = 8$) (Figure 5).

3.6. Influence of COS on the Activation of NF-κB in the ICB. The levels of IκB in the ICB were significantly reduced in the PBS-treated group at day 14. Treatment with COS significantly increased the expression of IκB, especially in the high-dose COS group ($P < 0.05$ low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group, $n = 5$) (Figure 6(a)). In contrast, the levels of p65 in the ICB were significantly increased in the PBS-treated group. Treatment with COS significantly decreased the expression of p65 in the ICB in a dose-dependent manner ($P < 0.05$ low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group, $n = 5$) (Figure 6(b)).

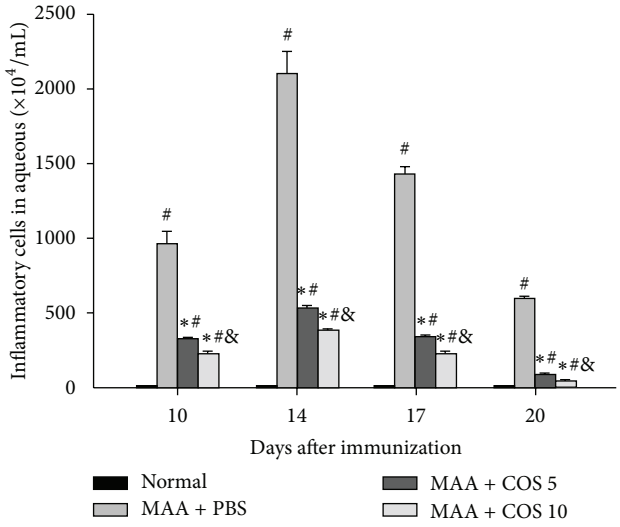
Increased staining of the NF-κB p65 subunit in the ICB was observed in the PBS-treated group at day 14. Treatment with low- and high-dose COS significantly reduced the relative density of p65 in the ICB ($P < 0.05$ low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group, $n = 3$) (Figure 6(c)).

The PBS-treated group had increased activity of NF-κB/DNA binding in the ICB at day 14. COS treatment significantly decreased the NF-κB/DNA binding activity, and this inhibitory effect was especially prominent in the high-dose COS group ($P < 0.05$ low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group, $n = 5$). Adding a 100-fold molar excess of the unlabeled NF-κB probe completely inhibited the binding of the labeled probe to the NF-κB/DNA complex (Figure 6(d)).

3.7. Effects of COS on Spleen-Derived Lymphocytes Chemotaxis toward AqH. The lymphocytes from the PBS-treated group exhibited markedly increased chemotaxis toward the aqueous humor obtained from the same group, more than that observed for the normal group. The chemotaxis was significantly decreased in the lymphocytes of the COS group, especially the high-dose COS group, compared with the PBS-treated group ($P < 0.05$ low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group, $n = 5$) (Figure 7(a)).



(a)



(b)

Figure 2: Effects of COS on histological changes in the iris/ciliary bodies (ICB) and leukocyte infiltration in the aqueous humor (AqH). (a) A representative H&E-stained ICB from normal or PBS-treated or high- or low-dose COS groups at day 14. The EAAU rats showed increased leukocyte infiltration in the ICB and the AqH, and the inflammation was attenuated by treatment with low-dose or high-dose COS. Original magnification 100x. (b) The quantification of leukocytes in the AqH at days 10, 14, 17, and 20. The EAAU rats treated with low-dose or high-dose COS showed significantly reduced leukocyte numbers in the AqH. The data are expressed as the mean ± SD ($^{*}P < 0.05$ compared with the PBS-treated group; $^{\#}P < 0.05$ compared with the normal group; $^{\&}P < 0.05$ compared with the low-dose group by Kruskal-Wallis H test with post hoc Dunn test; $n = 3$ for each group).

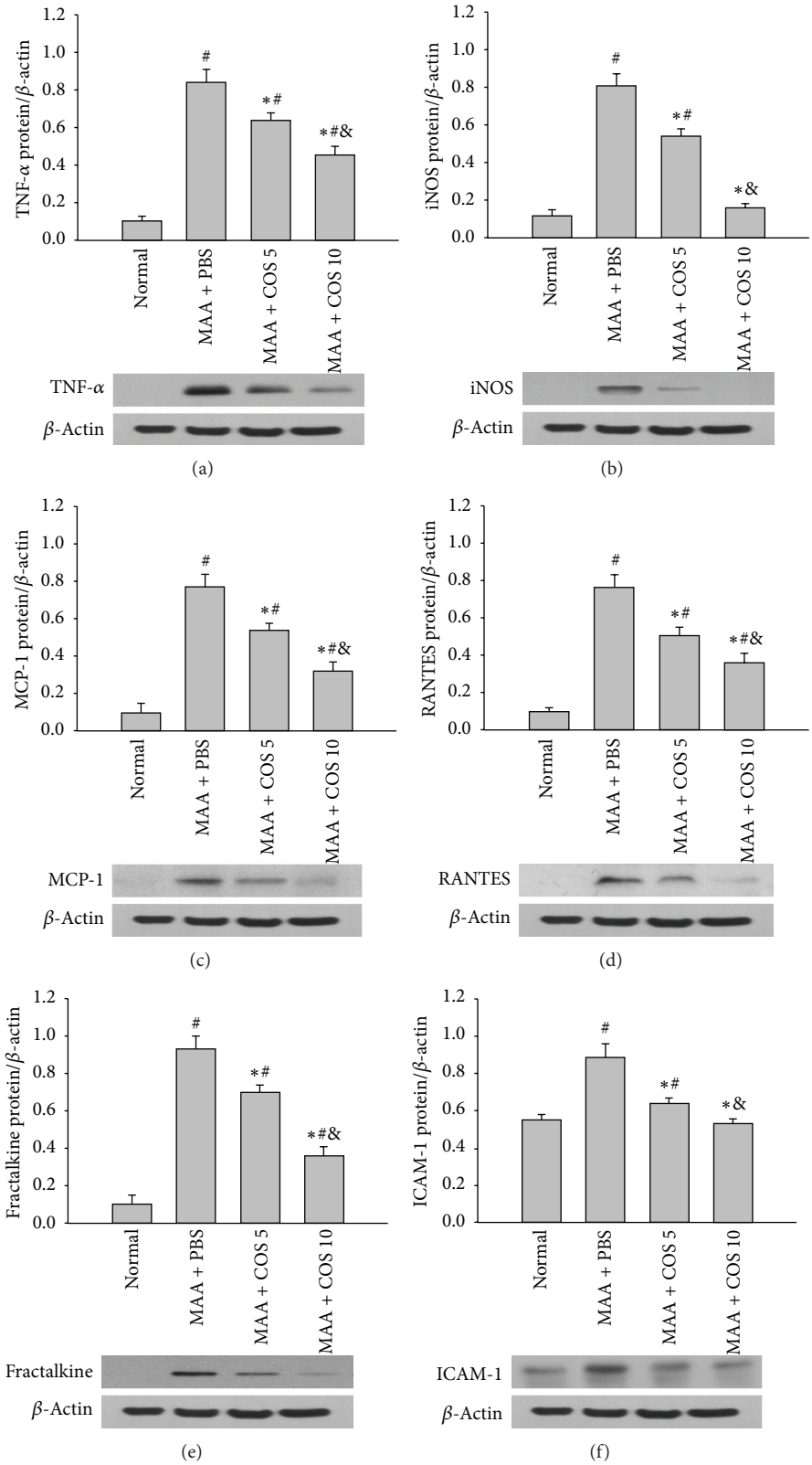
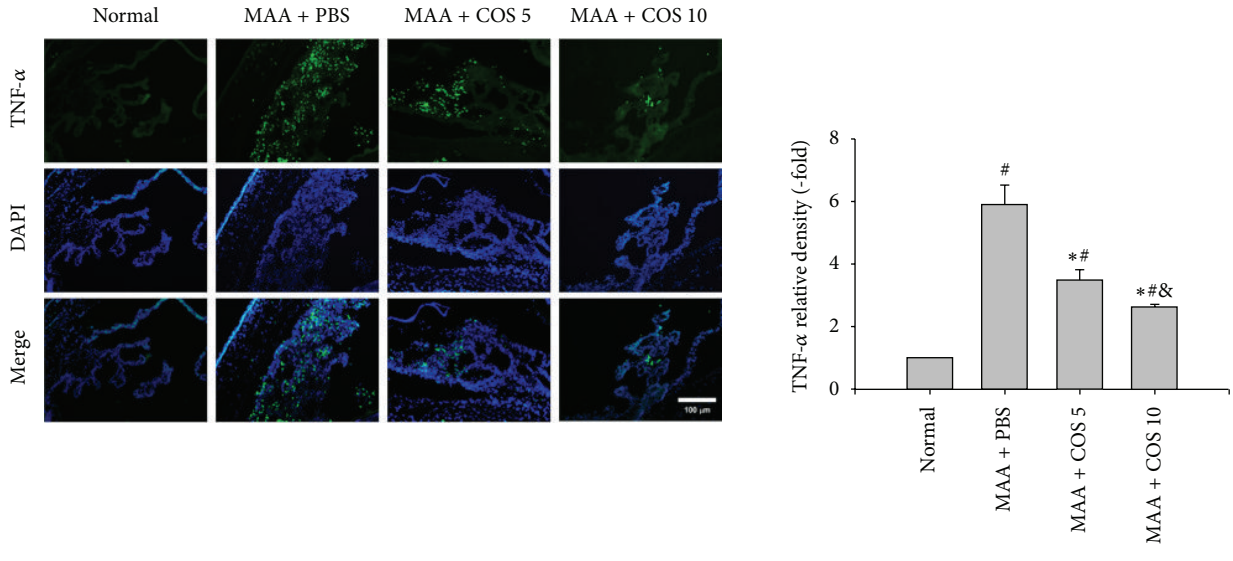
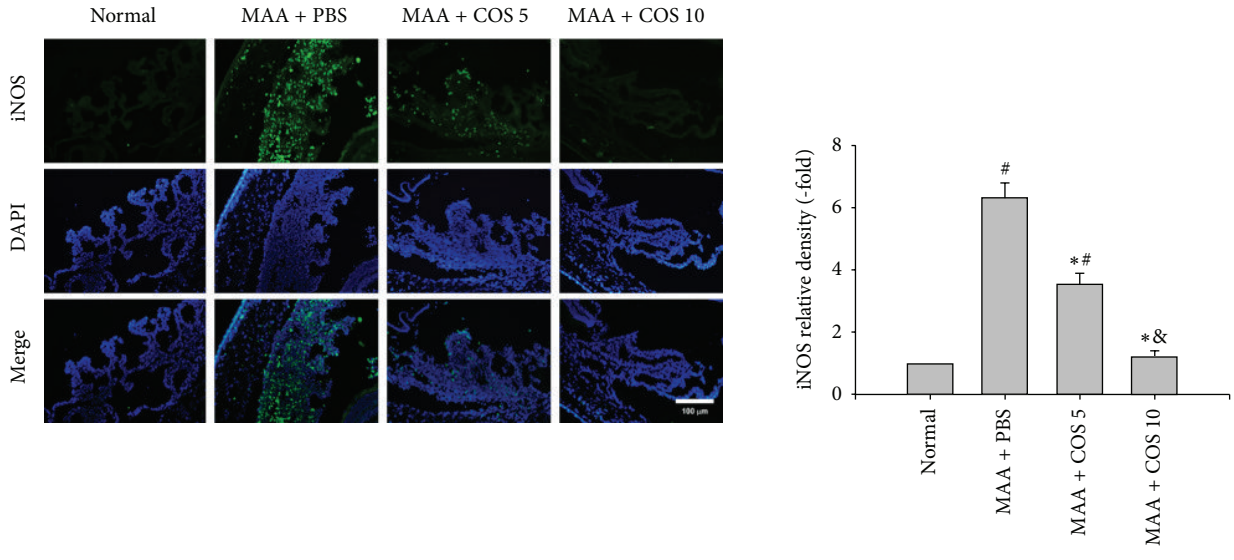


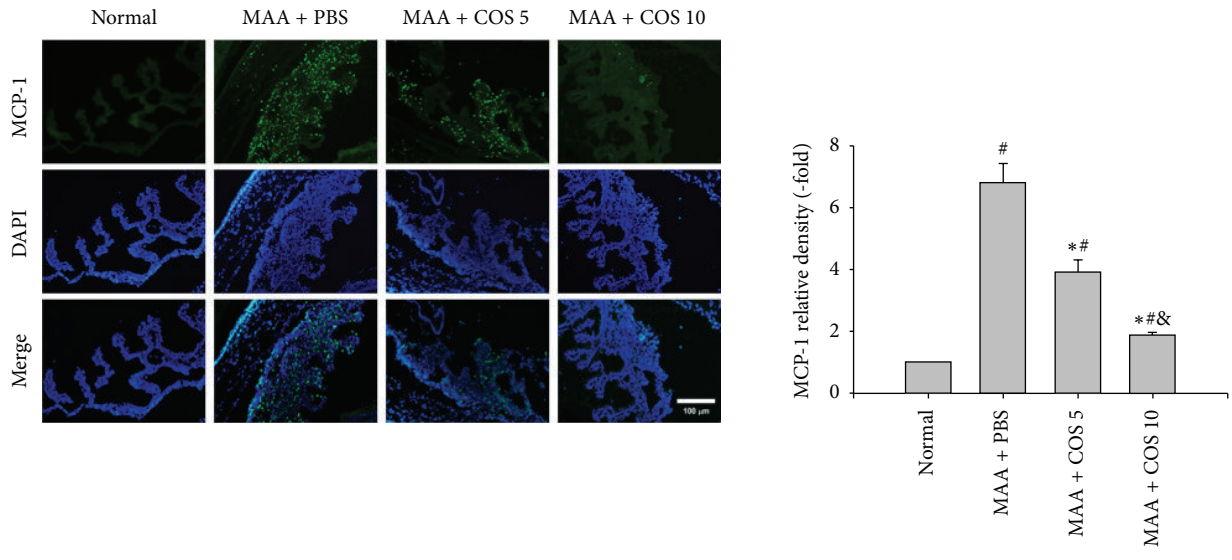
Figure 3: Evaluation of the protein expression levels of inflammatory mediators at day 14 by western blot analysis. The protein levels of (a) TNF-α, (b) iNOS, (c) MCP-1, (d) RANTES, (e) fractalkine, and (f) ICAM-1 were significantly higher in the PBS-treated group compared with the normal rats. In the COS-treated groups, especially in the high-dose group, the levels of inflammatory mediators were significantly lower than in the PBS-treated group. The data are expressed as the mean ± SD ($^{*}P < 0.05$ compared with the PBS-treated group; $^{\#}P < 0.05$ compared with the normal group; $^{\&}P < 0.05$ compared with the low-dose group by Kruskal-Wallis H test with post hoc Dunn test; $n = 8$ for each group).



(a)



(b)



(c)

Figure 4: Continued.

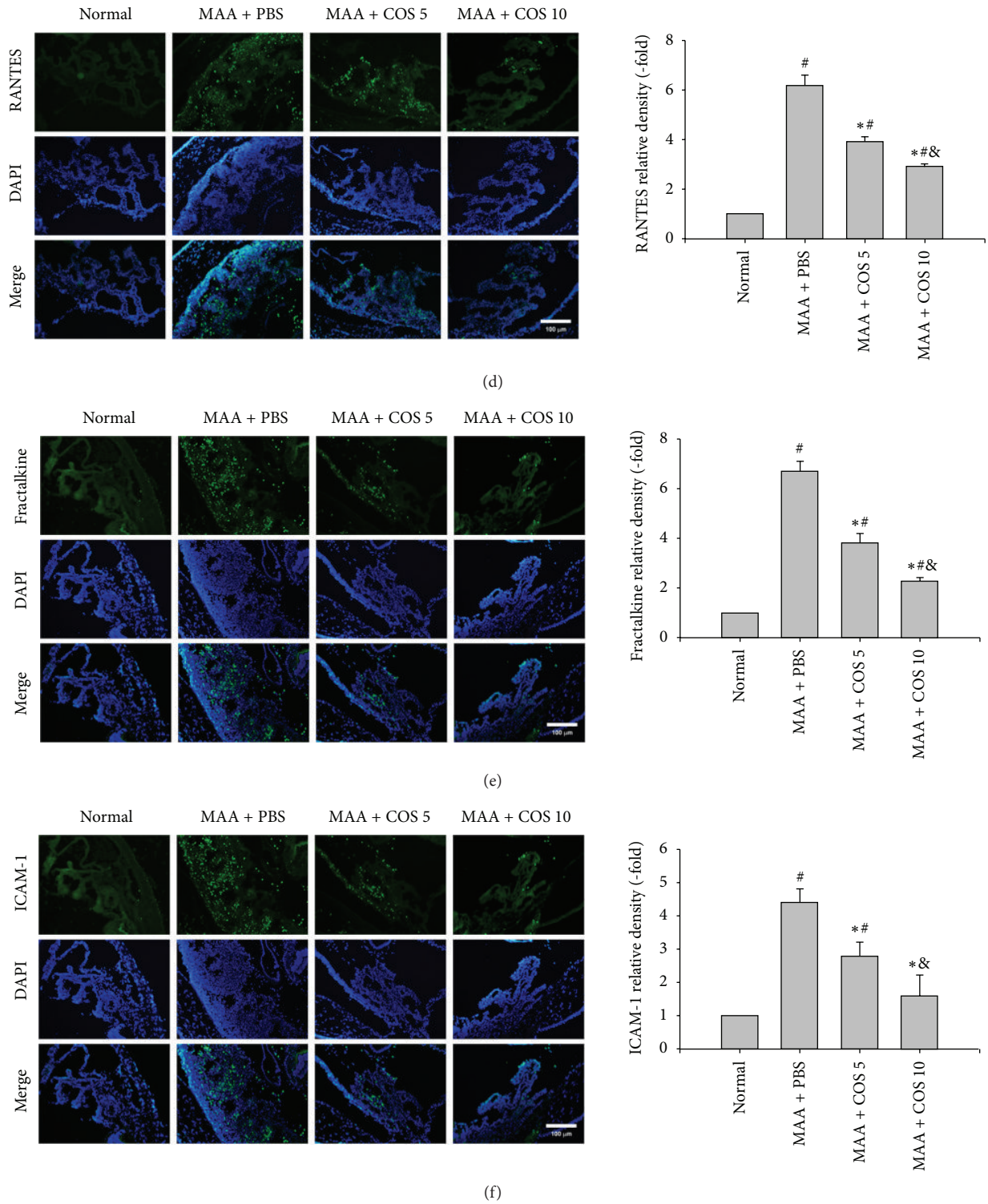


Figure 4: The evaluation of the expression levels of (a) TNF-α, (b) iNOS, (c) MCP-1, (d) RANTES, (e) fractalkine, and (f) ICAM-1 in the ICB at day 14 using IHC. For quantitation of immunostaining, we first determined the immunostaining index, which could be measured and calculated from the following formula: $\sum$[(immunostaining density − threshold) × area (pixels)]/total cell number. The relative density of immunostaining was defined as immunostaining index of PBS-treated, low-dose, or high-dose COS groups divided by immunostaining index of normal group. Treatment with low-dose or high-dose COS significantly decreased relative density of TNF-α, iNOS, MCP-1, RANTES, fractalkine, and ICAM-1 in the ICB, when compared with the PBS-treated group. The effects of decreased inflammatory mediators in the ICB were more noticeable in the high-dose COS group than in the low-dose group. There was little variation between the eyes in the same group. Original magnification 100x ($^{*}P < 0.05$ compared with the PBS-treated group; $^{\#}P < 0.05$ compared with the normal group; $^{\&}P < 0.05$ compared with the low-dose group by Kruskal-Wallis H test with post hoc Dunn test; $n = 3$ for each group).

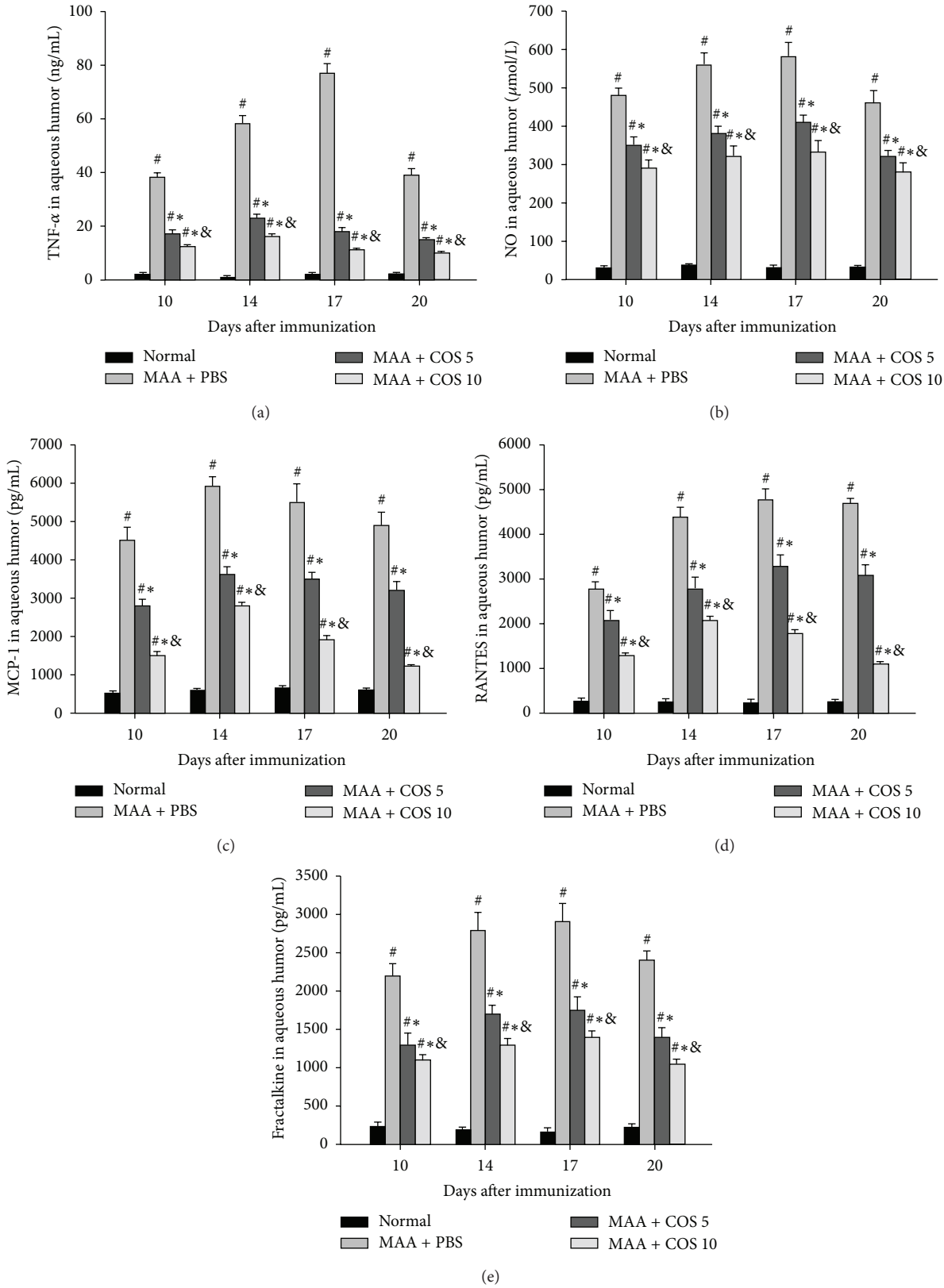


Figure 5: Quantification of the levels of (a) TNF-α, (b) iNOS, (c) MCP-1, (d) RANTES, and (e) fractalkine at days 10, 14, 17, and 20 in the AqH. Decreased expression levels of TNF-α, iNOS, MCP-1, RANTES, and fractalkine were observed in the low-dose or high-dose COS groups compared with the levels in the PBS-treated group. The AqH was pooled from one eye of five rats in each group. The data are expressed as the mean ± SD ($^{*}P < 0.05$ compared with the PBS-treated group; $^{\#}P < 0.05$ compared with the normal group; $^{\&}P < 0.05$ compared with the low-dose group by Kruskal-Wallis H test with post hoc Dunn test; $n = 5$ for each group in each time point).

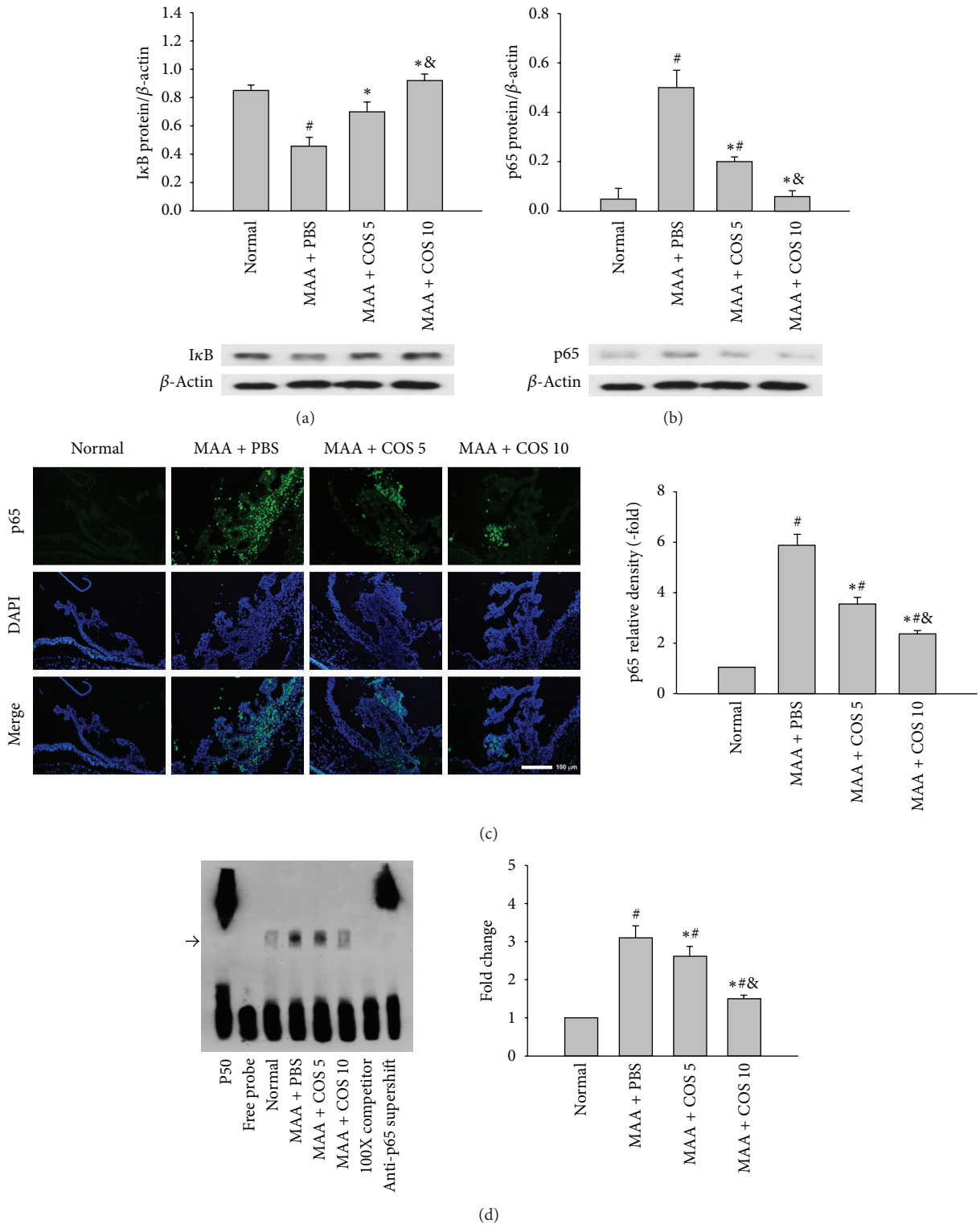


Figure 6: Effects of COS on the activation of NF-κB in the ICB at day 14. Evaluation of (a) IκB and (b) NF-κB p65 in each group using western blot analysis. The Y scale represents the ratio of the IκB or p65 blot density to the β-actin blot density ($n = 8$ for each group). (c) Immunohistochemical study of the expression of the NF-κB p65 subunit in retinas. The images represent three rats in each group. (d) The NF-κB/DNA binding activity in the ICB was measured using EMSA. "Fold change" was defined as optic density of the PBS-treated, low-dose, or high-dose COS group divided by optic density of the normal group ($n = 5$ for each group) ($^{*}P < 0.05$ compared with the PBS-treated group; $^{\#}P < 0.05$ compared with the normal group; $^{\&}P < 0.05$ compared with the low-dose group by Kruskal-Wallis H test with post hoc Dunn test).

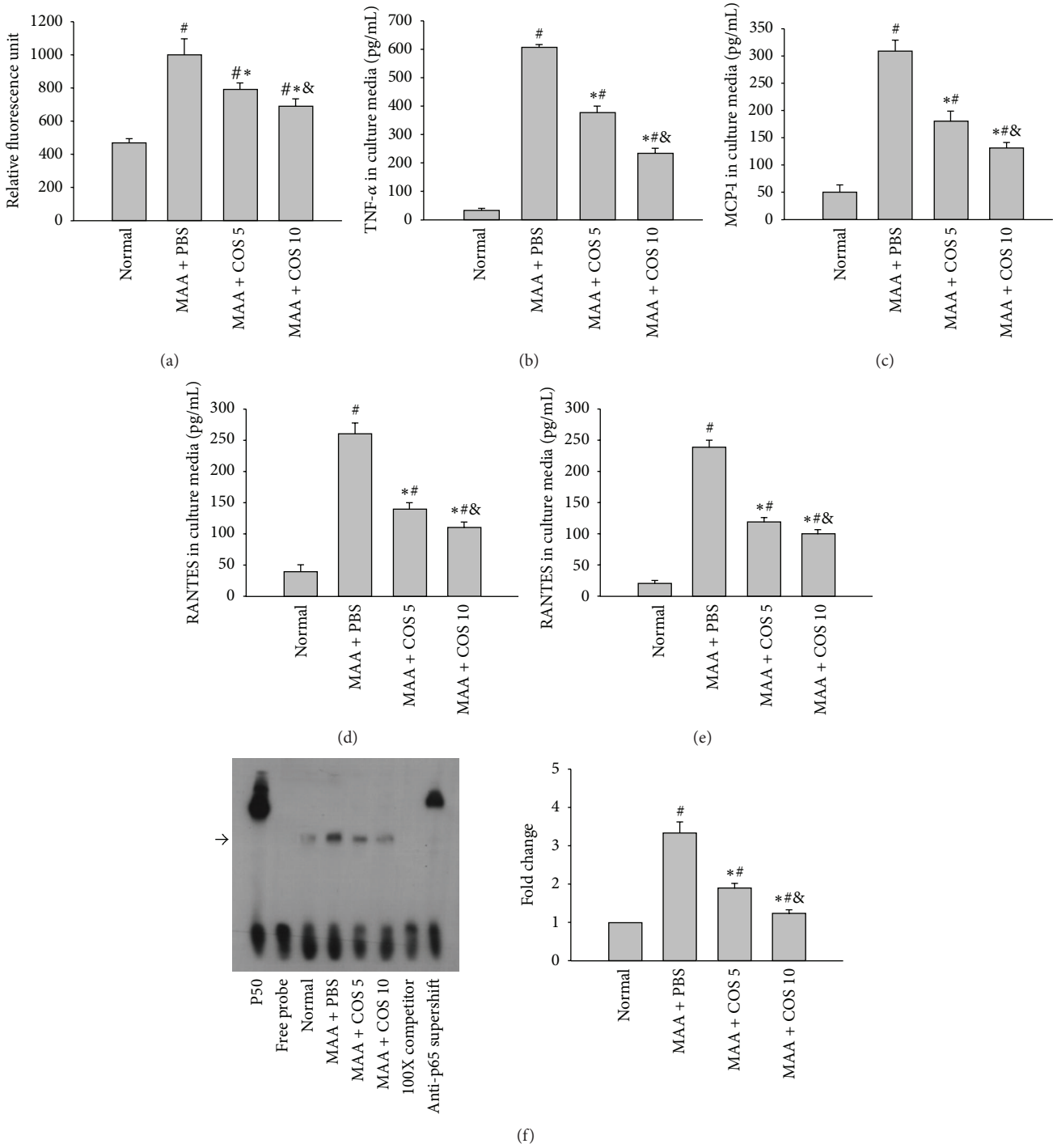


Figure 7: Effects of COS on spleen-derived lymphocyte chemotaxis, the secretion of inflammatory mediators, and the activation of NF-κB. (a) Spleen-derived lymphocytes of each group were harvested and exposed to 10 μg/mL MAA for 3 days. These cells were placed in the upper chamber of a QCM apparatus, and the aqueous humor of the same group was placed in the lower chamber of the QCM apparatus. After 24 hrs of incubation, the migrated cells were stained with a green fluorescent dye and subjected to ELISA and the relative fluorescence unit was calculated. The expression of (b) TNF-α, (c) MCP-1, (d) RANTES, and (e) fractalkine in the culture media of spleen-derived lymphocytes from each group was measured using ELISA. (f) The NF-κB/DNA binding activity of spleen-derived lymphocytes from each group was measured using EMSA. "Fold change" was defined as optic density of the PBS-treated, low-dose, or high-dose COS group divided by optic density of the normal group ($^{*}P < 0.05$ compared with the PBS-treated group; $^{\#}P < 0.05$ compared with the normal group; $^{\&}P < 0.05$ compared with the low-dose group by Kruskal-Wallis H test with post hoc Dunn test; $n = 5$ for each group).

3.8. Inflammatory Cytokine and Chemokine Secretion in the Culture Media by Spleen-Derived Lymphocytes after Stimulation with MAA In Vitro. When stimulated with MAA, the culture media of the lymphocytes from the PBS-treated group showed significantly increased expression of TNF-α, MCP-1, RANTES, and fractalkine compared with the culture media of the lymphocytes from the normal rats ($P < 0.05$ in all paired comparisons, $n = 5$). The levels of these inflammatory mediators were significantly reduced in the culture media of the lymphocytes from the COS group, especially in the high-dose COS group ($P < 0.05$ low-dose group versus PBS-treated group; $P < 0.01$, high-dose group versus PBS-treated group, $n = 5$) (Figures 7(b)–7(e)).

3.9. Influence of COS on the Activation of NF-κB in Spleen-Derived Lymphocytes. The spleen-derived lymphocytes of the PBS-treated group had increased activity of NF-κB/DNA binding. The lymphocytes of the COS-treated group showed markedly decreased NF-κB/DNA binding activity, and this inhibitory effect was especially prominent in the high-dose COS group (Figure 7(f)).

4. Discussion

In this study, we demonstrated for the first time that COS effectively attenuated the clinical severity, diminished inflammation in the ICB, and reduced the expression and production of inflammatory cytokines and chemokines in rats with EAAU. We found that COS suppressed NF-κB activation by inhibiting IκB degradation and p65 translocation, which might contribute to the decreased proinflammatory cytokine and chemokine production observed in EAAU. In an in vitro study, we verified the results obtained in vivo by showing that sensitized spleen-derived lymphocytes of COS-treated group showed less chemotaxis toward the aqueous humor obtained from the same group. Moreover, the lymphocytes of the COS-treated group showed decreased NF-κB activation and reduced secretion of the above proinflammatory mediators in the culture media. Our results suggested that COS most likely exerted anti-inflammatory effects in EAAU by the inhibition of NF-κB activation and caused a reduction of the expression of inflammatory mediators, which in turn decreased trafficking and the recruitment of inflammatory cells to the inflammatory sites, leading to protection of the ICB from damage. Our results demonstrated the therapeutic potentials of COS in the treatment of acute anterior uveitis.

