Cytokine Profile in Aqueous Humor of Patients with Ocular Toxocariasis

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Abstract

Purpose: Ocular toxocariasis (OT) is a vision-threatening disease with a largely unknown intraocular pathogenesis. Herein, we determined the cytokine expression profile in aqueous humor (AH) of patients with OT.

Methods: This is a prospective experimental case-control study of cytokine levels in AH of patients with OT and uveitis and control subjects. Thirty samples from eyes with OT, 23 from eyes with uveitis, and 25 from eyes with age-related cataract were analyzed using a multiplexed magnetic bead immunoassay. Thirty-one cytokines were detected and classified into 5 categories: T-helper type 1 (Th1)-associated cytokines, Th2-associated cytokines, Th17 cytokine, proinflammatory mediators, and growth factors.

Results: Higher expression in 19 and decreased expression in 3 cytokines in the OT group were identified, compared with controls. Levels of 17 cytokines were increased in the OT group, compared with the non-OT uveitis and control groups. Levels of 3 cytokines were decreased in the OT group compared with controls; no cytokine level was decreased in the OT and non-OT uveitis groups. Interleukin-10 was the only cytokine showing a significant difference in pairwise comparison among all groups. Th17 levels showed no change, while growth factors and proinflammatory mediators showed some significant changes.

Conclusions: We believe this is the first analysis of cytokine expression profile in OT. It revealed that Th2-associated cytokines, especially IL-5, IL-10 and IL-13, present a significantly higher concentration in OT compared with the non-OT uveitis and controls. These cytokines may be important for OT pathogenesis and help identify diagnostic markers and develop treatment strategies in future.

Introduction

Ocular toxocariasis (OT) is an intraocular parasitic infection caused by larvae of the roundworm *Toxocara*^{1,2}. It is reported to be an important cause of visual impairment during childhood with a mean age of onset of 7.5 years^{1,3}. OT is typically unilateral and includes clinical manifestations such as retinal granuloma, comorbidity with uveitis, epiretinal membrane, and retinal detachment. It leads to permanent retinal damage, visual loss, and strabismus^{4,5}. Furthermore, the diagnosis and treatment of OT are still challenging. In clinical practice, no signs or symptoms are pathognomonic for OT. Diagnosis is usually based on the combination of fundoscopy, imagological examination, and immunological tests^{1,4}.

Despite OT being a well-known disease, intraocular host response and concrete mechanisms remain largely unknown. OT is believed to be derived from the intraocular immune response to the presence of *Toxocara* larvae and their products^{6,7}. Cytokines play an important role in the coordination of the immune response, and their changing profile may unravel the underlying mechanism. To the best of our knowledge, no previous study examined the cytokine expression pattern associated with OT.

Therefore, it is important to elucidate the influences of multiple cytokines on the pathogenesis of OT. We used multiplex bead-based Luminex technology to detect 31 cytokines simultaneously in OT patients' AH samples, which were collected prior to any treatment, and compared them with those in eyes with non-OT uveitis and control subjects. The study clarified the cytokine profile in OT with a view to determining which cytokines are potential diagnosis biomarkers or treatment targets in the future.

Methods

Patients and control subjects

This is a prospective experimental case-control study of cytokine levels in AH of patients with OT and uveitis and control subjects. All procedures adhered to the tenets of the Declaration of Helsinki, and local approval was received from the Investigational Review Board of Zhongshan Ophthalmic Center, Sun Yat-sen University. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study.

Diagnosis of OT was based on the following criteria: (1) the typical and specific manifestations including unilateral chorioretinal granuloma in the peripheral or posterior pole, and diffuse nematode endophthalmitis; (2) positive specific anti-OT IgG levels and a Goldmann-Witmer coefficient larger than 3 in paired AH and serum samples⁸; (3) exclusion of other ocular diseases, such as ocular toxoplasmosis, sarcoidosis, ocular tuberculosis, and any other form of infectious uveitis. Patients diagnosed with non-OT uveitis, infectious uveitis of other etiologies, or idiopathic uveitis, were also recruited. Patients with age-related cataracts who underwent routine phacoemulsification surgery were selected as the control group. The exclusion criteria were as follows: (1) history of any other ocular diseases, apart from age-related cataracts; (2) any previous intraocular surgery; and (3) patients with serious systemic diseases, including diabetes, heart, lung, liver, and kidney dysfunction. Samples were collected and demographic data and ocular characteristics of the patients were recorded. The mean ages of the patients were

12.3 ± 3.43, 20.7 ± 4.29, and 75.5 ± 9.27 years in OT, non-OT uveitis, and control groups, respectively ($p = 0.016$). The male/female ratio was equal in three groups ($p = 0.658$).