Significant leukocyte infiltration in the iris/ciliary body, especially by T lymphocytes, is the pathological hallmark of EAAU [16]. To selectively recruit leukocytes to the inflammatory sites, chemokines and cytokines are released to attract certain cell types; this is the crucial step in the pathogenesis of uveitis [17]. It is widely accepted that multiple cytokines, chemokines, and adhesion molecules, such as TNF-α, MCP-1, RANTES, fractalkine, iNOS, and ICAM-1, are implicated in the pathogenesis of uveitis [18–20]. TNF-α is an early proinflammatory cytokine that might activate macrophages and stimulate the synthesis of other cytokines, NO, and adhesion molecules, particularly ICAM-1, in acute uveitis [21]. MCP-1 RANTES and fractalkine are potent chemoattractants for T lymphocytes, monocytes, and NK cells, all of which are the infiltrating cells observed in the ICB in rats with EAAU [22–24]. In this study, we showed that COS could decrease the production of TNF-α, MCP-1, RANTES, fractalkine, and NO in the ICB in a dose-dependent manner, which might counteract the inflammatory response by inhibiting leukocyte recruitment into the eye. Several studies have demonstrated that limited numbers of sensitized and in vitro stimulated CD4 T-lymphocytes from spleen and lymph nodes could adoptively transfer EAAU to naive rats, suggesting a good accessibility of the anterior uveal target antigen to these cells [5, 25]. In in vitro study, we try to simulate the recruitment of sensitized lymphocytes to AqH and ICB in vivo by showing that the decreased expression of inflammatory mediators in AqH may result in less chemotaxis of sensitized T lymphocytes. In addition, we found that the secretion of TNF-α, MCP-1, RANTES, fractalkine, and NO was significantly reduced in the sensitized lymphocytes of the COS-treated rats. Taken together, our results demonstrated that COS reduced the expression of inflammatory mediators and decreased chemoattraction of T lymphocytes to the ICB. Moreover, COS could also abate the ability of the recruited lymphocytes to secrete chemokines and cytokines, further blocking the attraction of leukocytes to the inflammatory sites.

NF-κB is a well-known transcription factor that could regulate gene expression involved in cellular proliferation, inflammation, and cell adhesion [26, 27]. Several studies have indicated that the expression of cytokines, chemokines, and adhesion molecules, such as TNF-α, MCP-1, RANTES, fractalkine, iNOS, and ICAM-1, is governed by NF-κB [28–31]. We previously demonstrated a significant activation of NF-κB in the ICB during EAAU, and the NF-κB inhibitor pyrrolidine dithiocarbamate effectively reduced ocular inflammation in EAAU [23]. In this study, we demonstrated that COS could inhibit NF-κB activation and decrease the expression and production of TNF-α, MCP-1, RANTES, fractalkine, iNOS, and ICAM-1, resulting in attenuated ocular inflammation. Previous studies have reported that COS exhibits anti-inflammatory activities by inhibiting NF-κB activation in vitro and in vivo [32–35]. Yousef et al. showed that oral administration of 20 mg/kg/day of COS inhibited NF-κB activation and the production of TNF-α and IL-6 in mouse models of experimental colitis and in human colonic epithelial cells (T84 cells) [36]. Wei et al. reported that COS at concentrations of 50 to 200 μg/mL suppressed the production of NO in LPS-induced N9 murine microglial cells mediated by inhibiting the activation of NF-κB and activator protein-1 [37]. In this study, we broadened the scope and demonstrated that COS treatment effectively inhibited NF-κB activation and the production of multiple chemokines and cytokines in rat models of EAAU and in sensitized lymphocytes.

Movement of leucocytes through the vascular endothelium into inflammatory sites occurs in a series of stages, including rolling, arrest, firm adhesion, and transmigration [38]. ICAM-1 is a key molecule involved in leukocyte adhesion and transmigration. Several studies indicated that reduced expression of ICAM-1 results in attenuation of leukocyte adhesion and/or transmigration [39–41]. Previous

studies have shown that increased expression of ICAM-1 is another important factor involved in the pathogenesis of EAAU [42]. In this study, we showed that the protein level of ICAM-1 in the ICB was upregulated and that COS significantly decreased the ICAM-1 expression in a dose-dependent manner. Our findings are consistent with a previous report by Li et al., who demonstrated that COS downregulated the expression of ICAM-1 by inhibiting the activation of NF-κB in LPS-treated porcine iliac artery endothelial cells [43].

5. Conclusions

Our studies suggest that COS dampened the inflammatory damage by affecting diverse components of the inflammatory response, including chemokine and cytokine production and adhesion molecule expression in EAAU rats. The COS anti-inflammatory effects are most likely associated with the inhibition of NF-κB activation. COS could be a promising agent for treating EAAU.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Review Article

The Role of Inflammation in the Pathogenesis of Macular Edema Secondary to Retinal Vascular Diseases

Francisco J. Ascaso,[1,2] Valentín Huerva,[3,4] and Andrzej Grzybowski[5,6]

[1] *Department of Ophthalmology, "Lozano Blesa" University Clinic Hospital, San Juan Bosco 15, 50009 Zaragoza, Spain*
[2] *Aragon Health Sciences Institute, Zaragoza, Spain*
[3] *Department of Ophthalmology, University Hospital Arnau de Vilanova, Lleida, Spain*
[4] *IRB-Lleida, Lleida, Spain*
[5] *Department of Ophthalmology, Poznań City Hospital, Poznań, Poland*
[6] *University of Warmia and Mazury, Olsztyn, Poland*

Correspondence should be addressed to Francisco J. Ascaso; jascaso@gmail.com

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Macular edema (ME) is a nonspecific sign of numerous retinal vascular diseases. This paper is an updated overview about the role of inflammatory processes in the genesis of both diabetic macular edema (DME) and ME secondary to retinal vein occlusion (RVO). We focus on the inflammatory mediators implicated, the effect of the different intravitreal therapies, the recruitment of leukocytes mediated by adhesion molecules, and the role of retinal Müller glial (RMG) cells.

1. Macular Edema: A Nonspecific Indication of Numerous Retinal Vascular Disorders

Macular edema (ME) is defined as an accumulation of either extracellular (mainly in the outer plexiform and the inner nuclear layers) or intracellular fluid (swelling of retinal Müller glial (RMG) cells) in the central part of the retina. Indeed, at times, a combination of these types of fluid accumulation occurs [1]. ME is a nonspecific sign of numerous retinal vascular diseases, such as diabetic retinopathy (DR) and retinal vein occlusions (RVO) [2, 3]. In these disorders, inflammatory processes have been considered to be critical [4–6], and breakdown of the blood retinal barrier (BRB) coupled to the subsequent increase in vascular permeability often causes ME and concomitant visual acuity impairment, secondary to an increased flux in the retinal capillary endothelial cells [7, 8]. Thus, the pathogenesis of diabetic macular edema (DME) includes several interrelated factors such as chronic hyperglycemia, hypoxia, accumulation of free radicals, activation of vascular endothelial growth factor (VEGF), alterations in endothelial intercellular junctions, pericyte loss, retinal vessel leukostasis, disruption of the BRB, and an increase in vascular permeability [9, 10]. Although the pathogenesis of ME when associated with RVO (RVO-ME) is not fully understood, increased rigidity of a crossing artery as a result of an atherosclerotic process has been suggested to cause compression of the underlying vein, provoking turbulent blood flow, endothelial damage, and thrombus formation [11]. Likewise, a common vitreous adhesion at the obstruction site has also been reported, suggesting a possible role of vitreovascular traction in the etiology of some cases of BRVO [12, 13].

Atherosclerosis is a chronic low-grade inflammatory disorder and inflammation within the vascular wall contributes to the development of ME [14–16]. Due to BRB breakdown secondary to damage at the tight junctions of endothelial cells, fluid diffusion from the occluded veins into the tissue can lead to ME [17]. In addition, through such mechanisms, inflammatory responses and vascular dysfunction can all interact to cause retinal ischemia, which induces the expression of VEGF [18]. DME and BRVO-ME may differ in terms of pathogenesis because the cytokine concentrations

in the aqueous humor are quite different, suggesting that the inflammatory reaction may be more activated in DME than in BRVO-ME, and ischemic insult may play a central role in the development of BRVO-ME [19].

2. The Role of Inflammatory Mediators in the Pathogenesis of Macular Edema

Since Vinores et al. [20] first described the role of VEGF in both ischemic and inflammatory ocular pathologies, it is well known that certain inflammatory mediators are present at the sites of ME, such as the aforementioned VEGF, together with cytokines, chemokines, angiotensin II, prostaglandins, matrix metalloproteinases, interleukins, selectins, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and inflammatory cells (macrophages and neutrophils), all of which participate in a complex chain of events that has yet to be fully defined [21, 22]. The vitreous levels of these inflammatory factors appear to be related to the pathological processes [23], although it remains to be seen what blood components are extravasated, how and where they flow into the retinal tissue, and from which vessels they are absorbed [24].

It is important to define which inflammatory mediators are enhanced or dampened in the clinical situation. Indeed, it is known that the concentration of several cytokines in the vitreous cavity increases in eyes with BRVO-ME [25–27], including VEGF and interleukin-6 (IL-6), and that such increases are related to the severity and prognosis of ME [28]. Likewise, increased vitreous fluid levels of interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), pigment epithelium-derived factor (PEDF), and particularly VEGF and ICAM-1 were related to retinal vascular permeability and the severity of DME [29].

However, whereas the aqueous humour is easily accessible and can be examined even in an outpatient setting, it is not possible to evaluate the vitreal levels of these cytokines in a routine examination [30]. When the vitreous levels of VEGF and interleukin-6 (IL-6) have been measured in patients with DME or with ME due to BRVO and CRVO, the vitreal VEGF concentration proved to be very similar in each group [31]. However, the level of IL-6 in the vitreous cavity was significantly higher in DME patients than in those with BRVO or CRVO. Noma et al. investigated whether VEGF or IL-6 contributes to the pathogenesis of ME in eyes with BRVO [26] and CRVO [32]. They found that the vitreous fluid level of VEGF was significantly higher in the patients with BRVO and CRVO than in controls. The vitreous fluid level of IL-6 was also significantly higher in the patients with both types of RVO than in the control subjects. In the BRVO and CRVO patients, there was a significant correlation between the vitreous levels of VEGF and IL-6. Vitreous fluid levels of both VEGF and IL-6 were significantly higher in patients with BRVO/CRVO patients with ischemia than in those without ischemia. In addition, the vitreous levels of both factors were significantly correlated with the severity of macular edema in the BRVO/CRVO patients. Nevertheless, further studies will be needed to fully understand the relationship of certain inflammatory mediators to DME and ME secondary to BRVO or CRVO.

3. Recruitment of Leukocytes Mediated by Adhesion Molecules

Chemokines are multifunctional mediators that can recruit leucocytes to sites of inflammation, promoting further inflammation [33, 34]. The vitreous levels of some chemokines, including MCP-1 and MIP-1a and MIP-1b, have been reported to be affected by different retinal diseases, including DME and RVO [35–37]. Mononuclear cell chemoattractants, such as MCP-1, IL-1, IL-6, IL-8, IL-12, and TNF-α, are also known to be expressed in ischemic areas, and these factors may induce the recruitment of leukocytes and their adhesion to the target tissue [38]. Thus, it may not be surprising that MIP-1b is expressed in eyes with DME and ME-RVO given that these disorders lead to retinal ischemia and inflammation [19, 36, 37, 39].

Leukocytes also play a role in increasing vascular permeability, along with VEGF. When they accumulate in the perivascular space, monocytes and lymphocytes initiate this process through leucocyte endothelial interactions [40]. These interactions are mediated by adhesion molecules (selectins, immunoglobulins, integrins, etc.) expressed by the vascular endothelium [41], which contribute to the disruption of tight junctions and the breakdown of the BRB [42, 43]. BRB breakdown may be initiated by different mechanisms, including leucocyte-mediated (recruitment and adhesion) endothelial injury, changes in endothelial cells, activation of protein kinase C, and the induction of fenestrations and vesiculovacuolar organelles [1].

4. The Role of Retinal Müller Glial (RMG) Cells

It is well known that ME develops due to vascular leakage and/or through cytotoxic events (e.g., glial cell swelling) [44, 45]. Although their importance in retinal vascular diseases is not fully known, RMG cells play a crucial role in regulating the volume of the extracellular space and water and ion homeostasis and in preserving the inner BRB [46].

Excess water is absorbed by retinal pigment epithelium (RPE) and RMG cells. RPE cells carry out the subretinal fluid, whereas RMG cells dehydrate the inner retinal tissue [44]. Transcellular water transport is linked to a transport of potassium and chloride ions [47]. Water flow through the RMG and RPE cells membranes is facilitated by water-selective channels: the aquaporins. The major water channel of RPE cells and photoreceptors is aquaporin-1, whereas RMG cells express aquaporin-4 [48, 49]. Water transport is coupled to the spatial-buffering potassium currents flowing through RMG cells [50]. Alteration of the transglial water transport after downregulation of Kir4.1 channels and osmotic swelling of RMG cells under pathologic conditions such as transient retinal ischemia-reperfusion and diabetes mellitus have been implicated in the development of ME [51, 52].

Moreover, they contribute to the survival of ganglion cell neurons and photoreceptors, they are responsible for the stabilization of retinal structure, and they modulate inflammatory and immune responses [53, 54]. Thus, the RMG cells can upregulate the expression of inflammatory mediators, including MCP-1, which recruit microglial cells and phagocytotic monocytes/macrophages to regions of damage [55, 56]. Distinct disorders are associated with BRB breakdown, which results in the extravasation of the blood constituents that inactivate Kir channels and that induce RMG cell depolarization [57, 58].

Vascular leakage is a crucial pathogenic mechanism involved in ME [59]. Retinal capillaries are closely ensheathed by glial processes [53] and RMG cells enhance the barrier function of the vascular endothelium [60–62]. Due to inflammation and hypoxia, RMG cells produce factors such as VEGF, TNF-α, IL-1β, and prostaglandins, all of which enhance retinal vascular permeability [62–74].

Fluid clearance is usually mediated by osmotic water transport through RMG cells, a process facilitated by Kir channels and water channels, especially AQP4 [75–78]. AQP4 acts in combination with K^+ channels to maintain osmotic retinal homeostasis. Indeed, Kir4.1 channel dysfunction, such as that observed in retinal vascular disorders, disturbs transcellular water transport [45, 79], resulting in water influx and RMG cells swelling [46]. Although a few studies have investigated the mechanisms of action of corticosteroids in ME, it has been shown that RMG cells express both the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) [44]. Moreover, the MR ligand aldosterone increases the expression of AQP4 and Kir4.1, and it induces retinal swelling [80]. Finally, the two main corticosteroids used in intravitreal therapies, TA and dexamethasone, the latter administered through a sustained-release implant, regulate AQP4 and Kir4.1 distinctly, indicating that they are not functionally equivalent [44].

5. The Effect of the Different Intravitreal Therapies

It is also important to determine whether there are any differences in the response to different therapies. Intravitreal injection of both triamcinolone acetonide (TA) and bevacizumab has been reported to be effective in reducing macular thickness in DME [39, 81]. Indeed, intravitreal injection of TA is effective in decreasing macular thickness in patients with ME due to BRVO or CRVO, reducing the ocular expression of inflammatory cytokines [31]. Recently, it was shown that intravitreal TA injection significantly diminished MCP-1 (monocyte chemotactic protein-1) and MIP-1b (macrophage inflammatory protein-1b) levels in the aqueous humour of eyes with BRVO-ME [28]. Moreover, the decrease in aqueous humour MIP-1b, a chemokine with proinflammatory activity, was correlated with the basal foveal thickness and its improvement following TA injection. Although the exact mechanism leading to the improvement in BRVO-ME following intravitreal TA injection has not been well established, several possible mechanisms have been considered. For example, TA could downregulate VEGF, which might prevent a decrease in occlusion as well as inhibiting any increase in glial fibrillary acidic protein (GFAP) expression in RMG cells [82]. Likewise, intravitreal TA prevents osmotic swelling of the RMG cells through the opening of K^+ (Kir) 4.1 channels and aquaporin-1 and aquaporin-4 (AQP-1 and -4) in the Müller cell membrane [83, 84]. These effects might reduce the BRB breakdown that occurs in BRVO, promoting the resolution of the ME. However, IL-6-independent VEGF secretion might also contribute to the persistence BRVO-ME after intravitreal TA injection [6].

Intravitreal injection of an anti-VEGF antibody has also been reported to be effective in reducing CRVO and DR associated with ME [39, 85]. Antiangiogenic drugs, such as ranibizumab, could be anti-inflammatory as well, and part of their actions could be through an anti-inflammatory process. They would need to be able to prevent the VEGF induced by TNF-α from acting on the RPE outside the cell. Inhibition of VEGF may act through both anti-inflammatory and antiangiogenic processes and human recombinant antiangiogenic isoforms such as VEGF-A_{165}b can be anti-inflammatory on RPE cells stimulated by TNF-α [86].

While intravitreal TA injection may have the same beneficial effects as bevacizumab in decreasing foveal thickness and improving visual acuity in the management of ME due to BRVO, TA seems to be more effective than anti-VEGF therapy in patients with DME [23, 34]. Therefore, regarding the improvement in DME, anti-VEGF therapy would be less beneficial than corticosteroid therapy. This suggests that the pathogenesis of DME can be attributed not only to VEGF alone but also to the other inflammatory molecules that are suppressed by corticosteroids [31, 87]. Although the pathogenesis of DME is not fully understood, steroids can modulate vascular permeability by suppressing the expression of VEGF and its receptor, as well as IL-6 and ICAM-1. In addition, they can also reduce the activity of inflammatory cells that release cytokines, stabilizing cell membranes and tight junctions, acting upstream of pigment epithelium-derived factor (PEDF) expression [88]. Therefore, TA has multiple actions compared with bevacizumab, which only diminishes the intraocular levels of free VEGF.

The use of anti-VEGF and steroid agents in ME secondary to retinal vascular diseases is an evolving field. There is an ongoing debate regarding the safety, efficacy, and economic concerns related to these intravitreal therapies to reduce the treatment burden [89]. The future of treatment for DME and macular edema associated with central and branch retinal vein occlusion will probably be some kind of combination: anti-VEGF inhibitors, steroids, and laser.

In conclusion, inflammatory processes can be considered crucial in the pathogenesis of ME related to retinal vascular disorders, thereby representing important therapeutic targets in these diseases.

Conflict of Interests

Costs of language proofreading and medical writer support were covered by Allergan.

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Research Article

Intraperitoneal Infusion of Mesenchymal Stem/Stromal Cells Prevents Experimental Autoimmune Uveitis in Mice

Joo Youn Oh,[1,2] Tae Wan Kim,[3] Hyun Jeong Jeong,[2] Hyun Ju Lee,[2] Jin Suk Ryu,[2] Won Ryang Wee,[1,2] Jang Won Heo,[1] and Mee Kum Kim[1,2,4]

[1] *Department of Ophthalmology, Seoul National University Hospital, Seoul 110-744, Republic of Korea*
[2] *Laboratory of Ocular Regenerative Medicine and Immunology, Seoul Artificial Eye Center, Seoul National University Hospital Biomedical Research Institute, Seoul 110-744, Republic of Korea*
[3] *Department of Ophthalmology, Seoul National University Boramae Medical Center, Seoul 156-707, Republic of Korea*
[4] *Department of Ophthalmology, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Republic of Korea*

Correspondence should be addressed to Mee Kum Kim; kmk9@snu.ac.kr

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Autoimmune uveitis is one of the leading causes of blindness. We here investigated whether intraperitoneal administration of human mesenchymal stem/stromal cells (hMSCs) might prevent development of experimental autoimmune uveitis (EAU) in mice. Time course study showed that the number of IFN-γ- or IL-17-expressing $CD4^+$ T cells was increased in draining lymph nodes (DLNs) on the postimmunization day 7 and decreased thereafter. The retinal structure was severely disrupted on day 21. An intraperitoneal injection of hMSCs at the time of immunization protected the retina from damage and suppressed the levels of proinflammatory cytokines in the eye. Analysis of DLNs on day 7 showed that hMSCs decreased the number of Th1 and Th17 cells. The hMSCs did not reduce the levels of IL-1β, IL-6, IL-12, and IL-23 which are the cytokines that drive Th1/Th17 differentiation. Also, hMSCs did not induce $CD4^+CD25^+Foxp3^+$ cells. However, hMSCs increased the level of an immunoregulatory cytokine IL-10 and the population of IL-10-expressing $B220^+CD19^+$ cells. Together, data demonstrate that hMSCs attenuate EAU by suppressing Th1/Th17 cells and induce IL-10-expressing $B220^+CD19^+$ cells. Our results support suggestions that hMSCs may offer a therapy for autoimmune diseases mediated by Th1/Th17 responses.

1. Introduction

Autoimmune uveitis of noninfectious origin is a vision-threatening disease that affects 115.3 per 100,000 people and accounts for 2.8–10% of all cases of blindness in the United States [1]. Corticosteroids are the first line of therapy for patients with autoimmune uveitis. However, long-term use of corticosteroids is associated with serious systemic and ocular adverse effects. Also, there are subtypes of autoimmune uveitis that are refractory to steroids [2]. For these reasons, efforts are being made to develop new therapies for autoimmune uveitis by modulating immune responses underlying the pathogenesis of uveitis. The pathogenesis of autoimmune uveitis is traditionally regarded as Th1-mediated [3, 4]. However, Th17 cell is recently identified as a novel subset of T cells that contributes to the development of autoimmune diseases including uveitis [3, 4].

Stromal progenitors of mesodermal cells, referred to as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs), have the remarkable capacity to protect tissues from various immune-mediated diseases. Studies demonstrate that MSCs exert immunosuppressive functions by modulating Th1, Th2, or Th17 immune responses, by inhibiting T cell proliferation, or by generating regulatory T (Treg) cells [5–7]. Also, clinical trials are in progress to capitalize on the immunomodulatory effects of MSCs for treatment of patients with autoimmune diseases such as multiple sclerosis, Crohn's disease, type 1 diabetes, systemic lupus erythematous, or systemic sclerosis [8].

In this study, we investigated whether an intraperitoneal (IP) administration of human bone marrow-derived MSCs (hMSCs) might prevent development of experimental autoimmune uveitis (EAU) in mice, a model for human autoimmune uveitis.

2. Materials and Methods

2.1. Animals. Six-week-old female B6 mice (C57BL/6J) were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained in a specific pathogen-free environment with continuously available water and food. Animals were treated in strict accordance with the ARVO statement for the use of animals in ophthalmic and vision research. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Biomedical Research Institute.

2.2. Preparation of hMSCs. Human bone marrow-derived MSCs were obtained from the Center for the Preparation and Distribution of Adult Stem Cells (http://medicine.tamhsc.edu/irm/msc-distribution.html) that supplies standardized preparations of MSCs enriched for early progenitor cells to over 300 laboratories under the auspices of an NIH/NCRR grant (P40 RR 17447-06). Animal experiments were performed with passage two hMSCs from one donor. The cells consistently differentiated into three lineages in culture, were negative for hematopoietic markers (CD34, CD36, CD117, and CD45), and were positive for mesenchymal markers CD29 (95%), CD44 (>93%), CD49c (99%), CD49f (>70%), CD59 (>99%), CD90 (>99%), CD105 (>99%), and CD166 (>99%). The cells were cultured in complete culture medium with 16% FBS until 70% confluence was reached and harvested with 0.25% trypsin/1 mM EDTA at 37°C for 2 min. After washing, the cells were resuspended in balanced salt solution (BSS; BioWhittaker, Walkersville, MD) at a concentration of 10,000 cells/μL for injection *in vivo*.

2.3. Induction and Treatment of EAU. EAU was induced in mice by subcutaneous injection into a footpad of 250 μg human interphotoreceptor retinoid binding protein (IRBP) peptide 1–20, GPTHLFQPSLVLDMAKVLLD (20 mg/mL; Peptron, Daejeon, Korea), that was emulsified in complete Freund adjuvant (Sigma, Saint Louis, MO) containing *Mycobacterium tuberculosis* (2.5 mg/mL; BD Difco, Franklin Lakes, NJ). Simultaneously, the mice received 0.7 μg Pertussis toxin (300 μL; Sigma) intraperitoneally. Immediately after immunization, either 1×10^6 hMSCs in 100 μL BSS or BSS (100 μL) alone was injected intraperitoneally.

2.4. Histology and Histological Scoring. On days 7, 14, and 21 after immunization, mice were humanely killed, and eyeballs, inguinal and popliteal LNs were collected for further assays. Eyeballs were fixed in 10% formaldehyde and paraffin-embedded. Serial 4 μm thick sections were cut and stained with either hematoxylin/eosin or TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) as the manufacturer's protocol (Chemicon International, Temecula, CA). The morphologic features of the retina were examined, and histological disease scores were assessed by two independent observes (Joo Youn Oh and Tae Wan Kim) in a blinded manner on a scale of 0 to 4 using the criteria previously defined by Caspi [9].

2.5. Flow Cytometric Analysis. The proportions of Th1, Th17, $\gamma\delta$ T, and Treg cells were determined by measuring IFN-γ, IL-17, $\gamma\delta$ TCR, or CD25 and Foxp3-expressing $CD4^+$ cells using flow cytometry. In addition, the population of IL-10 expressing cells was determined by staining the cells with CD4, CD25, Foxp3, CD19, B220, CD11b, CD11c, and IL-10. To collect cell suspensions, popliteal or inguinal nodes (LNs) were placed and minced between the frosted ends of two glass slides in RPMI media containing 10% FBS and 1% penicillin-streptomycin. The cells were immunostained with the following fluorescence-conjugated anti-mouse antibodies: CD4, CD25, Foxp3, IFN-γ, $\gamma\delta$ TCR, CD19, B220, CD11b, CD11c, IL-10 (eBioscience, San Diego, CA), and IL-17A (BD Pharmingen, San Diego, CA). For intracellular staining, the cells were stimulated for 4 h with 50 ng/mL phorbol myristate acetate and 1 μg/mL ionomycin in the presence of GolgiPlug (BD Pharmingen). The cells were then assayed for fluorescence using a FACSCanto flow cytometer (BD BioSciences, Mountain View, CA). The gate was set on $CD4^+$ or $CD19^+$ cell population, and further analysis of surface or intracellular markers was done within this gate. Data were analyzed using Flowjo program (Tree Star, Inc., Ashland, OR).

2.6. Real-Time RT-PCR. LNs were lysed in RNA isolation reagent (RNA Bee, Tel-Test Inc., Friendswood, TX) and homogenized using a sonicator (Ultrasonic Processor, Cole Parmer Instruments, Vernon Hills, IL). Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA) and used to synthesize double-stranded cDNA by reverse transcription (SuperScript III, Invitrogen, Carlsbad, CA). Real-time amplification was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA). An 18 s rRNA probe (TaqMan Gene Expression Assays ID, Hs03003631_g1) was used for normalization of gene expression. For all the PCR probe sets, TaqMan Gene Expression Assay kits were purchased from Applied Biosystems: IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IFN-γ (Mm01168134_m1) IL-17A (Mm00439618_m1), IL-10 (Mm00439614_m1), TGF-β (Mm01178820_m1), IL-12A (Mm00434165_m1), and IL-23 (Mm01160011_g1), and human GAPDH (Hs02758991_g1).

2.7. ELISA. For protein extraction, eyeballs were cut into small pieces and lysed in PRO-PREP Protein Extraction Solution (Intron Biotechnology, Seongnam, Korea). The samples were sonicated on ice using an ultrasound sonicator. After centrifugation at 12,000 rpm for 20 min, the supernatant was collected and assayed for IFN-γ by ELISA according to the manufacturer's protocol (DuoSet; R & D Systems, Minneapolis, MN).

2.8. Statistical Analysis. Values were compared *between* the groups using the one-way ANOVA (SPSS 12.0, Chicago, IL) or Student's t test and shown as the mean value ± standard error (SEM). Differences were considered significant at $P < 0.05$.

3. Results

3.1. hMSCs Reduced Retinal Damage in Mice with EAU. EAU was induced in mice by subcutaneous injection of IRBP in a footpad on day 0. Simultaneously, either hMSCs (1×10^6 cells/mouse) or BSS was injected intraperitoneally. On days 7, 14, and 21, the mice were humanely killed, and eyeballs and draining LNs (DLNs) were collected for assays (Figure 1(a)). ELISA showed that the level of the proinflammatory cytokine IFN-γ was markedly increased in the eyeball on day 14 and significantly reduced by treatment with hMSCs (Figure 1(b)). Histology demonstrated that the retinal structure including the photoreceptor layer was severely disorganized with massive infiltration of inflammatory cells in the vitreous cavity and in the retina of BSS-treated EAU mice on day 21 (histological score 2.13 ± 0.31, Figures 1(c) and 1(d)). In contrast, the retinal architecture was almost completely preserved with few inflammatory cells in hMSCs-treated mice on day 21 (histological score 0.25 ± 0.14, Figures 1(c) and 1(d)). Similarly, TUNEL staining indicated the presence of many dead cells in the photoreceptor layer in BSS-treated EAU mice on day 21, while there were few TUNEL-positive cells in mice treated with hMSCs (Figure 1(e)).

To determine whether hMSCs suppressed the intraocular inflammation by direct contact, we evaluated the presence of hMSCs in the eye by real-time RT-PCR assays for human-specific GAPDH. However, we did not detect any amplification of human GAPDH, indicating that hMSCs were not present in the eye on days 7, 14, or 21 after IP injection.

Therefore, the data clearly indicated that a single IP injection of hMSCs at the time of immunization prevented the development of EAU and protected the retina from inflammation-mediated damage.

3.2. hMSCs Decreased Th1/Th17 Cells in DLNs. We next investigated whether the tissue-protective effects of hMSCs might be due to the influence of hMSCs on the development of Th1, Th17, or $\gamma\delta$ T cells that are pathogenic effectors in uveitis [3, 4]. Time course study showed that the percentage of IFN-γ-expressing $CD4^+$ cells, IL-17A-expressing $CD4^+$ cells, and $\gamma\delta$ TCR-expressing $CD4^+$ cells was significantly increased in popliteal and inguinal LNs of EAU mice on day 7 and decreased thereafter to baseline levels (Figure 2, Supplemental Figure 1 (see Supplemental Figure 1 available online at http://dx.doi.org/10.1155/2014/624640)). Treatment with hMSCs significantly reduced the percentage of Th1 and Th17 cells in popliteal and inguinal LNs on day 7 (Figure 2). Consistently, the levels of IL-17A and IFN-γ transcripts were significantly reduced by hMSCs, compared to the BSS-treated controls (Figure 3(a)). However, the percentage of $\gamma\delta$ T cells was not affected by hMSC treatment (Supplemental Figure 1).

In order to determine whether hMSCs inhibited Th1 or Th17 cell generation by suppressing the production of Th1- or Th17-polarizing cytokines, we further measured the levels of IL-1β, IL-6, IL-12a, IL-23, and TGF-β which are the cytokines that drive the development of Th1 and Th17 cells [10–12]. However, hMSCs did not reduce the levels of IL-1β, IL-6, IL-12A, IL-23, and TGF-β in DLNs (Figures 3(b)–3(d)). Of note, hMSCs significantly increased the level of IL-10, an immunoregulatory cytokine that suppresses Th1/Th17 immune responses (Figure 3(b)).

3.3. hMSCs Induced IL-10-Expressing $B220^+CD19^+$ Cells in DLNs. To identify the IL-10-expressing cell population, we examined $CD4^+$, $CD19^+$, $B220^+$, $CD11b^+$, or $CD11c^+$ cells in DLNs for IL-10 expression. We found that the percentage of $IL\text{-}10^+B220^+CD19^+$ cells was markedly increased in popliteal LNs of hMSCs-treated EAU mice on day 7, compared to BSS-treated EAU mice (Figures 4(a) and 4(b)). However, IL-10 expression was not increased in $CD4^+$, $CD11b^+$, or $CD11c^+$ cells (data not shown). Also, hMSCs did not increase the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in popliteal or inguinal LNs at all examined time-points (Supplemental Figures 2(a) and 2(b)). Time course indicated that $CD4^+CD25^+Foxp3^+$ cells initially increased in popliteal and inguinal LNs on day 7 after EAU induction and gradually decreased to baseline on days 14 and 21. On the contrary, $CD4^+CD25^+Foxp3^+$ cells initially decreased in the spleen on day 7 and increased thereafter until day 21. The hMSCs significantly suppressed an increase of $CD4^+CD25^+Foxp3^+$ cells in the spleen on days 14 and 21 (Supplemental Figure 2(c)), reflecting that hMSCs might suppress early inflammation that is required for initial Treg expansion [11].

4. Discussion

Time course study revealed that Th1 and Th17 cells increased in DLNs after EAU immunization, and then the levels of proinflammatory cytokines markedly increased in the eyes. Subsequently, the retinal structure was severely and irreversibly disrupted. A single IP administration of hMSCs at the time of immunization significantly decreased Th1 or Th17 cells in DLNs and increased $IL10^+B220^+CD19^+$ cells on day 7. As a result, ocular inflammation was markedly repressed, and the retina was almost completely protected from damage. Therefore, our data suggest that hMSCs ameliorate autoimmune uveitis by suppressing the Th1/Th17 immune responses.

There are several possibilities that account for the mechanisms of hMSCs in repressing the Th1/Th17 immune responses. One is that hMSCs may directly inhibit differentiation or expansion of Th1/Th17 cells. In support of this hypothesis, previous reports demonstrated that MSCs suppressed T cell proliferation and inhibited differentiation of naïve $CD4^+$ T cells into Th1 or Th17 cells [13–18]. However, it is unclear *in vivo* whether enough number of systemically administered hMSCs reaches peripheral LNs or injured tissues to exert direct inhibitory effects on T cells.

Another possibility is that hMSCs may modulate host cells to suppress immune responses. This is likely because a single administration of MSCs that are short-lived has long-term effects in the host as shown in our study. In fact,

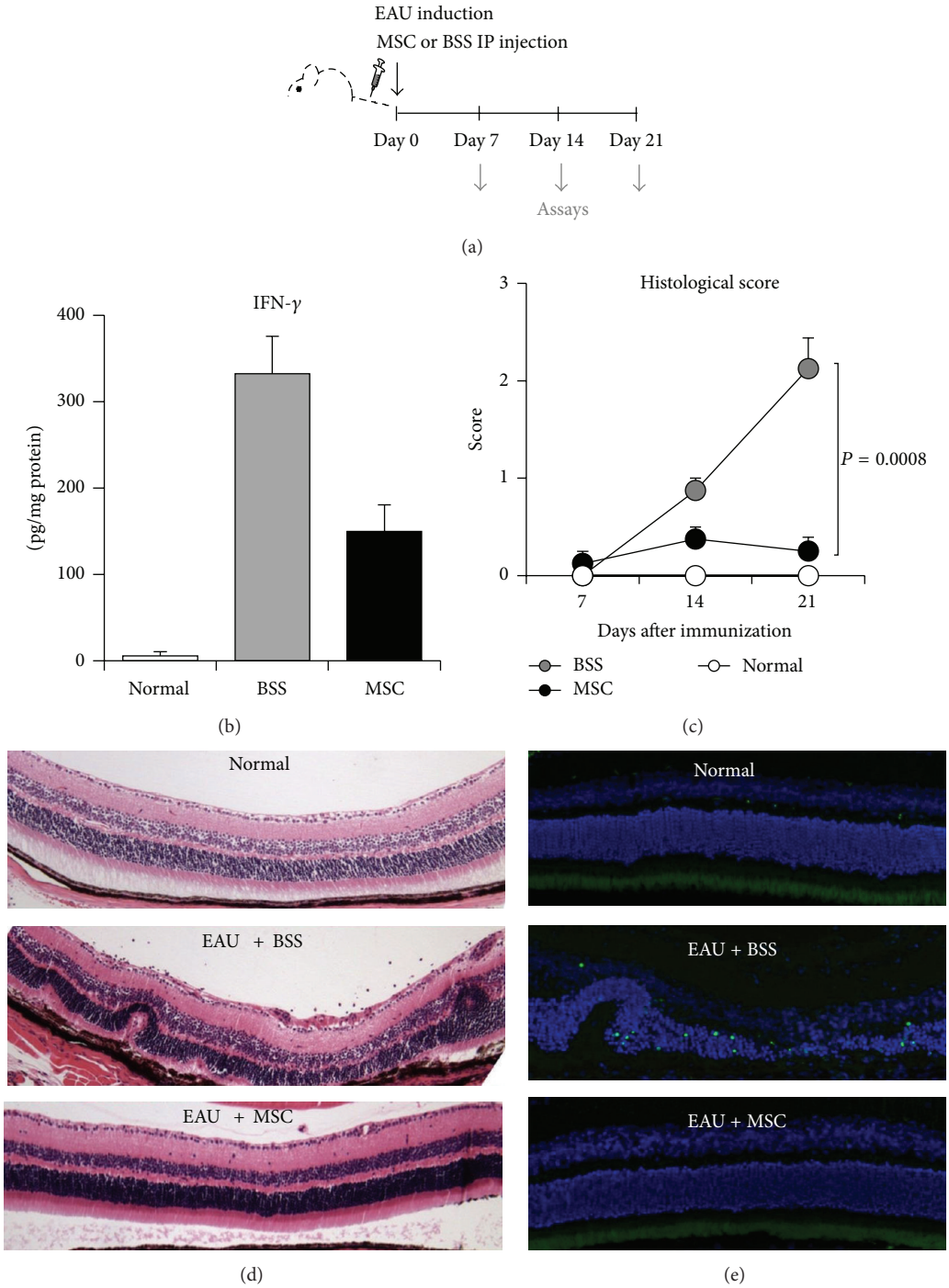

Figure 1: Histological findings of the eye. (a) EAU was induced in mice on day 0, and either hMSCs (1×10^6 cells in 100 μL BSS) or BSS (100 μL) were intraperitoneally (IP) injected immediately after EAU induction. On days 7, 14, and 21, the eyes or DLNs were collected for assays. (b) ELISA showed that IFN-γ in the eye was markedly increased in the eyeball on day 14 and significantly reduced by hMSCs. Data are presented in mean + SEM. $n = 5$ in each group. (c) Time course of histological disease scores demonstrated that the retinal pathology gradually developed with a peak at day 21. Histological scores were significantly lower in hMSCs-treated mice at all time-points, suggesting that hMSCs prevented the disease development. Data are presented in mean + SEM. $n = 5$ in each group. (d) Hematoxylin-eosin staining of the eye on day 21 showed severe disruption of the retinal structure including the photoreceptor layer with inflammatory cell infiltration in the vitreous cavity and in the retina of EAU mice. In contrast, the retinal structure was well-reserved, and few inflammatory cells were observed in EAU mice treated with hMSCs. (e) TUENL staining showed a number of dead cells in the disrupted photoreceptor layer of EAU mice. In contrast, no TUENL-positive cells were found in the retina of mice treated with hMSCs.

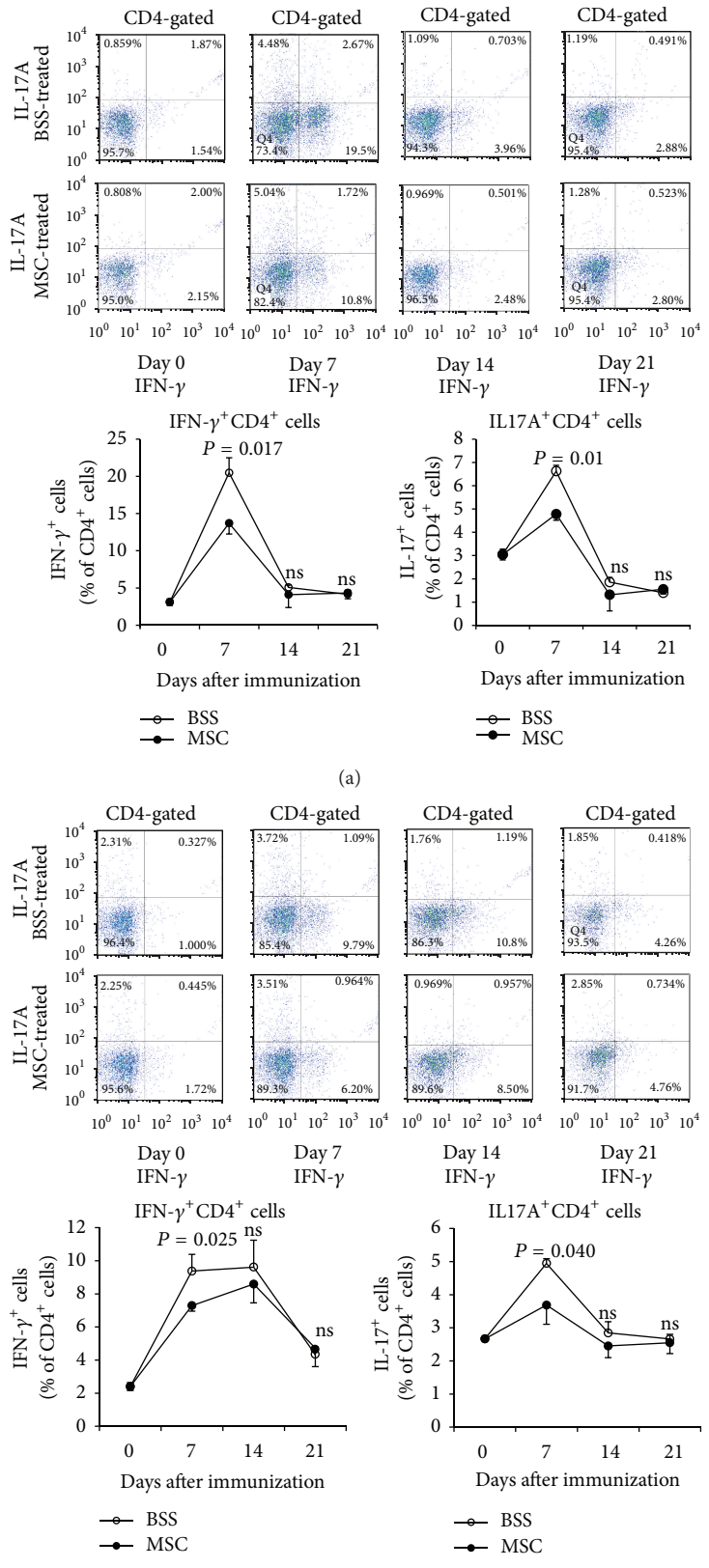

Figure 2: Flow cytometry for Th1 and Th17 cells in LNs. Time course study demonstrated that the percentages of IFN-γ-expressing $CD4^+$ cells or IL-17A-expressing $CD4^+$ cells were significantly increased in popliteal (a) and inguinal LNs (b) on day 7 after EAU induction and decreased thereafter to baseline until day 21. The percentages of both Th1 and Th17 cells were significantly lower in EAU mice treated with hMSCs, compared to BSS-treated mice. Data are presented in mean ± SEM. $n = 5$ in each group.

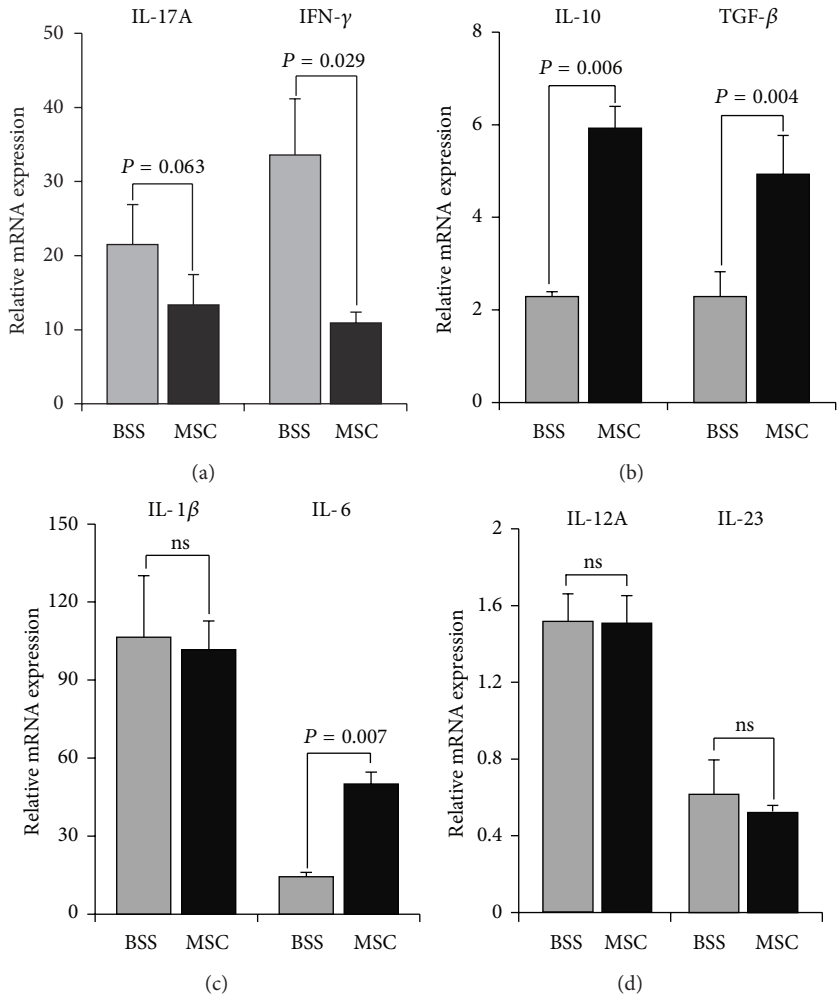

Figure 3: Assay for inflammation- and immune-related cytokines in LNs. Real-time RT-PCR analysis showed that the levels of IL-17A and IFN-γ transcripts were increased in popliteal LNs on day 7 after EAU induction and significantly reduced by hMSCs (a). However, the levels of IL-10, TGF-β, and IL-6 were significantly increased by hMSCs (b, c). The levels of IL-1β, IL-12A, or IL-23 were not different between BSS- and hMSC-treated EAU mice (c, d). Data are presented in mean + SEM. $n = 5$ in each group.

a number of previous studies showed that MSCs induced Treg cells [18, 19], switched microglia or macrophages toward anti-inflammatory or tissue-repairing phenotypes [20–22], or promoted generation of regulatory or tolerogenic dendritic cells [23–25]. Consistent with these reports, we found that the level of IL-10, an immunoregulatory cytokine that suppresses Th1/Th17 immune responses [26, 27], was markedly upregulated in DLNS of mice treated with hMSCs. Among the examined cell populations ($CD4^+$, $CD19^+$, $CD11b^+$, and $CD11c^+$ cells), IL10-expressing $B220^+CD19^+$ cells were dramatically increased. IL-10-producing B cells are known to be critical for suppression of autoimmune diseases, although B cells are both pathogenic and protective in autoimmune diseases [28]. Given that the effects of MSCs on B cells have been rarely studied so far, further characterization of IL10-expressing $B220^+CD19^+$ cells as a potential regulatory B cell subset [29, 30] would provide a mechanistic insight into the immunomodulatory mechanism of hMSCs.

Since the therapeutic efficacy of MSCs was first reported in experimental autoimmune encephalitis [31], there have been efforts to use MSCs for treating a variety of autoimmune diseases such as autoimmune arthritis, autoimmune myocarditis, or Sjögren's syndrome [5, 8, 16, 17, 19]. When it comes to autoimmune uveitis, two studies recently demonstrated that MSCs attenuated EAU in mice and rats. Tasso et al. [32] reported that a single IP injection of syngeneic mouse MSCs at the time of immunization almost completely reduced the incidence and severity of disease in mice with EAU induced by IRBP injection. They found that the percentage of $CD4^+CD25^+Foxp3^+$ cells was significantly higher in the spleen in MSC-treated EAU mice than in untreated controls. Another study by Zhang et al. [33] reported that an intravenous administration of syngeneic or allogeneic rat MSCs strikingly reduced the severity of EAU induced by IRBP in rats. MSCs were effective when the cells were administered before the onset or at the peak of disease, but

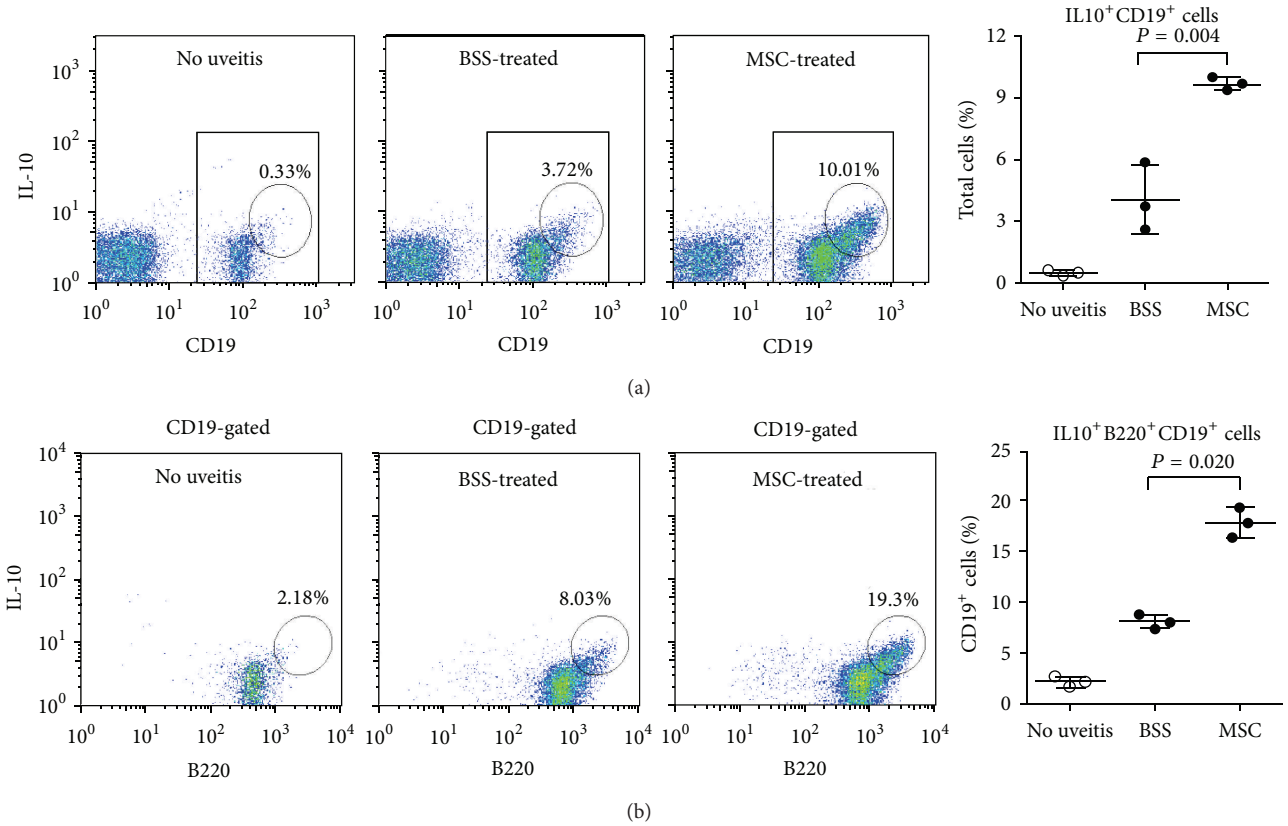

Figure 4: Assay for IL-10-expressing cells in LNs. Flow cytometric analysis of popliteal LNs on day 7 after EAU immunization showed that the percentages of IL-10$^+$CD19$^+$ (a) and IL-10$^+$B220$^+$CD19$^+$ cells (b) were significantly increased in mice treated with hMSCs, compared to mice without EAU or BSS-treated EAU mice. Data are presented in mean ± SEM. $n = 5$ in each group.

not after disease stabilization. They additionally found that the levels of IL-2, IL-17, and IFN-γ were lower in supernatants of T lymphocytes isolated from EAU mice treated with MSCs, compared to T lymphocytes from untreated EAU mice. However, either study did not directly evaluate the effects of MSCs on Th1 and Th17 cells in DLNs *in vivo* as in our study.

Another difference of our study is that we used human MSCs for the study. Mouse MSCs undergo spontaneous transformation during expansion in culture and occasionally become tumorigenic in the same manner as mouse fibroblasts [34–36]. Moreover, some of the immunomodulatory effects of MSCs are species-specific. For instance, indolamine 2, 3-dioxygenase (IDO) is involved in the immunosuppressive activity of human MSCs, whereas inducible nitric oxide synthase (iNOS) mediated the immunosuppressive activity of mouse MSCs [37]. Therefore we used hMSCs for this study in order to evaluate clinical efficacy of hMSCs and their mechanism(s) as a potential treatment of autoimmune uveitis in humans. As a result, we found that the number of Th1 and Th17 cells was markedly reduced by hMSCs and IL10$^+$B220$^+$CD19$^+$ cells, not classical CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, were increased by hMSCs. Further studies using a specific depletion of IL-10 production from B cells would be necessary to determine whether IL-10-producing B cells are a main mediator of hMSCs in suppressing EAU.

5. Conclusions

In conclusion, our data demonstrate that systemic administration of hMSCs almost completely prevented the development of EAU by suppressing Th1/Th17 immune responses and protected the retina from immune-mediated damage. The results provide a further rationale for the use of hMSCs to treat a variety of autoimmune or immune-mediated diseases involving the eye and other organs that are driven by excessive Th1/Th17 immune responses.

Conflict of Interests

There is no conflict of interests to declare.

Acknowledgments

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Review Article

Immune Recovery Uveitis: Pathogenesis, Clinical Symptoms, and Treatment

Beata Urban,[1] Alina Bakunowicz-Łazarczyk,[1] and Marta Michalczuk[2]

[1] *Department of Pediatric Ophthalmology and Strabismus, Medical University of Białystok, Białystok University Children's Hospital, Waszyngtona Street 17, 15-274 Białystok, Poland*
[2] *Department of Pediatric Ophthalmology and Strabismus, Białystok University Children's Hospital, Waszyngtona Street 17, 15-274 Białystok, Poland*

Correspondence should be addressed to Beata Urban; urbanbea@umb.edu.pl

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IRU is the most common form of immune reconstitution inflammatory syndrome in HIV-infected patients with cytomegalovirus retinitis who are receiving highly active antiretroviral therapy (HAART). Among patients with CMV in the HAART era, immune recovery may be associated with a greater number of inflammatory complications, including macular edema and epiretinal membrane formation. Given the range of ocular manifestations of HIV, routine ocular examinations and screening for visual loss are recommended in patients with CD4 counts <50 cells/μL. With the increasing longevity of these patients due to the use of HAART, treatment of IRU may become an issue in the future. The aim of this paper is to review the current literature concerning immune recovery uveitis. The definition, epidemiology, pathophysiology, clinical findings, complications, diagnosis, and treatment are presented.

1. Introduction

The initial human immunodeficiency virus (HIV) infection is characterized by polyclonal activation of both T-lymphocytes and B-lymphocytes with the release of inflammatory cytokines. Patients exhibit an increase in the production of both CD4+ and CD8+ T-lymphocytes. T-lymphocyte turnover is promoted by the production of interleukin-6, interleukin-1, interleukin-2, and tumor necrosis factor (TNF)-α, all of which promote HIV replication [1]. This cascade further accelerates the destruction of the immune system. Advancing infection is accompanied by further CD4+ T-lymphocyte destruction and worsening of the immune status. As the HIV infection progresses, a loss of CD4+ memory cells accompanied by an inability to activate and afterwards replicate new CD4+ cells is observed. The progressive loss of CD4+ clones puts the patient at increasing risk of opportunistic infections [2, 3].

HIV infection and consequent activation of immune system cells remain the most common cause of ocular damage. The ocular microenvironment is both immunosuppressive and anti-inflammatory in nature. The retina is protected by the blood-retinal barrier (BRB) which is composed of human retinal microvascular endothelial cells (HRMECs) and retinal pigment epithelium (RPE). RPE acts as an important outer barrier to prevent the movement of pathogenic microorganisms (including HIV-1) from the blood into the eye. Ocular complications HIV infection have been demonstrated to be closely related to the breakdown of the blood-retinal barrier (BRB); however, the underlying mechanism is not clear. Recently the role of Tat, the transactivator protein of HIV-1, which plays critical and complex roles in both the HIV-1 replication cycle and the pathogenesis of HIV-1 infection, is the object of research. HIV-1 Tat protein is released from HIV-infected cells and is found circulating in the blood of HIV-1-infected patients [4]. Findings of Chatterjee et al. indicated that the exposure of retinal neurosensory and glial cells to HIV Tat resulted in increased activation and release of proinflammatory mediators, predominantly the chemokine

and neurotoxic factor CXCL10 and the cytokine TNF-α [5]. In addition to pro-inflammatory mediators, they observed that retinal cells also demonstrated increased cell activation as evidenced by augmented expression of GFAP, which is due to the activation of Muller glial cells. Recent study by Che et al. also emphasizes the role of HIV-1 Tat protein in the development of ocular complications during HIV infection [6]. HIV-1 Tat protein induced the apoptosis of human retinal microvascular endothelial cells and retinal pigment epithelium cells. In addition, they found that the activation of N-methyl-D-aspartate receptors (NMDARs) was involved in the apoptosis of RPE cells, but it caused no changes in HRMECs. Furthermore, both cell types exhibited enhanced expression of Bak, Bax, and Cytochrome c. The inhibition of Tat activity protected against the apoptosis induced by NMDAR activation and prevented the dysregulation of Bak, Bax, and Cytochrome c, revealing an important role for the mitochondrial pathway in HIV-1 Tat-induced apoptosis.

Immune reconstitution inflammatory syndrome (IRIS), previously known as immune restoration disease (IRD) or immune reconstitution syndrome (IRS), is characterized by paradoxical worsening of treated opportunistic infection or the unmasking of previously subclinical, untreated infection in patients with HIV after initiation of antiretroviral therapy [7–11]. Some individuals who are infected with HIV rapidly deteriorate shortly after starting antiretroviral therapy, despite effective viral suppression. This reaction, referred to as IRIS, is characterized by tissue-destructive inflammation. The current definition of IRIS includes five fundamental criteria: (1) confirmed case of HIV, (2) temporal association between development of IRIS and initiation of HAART (highly active antiretroviral therapy), (3) specific host responses to HAART, such as decrease in HIV viral load (plasma levels of HIV RNA) and an increase in CD4+ cell count, (4) clinical deterioration characterized by an inflammatory process, and (5) exclusion of other causes that may lead to a similar clinical presentation [7]. There are various manifestations of IRIS. Among the clinical features, frequently reported pathogens associated with IRIS are *Mycobacterium tuberculosis*, atypical mycobacterium, cytomegalovirus, varicella zoster virus, and *Cryptococcus neoformans*. However, there are some less common pathogens including *Pneumocystis jirovecii* pneumonia, toxoplasmosis, hepatitis B and C virus, MC and genital warts, sinusitis, and AIDS-related lymphoma [7, 10]. It has been proposed that IRIS is caused by a dysregulation of the expanding population of CD4+ T cells specific for a coinfecting opportunistic pathogen [10]. The interval between the initiation of HAART and the beginning of IRIS is highly variable (from 1 week to more than 1 year), but, in the majority of the cases, it occurs during the first two months of HAART [8]. Increased IL-8, Th1, and Th17 cytokine levels in IRIS patients precede ART initiation and could help identify patient populations at higher risk for IRIS [11]. Ocular IRIS is referred to as immune recovery uveitis (IRU). It remains a leading cause of ocular morbidity.

2. Etiology of IRU

With initial HIV infection T-lymphocyte activation and the production of proinflammatory lymphokines (e.g., IL-2, TNF-α, and IL-6) can be observed. T-lymphocytes, particularly the CD4+ fraction, are a primary target for HIV infection. They are destroyed as the HIV infection progresses [2]. As HIV disease continues and the number of CD4+ T-lymphocytes continues to decrease, the patient is at higher risk for the development of opportunistic infections, such as CMV retinitis. With the occurrence of acquired immunodeficiency syndrome (AIDS) number of cytomegalovirus retinitides (CMV retinitis) significantly increased [2, 12]. CMV retinitis is a complication of late-stage human immunodeficiency virus (HIV) infection and is associated with CD4+ T cell counts less than 50/μL [13, 14]. CMV retinitis is the result of hematogenous spread of the virus to the retina through retinal blood vessels after systemic reactivation of a latent CMV infection. Patients with AIDS and CMV retinitis typically have minimal or no clinical vitritis or anterior chamber inflammation due to the underlying immunosuppressed state [15]. Even though anterior chamber is clinically quiet, a cytokine-mediated inflammatory response pattern is actively present in the aqueous humor. Cytokine analysis of aqueous humor in HIV patients with CMV retinitis revealed raised levels of IP-10, fractalkine, PDGF-AA, G-CSF, Flt-3L, and MCP-1 [16]. Higher median levels of these cytokines suggest a unique immunologic signature consistent with a combined Th-1 and monocyte macrophage-mediated response. The infection may initially be asymptomatic, but the retinal necrosis it produces may result in decreased visual acuity. A perivascular yellow-white retinal lesion frequently associated with retinal hemorrhage or a focal white granular infiltrate, often without hemorrhage, can be observed. Both lesions enlarge in a progressively expanding "brushfire" pattern [17].

In the era before highly active antiretroviral therapy, patients with CMV retinitis had minimal intraocular inflammation, and macular edema was rarely reported, but they required chronic suppressive anticytomegalovirus therapy to prevent relapse of the disease. If untreated, CMV retinitis spread throughout the entire retina, causing total retinal destruction and blindness. Highly active antiretroviral therapy (HAART) was introduced in 1996 to treat HIV-infected patients. It consists of combination of antiretroviral therapies. HAART includes one or two reverse transcriptase inhibitors and one or two protease inhibitors, recently expanded by an integrase inhibitor or an entry inhibitor (Table 1).

The most commonly used combination consists of one protease inhibitor and two reverse transcriptase inhibitors. This treatment leads to decreased plasma levels of HIV mRNA and increased CD4+ T-lymphocyte counts, resulting in increased patient survival and a decrease in the incidence of three major opportunistic infections: *Pneumocystis carinii* pneumonia, *Mycobacterium avium* complex disease, and cytomegalovirus retinitis [18, 19]. The first phase of immune recovery after initiation of HAART is characterized by a redistribution of both naïve and memory CD4+ T cells from lymphatic tissues, whereas the subsequent gradual CD4+ T cell recovery over time is primarily naïve CD4+ cells [20].

Table 1: Kinds of antiretroviral drugs used in HAART therapy.

Reverse transcriptase inhibitors (RTIs)
(1) Nucleoside and nucleotide analog reverse transcriptase inhibitors (NRTIs)
Abacavir (Ziagen)
Abacavir + lamivudine + zidovudine (Trizivir)
Abacavir + lamivudine (Epzicom/Kivexa)
Didanosine (Videx, Videx EC)
Emtricitabine (Emtriva)
Lamivudine (Epivir)
Lamivudine + zidovudine (Combivir)
Stavudine (Zerit)
Tenofovir (Viread)
Zalcitabine (Hivid)
Zidovudine (Retrovir)
(2) Nonnucleoside reverse transcriptase inhibitors (NNRTIs)
Delavirdine (Rescriptor)
Efavirenz (Sustiva, Stocrin, Efavir)
Efavirenz + tenofovir + emtricitabine (Atripla)
Etravirine (Intelence)
Nevirapine (Viramune)
Rilpivirine (Edurant)
Rilpivirine + tenofovir + emtricitabine (Complera)
Protease inhibitors (PIs)
Amprenavir (Agenerase)
Atazanavir (Reyataz)
Darunavir (Prezista)
Fosamprenavir (Lexiva, Telzir)
Indinavir (Crixivan)
Lopinavir (Kaletra)
Nelfinavir (Viracept)
Ritonavir (Norvir)
Saquinavir (Fortovase, Invirase)
Tipranavir (Aptivus)
Integrase inhibitors
Raltegravir (Isentress)
Entry inhibitors
Maraviroc (Selzentry)
Enfuvirtide (Fuzeon)

With HAART therapy the incidence of CMV retinitis has decreased by 80% to 90%, but it has not dropped to zero [21]. Before the availability of HAART, a diagnosis of CMV retinitis required anti-CMV therapy, which was associated with severe morbidity and was very expensive; the annual cost of oral ganciclovir for one patient was 17,000 $ in 1998 [18]. A response to HAART is defined as an increase in CD4+ T cell count of at least 50 cells/μL to a level of 100 cells/μL or more. Unfortunately, HAART fails in up to 50% of AIDS patients due to noncompliance, side effects of the drugs, adverse drug interactions, or HIV resistance.

3. Definition of IRU

In 1998 Karavellas et al. and Zegans et al. have described a new intraocular inflammatory syndrome, which develops in patients with AIDS and inactive CMV retinitis, who have experienced HAART-mediated increases in CD4+ T-lymphocyte levels [15, 22]. This syndrome was initially called immune recovery vitritis because the report by Zegans et al. describes transient vitritis as the principal manifestation. Karavellas et al. defined IRU as vitritis of 1+ or greater severity with visually important floaters, a decrease in vision of 1 or more lines, or both, with or without associated papillitis and macular changes [12].

Currently, there have been no definite criteria of IRU [23]. It is generally recognized by new or increased noninfectious intraocular inflammatory reaction in patients with AIDS and cytomegalovirus retinitis several weeks after starting HAART. The inflammation is associated with an increase in the CD4+ T-lymphocyte counts of at least 50 cells/mm^3 to the level of 100 cells/mm^3. Time from beginning of HAART till an increase in CD4+ count is approximately 2 months [22]. IRU is predominantly a posterior segment inflammatory disorder that results in decreased vision and floaters in the affected eye [22]. It is currently one of the most common causes of new vision loss in patients with AIDS-related CMV retinitis [24].

The current definition of IRU (similarly IRIS) includes at least five main criteria: (1) being a patient with AIDS, (2) receiving HAART, (3) achieving an immune reconstitution indicated by increased CD4+ T cell count over 100 cells/mm^3 for at least two months, (4) having preexisting CMV retinitis which is currently in the inactive state, and (5) developing an intraocular inflammation that cannot be explained by drug toxicity or a new opportunistic infection.

The severity of inflammation is related to various factors such as extent of CMV retinitis, amount of intraocular CMV antigen, degree of immune constitution, and previous treatment [19]. IRU usually develops in patients with inactive CMV retinitis but it rarely can occur in eyes with active CMV retinitis, especially at the onset of inflammation [25]. IRU usually affects all eyes with CMV retinitis, but sometimes patients had IRU in only one of the two eyes with CMV retinitis (26.3% of those with bilateral retinitis in the study of Kempen et al.) [26].

4. Pathogenesis of IRU

Although the pathogenesis of IRU is not entirely certain, it appears to represent an inflammatory reaction to either cytomegalovirus antigen in the eye or low or subclinical levels of cytomegalovirus replication and this inflammatory reaction occurs as the immune system recovers competency [13, 15, 27].

Since IRU in non-CMV retinitis eyes is not common, the ocular inflammation is postulated to be due to the CMV infection itself, which causes breakdown in the blood ocular barrier. This may allow CMV antigens to leak out of the eye and give the antigen access to lymphoid organs and stimulate an antigen-specific immune response [19, 28].

Immunohistological examination of epiretinal membrane associated with IRU showed evidence of chronic inflammation with predominant T-lymphocytes. This data, in conjunction with the finding of a positive correlation between IRU and surface area of inactive CMV retinitis, would suggest that IRU may be due to T cell-mediated reaction to CMV antigen present in inactive CMV retinitis [29].

According to Nussenblatt and Lane, as immune function after HAART improves, a threshold is reached at which the body can mount an intraocular inflammatory response to cytomegalovirus antigens present in the eye [2]. With continued recovery of immune function, a higher threshold is reached at which the immune system inactivates cytomegalovirus, production of antigen stops, and inflammatory reactions subside.

Speculation on the pathophysiology of IRU includes the fact that the intraocular inflammation is a reaction to antigenically altered retinal or glial cells adjacent to the healed CMV lesion or secondary to chronic subclinical viral replication along the border of healed CMV [30].

Although the pathologic immune reaction in IRU occurs in the eye, some kind of immune dysregulation that allows for the development of pathologic response is likely caused by faulty systemic immune cell reconstitution [31]. IRU, just like IRS, could be a result of unbalanced reconstitution of effector and regulatory T cells, leading to exuberant inflammatory response in patients receiving HART. Biomarkers, including interferon-γ (INF-γ); tumour necrosis factor-α (TNF-α); C-reactive protein (CRP); and interleukin- (IL-) 2, -6, and -7, are subject of intense investigation at present [32].

Schrier et al. examined aqueous and vitreous fluids from patients with IRU and active CMV retinitis for the presence of cytokines, using enzyme-linked immunosorbent assay techniques, and CMV DNA by polymerase chain reaction [28]. They observed that IRU eyes had the highest levels of IL-12 (median 48 pg/mL), moderate levels of IL-6 (median 146 pg/mL), and low interferon gamma (median 15 pg/mL) compared to control. Additionally all uveitis eyes were CMV DNA negative; in contrast eyes with active CMV retinitis were CMV DNA positive. They concluded that inflammatory IRU can be differentiated from active CMV retinitis by the presence of IL-12 and less IL-6 and absence of detectable CMR replication.

Hartigan-O'Connor et al. in a multicenter observational study studied T_{reg} cell control over T cell responses in peripheral blood mononuclear cells from 25 patients with CMV retinitis and IRU and 49 immunorestored by HAART control subjects with CMV retinitis who did not develop IRU [31]. They observed weak antiviral CD4+ T cell responses in patients with IRU, as compared with control subjects, whereas CD8+ T cell responses were comparable. They also found that patients with IRU were characterized by a smaller number of Th17 cells (identified by measuring IL-17 production) than control subjects. They speculate that lower numbers of Th17 cells among patients with IRU may reflect greater losses throughout the course of HIV disease and a greater level of immune dysfunction. In their opinion CD4 cell count and Th17 cell number may both be measures of the severity of HIV disease before the initiation of HAART.

Schrier et al. after examining aqueous and vitreous fluids from patients with IRU and active CMV retinitis observed that IRU can be differentiated from active CMV retinitis by the presence of IL-12 and less IL-6 and absence of detectable CMV replication [28]. Increased levels of proinflammatory cytokines have also been documented in tissues of patients who have recovered from CMV retinitis. The upsurge of macular and disc edema seems associated with the production of interleukin-4 and tumor necrosis factor alpha, whereas vitritis is associated with the production of interleukin-2 and interferon gamma [33].

A report of Modorati et al. suggested that all patients presenting with the clinical and ophthalmological characteristics of IRU showed the presence of HLA B 8–18 [34].