Sample Collection

Seventy-eight AH samples from 78 eyes were collected, including 30 samples from eyes with OT, 23 from eyes with uveitis, and 25 from eyes with age-related cataract. Sampling of AH was performed under a surgical microscope after sterilizing the surface of the cornea and conjunctiva with povidone-iodine. Approximately 100 µL of AH was collected via limbal paracentesis with the use of a 30-gauge needle. Each sample was centrifuged (3000 rpm for 5 minutes), separated into cellular component and supernatant components, and frozen at -80 °C until use.

Cytokine assays

The advent of bead-based multi-detection assays has made possible the measurement and correlation of multiple cytokine analytes from a single, small, AH sample. This technology utilizes internally color-coded magnetic microspheres, coupled to analyte-specific antibodies, allowing for the simultaneous measurement of up to 50 analytes within each sample. A customized bead panel kit which measures 31 cytokines in a 96-well format (Milliplex Human Cytokine/Chemokine Magnetic Bead Panel I kit, Cat. # HCYTMAG-60K; Billerica, MA, USA), was employed in this study⁹. These cytokines were classified into 5 categories: (1) T-helper type 1 cytokines: interleukin (IL)-2, IL-7, IL-12p40, IL-12p70, interferon alpha 2 (IFN-α2), IFN-γ, tumor necrosis factor alpha (TNF-α), TNF-β, and IL-15; (2) T-helper type 2 cytokines: IL-3, IL-4, IL-5, IL-9, IL-10, and IL-13; (3) T-helper (Th) 17 cytokine: IL-17A; (4) proinflammatory mediators: interleukin-1 receptor antagonist (IL-1ra), IL-1α, IL-1β, IL-6, IL-8, and soluble CD40 ligand (sCD40L); and (5) growth factors: granulocyte-colony stimulating factor (GCSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), fms-like tyrosine kinase 3 ligand (FLT3L), transforming growth factor alpha (TGF-α), platelet-derived growth factor composed of 2 A subunits (PDGF-AA), PDGF-AB/BB, and vascular endothelial growth factor (VEGF)^{10,11}.

The assay was performed according to the manufacturer's instructions. Undiluted samples (25 µL neat per well) were assayed in duplicate. Standard curves for each cytokine were generated from reference standards supplied with the kit. Data were analyzed by Luminex FlexMap 3D (Luminex, Austin, TX, USA). Cytokine concentrations were determined by Luminex Xponent 4.2 using 5-p log analysis. Concentrations above or below the detection limit were given as the highest or lowest detectable value. For statistical analysis, concentrations below the detection limit were converted to a value of 0.5 × the lowest point on the calibration curve.

Statistical analyses

Normally distributed data were determined by the Shapiro-Wilk test (mean ± standard deviation), while non-normal data, including the cytokine levels, were expressed as median and range, because outliers were present. Wilcoxon–Mann–Whitney rank sum test was used for the two-group comparison and Kruskal–Wallis test was used for more than two groups (OT, non-OT uveitis and controls) comparison. Heatmaps were generated using the heatmap.2 function in the GPLTS 2.11.0 library for R version 2.15.0 (available in the public domain at <http://cran.us.r-project.org>; Comprehensive R Archive Network) on log2-transformed values of cytokine concentrations for all samples in each groups, to provide an overall view of the changes in cytokine values for all patients. For all statistical analyses, $P < 0.05$ was considered statistically significant.

Results

Using a multiplexed magnetic bead immunoassay, we measured the levels of 31 cytokines in AH samples: 30 from OT affected eyes, 23 from eyes affected with non-OT uveitis, and 25 control cases. A complete dataset of cytokine expression is summarized in Table 1. Univariate logistic regression analysis identified 6 cytokines that have significantly different concentrations in OT patients compared with those in non-OT uveitis or controls.

Table 1
Summary of cytokines measured by multiplex bead immunoassay (ng/ml).