5. Occurrence of IRU

The median time from HAART initiation to develop IRU has varied from 20 to 43 weeks [4]. The median time to develop IRU in a study by Karavellas et al. was 45 weeks [27]. Hartigan-O'Connor et al. observed that median interval between diagnosis of CMV retinitis and IRU diagnosis was 47.5 months (range from 3 to 128 months) [31]. The study by Sudharshan et al. noted that interval between the start of HAART and onset of IRU was from 4 months to 2.5 years [35].

Kempen et al. evaluated the prevalence of immune recovery uveitis (IRU) in eyes of 374 patients with AIDS and CMV retinitis [26]. 36 patients (9.6%) were diagnosed with IRU in this 19-clinical-center cohort study. In the study by Karavellas et al. the prevalence of IRU varied from 38% to 63% in patients with CMV retinitis [27]. In India CMV retinitis still remains the commonest ocular manifestation in AIDS cases. In the study by Sudharshan et al. who examined 1000 HIV patients, the incidence of CMV retinitis remains high (36.2%) even in the era of HAART [35].

The occurrence of IRU appears to vary between studies, and the reason for this variability is unclear [13, 26, 27, 35–45]. Prevalence of IRU is presented in Table 2.

The degree of immune recovery may explain some of this variability [19]. Immediately after the introduction of HAART, the incidence of IRU based on large single-center cohort studies differs substantially, ranging from 0.11 per person-year (PY) to 0.83/PY [13, 27, 37]. One of the reasons of this disparity can be the role of intravitreal cidofovir, which is a major risk factor for IRU, and it was used in the treatment for CMV retinitis in the older studies [27]. The next reason is the time of starting HAART therapy in a patient with active CMV retinitis. Ortega-Larrocea et al. noted that early introduction of HAART in patient with CMV retinitis before completing induction therapy for CMV results in a higher incidence of IRU (71%) than among those who had suppressed CMV retinitis before starting HAART (31%) [38]. This data suggests that all patients with CMV retinitis should be treated for CMV and HAART therapy should be delayed until treatment of CMV retinitis is completed. The lower incidence of IRU in some studies might be related to more aggressive anticytomegalovirus therapy before and

Table 2: Occurrence of IRU in patients starting HAART for HIV infection.

Authors of the study	Country	Year of publication	Patients with IRU
Karavellas et al. [27]	USA	1999	63.3%
Nguyen et al. [13]	USA	2000	18.2%
Banker and Patel [36]	India	2002	41.7%
Arevalo et al. [37]	Wenezuela	2003	37.5%
Ortega-Larrocea et al. [38]	Mexico	2005	53.5%
Kempen et al. [26]	USA	2006	9.6%
Uemura et al. [39]	Japan	2006	30%
Dujić and Jevtović [40]	Serbia	2007	42.9%
Lin et al. [41]	Taiwan	2008	24.4%
Gharai et al. [42]	India	2008	5%
Shah et al. [43]	India	2009	3%
Hamamotoo et al. [44]	Japan	2012	1.5%
Sudharshan et al. [35]	India	2013	17.4%
Agarwal et al. [45]	India	2014	33.33%

immediately after initiation of potent antiretroviral therapy, thereby minimizing exposure to CMV antigens during a critical phase of immune recovery in the eye [30]. Jabs et al. tried to describe in the prospective, multicenter observational study the five-year outcomes of patients with CMV retinitis and AIDS in the era of HAART [21]. They observed that the rate of IRU was 1.7/100 PY and varied from 1.3/100 PY for those with previously diagnosed retinitis and immune recovery at enrollment to 3.6/100 PY for those with newly diagnosed retinitis who subsequently experienced immune recovery. Despite the availability of HAART, patients with AIDS and CMV retinitis are at increased risk for mortality, retinitis progression, complications of the retinitis, and visual loss over a 5-year period.

The other explanation for variability of IRU occurrence may be some genetic or environmental differences, which might influence the susceptibility to CMV retinitis and then IRU. It is not yet possible to identify at-risk patients on the basis of laboratory tests of immune function.

6. Signs and Symptoms of IRU

Immune recovery uveitis may dramatically change the clinical situation in some patients. Many investigators observed that, instead of CMV necrotizing retinitis, inflammatory involvement can lead to intraocular disorders. The clinical picture of IRU is still evolving. The severity of the inflammation depends on the degree of immune reconstitution, extent of CMV retinitis, amount of intraocular CMV antigen, and previous treatment.

Symptoms typically include floaters and/or vision loss, the latter usually of moderate degree, with visual acuities worse than 20/40 but better than 20/200 [12, 14, 19, 26, 46].

IRU manifests symptomatically with decreased vision and/or floaters. Within weeks after starting HAART and rising CD4+ T-lymphocyte count, an exudate in the anterior chamber and vitreous haze appears. Because of its transient nature, this stage can be missed by the clinician. The inflammatory reactions may improve, but in some patients the uveitis will develop and may be complicated by, for example, papillitis and macular changes [3, 13].

6.1. Mild to Severe Vitritis. Canzano et al. observed that, after resolution of vitritis, vitreomacular traction syndrome (VMT) may be developed [47]. They speculated that changes in immune status may permit an inflammatory response that can lead to VMT. Henderson and Mitchell reviewed charts of 80 patients with inactive CMV retinitis, who received HAART treatment [14]. In most of these patients a mild transient vitritis was observed, which did not require treatment. IRV developed in only 7 patients significantly enough (based on deteriorating visual acuity) to require therapy. The nine eyes involved with significant IRV had a mean visual acuity loss of 2.8 Snellen lines.

6.2. Cystoid Macular Edema (CME). CME is a complication that can result from this inflammation and is emerging as a major cause of visual loss in human immunodeficiency virus-(HIV-) infected patients. The main symptoms are decreased vision, metamorphopsia, and floaters [48, 49]. In the study by Kempen et al. eyes with IRU had a 20-fold higher risk of CME [26].

6.3. Epiretinal Membrane Formation. Kempen et al. observed that eyes with IRU had a 5- to 6-fold higher risk of epiretinal membrane than eyes without IRU [26]. Immunohistological examination of epiretinal membrane associated with IRU showed evidence of chronic inflammation with predominant T-lymphocytes [46].

6.4. Frosted Branch Angiitis. Frosted branch angiitis is essentially a severe form of vasculitis which affects the entire retina. It is commonly associated with cytomegalovirus

infection and the administration of anticytomegalovirus therapy without the need for corticosteroids [50]. In patients with IRU, frosted branch angiitis can occur in the eye with active CMV retinitis and can be unilateral or bilateral. Leeamornsiri et al. described a 40-year-old woman with AIDS and CMV retinitis, who was treated with intravitreal injection of 2 mg/0.04 mL ganciclovir, and retinitis had improved [23]. One week after HAART initiation, while cytomegalovirus was not completely resolved, extensive frosted branch angiitis was noted. 25 mg/day oral prednisolone was given with continuation of HAART and intravitreal ganciclovir and such treatment leads to significant improvement of perivascular infiltration within one week and this case has reported the earliest onset of IRU after HAART initiation. Recently Alp et al. described frosted branch angiitis associated with HAART in patient with immune recovery uveitis despite a low CD4+ T cell count (20 cells/mm^3) [51].

6.5. Papillitis. See [12, 17, 27].

6.6. Neovascularization of the Retina or Optic Disc [13, 52, 53]. Wright et al. reported extensive peripheral retinal neovascularization as a late finding of IRU in 3 HIV-infected patients with inactive CMV retinitis, 1 of whom developed recurrent vitreous hemorrhage that required vitrectomy [53]. The pathogenesis of fibrovascular membrane formation in patients with IRU may be hard to elucidate, because it is relatively uncommon and because surgically obtaining tissue specimens is not justified if the membrane follows a benign clinical course. Specific therapy for these fibrovascular membranes is not required unless they lead to recurrent vitreous hemorrhages and vision loss.

6.7. Proliferative Vitreoretinopathy with Retinal Detachment. In the study by Karavellas et al. 29 eyes of 21 patients with IRU and inactive CMV retinitis were followed up for median of 43 weeks after diagnosis of IRU [12]. Four eyes developed clinically important posterior segment complications. Two of these eyes had extensive proliferative vitreoretinopathy which developed within 2 to 3 days after rhegmatogenous retinal detachment. Immunostaining of proliferative vitreoretinopathy membranes from eyes with IRU revealed numerous lymphocytes, the majority of which were positive for T-lymphocytes cell markers, indicating that epiretinal proliferation in these eyes is the result of an inflammatory process in which T-lymphocytes play a role. All the eyes with this complication had a poor final visual outcome. One eye developed a vitreous hemorrhage from avulsion of a blood vessel secondary to contraction of the inflamed vitreous and partial posterior vitreous detachment. Additionally one eye developed extensive epiretinal and subretinal proliferation.

6.8. Anterior Segment Inflammation, Iris Synechiae, and Cataract. From the experience of Holland, patients with IRU-associated cataracts are particularly prone to postoperative problems such as posterior synechiae, papillary membranes, and inflammatory deposits on the lens implants [3]. In the study by Karavellas et al. anterior segment complications developed in seven eyes of twenty-nine eyes with IRU. These complications included progressive posterior subcapsular cataracts, anterior subcapsular cataract, and persistent postoperative anterior chamber inflammation with development of posterior synechiae and large visually important inflammatory deposits on the surface of the intraocular lens [12]. The authors assumed that subcapsular opacification of the lens in some eyes with IRU is a multifactorial process, involving such factors as corticosteroid therapy and previous surgery. It is also possible that patients with IRU may develop more severe and/or prolonged inflammation after intraocular surgery.

6.9. Panuveitis with Hypopyon. See [25, 54].

6.10. Macular Hole. See [37].

6.11. Cytomegalovirus Immune Recovery Retinitis (CMV-IRR). Recently, Ruiz-Cruz et al. reviewed charts of 75 patients with CMV retinitis on HAART initiation or during the 6 subsequent months [55]. 20 patients had improvement of CMV retinitis. The remaining 55 patients experienced CMV-IRR; 35 of those developed CMV-IRR after HAART initiation (unmasking CMV-IRR); and 20 experienced paradoxical clinical worsening of retinitis (paradoxical CMV-IRR). Nineteen patients with CMV-IRR had ≥50 CD4 T cells/mm^3. Six patients with CMV-IRR subsequently developed immune recovery uveitis (IRU). The authors propose definition for CMV-IRR as the condition which is likely to occur after successful initiation of HAART, even in patients with high CD4 T cell counts.

7. Risk Factors

The first well-known risk factor for IRU is immune recovery with a rapid rising in the number of CD4+ T-lymphocytes as a consequence of HAART [23]. The risk of IRU increases manyfold with increasing CD4+ T cell count to a level of ≥100 cells per microliter or decreasing HIV load [3, 22, 24, 26, 32].

Song et al. observed that the use of intravenous cidofovir is a primary risk factor in the subsequent development of immune recovery uveitis [56]. They assume that ongoing treatment of healed CMV retinitis after immune recovery does not appear to protect against the development of immune recovery uveitis. Kempen et al. reported that the use of intravitreous injections of cidofovir was associated with a 19-fold higher risk of IRU [26]. Similar observations were made by Kempen et al. [26].

Another risk factor for IRU includes surface area of retinal involvement due to CMV retinitis [26]. Karavellas et al. suggested that a higher antigen load in larger lesions would increase the likelihood that IRU would become clinically manifest [46]. In their study patients with >30% of retinal area affected had 4.5-fold higher risk of developing IRU when compared with eyes with a retinal CMV area of <18%. Effects of lesion size on the extent of blood-retinal barrier breakdown are also a potential explanation of this association.

In contrast, Arevalo et al. observed that eyes with IRU had a mean CMV surface area of 31.7% and eyes without IRU (control group) had a mean CMV surface area of 35%, so in their opinion CMV surface area does not seem to be a risk factor for the development of IRU [37]. Initiation of HAART should be delayed until after the induction phase of anti-CMV therapy, as the reduction of antigen load with anti-CMV agents may reduce potential risk of IRU [3].

On the other hand, the presence of a posterior pole lesion and male gender were found to be associated with reduced IRU risk [26].

Some other unidentified factors may influence susceptibility and severity of IRU, so further research to identify such factors is desired.

8. Treatment of IRU

The treatment of IRU depends on the location of the intraocular inflammation, the severity of the inflammation, and the presence of ocular complications, particularly CME.

8.1. Pharmacological Treatment. Inflammation in the anterior chamber is treated with topical corticosteroids in frequencies typical of treating other forms of anterior uveitis [14]. If IRU is an isolated mild vitritis without CME, these eyes may be observed, as the vitreous inflammation can be transient. Immune recovery uveitis with more severe vitreous inflammation and/or CME typically is treated with periocular corticosteroids (triamcinolone acetonide 40 mg), or short courses of oral corticosteroids, without recurrence of the CMV retinitis [46]. The main advantage of periocular corticosteroids is the production of therapeutic local drug levels to avoid the potential problems of systemic corticosteroids in these immunosuppressed patients [3, 21, 24].

Intravitreal corticosteroids have successfully treated eyes with IRU, refractory to less aggressive treatment; however, in addition to the usual complications of cataracts and glaucoma, reactivation of retinitis may occur [57]. To prevent CMV reactivation following corticosteroid treatment, some authors recommend restarting anti-CMV therapy [3]. Anti-CMV therapy is important during immune recovery because it has been proved to be protective against the development of IRU by reducing the amount of CMV antigens in the retina, although it has not shown a favorable cost-effectiveness ratio where there are no signs of CMV retinitis [3]. Kuppermann and Holland suggested that continued, aggressive anticytomegalovirus therapy for a prolonged time after initiation of potent antiretroviral therapy may reduce the rate or severity of IRU [30]. There are many anti-CMV drugs, which are available in intravenous, oral, and intravitreous therapy [13, 15, 19, 25, 26, 51]. Ganciclovir is the first anti-MCV drug, available since 1984. To achieve high tissue concentrations during induction, ganciclovir is administered intravenously (Cytovene) twice daily at a dose of 5 mg/kg. Foscarnet (Foscavir) is generally considered a second line intravenous therapy that is often administered to patients with ganciclovir-resistant viral strains or dose-limiting neutropenia. Cidofovir (Vistide), the third intravenous drug, because of its association with immune recovery uveitis, should not be used if immune recovery is expected. Oral ganciclovir was introduced in 1994 in attempt to lower costs, to eliminate the inconvenience of daily intravenous drug injections, and to improve patient quality of life. The primary indication for oral ganciclovir was prevention of contralateral retinitis and nonocular CMV disease in patients receiving intraocular therapy [30]. When oral valganciclovir (Valcyte) with its high bioavailability and convenient once-daily dosing was introduced, production of oral ganciclovir was discontinued. Intravitreal ganciclovir injections are given to patients who are intolerant of or refused systemic therapy. After cidofovir intravitreal injections uveitis occurred frequently, so this kind of administration is no longer recommended. The most popular in the industrialized world is intraocular implant of ganciclovir (Vitrasert), which produces intraocular levels of ganciclovir five times that of systemically administered ganciclovir and allows for avoidance of systemic toxicity [58].

Figueiredo et al. described IRU masked as endogenous endophthalmitis and hypopyon, treated with oral valganciclovir and topical dexamethasone [25]. The authors assume that maintenance treatment should be continued until immune recovery is achieved, because none of the anti-CMV drugs available eradicate ocular and systemic CMV antigens in the immunocompromised patient.

Vision loss in patients with IRU is usually caused by macular pathology, primarily cystoid macular edema. Several treatment options for patients with IRU and macular edema have been proposed. Physicians previously suggested the use of oral corticosteroids [17, 24]. However, Karavellas et al. reported the use of repository sub-Tenon steroid injections for the treatment of macular complications in IRU and they found only a modest effect [46]. Similarly, in the study by Nguyen et al. 4 eyes were reported to have IRU associated with CME, and CME improved in 2 of them (50%) [13]. In the other two patients, the CME persisted despite aggressive therapy with topical, periocular, and systemic corticosteroids. These results are also in agreement with other clinical studies [14, 37]. In the study of Kosobucki et al. 5 patients with chronic macular edema as a result of IRU were examined [29]. Fluorescein angiography, visual acuity, and CMV lymphoproliferative T cell function assays were obtained after receiving valganciclovir 900 mg daily for three months and again three months after withdrawal of therapy. Their vision improved by a mean of 11 letters, angiograms showed reduction of macular edema, and hematologic and CD4 count data remained stable. The authors assume that the lack of significant decrease in CMV lymphoproliferative response suggests that if valganciclovir is suppressing residual CMV replication, it is not reducing the cellular immune response to CMV. Morrison et al. used intravitreal injection of 20 mg decanted triamcinolone acetate (IVTA) for the treatment of macular edema secondary to IRU and visual acuity improved in all patients [59]. In total, 8 eyes of 7 patients received 13 injections. Visual acuity and OCT retinal volume and thickness improved in all patients, but longer follow-up is needed to assess the durability of the effect and to monitor for longer-term complications (the risk of the formation of cataract, glaucoma, and endophthalmitis). The authors

observed no cases of cytomegalovirus reactivation during a minimum follow-up of 9 months. This kind of therapy allows for avoidance of the side effects of systemic oral corticosteroid treatment. Mild inflammation with macular edema can sometimes be treated effectively with topical and periocular corticosteroids, but other eyes are refractory to treatment [14, 46, 57]. El-Bradey et al. after examining long-term results of treatment of macular complications in eyes with IRU noted that mild cases of immune recovery uveitis and macular edema may be observed [57]. They observed that, in the eyes with reduction of vision due to cystoids macular edema, there was only a modest treatment effect using repository corticosteroids. These patients with more severe inflammatory changes with VA of 20/30 or worse due predominantly to CME were treated with a series of posterior sub-Tenon injections of repository corticosteroids. In this study, repository corticosteroids appeared to improve vitritis with decline in inflammatory cells in 60% of treated eyes, but this treatment had a lesser effect on the visual acuity, which improved in only 40% of the treated eyes. In addition, macular edema was resistant to corticosteroid injections. Henderson and Mitchell reported successful treatment of nine eyes of seven patients of immune recovery vitritis with orbital floor injections of methylprednisolone acetate 40 mg or triamcinolone 20 mg [14]. Four of these nine eyes had CME, which showed improvement or disappearance after this treatment, and no complications were reported.

Recent studies have demonstrated that intravitreal immunosuppressant injections of methotrexate or anti-VEGF agents may not only lead to fewer intraocular side effects but also have a lower therapeutic activity for the reduction of macular edema in uveitis, because blockage of VEGF has not been shown to have an anti-inflammatory effect [60].

Recently, fluocinolone acetonide (Retisert) was used to treat cystoid macular edema resulting from IRU [61]. Improvement was observed in two of three eyes and no CMV reactivation was detected during the several-month follow-up period.

The most recent study by Krishnan and Chatterjee showed that endocannabinoids (N-arachidonoylethanolamide and 2-arachidonoylglycerol) could be used to alleviate Tat-induced cytotoxicity during HIV infection and rescue retinal cells [62]. The neuroprotective mechanism involved suppression in production of proinflammatory and increase in anti-inflammatory cytokines, mainly through the MAPK pathway. Both endocannabinoids regulated cytokine production by affecting at the transcriptional level the NF-κB complex, including IRAK1BP1 and TAB2. These findings have direct relevance in immune recovery uveitis where antiretroviral therapy has helped immune reconstitution. In their opinion endocannabinoids and their agonists may be thought of as neurotherapeutic during certain conditions of HIV-1-induced inflammation. Also recent findings of Che et al. related to the role of HIV-1 Tat protein in breaking the blood-retinal barrier suggest that the inhibition of HIV-1 Tat activity could be essential in the future therapy of CME secondary to IRU [6].

8.2. Surgical Treatment of IRU. Treatment with corticosteroids (subtenon or systemic or intravitreal) is effective in controlling inflammation and improving vision in some cases. However, surgery may be required in patients with vitreomacular traction syndrome, epiretinal membrane formation, cataract, and proliferative vitreoretinopathy. El-Bradey et al. observed that, in eyes with structural macular changes secondary to IRU, such as dense epiretinal membrane (ERM), trans- pars plana vitrectomy with peeling of ERM resulted in vision improvement in three of four eyes, but the cystoid macular edema persisted despite surgery [57]. The effect of vitrectomy on inflammatory cystoid macular edema is not yet clear and might become more important in the future [63].

9. Summary

Among patients with CMV in the HAART era, immune recovery may be associated with a greater number of inflammatory complications, including macular edema and epiretinal membrane formation. Given the range of ocular manifestations of HIV, routine ocular examinations and screening for visual loss are recommended in patients with CD4 counts <50 cells/μL. As studies on HIV disease after the introduction of HAART continue to become available, more thorough descriptions of treated patients with ocular opportunistic infections will include side effects and toxicities on therapy. As increasing number of HIV-infected individuals present with treatment failure in developing countries, the risk of ophthalmic complications may increase. With the increasing longevity of these patients due to the use of HAART, treatment of IRU may become an issue in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publishing of this paper.

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Research Article

Decreased Expression of the Aryl Hydrocarbon Receptor in Ocular Behcet's Disease

Chaokui Wang,[1] Zi Ye,[1] Aize Kijlstra,[2] Yan Zhou,[1] and Peizeng Yang[1]

[1] *The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Lab of Ophthalmology, Chongqing Eye Institute, Youyi Road 1, Chongqing 400016, China*
[2] *University Eye Clinic Maastricht, Maastricht, The Netherlands*

Correspondence should be addressed to Peizeng Yang; peizengycmu@126.com

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Recent studies show that the aryl hydrocarbon receptor (AhR) is involved in immune responses. AhR is activated following interaction with its ligands, such as 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE). In this study, we investigated the role of AhR activation by its endogenous ligands in the pathogenesis of ocular Behcet's disease (BD). The expression of AhR was significantly decreased in active BD patients as compared to inactive BD patients and normal controls. Both FICZ and ITE inhibited Th1 and Th17 polarization and induced the expression of IL-22 by PBMCs and by $CD4^+$T cells in active BD patients and normal controls. Stimulation of purified $CD4^+$T cells with FICZ or ITE caused a decreased expression of RORC, IL-17, IL-23R, and CCR6 and an increased phosphorylation of STAT3 and STAT5. The present study suggests that a decreased AhR expression is associated with disease activity in BD patients. The activation of AhR by either FICZ or ITE was able to inhibit Th1 and Th17 cell polarization. Further studies are needed to investigate whether modulation of AhR might be used in the treatment of BD.

1. Introduction

Behcet's disease (BD) is a chronic systemic inflammatory disease affecting the eye, skin, oral mucosa, gastrointestinal tract, and central nervous system [1]. It is a relatively common uveitis entity in China and the clinical ocular features have been described extensively elsewhere [2]. Although the pathogenesis of BD is still not completely understood, it is currently thought that environmental factors may trigger the development and recurrence of this disease in a genetically susceptible host [3]. It is classified as an example of an autoinflammatory disorder with marked involvement of both Th1 and Th17 lymphocyte subsets [4–7]. Consistently, strategies aimed at suppressing the abnormal Th1 and Th17 cell response have been reported as a therapeutic approach in BD patients, which is supported by findings in experimental autoimmune uveitis (EAU) models in mice [8, 9].

The Aryl hydrocarbon receptor (AhR) is ubiquitously expressed in vertebrate cells and is well known to mediate toxic effects of several environmental pollutants, including polycyclic- and halogenated aromatic hydrocarbons such as benzo(a)pyrene (B(a)P) and 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) [10]. Other ligands include natural dietary substances, heme metabolites, and tryptophan photoproducts [11, 12]. AhR is a ubiquitous transcription factor present in the cytoplasm, which, after binding to its ligands, translocates into the nucleus where it attaches to its dimerization partner AhR nuclear translocator. The AhR/AhR nuclear translocator complex initiates transcription of a variety of genes with promoters containing a so-called dioxin-responsive element consensus sequence, which ultimately results in a variety of toxic or biochemical responses [10]. In addition to its role in mediating toxic responses, the AhR pathway has many other physiological roles. Mouse strains deficient in the AhR protein showed defects in neuronal development and photoreceptor development, decreased animal weights, fatty metamorphosis and portal tract fibrosis in the liver, and poor fecundity [13, 14]. It has also been reported that AhR plays an important role in vascular development. The most overt phenotype of the AHR knockout mouse is

a markedly reduced liver size, which is owing to defects in the resolution of fetal vascular structure. AhR-null mice also showed abnormalities in the vascular architectures of the kidney, liver sinusoids, and eye, including persistence of the embryonic hyaloid artery. In adults, the knockout of AhR is linked to cardiac hypertrophy, hypertension, and elevated levels of the potent vasoconstrictors endothelin-1 and angiotensin II [14]. Taken together, AhR plays a critical role in the normal physiological function.

More recent studies have shown that the AhR also plays a critical role in the immune response. AhR-deficient mice, for instance, develop a more severe form of experimental autoimmune encephalomyelitis (EAE) as compared to wild-type mice [15, 16]. Studies in mice also showed that AhR activation can alter the differentiation of Treg cells and Th17 cells in a ligand-specific manner [15]. AhR activation by TCDD was able to suppress EAU and EAE by inducing $CD4^+CD25^+$Foxp3 Treg cell differentiation. On the other hand, AHR activation by the ligand 6-formylindolo[3,2-b]carbazole (FICZ) aggravated the severity of EAE by inhibiting the development of Treg cells and promoting the differentiation of Th17 cells [15]. Although TCDD may block the autoimmune response, it also causes various toxic responses, including cellular damage and carcinogenesis [17]. Treating EAE mice with another AHR ligand, 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), which is an endogenous nontoxic tryptophan-derived AhR ligand, resulted in a significant reduction of the inflammatory response, increased $FoxP3^+$Treg cells, and resulted in an enhanced production of tolerogenic dendritic cells [18]. In the animal models of collagen-induced arthritis (CIA), it was shown that AhR deficiency ameliorated the severity of CIA by inhibiting the proinflammatory cytokines as IL-1β and IL-6 as well as Th17 cell response [19]. In human studies, AhR activation by FICZ or TCDD in $CD4^+$T cells was shown to inhibit the production of IL-17A while simultaneously promoting the production of the immunoprotective cytokine IL-22 [20, 21]. Taken together these data indicate that, depending on the cell type analyzed and the AhR ligands used, AhR activation can differentially modulate the Th cell response and act as initiator or attenuator of T cell-driven autoinflammatory responses.

Given the deregulated Th1 and Th17 cell response in BD patients, we investigated whether AhR signaling could be exploited to inhibit the development of T cell responses in BD patients. Here we found that the gene expression of AhR was decreased in active BD patients. AhR activation by either FICZ or ITE inhibited the Th1 and Th17 cell polarization and induced IL-22 production by PBMCs and $CD4^+$T cells. The effect of FICZ and ITE on $CD4^+$T cells was associated with a decreased expression of RORC, IL-17, IL-23R, and CCR6 and an increased phosphorylation of STAT3 and STAT5.

2. Materials and Methods

2.1. Subjects. Thirty active BD patients (18 men and 12 women, with an average age of 34.3 years) and nineteen inactive BD patients (11 men and 8 women, with an average age of 36 years) were included in this study. Forty-nine age- and gender-matched healthy volunteers were included as normal controls. The criteria of the International Study Group for BD were used to diagnose BD [22]. Active intraocular inflammation was defined by the presence of nongranulomatous keratic precipitates (100%), flare and cells in the anterior chamber (100%), vitreous cells (75%), and retinal vasculitis (100%) as shown by fundus fluorescein angiography (FFA). The extraocular manifestations were recurrent oral aphthous ulcers (100%), multiform skin lesions (80%), arthritis (36%), and recurrent genital ulcers (30%). The most frequent skin symptom in the patients included in our study was erythema nodosum, and the most frequent combination of skin lesions was erythema nodosum with papulopustular lesions. Active BD patients included in our study refer to the patients with Behcet's disease when visiting us all showed active intraocular inflammation; meanwhile, some of them had active extraocular findings, such as oral ulcers, genital ulcers, or skin lesions and some had a previous history of extraocular findings. The active BD patients enrolled in this study were all on their first visit to our hospital. These patients did not use any immunosuppressive agents or have received a low dose of immunosuppressive agents or prednisone but have stopped at least 2 weeks prior to blood sampling. We normally treated these active BD patients using systemic corticosteroids in combination with cyclosporine, cyclophosphamide, or chlorambucil for more than one and a half years. The drug dose was gradually tapered after the intraocular inflammation and other extraocular findings were controlled and the treatment usually stopped 6 months after complete control of the intraocular inflammation. After termination of all medications for at least 2 months we collected the blood sample from those inactive BD patients. The inactive patients were those who did not have any active inflammation in the eye as well as extraocular organs following a long term treatment with systemic corticosteroids combined with other immunosuppressive agents. We collected the peripheral blood sample of the outpatients with Behcet's disease and corresponding controls mostly in the afternoon. Written and informed consent was obtained from all patients and normal controls. All procedures followed the tenets of the Declaration of Helsinki and were approved by the Clinical Ethical Research Committee of Chongqing Medical University.

2.2. Cell Culture. Peripheral blood samples were obtained from BD patients and healthy volunteers. Preparation of PBMCs and $CD4^+$T cells was performed as described earlier [23]. Human $CD4^+$T cells were isolated from PBMCs by human CD4 microbeads (purity > 90%; Miltenyi Biotec, Palo Alto, CA) according to the manufacturer's instructions. The PBMCs and purified $CD4^+$T cells were cultured at 1×10^6 in 24-well plates in RPMI1640 medium supplemented with 10% FBS. A combination of anti-CD3 and anti-CD28 antibodies (2 ug/mL) (eBioscience, San Diego, Calif) was used to activate PBMCs for 3 days, and a combination of anti-CD3/CD28 (2 ug/mL) and IL-23 was used to activate $CD4^+$T cells for 6 days. When used, 0.05% DMSO (control), FICZ (100 nmol/L) (Enzo Life Sciences, USA), and ITE (100 nmol/L, Tocris Bioscience, USA) were added at the beginning of the culture. The supernatants were collected for cytokine measurement

by ELISA and the cells were harvested and used for FACS analysis or mRNA quantification.

2.3. Flow Cytometry. AnnexinV-FITC/PI Kit (KeyGen Biotechnology, Nanjing, China) was used to evaluate the effect of FICZ and ITE on the apoptosis of PBMCs. For analysis of the frequency of Th1 and Th17, the cells were stimulated by adding PMA (50 ng/mL, Sigma-Aldrich, St. Louis, MO) and ionomycin (1 ug/mL, Sigma) for 1 h at 37°C. Then, brefeldin A (10 ug/mL, Sigma) was added for another 4 h; the cells were fixed and permeabilized using the eBioscience Cytofix/Cytoperm kit according to the manufacturer's instructions and then incubated with CD3-(PerCP)-Cy5.5, anti-human CD8-APC, anti-human IL-17A-PE, and anti-human IFN-γ-FITC (BD Biosciences). For phosphorylated STAT staining, stimulated $CD4^+$T cells were fixed with Fix buffer (BD Biosciences) for 10 min at 37°C, permeabilized with Perm buffer (BD Biosciences) for 30 min on ice, and stained with anti-human pSTAT3-PE, anti-human pSTAT4-Per-cy5.5, anti-human pSTAT4-Per-cy5.5, anti-human pSTAT5-PE, or isotype control mAbs (BD Biosciences). Flow cytometric analysis was performed on a FACScan flow cytometer (BD Biosciences) to measure mean fluorescence intensity (MFI). Results were expressed as the percentage difference compared with isotypic control (IC) using the formula [mean fluorescence intensity (MFI) of sample − MFI of IC]/MFI of IC.

2.4. Quantitative RT-PCR. To detect the expression of AhR mRNA in BD patients and normal controls, total RNA was extracted from PBMCs of active BD patients, inactive BD patients, and normal controls by using a commercially available kit (RNeasyPlus Mini kit; Qiagen, Valencia, California) according to the manufacturer's instructions. To investigate the effect of AhR activation by FICZ or ITE on the expression of associated molecules of Th1 and Th17 cells, isolated $CD4^+$T cells (purity > 90%) were stimulated with antiCD3/CD28 and IL-23 with or without AhR ligands for 6 days; then, the cells were harvested for T-bet, RORC, IL-17, CCR6, and IL-23R mRNA quantification. Total RNA was isolated from stimulated $CD4^+$T cells using a commercially available kit (RNeasyPlus Mini kit; Qiagen, Valencia, California). Reverse transcription of RNA of the PBMCs or stimulated $CD4^+$T cells was performed using the Superscript III Reverse Transcriptase system (Invitrogen, Carlsbad, CA, USA). Amplification of transcripts was performed using SYBR RT-PCR (Takara, Dalian, China) and run on AB 7500 Fast System (Applied Biosystems). The following primers were used for real-time PCR: T-bet forward: 5′-GATGCTGCCAGGAAGTTTCAT-3′ and reverse: 5′-GCACAATCATCTGGGTCACATT-3′ and β-actin forward: 5′-GGATGCAGAAGGAGATCACTG-3′ and reverse: 5′-CGATCCACACGGAGTACTTG-3′. The primer sequences of IL-23R, IL-17, RORC2, and CCR6 were used as described elsewhere [19]. For AhR, Quantitect Primers (Qiagen, Valencia, CA) were used. The expression of each gene was normalized to the expression of β-actin using the $2^{-\Delta\Delta CT}$ method as described previously [24].

2.5. ELISA. IL-17, IFN-γ, and IL-22 levels in the cell culture supernatants were measured with ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

2.6. Statistical Analysis. The statistical significance of differences was determined by the Kruskal-Wallis test and Mann-Whitney test, Independent-Sample *t* test, Paired-sample *t* test, or Wilcoxon's matched-pairs test. $P < 0.05$ was considered statistically significant. All analyses were performed using commercially available statistical software (SPSS 12.0; SPSS Inc., Chicago, Illinois).

3. Results

3.1. Decreased AhR mRNA Expression in PBMCs from Active BD Patients. PBMCs from active BD patients, inactive BD patients, and normal controls were used to assay the mRNA expression of AhR. The results showed that AhR mRNA expression was significantly decreased in active BD patients as compared to inactive BD patients ($P = 0.006$) and normal controls ($P < 0.001$). There was no significant difference concerning AhR mRNA expression between inactive BD patients and normal controls (Figure 1(a)).

3.2. FICZ and ITE Inhibit Th1 and Th17 Cell Polarization and Induce IL-22 Expression by PBMCs from BD Patients and Normal Controls. Because an increased frequency of Th1 and Th17 cells has been shown to be associated with the inflammatory activity of BD and since AhR has been reported to be involved in T cell immune responses, we next determined the effect of endogenous AhR ligands on the Th1 and Th17 cell response in active BD patients and normal controls. PBMCs were stimulated with anti-CD3/CD28 to mimic antigen presentation in the presence or absence of FICZ or ITE. The apoptotic effect of FICZ and ITE was first evaluated by flow cytometry. Annexin V and PI double staining showed that FICZ and ITE had no significant influence on the apoptosis of PBMCs (Supplementary Figure 1, available online at http://dx.doi.org/10.1155/2014/195094). IFN-γ, IL-17, and IL-22 production in cell culture supernatants by these stimulated PBMCs from active BD patients were higher as compared to normal controls. Addition of FICZ or ITE significantly inhibited the production of IFN-γ and IL-17 but enhanced IL-22 production in both the BD and the control groups (Figures 1(b)–1(g)). Flow cytometry analysis showed an increased frequency of IL-17 and IFN-γ-expressing $CD4^+$T cells in PBMCs obtained from active BD patients as compared to normal controls. FICZ and ITE significantly inhibited the frequency of IL-17-expressing $CD4^+$T cells in both BD patients and in the controls, whereas no detectable effect was observed on the percentage of IFN-γ-expressing $CD4^+$T cells (Figure 2).

3.3. FICZ and ITE Directly Inhibit Th1 and Th17 Cell Polarization and Induce IL-22 Expression by $CD4^+$ T Cells from BD Patients and Normal Controls. The aforementioned results showed that FICZ and ITE can inhibit Th1 and Th17 cell polarization and induced the expression of IL-22 by PBMCs. As

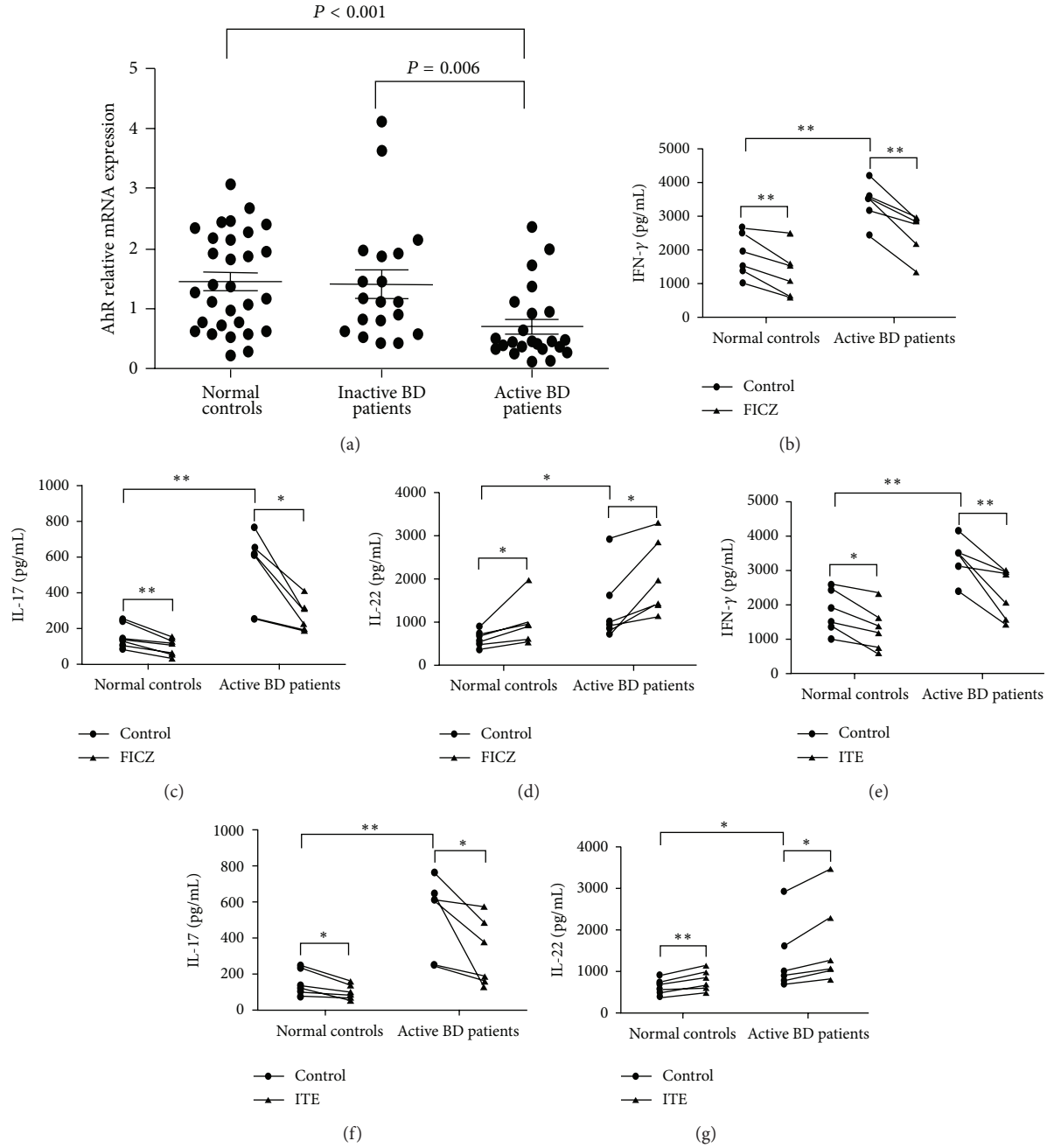

Figure 1: AhR mRNA expression is decreased in active BD patients and AhR activation by FICZ and ITE inhibits IFN-γ and IL-17 and induces IL-22 production by PBMCs. (a) AhR mRNA was evaluated in PBMCs from active BD patients (n = 23), inactive BD patients (n = 19), and normal controls (n = 30) by real-time PCR and normalized to β-actin. The Kruskal-Wallis test and Mann-Whitney test were used to analyze the statistical difference between the various groups. Data are expressed as mean ± s.e.m. (b–g) PBMCs from active BD patients (n = 6) and normal controls (n = 6) were stimulated with anti-CD3/CD28 in the presence or absence of FICZ (100 nmol/L) or ITE (100 nmol/L) for 3 days. The supernatants were harvested for detection of IFN-γ (b and e), IL-17 (c and f), and IL-22 (d and g) by ELISA. Paired-sample t test or Wilcoxon's matched-pairs test for related samples and Independent-Sample t test for independent samples were used for statistical analyses. $^{*}P < 0.05$, $^{**}P < 0.01$.

$CD4^{+}$T cells play a critical role in the pathogenesis of Behcet's disease [4], we next investigated whether FICZ and ITE were able to influence the development of Th1 and Th17 cells. The results showed that stimulation of purified $CD4^{+}$T cells from active BD patients resulted in a higher level of IFN-γ, IL-17, and IL-22 production in the cell culture supernatants as compared to normal controls. The addition of FICZ and ITE significantly inhibited the production of IFN-γ and IL-17 but induced IL-22 production by stimulated $CD4^{+}$T cells (Figure 3). Consistent with the ELISA, intracellular cytokine analysis by flow cytometry revealed a significantly increased percentage of IFN-γ- and IL-17-expressing $CD4^{+}$T cells in

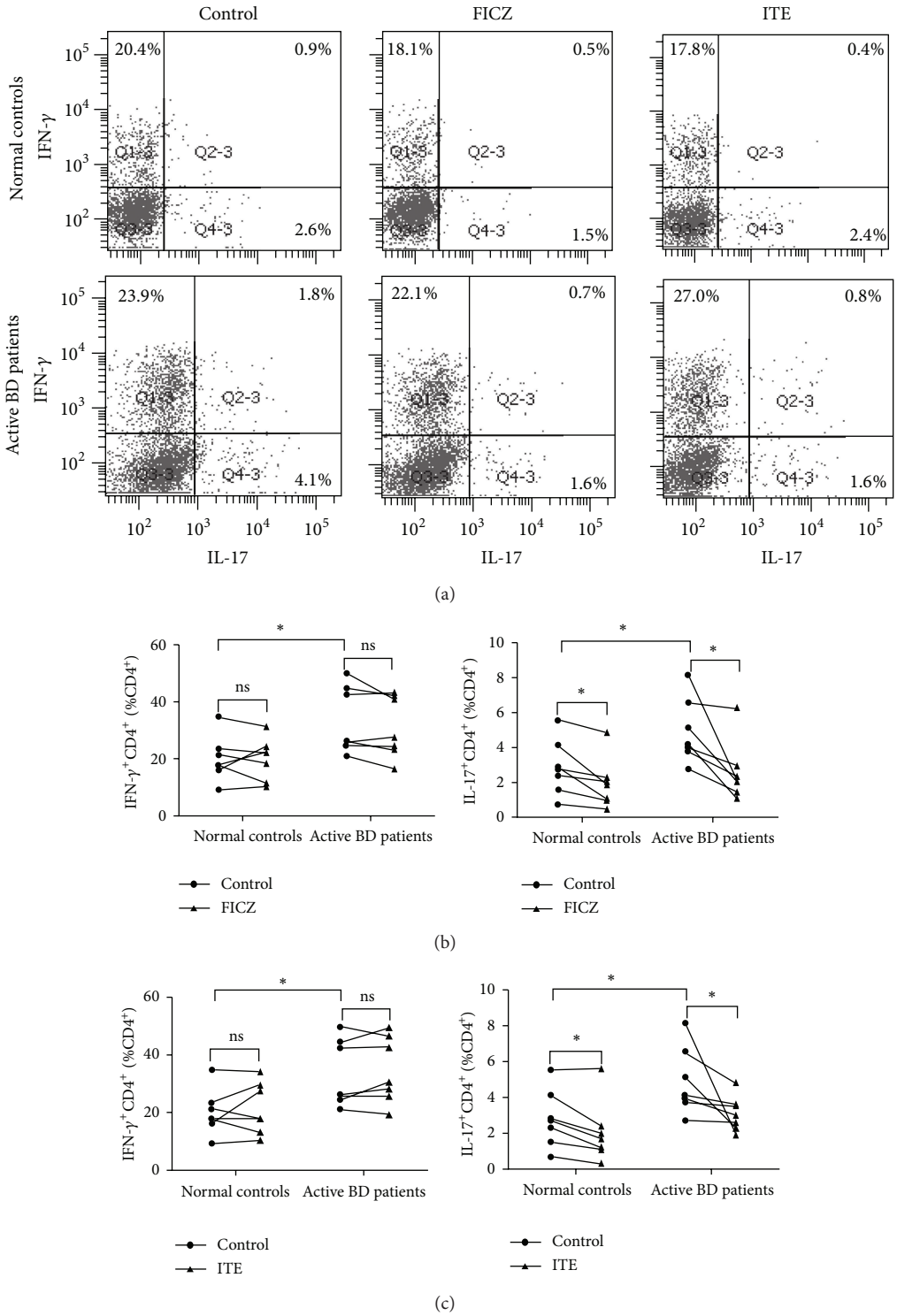

Figure 2: FICZ and ITE inhibit the frequency of IL-17-expressing $CD4^+$T cells but have no effect on IFN-γ-expressing $CD4^+$T cells in PBMCs. PBMCs from active BD patients ($n = 7$) and normal controls ($n = 7$) were stimulated with anti-CD3/CD28 in the presence or absence of FICZ (100 nmol/L) or ITE (100 nmol/L) for 3 days. The cells were analyzed for intracellular expression of IFN-γ and IL-17 by flow cytometry. (a) Dot plots of a representative subject for each group are shown. (b and c) Quantitative analysis of the percentage of IFN-γ- and IL-17-expressing $CD4^+$T cells. Paired-sample t test or Wilcoxon's matched-pairs test for related samples and Independent-Sample t test for independent samples were used for statistical analyses. $^*P < 0.05$, ns: not statistically different.

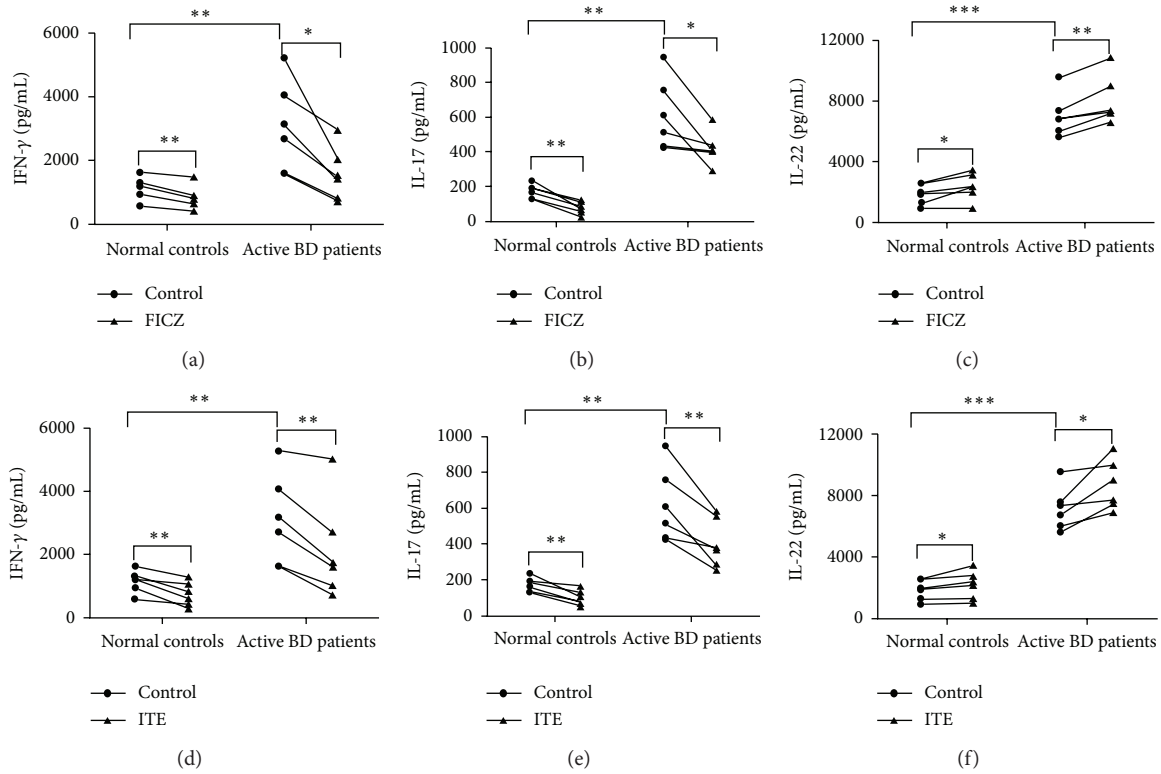

Figure 3: FICZ and ITE inhibit IFN-γ and IL-17 but induce IL-22 expression by $CD4^+$T cells from BD patients and normal controls. Purified $CD4^+$T cells from active BD patients ($n = 6$) and normal controls ($n = 6$) were stimulated with anti-CD3/CD28 and rIL-23 in the presence or absence of FICZ (100 nmol/L) or ITE (100 nmol/L) for 6 days. IFN-γ (a and d), IL-17 (b and e), and IL-22 (c and f) production in the supernatants were determined by ELISA. Paired-sample t test or Wilcoxon's matched-pairs test for related samples and Independent-Sample t test for independent samples were used for statistical analyses. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

active BD patients as compared to normal controls. AhR activation by either FICZ or ITE inhibited the frequency of IFN-γ- and IL-17-expressing $CD4^+$T cells in active BD patients and normal controls. No detectable difference was observed concerning the inhibitory effect between the two tested ligands (Figure 4).

To investigate the mechanism by which AhR signaling in $CD4^+$T cells might modulate the expression of IFN-γ, IL-17, and IL-22, we assessed the signaling activity of STAT pathways, which are critically involved in the differentiation of different Th cell subsets. The results showed that AhR activation by FICZ or ITE in $CD4^+$T cells cultured in the presence of antiCD3/CD28 and rIL-23 induced STAT3 and STAT5 phosphorylation. However, phosphor-STAT1 and phosphor-STAT4 were only detected at low levels and we could not detect an effect of AhR activation by FICZ or ITE on STAT1 and STAT4 phosphorylation (Figure 5).

As polarization of $CD4^+$T cell subsets is regulated by transcription factors [25], we next investigated whether FICZ or ITE had an influence on the transcription factors of Th1 and Th17 cells. We found that AhR activation by FICZ or ITE significantly inhibited the expression of T-bet and RORC mRNA in $CD4^+$T cells. Furthermore, we observed that FICZ or ITE not only inhibited the gene expression of IL-17 but also suppressed the gene expressions of IL-23R and CCR6 which are essential to both the maintenance and function of Th17 cells, indicating that FICZ and ITE interfere with Th17 polarization and function (Figure 6).

4. Discussion

In this study we found a decreased gene expression of AhR in PBMCs of active BD patients as compared to inactive BD patients and normal controls. These findings suggest a role for AhR expression in the pathogenesis of BD. Most studies in the past on AhR have been dedicated to its role in mediating toxicity [10] by dioxins but it is now emerging that AhR plays a physiological role in the immune response and that it is highly expressed on dendritic cells as well as on Th17 cells [26]. The role of AhR in clinical autoimmune disease is a novel area of research and expression of AhR on PBMCs obtained from active BD patients has, to our knowledge, not yet been addressed before. Interestingly, we found a significantly decreased AhR expression in active BD patients as compared to inactive BD patients and normal controls. It is well known that some immunosuppressive agents or prednisolone has a long effect on the immune system. The decreased AhR expression in active BD patients treated by a low dose of immunosuppressive agents or prednisolone seems to exclude the influence of these immunosuppressive agents or prednisolone on AhR expression. However, it is interesting to address the influence of immunosuppressive

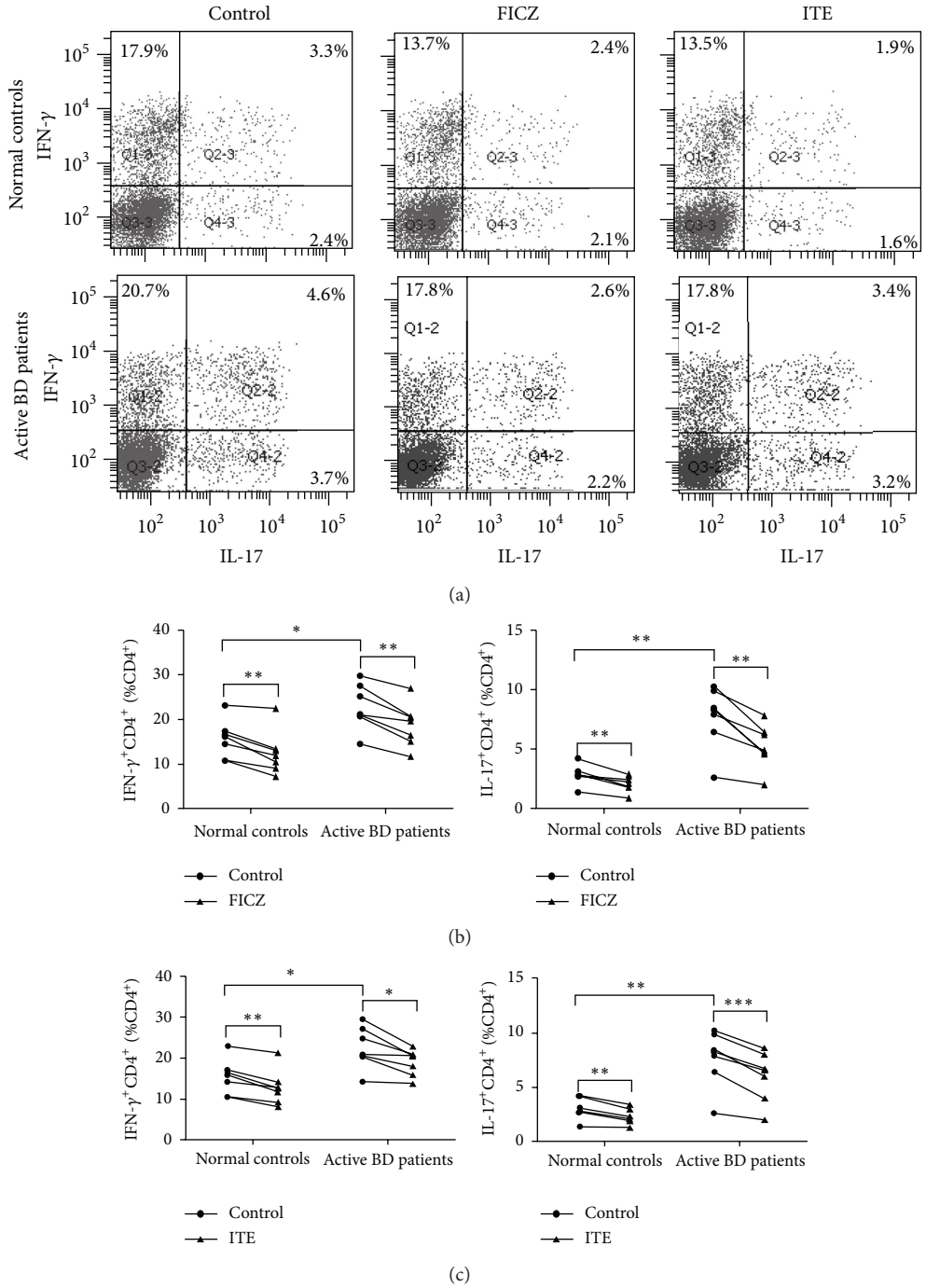

Figure 4: FICZ and ITE inhibit Th1 and Th17 cell polarization by $CD4^+$T cells from BD patients and normal controls. Purified $CD4^+$T cells from active BD patients ($n = 7$) and normal controls ($n = 7$) were stimulated with anti-CD3/CD28 and rIL-23 in the presence or absence of FICZ (100 nmol/L) or ITE (100 nmol/L) for 6 days. The cells were collected and analyzed for intracellular expression of IFN-γ and IL-17 by flow cytometry. (a) Dot plots of a representative subject for each group are shown. (b and c) Quantitative analysis of the percentage of IFN-γ- and IL-17-expressing $CD4^+$T cells. Paired-sample t test or Wilcoxon's matched-pairs test for related samples and Independent-Sample t test for independent samples were used for statistical analyses. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

agents or prednisolone on AhR expression; this can be achieved by our further studies. It is well established that male BD patients run a more active course as compared to female patients and that this disease may involve different organs [2]. Therefore, it is necessary to clarify whether there is difference in male and female patients as well as among those with different organ involvement using more prehensive studies in the future.

To provide further evidence for a possible role of the AhR in the pathogenesis of BD we performed a series of

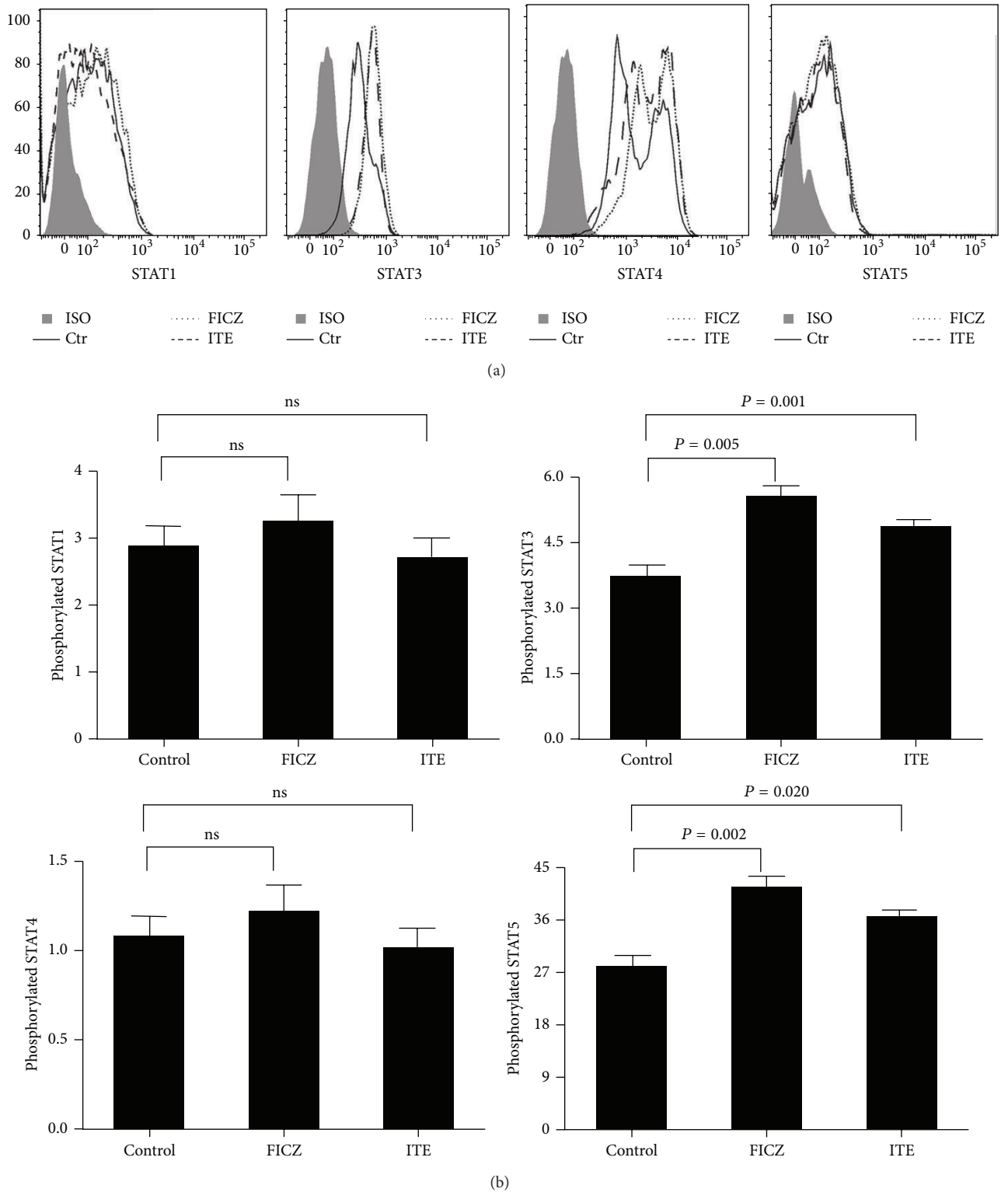

Figure 5: Effect of AhR activation by FICZ or ITE on phospho-STAT1, -3, -4, and -5 in $CD4^+$T cells. (a) $CD4^+$T cells from normal controls ($n = 7$) were stimulated with anti-CD3/CD28 and rIL-23 in the presence or absence of FICZ or ITE for 30 min. Intracellular phosphorylated STAT1, -3, -4, and -5 were analyzed by flow cytometry. A representative histogram from each group along with the isotype control is shown. (b) Statistical results of phosphorylated STAT1, -3, -4, and -5 in stimulated $CD4^+$T cells. Paired-sample t test for related samples was used for statistical analyses. The data are expressed as mean ± s.e.m.

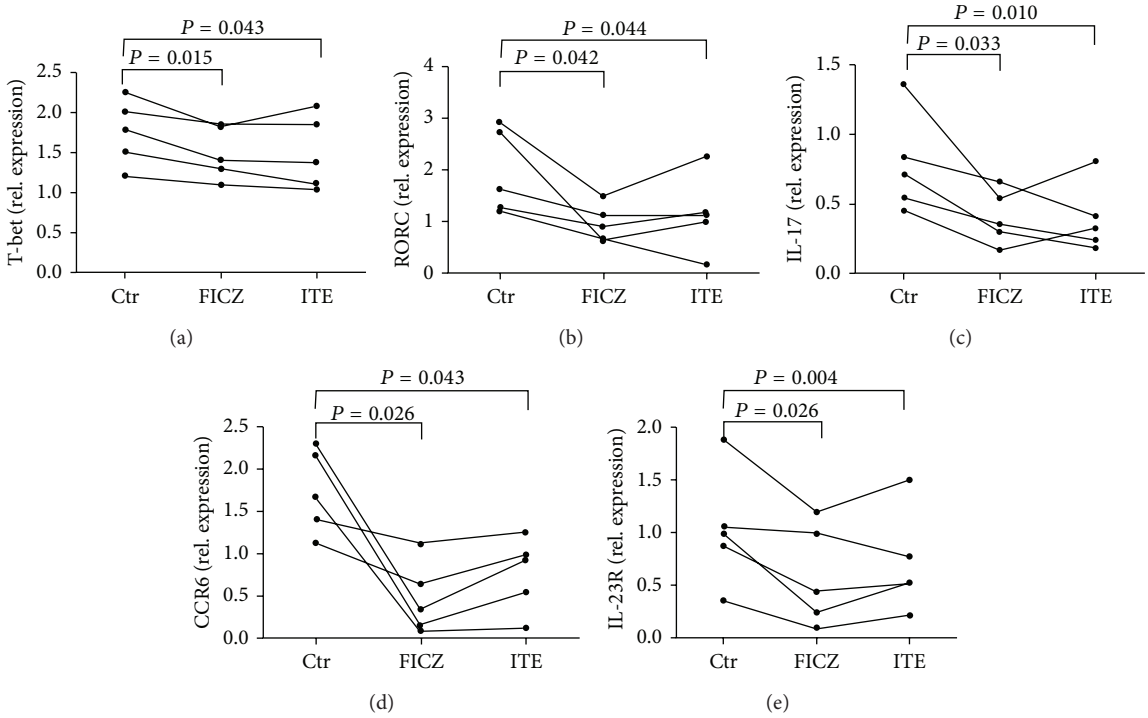

Figure 6: FICZ and ITE inhibit the molecules associated with Th1 and Th17 cell effector function in stimulated CD4$^+$T cells. CD4$^+$T cells from healthy controls (n = 5) were cultured with anti-CD3/CD28 and rIL-23 in the presence or absence of FICZ or ITE for 6 days. The cells were harvested for mRNA analysis of T-bet, RORC, IL-17, IL-23R, and CCR6 expression by real-time PCR. Paired-sample t test for related samples was used for statistical analyses.

experiments whereby we investigated the role of two known endogenous AhR ligands on the function of T cells. Consistent with previous results from our group, we observed that PBMCs obtained from active BD patients showed an elevated production of IFN-γ, IL-17, and IL-22 as compared to normal controls [4, 27]. Addition of the AhR ligands FICZ and ITE to these PBMC cultures resulted in a significant inhibition of the production of IFN-γ and IL-17 and an increased expression of IL-22. Because CD4$^+$T cells play a critical role in the development of autoimmune diseases [25], including BD [4], we further tested whether there was a direct effect of FICZ and ITE on cytokine production using purified CD4$^+$T cells. CD4$^+$T cells showed the same result as obtained earlier with PBMCs. Intracellular staining confirmed the above results except that the ligands did not affect the frequency of IFN-γ-producing CD4$^+$T cells from PBMCs. The reasons for the discrepancy between intracellular and extracellular IFN-γ expression is not clear and warrants further study.

Our findings with FICZ are consistent with data of recent studies which showed that AhR activation was able to induce production of IL-22 and that it could inhibit IL-17 production [20]. The findings are however in disagreement with results showing that FICZ induced IL-17 production in the mouse [28, 29]. Our findings with ITE were consistent with the in vivo inhibitory effect on the production of IFN-γ and IL-17 in mice [18]. All together, these data provide evidence that FICZ and ITE may act as a negative regulator for the adaptive Th1 and Th17 cell response, providing a possible novel therapeutic target for Behcet's disease. The observed discrepancies seem to suggest that the response of CD4$^+$T cells to AhR activation may differ among species [30].

In mouse studies it has been reported that the effect of FICZ on Th17 cell differentiation was associated with a reduction in Stat5 phosphorylation [29]. Kimura et al. found that AhR participates in Th17 cell differentiation by regulating Stat1 activation [28]. However, the exact mechanism whereby AhR activation by FICZ or ITE affects CD4$^+$T cell polarization is not known in humans. We found that AhR activation of CD4$^+$T cells by FICZ or ITE in the presence of antiCD3/CD28 and rIL-23 induced STAT3 and STAT5 phosphorylation, whereas no effect on STAT1 and STAT4 phosphorylation could be detected. It has been shown that Th17 differentiation is inhibited by IL-2 signaling via induction of Stat5 [31] and IL-22 production is associated with STAT3 phosphorylation [32–35]. We hypothesize that in our experiments Stat5 phosphorylation in CD4$^+$T cells that were stimulated with FICZ or ITE may lead to an inhibition of IL-17 expression, whereas STAT3 phosphorylation may be associated with the induction of IL-22. Additional studies are needed to support this assumption.

We further showed that the AhR ligands induced a decreased gene expression of T-bet and RORC in CD4$^+$T cells. T-bet and RORC are the Th1 and Th17 cell master transcription factors, respectively. The FICZ- or ITE-mediated suppression on Th17 cell polarization was also associated with a decreased gene expression of CCR6 and IL-23R. These

findings suggest that AhR activation by FICZ or ITE inhibited not only the development of Th17 cells but also the molecules that are relevant to Th17 cell function and migration.

5. Conclusion

Our findings suggest that AhR activation by FICZ or ITE can potentially modulate an aberrant immune response by inhibiting Th1 and Th17 cell responses. Further studies are needed to show whether modulation of the AhR pathways may offer a possible novel therapeutic approach for BD and other autoimmune diseases which are mediated by an aberrant Th1 and Th17 immune response.

Conflict of Interests

None of the authors has a proprietary or financial interest in any product mentioned.

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Research Article

Effect of the Toll-Like Receptor 4 Antagonist Eritoran on Retinochoroidal Inflammatory Damage in a Rat Model of Endotoxin-Induced Inflammation

Feyzahan Ekici,[1] Emine Esra Karaca,[2] Şafak Korkmaz,[3] Osman Yüksel,[4] Özlem Gülbahar,[5] Murat Alper,[6] Sevim Ercan,[7] and Meral Or[8]

[1] *Department of Ophthalmology, Recep Tayyip Erdogan University Medical School, 53020 Rize, Turkey*
[2] *Department of Ophthalmology, Sorgun State Hospital, 66700 Yozgat, Turkey*
[3] *Department of Ophthalmology, Duzce State Hospital, 81100 Duzce, Turkey*
[4] *Department of General Surgery, Gazi University Medical School, 06560 Ankara, Turkey*
[5] *Department of Medical Biochemistry, Gazi University Medical School, 06560 Ankara, Turkey*
[6] *Department of Pathology, Dışkapı Training and Research Hospital, 06330 Ankara, Turkey*
[7] *Department of Medical Pharmacology, Gazi University Medical School, 06560 Ankara, Turkey*
[8] *Department of Ophthalmology, Gazi University Medical School, 06560 Ankara, Turkey*

Correspondence should be addressed to Feyzahan Ekici; feyzahan@gmail.com

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Purpose. We investigated the effect of eritoran, a Toll-like receptor 4 antagonist, on retinochoroidal inflammatory damage in an endotoxin-induced inflammatory rat model. *Methods*. Endotoxin-induced inflammatory model was obtained by intraperitoneal injection of 1.5 mg/kg lipopolysaccharide (LPS). Group 1 had control rats; in groups 2-3 LPS and 0.5 mg/kg sterile saline were injected; and in groups 4-5 LPS and 0.5 mg/kg eritoran were injected. Blood samples were taken and eyes were enucleated after 12 hours (h) (groups 2 and 4) or 24 hours (Groups 3 and 5). Tumor necrosis factor-α (TNF-α) and malondialdehyde (MDA) levels in the serum and retinochoroidal tissue and nuclear factor kappa-B (NFκB) levels in retinochoroidal tissue were determined. Histopathological examination was performed and retinochoroidal changes were scored. *Results*. Eritoran treatment resulted in lower levels of TNF-α, MDA, and NFκB after 12 h which became significant after 24 h. Serum TNF-α and retinochoroidal tissue NFκB levels were similar to control animals at the 24th h of the study. Eritoran significantly reversed histopathological damage after 24 h. *Conclusions*. Eritoran treatment resulted in less inflammatory damage in terms of serum and retinochoroidal tissue parameters.

1. Introduction

Toll-like receptors (TLRs) are a family of pattern recognition receptors that recognize distinct molecular patterns associated with microbial pathogens [1]. TLR4 binding is required for the recognition of Gram-negative LPS. Once the TLR4 signaling pathway is triggered, inactive cytosolic NFκB is activated and induces the synthesis and release of proinflammatory mediators, including TNF-α, cytokines, chemokines, adhesion molecules, reactive oxygen species, and reactive nitrogen radicals [2, 3]. During this inflammatory response, MDA is produced as a result of lipid peroxidation [4]. MDA is a degradation product of free oxygen radicals and arachidonic acid pathway metabolites [5] and was suggested as a marker of oxidative stress [6].

Recent studies have demonstrated the expression and the function of TLRs in the eye, with significant implications for a better understanding of ocular immunity and the pathogenesis of inflammatory eye diseases affecting the cornea, uvea, and retina. TLR4 is expressed on the corneal epithelium [7],

corneal stromal fibroblasts [8], ciliary body, iris endothelial cells [9], uveal resident antigen presenting cells [10], and retina pigment epithelium [11].

Endotoxin-induced uveitis (EIU) is an important ocular inflammatory model [12]. Initially, EIU was described as an anterior uveitis model affecting the anterior chamber, iris, and ciliary body [12, 13]. Subsequently, retinal and choroidal involvement were demonstrated [14, 15]. Inflammatory changes such as endothelial cell damage, blood-retinal barrier (BRB) dysfunction, adhesion/migration/infiltration of polymorphonuclear leukocytes, protein leakage from vessel walls, and retinal cell damage were described in the retina and retinochoroidal microvascular system in EIU models [16]. When an inflammatory response is initiated using LPS to trigger the TLR pathway, proinflammatory mediators, including TNF-α, interleukins, adhesion molecules, and chemokines, are released. TNF-α, in particular, modulates BRB damage in the EIU model, which is related to polymorphonuclear leukocyte adhesion, endothelial cell apoptosis, and microvascular damage [17]. Eritoran tetrasodium (E5564) is suggested for sepsis therapy, belonging to a new class of drugs which inhibits LPS-induced inflammation by blocking TLR4 [3].

We investigated the changes in serum and tissue levels of TNF-α, MDA, and NFκB and the histopathological scores in a rat model of ocular inflammation induced by systemic LPS administration and determined the improvement of these ocular inflammation parameters induced by eritoran, a TLR4 antagonist.

2. Materials and Methods

2.1. Study Design. This was a prospective randomized controlled experimental animal study. Wistar albino male rats weighing 240–310 g and at the age of 8 weeks were used. The animals were housed under a 12 h light-dark cycle and were fed on a standard diet until the experiment. The criteria of the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals were applied and the study was approved by the Gazi University Ethic Committee for Experimental Animals.

2.2. Study Procedures. Endotoxin-induced inflammatory model was obtained by the intraperitoneal injection of 1.5 mg/kg LPS (Sigma-Aldrich, Munich, Germany, *Escherichia coli*, serotype O111:B4). Eritoran (E5564, Eisai, Inc.) was used as a TLR4 antagonist. After establishing anesthesia with intramuscular 100 mg/kg ketamine (Ketalar, Eczacıbaşı, Istanbul, Turkey), blood samples were obtained from the vena cava. Eritoran and saline injections routed through tail vein concomitantly. Enucleation was performed after peritomy and the animals were sacrificed. The right eyes were used for the biochemical parameters and the left eyes were kept for the histopathological evaluation.

2.3. Study Groups. Fifty rats were investigated in five groups ($n = 10$). To assign the rats to the individual study groups, the Random Allocation Software program (Version 1.0.0© Mahmood Saghaei, Isfahan University of Medical Sciences, Isfahan, Iran) was used. The study groups were as follows. Group 1 has control rats, no intervention; in group 2, 1.5 mg/kg LPS and 0.5 mg/kg (11 μL) saline were injected, blood samples were taken, and enucleation was performed after 12 h; in group 3, 1.5 mg/kg LPS and 0.5 mg/kg saline were injected, blood samples were taken, and enucleation was performed after 24 h; in group 4, 1.5 mg/kg LPS and 0.5 mg/kg eritoran were injected, blood samples were taken, and enucleation was performed after 12 h; in group 5, 1.5 mg/kg LPS and 0.5 mg/kg eritoran were injected, blood samples were taken, and enucleation was performed after 24 h. Inclusion of the 12 h and 24 h groups allowed us to monitor the changes in the inflammation parameters within time in the serum and the retinochoroidal tissue as well as the change in eritoran efficacy.

2.4. Serum and Tissue Cytokine Levels. The levels of TNF-α (pg/mL) and MDA (U/mL) in the serum and the retinochoroidal tissue were assessed with commercially available enzyme-linked immunosorbent assay (ELISA) kits: Rat TNF-α ELISA (Sigma-Aldrich, Munich, Germany) and lipid peroxidation (MDA) ELISA (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions. Retinochoroidal tissue was rinsed with 1X phosphate buffer solution (PBS) to remove excess blood, homogenized in 20 mL of 1X PBS, and stored overnight at $\leq -20^{\circ}$C. After two freeze-thaw cycles to get rid of the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 ×g. The MDA and TNF-α assays were performed using the OxiSelect MDA and TNF-α adduct ELISA kit. Tissue samples were diluted to 10 mg/mL in 1X PBS and 100 μL from each sample was added in duplicate to the 96-well protein binding plate. Then, 200 mL of assay diluent was added per well and incubated for 1 h at room temperature (RT) on an orbital shaker. The plate was washed 3 times with 1X wash buffer before adding the diluted anti-MDA and anti-TNF-α antibody and incubated for 1 h at RT on the orbital shaker. Finally, after warming, the substrate solution was added to each well and the plate was incubated at RT. The absorbance of each sample was measured at 450 nm after the addition of stop solution using MultiSkan Go spectrophotometer (Thermo Scientific, Finland). The concentration of MDA (U/mL) and TNF-α (ng/mg protein) in the samples is then determined by comparing the optical density of the samples to the standard curve.

For the measurement of NFκB (ng/mg protein) levels, retinochoroidal tissues from each group were centrifuged at 4°C, 1000 g for 5 minutes. The supernatant fluid was discarded and the cells were washed by PBS twice. The tissues were incubated in the complete lysis buffer for 30 min. Then lysis buffer was centrifuged at 4°C, 10.000 g for 10 min, and the supernatants were collected. Protein concentration was measured by using bicinchoninic acid method. Aliquots of each sample containing lg protein were fractionated on 10% polyacrylamide-sodium dodecyl sulfate gel (70 V at first, when the protein ran out of concentration gel, turned to 90 V for 1.5 h) and transferred to polyvinylidene fluoride

(350 mA, 90 min) membrane. The membrane was blocked with 10% nonfat milk in PBS containing 0.9% saline and 0.05% Tween 200 (PBST) for 1 h at room temperature and then incubated with primary antibodies (NFκB antibody diluted at 1 : 200, Santa Cruz, USA; b-actin antibody diluted at 1 : 1000, Santa Cruz, USA) at 4°C overnight. After washing for 5 min and 3 times with PBST, the blots were incubated with horse radish peroxidase conjugated anti-rabbit IgG second antibody (1 : 1000) for 1 h at RT. Immunoreactive band was visualized with enhanced chemiluminescence staining. The intensity of each band was scanned by software. The ratio of NFκB/b-actin was considered as the relative expressional level of NFκB.

2.5. Histopathological Analysis. The enucleated eye specimens were fixed in 10% formalin for 72 h and embedded in paraffin, and 5 mm tissue sections were taken through posterior pole and stained with hematoxylin-eosin staining for light microscopy. Histopathological findings assessed in the retina were scored as follows: *minimal* (few infiltrating cells, pigment disturbance, or minimal degeneration), *mild* (infiltration of anterior retina and thickening of the inner limiting membrane), *moderate* (retinal detachment and infiltration, some retinal folds), *severe* (retinal degeneration), and *very severe* (no retinal tissue observable) [18].

2.6. Statistical Analysis. The sample size required for this study and the power were calculated using the G*Power program (Version 3.0.10, Franz FAUL, Kiel University, Germany, http://www.gpower.hhu.de/). To obtain 80% power, with effect extent $f = 0.30$, $\alpha = 0.05$ type I error, and $\beta = 0.20$ type II error ratio, it was calculated that a minimum of 10 rats per group was needed.

The Shapiro-Wilk test indicated that only the serum TNF-α and tissue MDA levels after 12 h and 24 h showed a normal distribution. Results were presented as the minimum and the maximum values, the means ± standard deviation (SD), or the medians (interquartile range, [IQR]). To compare the levels of serum TNF-α and tissue MDA after 12 h and 24 h, one-way analysis of variance (ANOVA) and the Bonferroni test for post hoc pair comparison were used. For other comparisons, the Kruskal-Wallis nonparametric variance analysis was used. The variance among groups and pairwise comparisons were determined using the Mann-Whitney U test with Bonferroni correction. A paired-sample t-test for serum TNF-α and tissue MDA and a Wilcoxon signed rank test for the other parameters were used to evaluate the change between 12 h and 24 h for the same treatment. Histopathological scores were evaluated by Chi-square likelihood ratio. All statistical analyses and calculations were made with the MS Excel 2003 and SPSS programs (Statistical Package for Social Sciences version 15.0, SPSS Inc., Chicago, Illinois, USA). A statistical level of significance was defined as $P \leq 0.05$.

3. Results

The TNF-α and MDA levels in the serum and retinochoroidal tissue and the NFκB level in the retinochoroidal tissue in control rats with no intervention (group 1), rats not treated with eritoran (groups 2 and 3), and rats treated with eritoran (groups 4 and 5) are shown in Table 1. The levels of these markers are also schematized in Figures 1–5 for the 12 h (groups 2 and 4) and 24 h (groups 3 and 5) measurements.

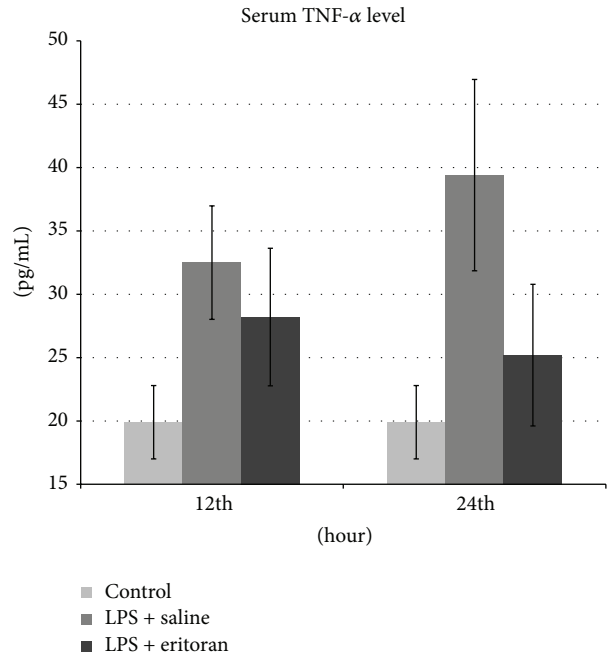


Figure 1: The tumor necrosis factor-α (TNF-α) levels in serum after 12 h and 24 h in control group with no intervention, rats with sepsis but without eritoran, and rats with sepsis treated with eritoran. Vertical lines show standard deviation. LPS: lipopolysaccharide.

3.1. Serum and Tissue TNF-α Levels. The serum TNF-α levels were significantly higher in groups 2, 3, and 4 compared to the control group ($P < 0.001$), which confirmed that inflammation was present in groups 2, 3, and 4. The serum TNF-α level was significantly different between groups 3 and 5 ($P < 0.001$). Although the serum TNF-α level was higher in group 2 than group 4, this was not statistically significant ($P = 0.112$). Similarly, serum TNF-α levels were not significantly different between groups 1 and 5 ($P = 0.139$), indicating that treatment with eritoran caused similar serum TNF-α levels with control rats after 24 h (Table 1, Figure 1).

The tissue TNF-α levels in all groups were significantly different than the control group (group 1) ($P < 0.001$) and were also different between groups 3 and 5 ($P < 0.001$), but no significant difference was found between groups 2 and 4 ($P = 0.353$). These tissue TNF-α levels confirmed the inflammatory status of the animals (Table 1, Figure 2).

3.2. Serum and Tissue MDA Levels. Serum and tissue MDA levels were significantly different in all study groups (groups 2–5) compared to the control group (group 1) ($P < 0.001$) and between groups 3 and 5 ($P < 0.001$), but no significant difference was found between groups 2 and 4 ($P = 0.105$ for serum, $P = 0.334$ for tissue) (Table 1, Figures 3 and 4).

Table 1: TNF-α, MDA, and NFκB levels of study groups.

Variable	Group 1 (control)	Group 2 (12 h) (LPS + saline)	Group 3 (24 h) (LPS + saline)	P value (group 2 versus 3)	Group 4 (12 h) (LPS + eritoran)	Group 5 (24 h) (LPS + eritoran)	P value (group 4 versus 5)	P value (group 1 versus 5)	P value (group 2 versus 4)	P value (group 3 versus 5)
Serum TNF-α	19.90 ± 2.89 (15.00–24.00)	32.50 ± 4.48 (25.00–39.00)	39.40 ± 7.55 (22.0–48.00)	0.008[a]	28.20 ± 5.43 (19.00–36.00)	25.20 ± 5.59 (15.00–34.00)	0.069[a]	0.139[c]	0.112[c]	<0.001[c]
Tissue TNF-α	0.75, 0.43 (0.30–1.00)	2.15, 0.53 (1.90–2.70)	2.90, 0.80 (2.20–3.60)	0.004[b]	2.15, 0.33 (1.70–2.40)	1.95, 0.43 (1.40–2.90)	0.057[b]	<0.001[c]	0.353[c]	<0.001[c]
Serum MDA	8.00, 8.00 (8.00-8.00)	17.50, 5.50 (14.00–25.00)	29.00, 6.00 (24.00–35.00)	0.004[b]	15.50, 4.75 (11.00–20.00)	13.00, 4.25 (8.00–18.00)	0.007[b]	<0.001[c]	0.105[c]	<0.001[c]
Tissue MDA	9.94 ± 1.71 (7.70–12.10)	24.50 ± 6.75 (20.00–34.00)	28.40 ± 4.74 (21.00–37.00)	0.003[a]	22.50 ± 4.62 (16.00–30.00)	20.00 ± 3.80 (15.00–27.00)	0.002[a]	<0.001[c]	0.334[c]	<0.001[c]
Tissue NFκB	0.30, 0.02 (0.27–0.32)	0.38, 0.03 (0.32–0.77)	0.41, 0.04 (0.35–0.46)	0.073[b]	0.34, 0.05 (0.28–0.37)	0.31, 0.05 (0.24–0.36)	0.006[b]	0.529[c]	0.218[c]	<0.001[c]

[a]Paired-sample t-test, [b]Wilcoxon signed rank test, [c]Mann-Whitney U test.

TNF-α: tumor necrosis factor-α, MDA: malondialdehyde, NfκB: nuclear factor kappa B.

Data are presented as mean ± standard deviation (min–max) or median, interquartile range (min–max).

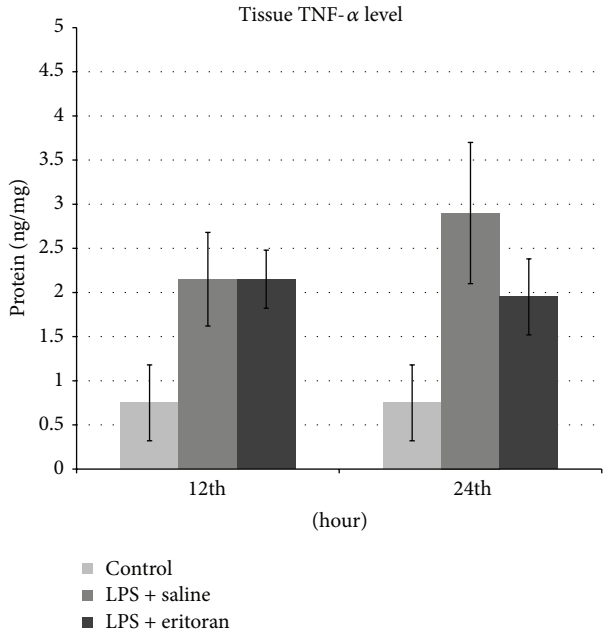


Figure 2: The tumor necrosis factor-α (TNF-α) levels in retinochoroidal tissue after 12 h and 24 h in control group with no intervention, rats with sepsis but without eritoran, and rats with sepsis treated with eritoran. Vertical lines show standard deviation. LPS: lipopolysaccharide.

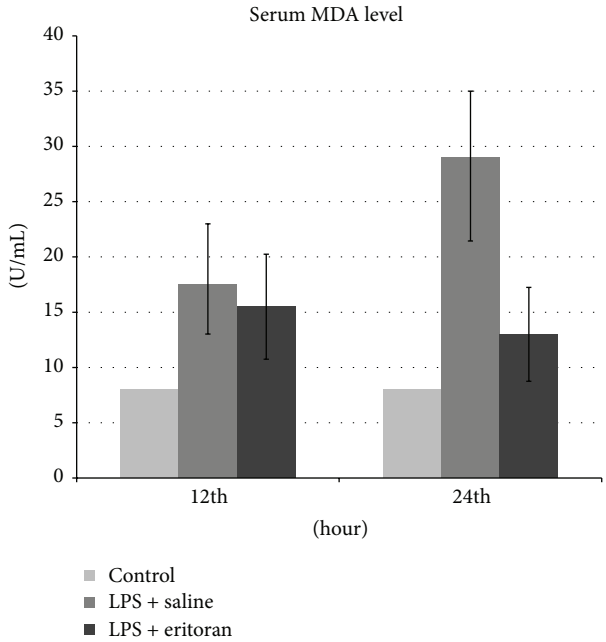


Figure 3: The malondialdehyde (MDA) levels in serum after 12 h and 24 h in control group with no intervention, rats with sepsis but without eritoran, and rats with sepsis treated with eritoran. Vertical lines show standard deviation. LPS: lipopolysaccharide.

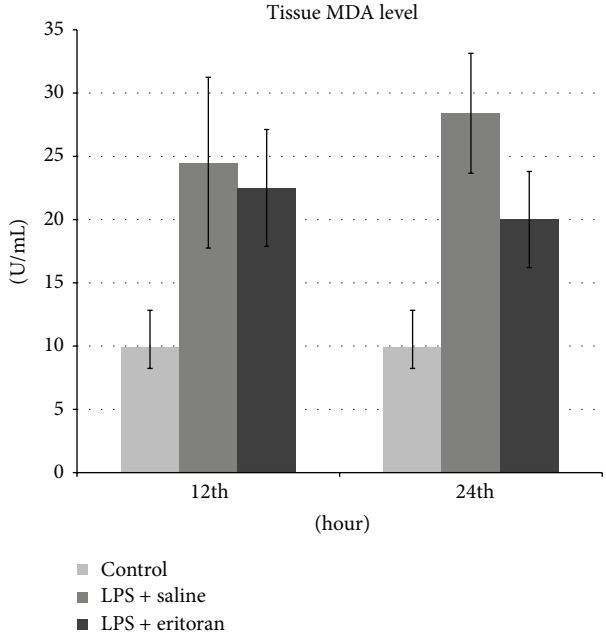


Figure 4: The malondialdehyde (MDA) levels in retinochoroidal tissue after 12 h and 24 h in control group with no intervention, rats with sepsis but without eritoran, and rats with sepsis treated with eritoran. Vertical lines show standard deviation. LPS: lipopolysaccharide.

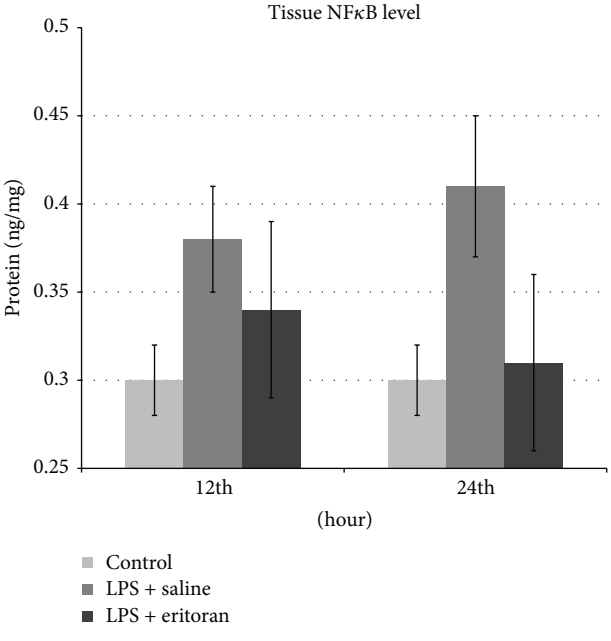
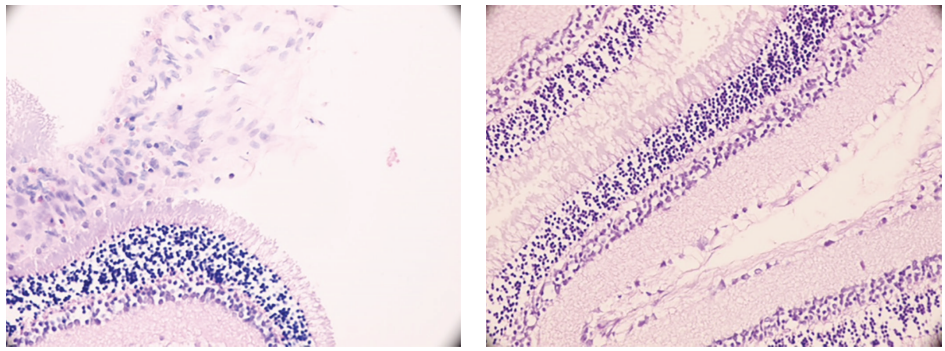


Figure 5: The nuclear factor kappa B (NfκB) levels in retinochoroidal tissue after 12 h and 24 h in control group with no intervention, rats with sepsis but without eritoran, and rats with sepsis treated with eritoran. Vertical lines show standard deviation. LPS: lipopolysaccharide.



(a) (b)

Figure 6: In histopathological section (H&E, ×400) of an eye from (a) one of group 3 rats (saline injected) at 24th h, see moderate retinal infiltration and some retinal folds and from (b) one of group 5 rats (eritoran injected) at 24th h, see minimal infiltrating cells.

3.3. Tissue NFκB Levels. Tissue NFκB levels were significantly different in both 12 h groups (groups 2 and 4) and group 3 compared to the control group (group 1) ($P < 0.001$). The difference between groups 2 and 4 was not statistically significant ($P = 0.218$). Tissue NFκB levels were not significantly different between groups 1 and 5 ($P = 0.529$), indicating that treatment with eritoran induced as lower tissue NFκB levels as the control rats after 24 h (Table 1, Figure 5).

3.4. Comparison of 12- and 24-Hour Levels of TNF-α, MDA, and NFκB. Serum and tissue TNF-α levels were significantly higher at 24th h of the inflammatory process (group 3) when compared to 12th h (group 2) ($P = 0.008$ and $P = 0.004$, resp.). In contrast, treatment with eritoran induced nonsignificant lower serum and tissue TNF-α levels after 24 h (group 5) compared to those measured after 12 h (group 4) ($P > 0.05$) (Table 1).

Serum and tissue MDA levels after 24 h of inflammation in group 3 were significantly higher when compared to group 2 ($P = 0.004$ and $P = 0.003$, resp.). In contrast, serum and tissue MDA levels were significantly lower with eritoran treatment after 24 h in group 5 compared to those measured after 12 h in group 4 ($P = 0.007$ and $P = 0.002$, resp.) (Table 1).

NFκB levels in tissue were higher after 24 h in group 3 than after 12 h in group 2, although this difference was not significant ($P = 0.073$). On treatment with eritoran, the NFκB levels were significantly lower after 24 h in group 5 compared to 12 h in group 4 ($P = 0.006$) (Table 1).

3.5. Histopathological Analysis. The retinochoroidal sections were examined and the severity of inflammatory changes was scored as described before. The results of the scores were shown in Table 2. For statistical purposes, "minimal" results were labeled as "not affected" and mild or worse results were labeled as "affected." Histopathological scores at 12 h were not significantly different between saline and eritoran groups. At 24 h of study LPS-induced inflammation related damage became more prominent in LPS + saline group. Also at 24th h, all saline injected rats were "affected"; however, 60% of eritoran treated rats remained "not affected" and this difference was statistically significant ($\chi^2 = 10.974$; $P = 0.001$) (Table 3) (Figure 6).

4. Discussion

We investigated the effect of systemic LPS injection on retinochoroidal inflammation by assessing TNF-α, MDA, and NFκB levels in serum and tissue and the changes in the histopathological scores. We further studied the effect of eritoran on retinochoroidal inflammation in this LPS-induced inflammation model. We found an improvement in all parameters upon treatment with eritoran. Therefore, we believe eritoran might be effective in preventing retinochoroidal inflammatory damage.

Eritoran is a TLR4 antagonist that binds to the MD-2 region, thus inhibiting LPSTLR4 binding and consequently NFκB activation [19]. In vitro studies indicate that eritoran, without endotoxin-like activity upon binding to the TLR4 molecule [20], inhibits cytokine production in human myeloid and macrophage cultures [21] and downregulates intracellular cytokine production [22]. Recent studies using EIU models showed that the iris endothelium and ciliary body express TLR4, and TLRs play an important role in the pathogenesis of anterior uveitis [23]. Li et al. showed that in the iris-ciliary body complex expression of NFκB was increased in a rat model of EIU [24]. TLR4 mutant rats did not respond to LPS and were protected from the lethal effect [25].

Eritoran prevents the uncontrolled inflammatory response and inhibits the proinflammatory mediator production during inflammatory process. TLR4 molecule is expressed in retinochoroidal tissue and may play a potential role in the ocular inflammatory processes. So, the main aim of this study with regard to TLR4 antagonism was whether retinochoroidal damage could be prevented with eritoran.

In this study, we observed significant high TNF-α levels in both serum and retinochoroidal tissue after the induction of inflammation with LPS. Serum and tissue TNF-α levels were significantly lower in eritoran injected animals; even serum TNF-α levels were the same as the control rats after 24 h. TNF-α is an important mediator of sepsis and is responsible

Table 2: Histopathological scores of study groups.

Histopathological scores			LPS + saline	LPS + eritoran	Total
12th hour	Minimal	*n* (%)	7 (70.0)	9 (90.0)	16 (80.0)
	Mild	*n* (%)	3 (30.0)	1 (10.0)	4 (20.0)
	Moderate	*n* (%)	0 (0.0)	0 (0.0)	0 (0.0)
	Severe	*n* (%)	0 (0.0)	0 (0.0)	0 (0.0)
	Very severe	*n* (%)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	***n* (%)**	**10 (100.0)**	**10 (100.0)**	**20 (100.0)**
24th hour	Minimal	*n* (%)	0 (00.0)	6 (60.0)	6 (30.0)
	Mild	*n* (%)	9 (90.0)	4 (40.0)	13 (65.0)
	Moderate	*n* (%)	1 (10.0)	0 (0.0)	1 (5.0)
	Severe	*n* (%)	0 (0.0)	0 (0.0)	0 (0.0)
	Very severe	*n* (%)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	***n* (%)**	**10 (100.0)**	**10 (100.0)**	**20 (100.0)**

LPS: lipopolysaccharide.

Table 3: Relabeled statistical analysis of histopathological scores.

Histopathological score			LPS + saline	LPS + eritoran	Total	χ^2	*P* value
12 h	Not affected	*n* (%)	7 (70.0)	9 (90.0)	16 (80.0)	1.297	0.255
	Affected	*n* (%)	3 (30.0)	1 (10.0)	4 (40.0)		
24 h	Not affected	*n* (%)	0 (0.0)	6 (60.0)	6 (30.0)	10.974	**0.001**
	Affected	*n* (%)	10 (100.0)	4 (40.0)	14 (70.0)		

LPS: lipopolysaccharide; χ^2: Chi-square likelihood ratio; bold: statistically significant.

for acute phase reactions, endothelial activation, capillary permeability changes, end organ damage, and shock-like syndrome [26]. Therefore, TNF-α is a target molecule for the treatment of sepsis and ocular inflammatory disease processes [27]. Evereklioglu et al. reported that TNF-α levels correlated with disease activity in Behcet's disease [28]. TNF-α expression was observed in the retina after 4 h of LPS injection and peaked at 22–24 h [29]. Because TNF-α plays an important role in the ocular inflammatory disease process, recent studies have reported successful results of anti-TNF-α therapies [30, 31]. Although there is no published report of changes in TNF-α levels in retinochoroidal tissue with TLR4 antagonism in the literature, we believe that through TLR4 inhibition the TNF-α load could be diminished in the retina and thus further tissue injury could be ameliorated.

MDA is a metabolite of free oxygen radicals and some inflammatory mediators, including thromboxane A2, and may be used as a marker of oxidative stress [4, 5]. MDA forms covalent bonds with intracellular proteins and DNA and also forms advanced toxic peroxidation end products [6]. Studies have shown that lipid peroxidation is responsible for retinal cell damage in experimental autoimmune uveitis models [32, 33]. Evereklioglu et al. reported that MDA levels correlated with disease activity in Behcet's disease [28]. Retinal damage could be prevented using antioxidant molecules against these peroxidation products [34, 35].

Similar to TNF-α, serum MDA levels were significantly higher in group 2 rats when compared to controls and continued to be higher in group 3. On treatment with eritoran, serum MDA levels were lower than saline injected groups at 12th h in group 4 rats and were significantly lower after 24 h in group 5 rats. However, in contrast to TNF-α, these levels did not return to those of the control rats after 12 h or 24 h. MDA is a metabolite of various proinflammatory molecules and thus the MDA load may be greater than that of TNF-α; hence, the serum MDA levels were not as low as the TNF-α levels when treated with eritoran. In addition, MDA forms covalent bonds with proteins; thus, this may be a cause for higher levels of MDA than that of TNF-α. We achieved a significant lower serum and tissue MDA levels with eritoran, suggesting that lipid peroxidation related retinochoroidal damage may be prevented by TLR4 antagonism.

NFκB stimulates proinflammatory genes when the TLR signaling pathway is triggered and constitutes the most important step of the inflammatory process [36]. NFκB correlates with the mortality rates in sepsis patients [37, 38], and with retinal damage in ocular inflammatory diseases, while NFκB inhibition prevented further tissue damage in experimental models of autoimmune uveitis [39, 40].

Similar to serum TNF-α levels, NFκB levels after 12 h in group 4 were nonsignificantly lower than saline injected group (group 2) and significantly lower at 24 h in group 5. Additionally, group 5 levels were nonsignificantly different than those of control rats after 24 h. This indicates that NFκB production and further cascade of the inflammatory response could be inhibited by TLR4 antagonism. Although we clearly showed that NFκB inhibited the excessive inflammatory response in a LPS-induced inflammation model, further studies are required to evaluate the immune response when an infectious agent exists.

In the saline injected groups (groups 2 and 3), inflammatory response worsened by time, and serum and retinochoroidal tissue TNF-α and MDA levels were significantly higher at 24 h when compared to 12 h. NFκB levels were already high starting from the early stages of inflammation; thus no significant difference between 12 h and 24 h was identified. In the eritoran treated groups, although inflammation parameters were worse after 24 h than after 12 h, group 5 showed a better response to eritoran than group 4. This may be explained by the BRB effect. Eritoran is a large molecule (1400 kDa) and its capability to pass through the BRB is still unknown. We hypothesized that when inflammation worsened by time, disruption of the BRB might be increased, and thus the passage of eritoran may be more likely. Similarly, the histopathological evaluation suggested that systemically administered LPS caused retinochoroidal damage especially more severe at 24 h and some of this damage could be reversed by eritoran. As we compared 12 h and 24 h study groups, eritoran caused a nonsignificant reversal of inflammatory damage at 12 h but this trend was more prominent and significant at 24 h although sepsis parameters in the tissue have worsened. This explains at least in part why inflammatory parameters have worsened after 24 h, while at this time the passage of the drug would be easier and so a better response would be expected. As a limitation of our study, since the local behaviour of eritoran in the retinochoroidal tissue and BRB is unknown, we probably demonstrated the potential systemic beneficial effects of eritoran rather than a local effect. Further studies are needed to investigate the local effect of eritoran when administered intravitreally and the passage of eritoran through the BRB.

The change in the TNF-α level in the eritoran treated groups at 12 h (group 4) and 24 h (group 5) was not significant, whereas the changes in MDA levels were significant. Although the mechanism is unclear, this indicates that TNF-α levels were lowered more rapidly than that of MDA. As indicated above, this may be associated with a greater MDA load in tissues or covalent bonds between MDA and intracellular proteins.

This present study has several limitations. The effect of eritoran was tested only after one injection at 12th and 24th h of the study. The effect of eritoran on retinochoroidal damage should be investigated for a longer time period and with multiple injections as well.

In conclusion, both serum and retinochoroidal tissue levels of TNF-α and MDA and tissue levels of NFκB improved in a rat model of LPS-induced inflammation when treated with eritoran, a TLR4 antagonist. To the best of our knowledge, this is the first report of the effect of eritoran on retinochoroidal inflammatory damage. Our data suggests that investigation of the effect of eritoran in other ocular diseases in which inflammatory processes may play a role including retinochoroiditis, posterior uveitis, senile macular degeneration, and diabetic retinopathy is warranted. Further investigations should evaluate the passage of eritoran through biological barriers, the toxic doses, and the results upon intravitreal administration.

Conflict of Interests

The authors report no conflict of interests or any financial interests to disclose. The authors alone are responsible for the content and writing of the paper.

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Research Article

Proinflammatory Cytokines and C-Reactive Protein in Uveitis Associated with Behçet's Disease

Marina Mesquida,[1] Blanca Molins,[1,2] Victor Llorenç,[1] Maite Sainz de la Maza,[1] María Victoria Hernandez,[3] Gerard Espinosa,[4] and Alfredo Adán[1]

[1] *Institut Clínic d'Oftalmologia, Hospital Clinic de Barcelona, University of Barcelona, Sabino de Arana 1, 08028 Barcelona, Spain*
[2] *Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain*
[3] *Rheumatology Department, Hospital Clinic de Barcelona, University of Barcelona, Barcelona, Spain*
[4] *Autoimmune Disease Department, Hospital Clinic de Barcelona, University of Barcelona, Barcelona, Spain*

Correspondence should be addressed to Blanca Molins; bmolins@clinic.ub.es

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The aim of the present study was to determine the serum cytokine profile and levels of high sensitivity C-reactive protein (hsCRP) in patients with uveitis associated with Behçet's disease (BD) and to compare them with those obtained from healthy control subjects. We determined the serum concentration of interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-12p70, IL-17A, tumor necrosis factor-α (TNF-α), and hsCRP in 13 patients with active uveitis associated to BD, 24 inactive BD patients, and 20 controls. In a subgroup of 10 active patients, a second serum sample was obtained when the disease was inactive. Cytokine profiles and hsCRP levels were correlated with disease activity, severity, complications, and visual outcome. Levels of IFN-γ and TNF-α were significantly increased in patients with active uveitis associated to BD compared to controls ($P < 0.05$). IFN-γ, TNF-α, and hsCRP were significantly higher during active uveitis associated to BD compared to inactive disease ($P < 0.05$). Furthermore, IL-17A was significantly increased in patients with active BD without pharmacological treatment compared to controls ($P < 0.05$). No significant correlations were found with specific cytokine profiles and disease severity, visual outcome, or complications. In summary, increased serum levels of IFN-γ, TNF-α, IL-17A, and hsCRP were associated with active uveitis associated with BD and might serve as markers of disease activity.

1. Introduction

Behçet's disease (BD) is a chronic systemic inflammatory disorder at the crossroad between autoimmune and autoinflammatory syndromes. Major symptoms include oral aphthous ulcers, genital ulcerations, skin lesions, and ocular lesions [1]. Uveitis, associated with BD, is a sight-threatening condition that affects 60–80% of BD patients and is characterized in its more severe form by posterior or panuveitis including occlusive retinal vasculitis [2]. Although the pathogenesis of BD remains poorly characterized, it is currently believed that certain infectious and/or environmental factors are able to trigger symptomatology in predisposed individuals [2]. Association with Class I MHC (HLA-B*51) may predispose to inflammation with engagement of the innate immune system, and further perpetuation by the adaptive T cell responses against infectious antigens and/or autoantigens [1, 3]. The aim of therapeutic strategy in BD is to prevent the recurrent inflammatory attacks in order to minimize potential irreversible damage. Systemic corticosteroids (CS) and immunosuppressive (IS) drugs are the first line of treatment for severe manifestations of BD [4, 5] but often do not result in stable remission and a subgroup of patients keep on suffering flare-ups despite conventional therapy [6]. In these cases, novel biologic response modifier (BRM) drugs, particularly TNF-α antagonists, have emerged as a valid option resulting in substantial improvement of long-term visual prognosis and patients' quality of life [7].

The current hypothesis for the pathogenesis of BD states that genetic factors induce a general hyperactivity of the

immune system and bacterial or viral infection stimulates the expression of heat shock proteins (HSP) and MICA (MHC Class I chain-related molecules A). This induces the upregulation of adhesion molecules in the endothelium (ICAM-1 and VCAM-1), activation of coagulation, and stimulation of T cells by secreting IL-6, IL-8, and MCP-1 among others, continuing elevation of the cytokine production eventually leading to tissue damage and vasculitis (reviewed in [8]). Previous studies suggested that BD pathogenesis is predominated by a T helper (Th)1 and Th17 immune response [9–13] as increased levels of IFN-γ, IL-12, and TNF-α have been found in patients with BD [10]. Active BD was also shown to be characterized by increased levels of IL-17A [11–13]. Furthermore, genetic studies including genome-wide association studies, identified IL23R-IL12RB2 and IL10 as BD susceptibility loci [14, 15]. Therefore, Th1/Th17-type immune responses may play a critical role in BD. However, it is unclear whether Th1/Th17-related cytokines could serve as biomarkers of disease activity and the effect of both standard and novel therapies on the circulating levels of these cytokines in BD patients is unknown. Moreover, it is also ill-defined whether uveitis associated with BD is characterized by altered levels of acute phase reactants such as high-sensitivity (hs) C-reactive protein (CRP) that may eventually exacerbate the inflammatory burst further modifying serum inflammatory cytokine levels.

In the present study, we used a multiplex assay to study the profile of 5 cytokines and hsCRP in serum samples obtained from patients with active and inactive BD-associated uveitis and an age-matched healthy control group. In a subset of patients, the cytokine profile was analyzed longitudinally during active and inactive stages of the disease. Additionally, cytokine profiles were correlated with visual outcome, disease severity, and ocular complications.

2. Materials and Methods

2.1. Subjects. Patients were recruited from the Ophthalmology Department, Hospital Clinic of Barcelona (Spain), between January 2011 and July 2013. Thirty-seven patients aged 22–71 years (mean age: 40.6 ± 10.8) with uveitis associated with BD were invited to participate in the study. Twenty-two patients were male and 15 were female. Thirty-two patients were Caucasians and 5 were from North of Africa.

Inclusion criteria were patients diagnosed with BD fulfilling the diagnostic criteria of the International Study Group [16] who presented with intraocular inflammation. The diagnosis of active disease followed the clinical criteria based on inflammatory cell reaction in the anterior chamber or vitreous > 0.5+ as per Standardization of Uveitis Nomenclature (SUN) and National Eye Institute (NEI) grading system [17, 18]. Active retinal lesions and retinal vasculitis were evaluated by indirect ophthalmoscopy, fundus autofluorescence, and/or fundus fluorescein angiography. Any mentioned inflammatory sign (i.e., anterior chamber cell > 0.5+, vitreous cells > 0.5+, active retinal vasculitis, or active chorioretinal lesions) was enough to be eligible. Ocular complications included macular edema; tractional, serous, or rhegmatogenous retinal detachment; retinal tear; retinal neovascularization; epiretinal membrane; persistent vitreous opacities; vitreous hemorrhage; and optic atrophy. BD was considered severe when it included any of the mentioned complications.

Visual acuity was measured with Snellen charts and was converted to logarithm of the minimum angle of resolution (log-MAR) values for statistical analysis. Table 1 summarizes different features of patients and controls. Immunomodulatory treatments that patients were receiving (i.e., CS, IS, and/or BRM) were also gathered.

For comparison, 20 age-matched (mean age: 40.1 years) and gender-matched (10 women and 10 men) healthy subjects (by clinical anamnesis, ocular examination, and complete blood analysis), with no evidence of active ocular disease, who were waiting for unrelated ophthalmic surgery (cataract, eyelid benign lesions, etc.), were recruited as a control group.

All patients and controls provided informed consent and the research followed the tenets of the Declaration of Helsinki. The Hospital Clinic of Barcelona Institutional Review Board (IRB) approved this study according to local and national IRB guidelines.

Blood was collected aseptically just after ophthalmological evaluation and serum was prepared and stored at −70°C until further cytokine analysis. A total of 13 samples were obtained from patients with active uveitis and 24 samples were collected from patients with inactive disease. In a subset of 10 patients, the cytokine profile was analyzed longitudinally during active and inactive stages of the disease during a mean follow-up of 7.2 ± 1.1 months.

2.2. Serum Cytokine Determination. The Luminex platform (Millipore's MILLIPLEX Human Cytokine/Chemokine kit) was used to measure serum cytokine levels as recommended by the manufacturer. Five immune mediators associated with Th1 and Th17 responses (IFN-γ, IL-12p70, IL-17A, IL-1β, and TNF-α) were determined. These cytokines were chosen based on the results of a preliminary study (data not shown) where 10 cytokines were analyzed (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-17A, and TNF-α). The cytokine and chemokine assay plate layout consisted of 7 standards in duplicate (3.2–2,000 pg/mL), 1 blank well (for background fluorescence subtraction), 2 internal quality control samples in duplicate, and 25 μL duplicates of each serum sample.

2.3. Determination of Levels of C-Reactive Protein. Levels of hsCRP in serum samples were determined by CRP high sensitivity ELISA (hsCRP, IBL International GMBH) following the manufacturer's instructions.

2.4. Statistical Analysis. Nonparametric analysis was performed using the Mann-Whitney test for comparison of unpaired data from two groups and Wilcoxon test for comparison of paired data. Differences among the three groups were evaluated using the Kruskal-Wallis test. Spearman's correlation analysis was carried out to determine the association of serum cytokine levels with clinical parameters. Statistical

Table 1: Clinical characteristics of BD patients and normal controls.

Parameters	Normal controls ($n = 20$)	Active BD patients ($n = 13$)	Inactive BD patients ($n = 24$)
Age (years)	40,1 ± 4,7	31,7 ± 6,8	44,9 ± 9,7
Female/male (n)	10/10	6/7	9/15
Anterior uveitis	NA	0/13	4/24
Posterior uveitis	NA	5/13	12/24
Panuveitis	NA	8/13	8/24
Cells in anterior chamber	NA	8/13	0/24
Vitreous cells	NA	13/13	2/24
Retinal vasculitis	NA	10/13	0/24
Oral ulcers*	NA	11/13	2/24
Genital organ ulcers*	NA	9/13	1/24
Erythema nodosum*	NA	4/13	0/24
Arthritis*	NA	2/13	0/24
Visual acuity (logMAR)	ND	0,36 ± 0,30	0,45 ± 0,73

Data are shown as mean ± SD or absolute numbers.
*Extraocular lesions observed at the time of blood sampling are shown in this table. Patients whose extraocular lesions were negative may have experienced these symptoms during an earlier phase of the disease.
BD: Behçet's disease; NA: not applicable; ND: not determined.

significance was set at $P < 0.05$. All calculations were performed using SPSS Version 18.0 (SPSS, IBM Corporation, New York).

3. Results

Thirty-seven patients (22 male and 15 female) with uveitis associated with BD were included in the study. When serum sample was obtained, patients with active uveitis associated with BD were receiving the following therapies: 3/13 patients were not receiving any immunomodulatory treatment (naïve subjects with first episode of uveitis attack), 7/13 were treated with BRM (TNF-α antagonists), 2/13 were receiving CS, and 1/13 was receiving conventional IS therapy. With regard to the inactive patients group, 13/24 were not receiving any immunomodulatory treatment due to long-standing remission, whereas 2 patients were under low-dose CS, 8 patients were receiving BRM (TNF-α antagonists), and one patient was on IS therapy.

Firstly, we analyzed differences in the serum cytokine profile among patients with active disease, inactive disease, and healthy subjects. For this purpose the cytokine levels of 13 patients with active ocular BD, 24 different patients with inactive BD, and 20 controls were determined. Figure 1 shows IFN-γ, IL-12p70, IL-17A, IL-1β, and TNF-α serum cytokine levels from each group. Active patients showed the highest levels of IFN-γ, IL-17A, IL-1β, and TNF-α, whereas the control group had the lowest levels of these cytokines. IFN-γ and TNF-α levels were significantly higher in active patients compared to control subjects (Figures 1(a) and 1(e); $P < 0.05$). In fact, IFN-γ correlated well with IL-17A (Spearman's rho $r = 0.833$; $P < 0.01$) and with TNF-α ($r = 0.855$; $P < 0.01$) in active patients. TNF-α also correlated well with IL-17A ($r = 0.663$; $P < 0.05$). Patients with active disease also showed significantly higher levels of hsCRP compared to inactive patients (5.91 ± 1.65 mg/L active versus 2.19 ± 0.53 mg/L inactive; $P = 0.029$).

In a subgroup of 10 patients, serum samples were obtained during both active and inactive phases of the disease and, as shown in Figure 2, IFN-γ and TNF-α levels were significantly higher during the active phase compared to the inactive period.

Interestingly, as shown in Figure 3, when considering only those samples from patients without any pharmacological treatment ($n = 3$ active and $n = 13$ inactive), IL-17A levels were also significantly higher in active patients compared to healthy subjects (Figure 3(b), $P < 0.05$). In addition, untreated inactive patients also showed significantly higher levels of TNF-α compared to healthy subjects (Figure 3(c), $P < 0.05$).

We then analyzed the effect of pharmacological treatment on the circulating levels of the different cytokines. Thus, serum cytokine levels in active and inactive patients were compared between patients with or without treatment. As observed in Figure 4, IFN-γ, IL-17A, and TNF-α levels were higher in patients without treatment, although only TNF-α levels in active patients with no treatment were statistically different from those of active patients with treatment (Figure 4(c)). However, we were unable to observe differences in any of the cytokines among the different treatments (data not shown).

Finally, none of the analyzed cytokines correlated with visual acuity or disease severity in neither active nor inactive BD patients and there were no significant differences between cytokine or hsCRP levels of patients with occlusive vasculitis ($n = 5$) and those without complications (data not shown).

4. Discussion

Behçet's disease (BD) is a chronic multisystemic inflammatory disease, where autoimmunity seems to play a crucial role [19, 20]. Ocular involvement affects around 70% of BD patients [21] and is characterized by relapsing nongranulomatous uveitis involving both the anterior and the posterior

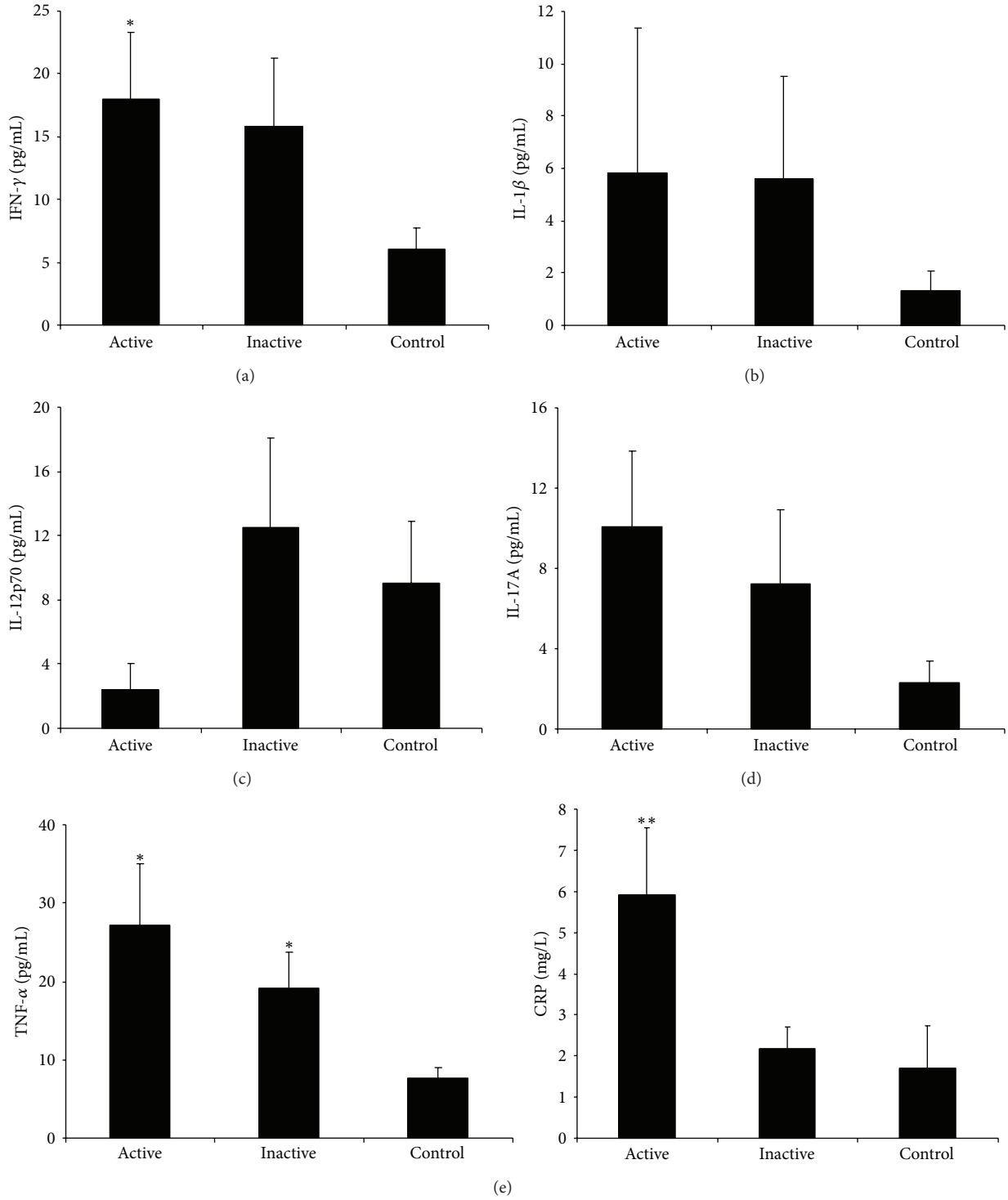

Figure 1: Cytokine profile and hsCRP levels of serum samples of patients with active BD ($N = 13$), inactive ocular BD ($N = 24$), and control subjects ($N = 20$). Results are expressed as mean values of cytokine levels (pg/mL) ± SEM. Statistical analysis was performed by Kruskal-Wallis test ($^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus control and inactive).

segments of the eye. These ocular lesions are often sight-threatening, requiring prompt and aggressive treatment to preserve vision [22]. Although several reports have suggested involvement of Th1 and Th17 immune responses in BD [9, 10], it is still unclear whether Th1- and Th17-related cytokines may serve as markers of disease activity, severity, and/or visual outcome. In the present study we aimed to study the serum cytokine profile and hsCRP levels in patients with uveitis

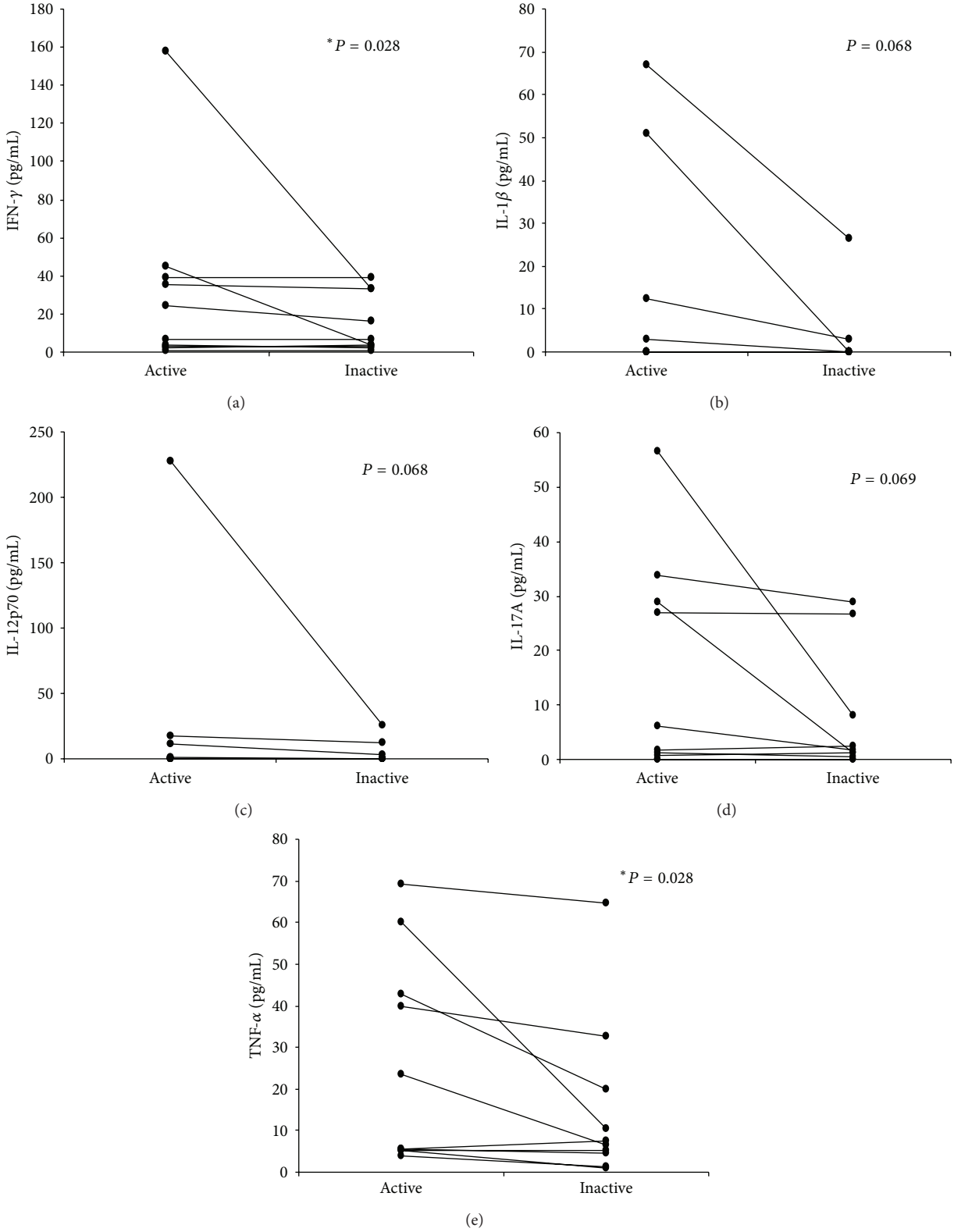

Figure 2: Levels of IFN-γ, IL-12p70, IL-17A, IL-1β, and TNF-α (pg/mL) ± SEM in the serum of patients with uveitis associated with BD during the active and inactive stages ($N = 10$). Statistical analysis was conducted using the Wilcoxon test ($^*P < 0.05$).

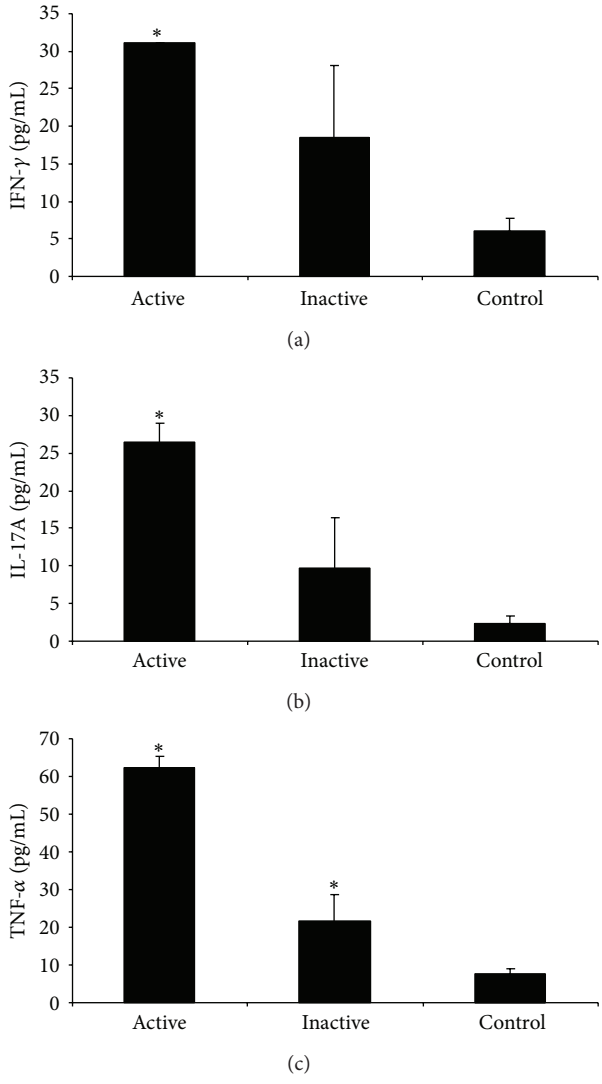

Figure 3: Circulating levels of IFN-γ, IL-17A, and TNF-α in patients with active BD ($N = 3$) and inactive BD ($N = 13$) without treatment and control subjects ($N = 20$). Results are expressed as mean values of cytokine levels (pg/mL) ± SEM. Statistical analysis was performed by Kruskal-Wallis test ($^*P < 0.05$).

associated with BD and its relationship with visual outcome and disease severity. We showed that IFN-γ, IL-17A, TNF-α, and hsCRP are increased in active uveitis associated with BD.

We observed that BD patients with active uveitis showed significantly higher levels of IFN-γ and TNF-α compared to healthy subjects. These results are in agreement with previously published observations that reported increased levels of IFN-γ and TNF-α in active BD without treatment, thus supporting a Th1 immune response [9, 10]. Interestingly, we observed these effects not only in patients without any treatment but also in those undergoing pharmacological treatment. Moreover, the analysis of the cytokine profile in the subgroup of patients to whom we obtained serum during both active and inactive stages also revealed that IFN-γ and TNF-α significantly increased during the active stage of the disease. Similarly, IL-17A was increased in patients with active uveitis compared to healthy controls. However, this increase was statistically significant only in those patients without treatment. These observations are in agreement with other groups who found increased levels of circulating IL-17A in patients with active BD compared to healthy subjects [11, 12], supporting the involvement of Th17 response in BD. We observed that IFN-γ correlated well with TNF-α and IL-17A in patients with active BD. Additionally, TNF-α also correlated well with IL-17A in these patients. These observations support the idea that BD is mediated simultaneously by both Th1 and Th17 responses. On the other hand, although IL-1β and IL-12 seemed to be increased in active BD, the difference did not reach statistical significance and none of the analyzed mediators correlated with visual outcome or disease severity. Similarly, we were unable to detect differences in the cytokine profile of BD patients with occlusive vasculitis and in those without.

In the present work we also observed significantly higher levels of circulating hsCRP in patients with active uveitis associated with BD compared to inactive patients. CRP is the prototypical acute phase reactant and an active regulator of the innate immune system. It is considered to be a serum biomarker for chronic inflammation [23]. Indeed, CRP was significantly higher in active patients compared not only to healthy subjects but also to inactive patients. Among the many functions ascribed to CRP are activation of the classical complement pathway and inactivation of the alternative pathway [24]. In plasma, CRP exists as a cyclic pentamer. However, CRP can undergo dissociation, upon exposure to acidic or inflammatory conditions, thereby acquiring distinct functionality [25, 26]. In fact, dissociated monomeric CRP has been shown to display a proinflammatory phenotype [27–30]. Therefore, the increased levels of circulating hsCRP in patients with active uveitis associated with BD could eventually contribute to the exacerbation of the inflammatory burst increasing the secretion of Th1- and Th17-proinflammatory cytokines.

The aim of therapeutic strategy in BD is to prevent recurrent inflammatory attacks in order to minimize potential irreversible damage. As mentioned above, classical treatment with systemic CS and conventional IS therapies may be effective in some cases [5]. Nonetheless, there is a subgroup of patients who keep on suffering flare-ups despite standard therapy. Thus, in the present work, we also aimed to analyze the effect of pharmacological treatment on the cytokine profile of BD. Indeed, we observed that IFN-γ, TNF-α, and IL-17A levels were higher in patients without treatment than in those with pharmacological treatment in both active and inactive patients. Nevertheless, we did not observe differences in the cytokine profile among the different treatments. Our observations suggest that successful therapies should be able to prevent recurrent inflammatory attacks by keeping under check the circulating levels of these proinflammatory cytokines. However, inactive patients maintained significantly higher levels of TNF-α than controls, thus keeping a proinflammatory background.

Our cohort of patients with uveitis associated with BD was very representative as it included patients with different demographic origins. In fact, we did not observe differences

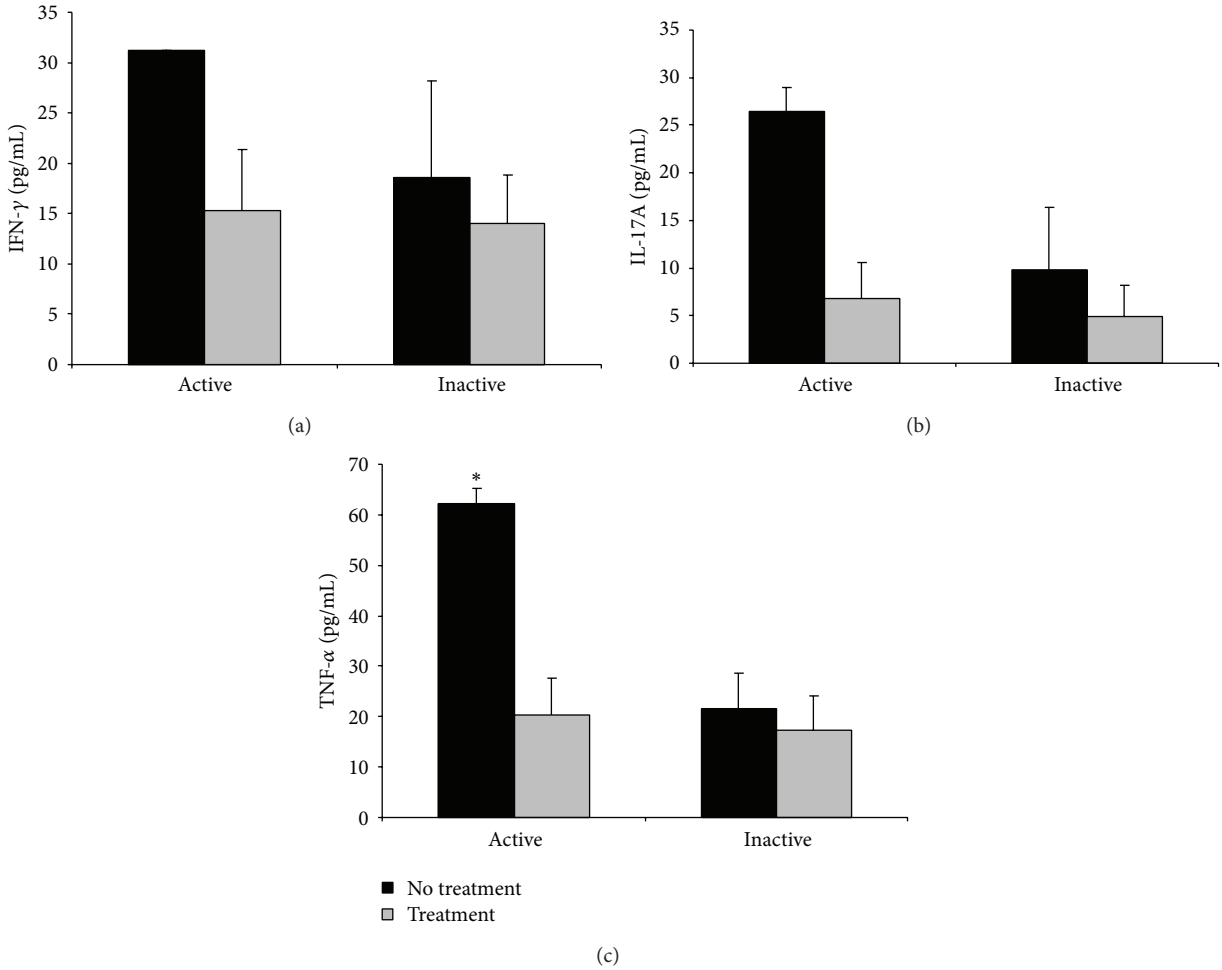

Figure 4: Effect of treatment on circulating levels of IFN-γ, IL-17A, and TNF-α (pg/mL) ± SEM in patients with active and inactive BD. Results are expressed as mean values of cytokine levels (pg/mL) ± SEM. Statistical analysis was performed by Mann Whitney U-test ($^{*}P < 0.05$).

in the cytokine profile between Caucasian and African patients (data not shown), which allows us to speculate that the observed increased levels of IFN-γ, TNF-α, and IL-17A may apply to uveitis associated with BD from any origin. The main limitation of the present work is the sample size, specially the group of active patients without treatment as well as the heterogeneity of treatment modalities. Nevertheless, the fact that our results were observed across such a heterogeneous group also reinforces our findings.

5. Conclusions

In conclusion, our study shows that active BD is associated with increased serum levels of IFN-γ, TNF-α, IL-17A, and hsCRP compared to inactive disease or healthy controls. Although further research is warranted to elucidate the role of these mediators in BD, serum cytokine profiling may contribute to the understanding of the physiopathology processes underlying retinal damage in BD and provide tools for new biomarkers and/or personalized treatment targets.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Marina Mesquida and Blanca Molins equally contributed to the paper.

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Research Article

Regulation of Toll-Like Receptor Expression in Human Conjunctival Epithelial Cells

Jing Li,[1] Melina Setiawan,[1] Hong Wu,[2] Roger W. Beuerman,[1,3] and Peiquan Zhao[1]

[1] *Department of Ophthalmology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, 1665 Kong Jiang Road, Shanghai 200092, China*
[2] *Department of Ophthalmology, Second Hospital of Jilin University, Changchun 130041, China*
[3] *Singapore Eye Research Institute, 11 Third Hospital Avenue, Singapore 168751*

Correspondence should be addressed to Peiquan Zhao; zhaopeiquan@126.com

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Previous studies showed marked decrease of multiple Toll-like receptor (TLR) expression in corneal and conjunctival epithelial cells upon culture in vitro. The aim of this study was to identify factor(s) which regulate TLR expression. Primary human conjunctival epithelial cells and immortal conjunctival (IOBA-NHC) and corneal epithelial cell lines (HCET) were used. The effect of various cytokines, hypoxia, mechanical wounding, and airlifting culture on TLR expression was examined by quantitative PCR and western blot analysis. Ligand stimulated TLR activation was analyzed. TLR mRNA expression increased modestly when cultured monolayered cells were stimulated by TNF-α, IL-1α, IL-1β, IL-6, IL-8, IFN-γ (about 2-fold), hypoxia (2.1- to 4.8-fold selectively), and wounding (3.1- to 9.3-fold). In airlifted multilayered cells, TLR expression increased 7.8- to 25.9-fold compared to monolayered cells. Airlifted cells showed increased response to low concentrations of lipopolysaccharide (LPS) and peptidoglycan (PGN) stimulation. NFκB inhibition prevented the formation of cell sheets and led to the collapse of already-formed multilayered structure and the simultaneous reduction of TLR mRNA level. In conclusion, our study showed that the conjunctival epithelial cell expressed TLR was sensitive to various stimulants, and a multilayered epithelium-like structure was needed to maintain TLR expression.

1. Introduction

Toll-like receptors (TLRs) are a family of pattern-recognition receptors [1]. Ten TLR proteins have been identified in human cells. Each TLR binds specific pathogen-associated molecular patterns (PAMPs) of viruses, bacteria, fungi, and parasites. Some TLRs, such as TLR3 and TLR4, also recognize damage-associated molecular patterns (DAMPs) released by injured cells [2]. The activation of TLR and its associated signaling pathway leads to a broad range of inflammatory responses mediated mainly by increased secretion of cytokines [3].

Ocular surface epithelial cells, namely, conjunctival and corneal epithelial cells, were known to express multiple TLRs [4–6]. Cultured monolayer cells were most often used to study the expression, function, and regulation of TLR in epithelial cells. While the most TLR expressed in epithelial cells showed responses to its ligands in cultured cells, contradictory results were reported on the biological activity of TLR2 and TLR4 in corneal and conjunctival epithelial cells [7–9]. In a previous study, we quantified and compared the expressions of multiple TLRs in primary cultured human conjunctival epithelial cells and conjunctival epithelium tissue and found that TLR expressions in cultured cells were much lower than those in undigested tissue [6]. Here, we explored the effect of multiple factors on TLR expression and report that the multilayered structure was a key factor to maintain a normal level of TLR expression in conjunctival and corneal epithelial cells.

2. Materials and Methods

2.1. Reagents. Unless otherwise specified, all cell culture medium and supplements were purchased from Life Technologies (Carlsbad, CA, USA). All plasticware for cell culture was purchased from Greiner Bio-One (Frickenhausen,

Germany). General reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot were purchased from Bio-Rad (Hercules, CA, USA). Lipopolysaccharide (LPS) isolated from *Pseudomonas aeruginosa* and peptidoglycan (PGN) isolated from *Bacillus subtilis* were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-Aminosalicylic acid (5-ASA, NFκB inhibitor) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Primary Conjunctival Epithelial Cell Isolation and Culture. Primary human conjunctival epithelial cells were isolated from cadaver conjunctival tissue obtained from Singapore Eye Bank as described before [10]. Briefly, after antibiotics/PBS washing, the conjunctival tissue was cut into small pieces and placed on cell culture plate with one drop of full medium which contained equal volume of DMEM and F12, 10% fetal bovine serum (FBS), 0.5 μg/mL hydrocortisone, 10 nM cholera toxin, 10 ng/mL human epidermal growth factor (hEGF), 5 μg/mL insulin, and antibiotics. Epithelial cell outgrowth was observed 2-3 days later, and the culture was maintained for 4-5 days before the tissues were discarded. The cells were then submerged-cultured in the same medium for further propagation. Passage 2 to 3 cells were used in this study. The study protocol was approved by the Institutional Review Board of Singapore Eye Research Institute and followed the tenets of the Declaration of Helsinki.

2.3. Conjunctival and Corneal Epithelial Cell Lines. Immortalized human conjunctival epithelial cell (IOBA-NHC) was a gift from Dr. Diebold at the University of Valladolid, Spain [11]. SV40 large T-antigen immortalized human corneal epithelial cell (HCET) was purchased from Riken Cell Bank (Ibaraki, Japan) [12]. Both cells were cultured in the same medium as the primary conjunctival epithelial cells.

2.4. Cell Treatment

2.4.1. Culture Supplement. A basic medium was prepared first which contained equal volume of DMEM and F12 with antibiotics. The following supplements were added individually to the basic medium: 1 μg/mL bovine insulin, 2 ng/mL recombinant hEGF, 0.5 μg/mL hydrocortisone, 10% FBS, and 0.1 μg/mL cholera toxin. The isolated primary human conjunctival epithelial cells were submerged in the above medium for 24 hrs, and TLR mRNA expression was compared to cells grown in full and basic medium.

2.4.2. Airlifted Culture. Airlifted culture refers to the culture condition in which cells were grown at the air-medium interphase. To achieve this, submerged-cultured cells were trypsinized and seeded on BioCoat collagen I coated 6-well inserts with 3.0 micron pore (BD Biosciences, San Jose, CA, USA). Cells in the inserts were submerged in the full medium for the first 24 hrs and continued in a medium which contained DMEM : F12 (3 : 1, V : V) with the same supplement as in the full medium, and the volume of medium was reduced so that only the bottom of the insert was in contact with the medium (about 1 mL per well). Epithelial cells started to form multilayered sheet after 3 days. The culture was maintained for 7–12 days depending on the progression of cell stratification, and the culture medium was changed every day.

2.4.3. Cytokine Stimulation. Selected cytokines were added to 80–90% confluent submerged cells for 24 hrs before the supernatant was harvested for cytokine secretion analysis.

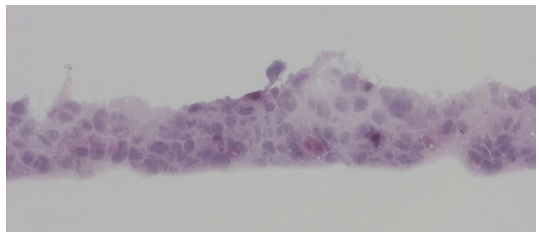
2.4.4. Cell Wounding. Eight to ten parallel scratches were made to 80–90% confluent submerged cells at about 1 cm space using a sterile surgical blade. Cells were then washed with PBS and given fresh medium after wounding. Supernatant was harvested 24 hrs later for cytokine secretion analysis.

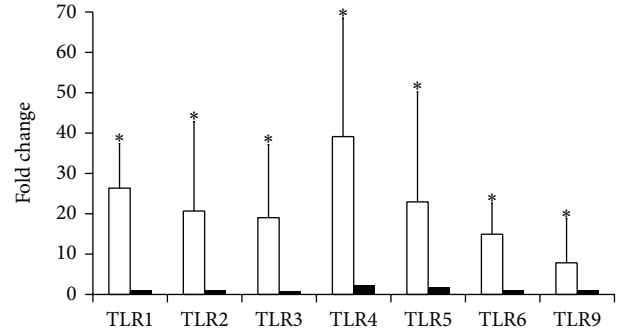
2.4.5. Hypoxia Stimulation. Hypoxia stimulation was achieved by culturing submerged cells in 37°C incubator with 95% nitrogen and 5% carbon dioxide for 24 hrs.

2.5. Quantitative PCR (qPCR) Analysis. Total RNA was extracted using NucleoSpin RNA II (Macherey-Nagel, Germany) and transcribed into cDNA using SuperScript III Reverse Transcriptase from Life Technologies. qPCR was performed using Taqman real-time PCR reagents as previously described [6]. β-Actin was used as internal control. The mRNA expression was calculated by the comparative expression methods of $2^{(-\delta\delta Ct)}$, where δCt is the threshold cycle and $\delta\delta Ct = \delta Ct_{\text{airlifted}} - \delta Ct_{\text{submerged}}$.

2.6. Western Blot Analysis. Cells were lysed in RIPA buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, and a protease inhibitor cocktail (Roche Diagnostics Asia Pacific, Singapore). Protein concentration was measured by Pierce Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Thirty micrograms of the total lysates was resolved on SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed with the following antibodies at 4°C overnight: goat anti-TLR1 antibody (R&D Systems, Minneapolis, MN) at the concentration of 2 ng/lane; monoclonal anti-TLR2 and anti-TLR3 at the dilution of 1 : 100 (Imgenex, San Diego, CA); rabbit anti-TLR4, rabbit anti-TLR5, and goat anti-TLR6 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at the dilution of 1 : 200; and monoclonal anti-TLR9 antibody (Abcam, Cambridge, UK) at the concentration of 1 μg/mL. Horseradish peroxidase conjugated species-specific secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) incubation was carried out at room temperature for 1 hr (1 : 2000 for anti-rabbit antibody sc-2030; 1 : 2000 for anti-mouse antibody sc-2005; and 1 : 5000 for anti-goat antibody sc-2350). The resulting immune complex was visualized using SuperSignal chemiluminescent substrates (Thermo Scientific, Rockford, IL, USA) and exposed to X-ray film.

2.7. IL-6 and IL-8 Secretion Analysis. IL-6 and IL-8 secretion was analyzed in the culture medium using OptEIA (BD Biosciences, USA) and corrected by total cellular protein and





(a) (b)

Figure 1: TLR mRNA expression in airlifted primary human conjunctival epithelial cells. (a) Hematoxylin and eosin staining of a representative cell sheet 7 days after airlifting culture. Typically, the cell sheet was composed of 3-4 layers of cells. Cells at the bottom were mostly cuboidal and more compact than the cells at the upper layers. (b) Averaged fold increase of individual TLR mRNA in airlifted (white bars) and replated conjunctival epithelial cells (black bars). Cells were harvested 10 days after airlifting culture. Half of the cells were replated to 6-well plate and submerged-cultured for 24 hrs in 1 : 1 medium with full supplement before being lysed for RNA extraction. TLR mRNA level was determined by qPCR and compared to the same batch of cells submerged-cultured. Three different batches of cells were analyzed, and the fold increase was averaged. Y error bars represent the standard deviation of the averaged results. Asterisks (∗) denote significant difference ($P < 0.05$) between airlifted and replated cells.

total medium volume. For each experiment, an aliquot of the culture medium was saved, briefly spun, and stored at −80°C for ELISA analysis. Total medium volume (mL) at the time of harvesting was recorded. Cells were washed in PBS twice and lysed, and total protein content was measured with Pierce Micro BCA Protein Assay Kit. IL-6 and IL-8 secretion was expressed as follows: cytokine concentration (pg/mL) multiplied by total medium (mL) volume divided by total cell protein content (mg). The final unit was pg cytokine/mg cell protein.

2.8. NFκB Activity Analysis. The activities of p65 and p50 subunits of NFκB were measured by ELISA analysis using 96-well plates precoated with NFκB-binding DNA consensus sequence (Pierce, Rockford, IL). Only the active form of p65 and p50 binds to the immobilized DNA sequence, and the bound protein was subsequently detected by specific primary antibody against p65 and p50 followed by HRP conjugated secondary antibody. The chemiluminescence signal was measured by Victor X3 microplate reader (Perkin Elmer, China). Data were expressed as the mean ± SE.

2.9. Statistical Analysis. Data were expressed as mean ± standard deviation and analyzed by analysis of variance (ANOVA) after Levene's test for homogeneity, followed by the Fisher least significant difference (LSD) test. A probability level of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Culture Medium Supplements Had No Effect on TLR Gene Expression in Primary Cultured Conjunctival Epithelial Cells. No changes in TLR mRNA expression were measured by qPCR in monolayered cells cultured in the basic medium with additional insulin, recombinant hEGF, hydrocortisone, FBS, or cholera toxin (data not shown).

3.2. Airlifting Culture Stimulated TLR mRNA and Protein Expression. Primary conjunctival epithelial cells formed multilayered, stratified structure when airlifted (Figure 1(a)). Increased TLR mRNA expression was first detected by qPCR in cells 3 days after airlifting and continued to increase as cells form stratified sheets. Table 1 lists the δCt values for TLR mRNA in submerged and 10-day airlifted primary human conjunctival cells. The averaged fold increase of each TLR mRNA is presented in Figure 1(b). TLR4 mRNA expression as detected in only one batch of submerged primary cells but in all airlifted cells.

Next, we replated the airlifted primary cells on 6-well plate and maintained at submerged condition. Less than 10% of the cells adhered to the culture dish after 24 hrs. TLR mRNA expression decreased to levels prior to airlifting culture (Figure 1(b)). In cells which had no detectable amount of TLR4 mRNA, it remained positive when the airlifted cells were replated.

The same experiments were repeated in immortalized human corneal (HCET) and conjunctival epithelial cells (IOBA-NHC). Differences in TLR mRNA expression were noticed (Table 1). TLR6 mRNA expression was not detected in HCET cells by qPCR. Airlifting culture induced further increase of all TLR mRNA in both cell lines, however, at a smaller magnitude than the primary cells (Figures 2(a) and 2(b)).

Due to the limited availability of primary human cells, we used IOBA-NHC cells to compare the changes of TLR protein expression before and after airlifting culture. Western blot analysis revealed increased TLR1, TLR2, TLR3, TLR4, and TLR5 expression, which largely matched the increase of the respective mRNA (Figures 2(c) and 2(d)). Because TLR2 and TLR5 were barely detectable in submerged-cultured

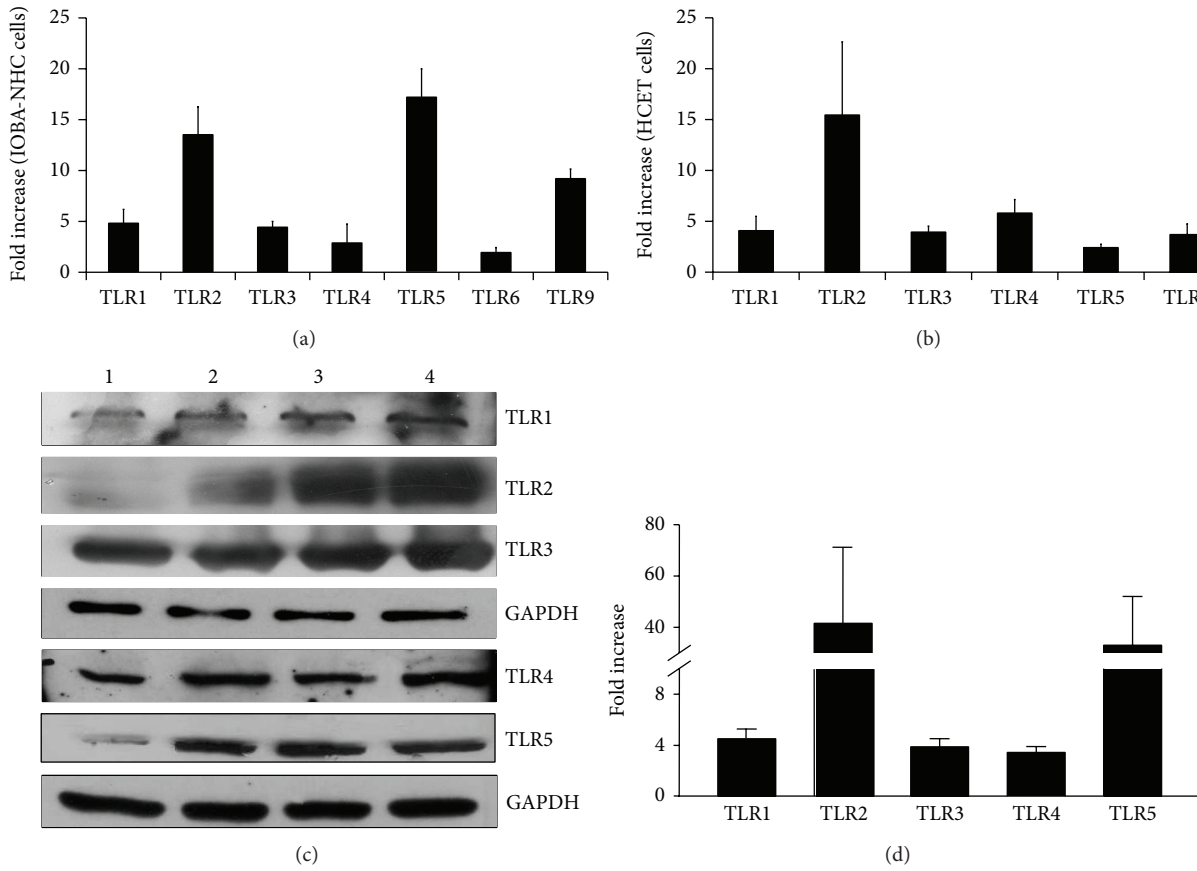


Figure 2: TLR mRNA and protein expression in airlifted immortal human corneal (HCET) and conjunctival epithelial cells (IOBA-NHC). (a) Averaged fold increase of TLR mRNA in IOBA-NHC cells. The increase was statistically significant for each TLR. (b) Averaged fold increase of TLR mRNA in HCET cells. The increase was statistically significant for each TLR. Cells were airlifted for 10 days. TLR mRNA level was determined by qPCR and compared to submerged cells. The experiment was repeated 3 times. *Y* error bars represent the standard deviation of the averaged results. (c) Micrographs of a representative western blot showing TLR protein expression in IOBA-NHC cells. Lane 1: submerged-cultured; lane 2: 3 days after airlifting culture; lane 3: 7 days after airlifting culture; lane 4: 10 days after airlifting culture. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was probed as loading control. (d) Averaged TLR protein increase in IOBA-NHC cells 10 days after airlifting culture compared to submerged-cultured cells. X-ray films from 2 independent western blot experiments were scanned in a densitometer, and the results were averaged. The increase was statistically significant for each TLR protein tested.

Table 1: ΔCt of individual TLR mRNA in submerged and airlifted primary human conjunctival epithelial cells, immortalized human conjunctival epithelial cells (IOBA-NHC), and immortalized human corneal epithelial cells (HCET).

mRNA	Primary conjunctival epithelial cells ($n = 3$)		IOBA-NHC cells		HCET cells	
	Submerged	Airlifted	Submerged	Airlifted	Submerged	Airlifted
TLR1	10.97 ± 1.08	6.27 ± 0.72	7.02 ± 0.36	4.77 ± 0.42	10.58 ± 0.03	8.55 ± 0.14
TLR2	10.51 ± 1.84	6.14 ± 1.32	8.92 ± 0.24	5.17 ± 0.56	5.93 ± 0.38	1.98 ± 0.18
TLR3	9.13 ± 1.93	4.88 ± 1.23	4.69 ± 0.18	2.57 ± 0.38	4.63 ± 0.17	2.65 ± 0.26
TLR4	14.93 ($n = 1$)	9.74 ± 1.01	3.06 ± 0.14	1.57 ± 0.30	7.25 ± 0.04	4.71 ± 0.10
TLR5	10.61 ± 2.08	6.28 ± 1.13	10.85 ± 1.79	6.75 ± 0.18	5.64 ± 0.17	4.36 ± 0.09
TLR6	12.41 ± 1.02	8.51 ± 0.53	5.39 ± 0.48	4.48 ± 0.51	ND	ND
TLR9	13.32 ± 1.19	10.36 ± 0.78	9.54 ± 0.73	6.35 ± 0.16	10.10 ± 0.46	8.21 ± 0.79

ΔCt was calculated using β-actin as internal control. Data was presented as average ± standard deviation. The experiment was performed three times for each condition. For primary cells, the results shown were the averaged δCt of three different batches of cells. "Submerged" refers to submerged-cultured cells at 80–90% confluence. "Airlifted" refers to airlifted culture at 10 days. ND stands for nondetected.

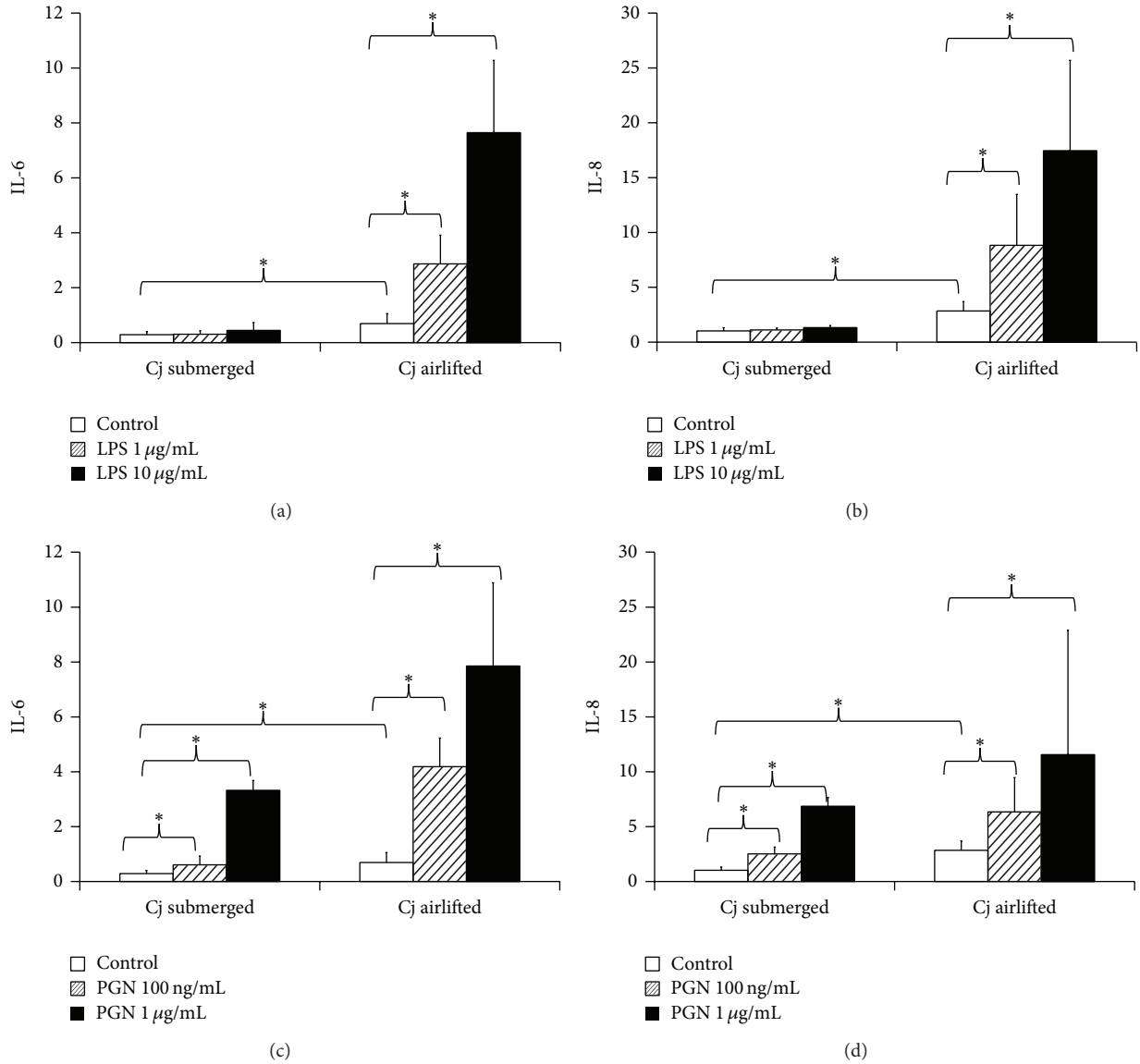


Figure 3: LPS and PGN stimulated IL-6 and IL-8 secretion in submerged and airlifted primary conjunctival epithelial cells. (a) LPS stimulated IL-6 secretion. (b) LPS stimulated IL-8 secretion. (c) PGN stimulated IL-6 secretion. (d) PGN stimulated IL-8 secretion. IL-6 and IL-8 concentrations were expressed as pg/mL medium/mg total cellular protein. Asterisks (∗) denote significant difference ($P < 0.05$ by paired t-test) between two groups. Y error bars represent the standard deviation of the averaged results from 3 different experiments.

IOBA-NHC cells, the calculated increase for the protein was greater than that for its respective mRNA. Similar results were obtained from HCE cells (data not shown).

3.3. Airlifted Conjunctival Epithelial Cells Responded to Low Concentrations of LPS and PGN Stimulation. To test the effect of increased TLR expression in airlifted cells, we compared LPS and PGN stimulated IL-6 and IL-8 secretion in primary conjunctival epithelial cells cultured under submerged and airlifted conditions.

Compared to submerged culture, airlifting required less medium level and had higher cell density. To correct for these differences, we calculated cytokine concentrations in the unit of pg/mL medium/mg total cell protein. We found that both IL-6 and IL-8 concentrations were significantly higher in the airlifting culture medium than in the submerging culture medium without added ligands (Figure 3).

When incubated with 1 and 10 μg/mL LPS for 24 hrs, only airlifted cells showed increased IL-6 and IL-8 secretion. No significant changes were measured in the submerged-cultured cells (Figures 3(a) and 3(b)).

When incubated with 100 ng/mL and 1 μg/mL PGN for 24 hrs, the airlifted cells showed more increase of both IL-6 and IL-8 compared to the submerged cells (Figures 3(c) and 3(d)).

3.4. Airlifted Conjunctival Epithelial Cells Were Sensitive to NFκB Inhibition. In an attempt to explore the mechanisms

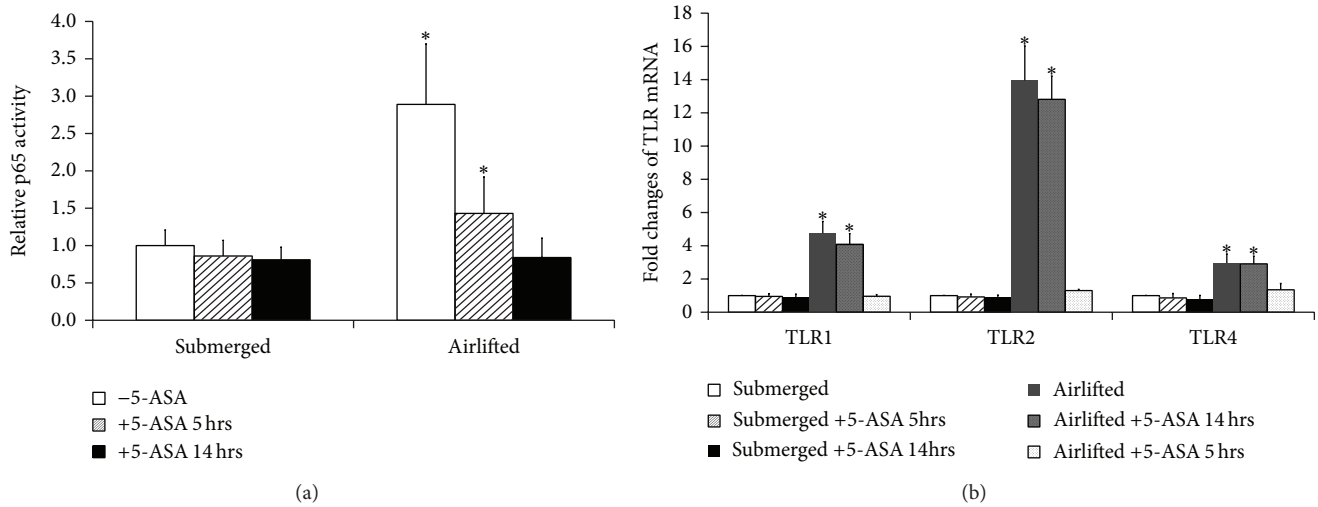


Figure 4: The effect of NFκB inhibition on airlifted IOBA-NHC TLR expression. (a) Relative activity of p65 in submerged and airlifted IOBA-NHC cells with and without 5-ASA. p65 activity in submerged monolayer cells without 5-ASA was set as 1, and the activities of other cell conditions were compared to it. Asterisks (∗) denote significant difference ($P < 0.05$) between airlifted and submerged cells under the same treatment. (b) Fold changes of selective TLR mRNA expression by qPCR. Individual TLR expression in submerged monolayer cells without 5-ASA was used as control to calculate the fold change. Asterisks (∗) denote significant difference ($P < 0.05$) between airlifted and submerged cells under the same treatment. All experiments were repeated three times, and the data were averaged. *Y* error bars represent the standard deviation of the averaged results.

underlying increased TLR expression in airlifted epithelial cells, we studied the response of submerged and airlifted primary cultured conjunctival epithelial cells and IOBA-NHC cells to the treatment of 5-ASA, an inhibitor of NFκB.

Neither primary cells nor immortal cells were able to form multilayered sheets in the presence of 5-ASA. When 5-ASA was added to the airlifted multilayered cells (4-5 days after airlifting culture), we observed an almost complete collapse of the multilayered structure 14 hrs after the drug treatment. Airlifted IOBA-NHC cells showed higher NFκB activity than submerged cells (Figure 4(a)). At 5 hrs after 5-ASA treatment, IOBA-NHC cells showed decreased NFκB activity but little changes in TLR expression. TLR mRNA was reduced to close to the pre-airlifting level at 14 hrs after the treatment (Figure 4(b)). However, we found no significant changes of TLR mRNA with 5-ASA treatment in monolayered cells.

3.5. Proinflammatory Cytokines Stimulated TLR mRNA Expression in Conjunctival Epithelial Cells. We examined TLR mRNA expression in submerged primary cultured conjunctival epithelial cells in the presence of 10 ng/mL TNF-α, IL-1α, IL-1β, IL-6, and IL-8; 40 ng/mL IFN-γ alone; and 50 ng/mL of LPS. Different batches of primary cells showed different responses to cytokine stimulation. Overall, no consistent or significant changes of TLR4 and TLR6 mRNA (larger than 2-fold increase in all cells tested) were detected when cells were stimulated with the above cytokines. Small increase (2-3-fold) of TLR1, TLR2, TLR3, TLR5, and TLR9 mRNA was observed when stimulated with TNF-α and IL-1α, but not with IL-1β, IL-6, or IL-8. On average, IFN-γ stimulated the expression of TLR3 and TLR9 mRNA by more than 2-fold, but not other TLRs. While 50 ng/mL LPS alone did not have significant effect on TLR mRNA expression, it showed synergistic effect when costimulated with some cytokines. The changes of TLR1 and TLR2 mRNA were shown as representatives (Figure 5).

3.6. Hypoxia Stimulated TLR mRNA Expression in Conjunctival Epithelial Cells. Hypoxic culture stimulated TLR2 and TLR3 mRNA expression in submerged-cultured primary human conjunctival epithelial cells (Figure 6). A 4.81-fold increase of TLR2 mRNA was observed in cells 16 hrs after hypoxia culture and remained increased by 4.00-fold at 24 hrs (Figure 6). TLR3 mRNA increased 2.04-fold at 4 hrs after hypoxia, peaked at 16 hrs, and remained elevated at 24 hrs. No changes of TLR1, TLR4, and TLR5 mRNA expression were observed.

3.7. Wounding Stimulated TLR mRNA Expression in Conjunctival Epithelial Cells. Wounding stimulated the expression of most TLR mRNA in submerged-cultured cells (Figure 7). Specifically, TLR3 mRNA increased 9.3-fold; TLR9, TLR2, and TLR6 increased 6.2-, 4.8-, and 4.2-fold, respectively. TLR5 and TLR2 mRNA increased 3.3- and 3.1-fold. However, TLR4 mRNA remained unexpressed in all three batches of primary cells tested.

4. Discussion

In this study, we showed that TLR expression in cultured human conjunctival epithelial cells could be stimulated by proinflammatory cytokines, hypoxia, wounding, and airlifting culture, but not by culture medium supplement. Among these, airlifting culture had the most powerful effect on TLR expression. In airlifted conjunctival cells, the expression

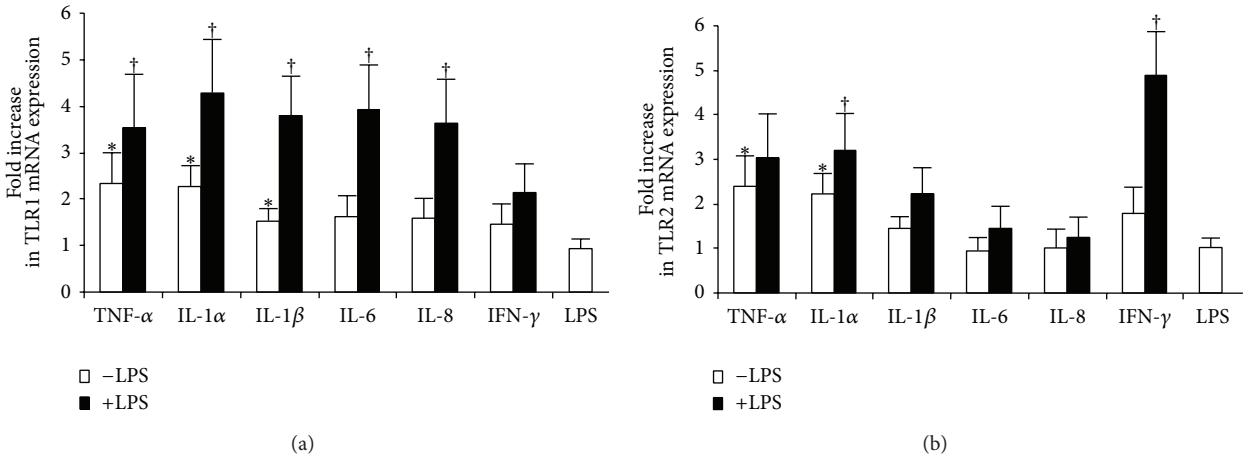


Figure 5: TNF-α (10 ng/mL), IL-1α (10 ng/mL), IL-1β (10 ng/mL), IL-6 (10 ng/mL), IL-8 (10 ng/mL), and IFN-γ (40 ng/mL) stimulated TLR1 and TLR2 mRNA expression in submerged primary conjunctival epithelial cells with and without 50 ng/mL LPS. TLR expression in cells cultured in normal medium was used as reference for the calculation of fold increase. Open bars represent the averaged increase when cells were stimulated with cytokine alone. Solid black bars represent the averaged increase when cells were costimulated with indicated cytokines and LPS. The experiments were repeated in three different batches of cells, and the averaged results were presented. *Y* error bars represent the standard deviation of the averaged results. Asterisks (∗) denote significant difference when cytokine alone was compared to that without stimulation, and (†) denotes significant difference when cytokine alone was compared to that with LPS.

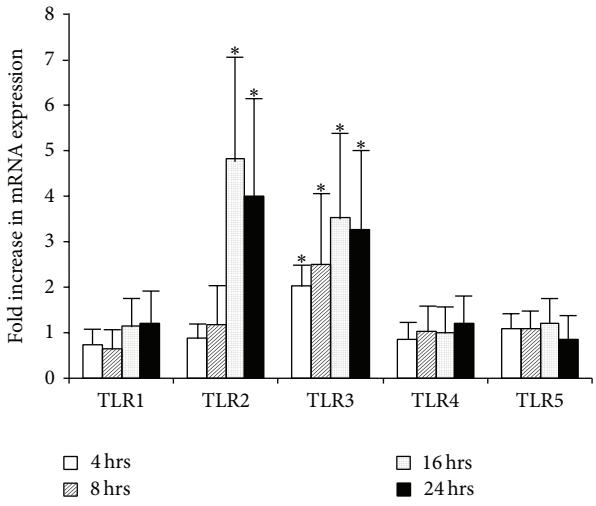


Figure 6: Hypoxia stimulated TLR mRNA expression in submerged primary conjunctival epithelial cells. Cells were cultured in 95% N_2 and 5% CO_2 at 37°C for indicated duration before being harvested for RNA extraction and qPCR. The experiment was repeated in 3 different batches of primary cells, and the averaged changes were shown. *Y* error bars represent the standard deviation of the averaged results. Asterisks (∗) denote significant difference when compared to cells under normoxia condition.

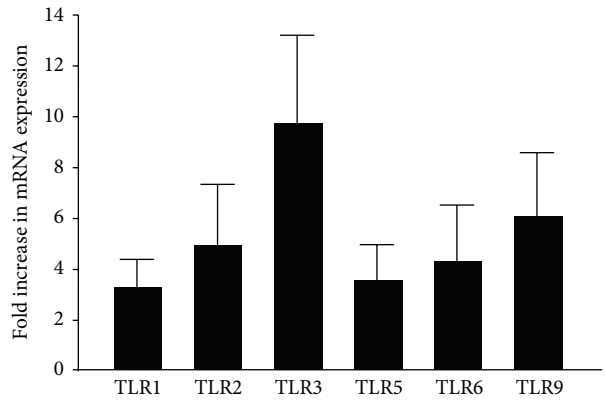


Figure 7: Wounding stimulated TLR expression in submerged primary conjunctival epithelial cells. Confluent cells were scratched by sterile surgical blade at about 1 cm interval and harvested 24 hrs later for RNA extraction and qPCR analysis. The experiment was repeated in 3 different batches of primary cells, and the averaged changes were shown. *Y* error bars represent the standard deviation of the averaged results. The increase was statistically significant for each TLR.

of TLR mRNA and protein was similar to what we have previously reported in vivo. Increased TLR expression in airlifted cells led to enhanced cytokine responses to low concentrations of TLR ligands, including LPS. Our study also showed that both the formation of multilayered cell sheet and increased TLR expression were sensitive to the inhibition of NFκB.

Our data suggested that TLR expression was intrinsically associated with multilayered structure formed under airlifting culture, which required NFκB activation. Airlifting culture of conjunctival and limbal epithelial cells is an effective and commonly used approach to achieve a multilayered epithelium-like structure in vitro. The multilayered epithelial cell sheets resulting from airlifting culture have

been used as tissue graft on various ocular surface engineering applications, mostly using amniotic membrane as support [13, 14]. Significant changes occur when epithelial tissue was digested and cells were cultured in monolayer in vitro, especially the loss of cell-cell connection. Our results indicated that the formation of multilayered structure was needed for the proper expression of TLR in conjunctival epithelial cells. Activation of NFκB was required for the formation of multilayered cell sheets. Due to the sensitive nature of airlifting culture, we were not able to further dissect the association between the formation of cell-cell connection and TLR expression at this time. However, this is not the first study which showed association between TLR expression and intact epithelial cell structure. Previous studies reported that the intestinal epithelium of TLR2 and MyD88 knockout mouse was prone to stress-induced tight junction disruption, suggesting a relationship between functional TLR and the integrity of the epithelium [15, 16]. Low levels of TLR expression were also reported in cultured intestinal and lung epithelial cells. Unlike ocular surface, intestinal epithelium and lung epithelium contain polarized single layer epithelial cells. It would be interesting to investigate the changes of TLR in these cells under similar culture conditions.

The stimulation by proinflammatory cytokines, hypoxia, and mechanical wounding mimics the stress to which ocular surface epithelial cells may be exposed in vivo. The changes of TLR mRNA expression in cultured primary conjunctival cells demonstrated in this study suggested that epithelial cell-expressed TLR was responsive to hypoxia and wounding. The effect of proinflammatory cytokines on individual TLR mRNA expression was relatively small. We also found several studies which supported our results. For example, two recent studies described increased multiple TLR expression in mouse corneal and conjunctival epithelial cells of dry eye disease models [17, 18]. Proinflammatory cytokines also showed stimulatory effect on human airway epithelial cell TLR2 expression [19]. However, the effect of hypoxia on individual TLR expression seemed to be different; that is, the expression of TLR2 and TLR3 was increased, while TLR1, TLR4, and TLR5 were not changed. Contradictory results were also reported on the effect of hypoxia on TLR4 expression in macrophage, endothelial cells, and corneal epithelial cells [20–22]. Further studies were needed to clarify the mechanism which mediates the effect of hypoxia on TLR expression.

In conclusion, this study identified multiple factors which regulated TLR expression in ocular conjunctival epithelial cells. Most importantly, our data suggested the existence of a coordinated reinforcement of both physical and molecular defense mechanisms during the formation of epithelial structure. Therefore, we propose that multilayered cell sheet, instead of submerged-cultured monolayer cells, is a better system to evaluate the biological activities of epithelial cell-expressed TLR.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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