Categories	Cytokines	OT Median (Q25,Q75)	Non-OT Uveitis Median (Q25,Q75)	Control Median (Q25,Q75)	P value (K_W test)	P value OT vs Control	P value OT vs Uveitis	P value Uveitis vs Control
T helper type 1	TNF- α	2.04 (0.85,3.93)	0.85 (0.85,1.88)	0.85 (0.85,0.85)	< 0.001	< 0.001	0.079	0.007
	IFN- α 2	6.86(3.45,13.93)	5.99 (3.45,11.28)	5.12 (3.45,5.12)	0.025	0.005	0.626	0.103
	IFN- γ	1.39(1.39,1.39)	1.39 (1.39,3.67)	1.39 (1.39,1.39)	0.027	0.061	0.237	0.007
	IL-2	0.74(0.74,0.74)	0.74(0.74,0.74)	0.74(0.74,0.74)	0.104			
	IL-12p70	1.50(1.50,1.50)	1.50(1.50,1.50)	1.50(1.50,1.50)	0.168			
	IL-7	2.34(1.00,3.77)	2.02(1.00,2.66)	2.66 (2.34,2.98)	0.198			
	TNF- β	1.32 (1.32,1.32)	1.32(1.32,1.32)	1.32(1.32,1.32)	0.449			
	IL-12p40	6.59(4.33,11.99)	6.58 (3.83,12.57)	5.87 (4.16,7.49)	0.450			
	IL-15	3.29(2.21,5.81)	3.18 (0.83,7.01)	2.98 (2.31,3.59)	0.543			
T helper type 2	IL-10	26.18(5.30,98.98)	3.19 (1.31,20.77)	1.31(1.31,1.31)	< 0.001	< 0.001	0.012	< 0.001
	IL-13	14.11(2.09,42.70)	1.31 (0.32,2.36)	1.07 (0.84,1.31)	< 0.001	< 0.001	< 0.001	0.353
	IL-9	2.88(1.18,7.71)	1.18(1.18,1.18)	1.18(1.18,1.18)	< 0.001	< 0.001	< 0.001	1.000
	IL-5	39.58(6.61,78.53)	1.47(1.47,1.47)	1.47(1.47,1.47)	< 0.001	< 0.001	< 0.001	0.297
	IL-4	9.72(6.29,21.38)	9.72(2.40,13.43)	6.29(6.29,9.72)	0.010	0.002	0.218	0.144
	IL-3	0.99(0.99,0.99)	0.99(0.99,0.99)	0.99(0.99,0.99)	1.000			
T helper (Th) 17 cytokine	IL-17A	1.54(1.54,.54)	1.54(1.54,1.54)	1.54(1.54,1.54)	1.000			
Proinflammatory mediators	sCD40L	3.48(1.53,7.71)	1.53(1.53,4.87)	1.53(1.53,1.53)	< 0.001	< 0.001	0.228	0.005
	IL-6	55.65(25.03,151.69)	49.58(1.33,168.77)	4.36(1.33,20.52)	< 0.001	< 0.001	0.258	0.029
	IL-1a	1.18(1.18,2.49)	1.18(1.18,4.17)	1.18(1.18,1.18)	0.009	0.003	0.675	0.003
	IL-8	17.60(6.87,58.33)	8.58(1.32,38.23)	8.88 (6.27,10.59)	0.085			
	IL-1ra	20.03(9.54,33.72)	13.30(5.95,24.56)	12.21(10.06,15.52)	0.267			
	IL-1b	1.34(1.34,1.34)	1.34(1.34,1.34)	1.34(1.34,1.34)	0.303			
growth factors	PDGF-AA	48.42(31.52,66.53)	22.80 (8.97,44.57)	22.76(20.25,27.35)	< 0.001	< 0.001	0.001	0.975
	PDGF-AB/BB	3.72(0,8.08)	1.48(1.48,12.12)	1.48(1.48,1.48)	< 0.001	< 0.001	0.637	< 0.001
	FLT3I	30.06(21.64,47.92)	18.43 (9.88,47.25)	11.00 (7.52,12.08)	< 0.001	< 0.001	0.082	0.005
	GCSF	23.17(11.52,55.24)	16.02 (4.57,25.48)	14.39 (11.93,16.83)	0.030	0.017	0.038	0.967
	EGF	5.20(3.89,5.82)	4.55 (3.89,5.82)	3.89 (3.89,4.55)	0.035	0.006	0.329	0.280
	VEGF	32.80 (11.13,71.45)	28.96 (14.83,50.22)	58.81 (34.95,75.00)	0.049	0.059	0.808	0.018

Categories	Cytokines	OT Median (Q25,Q75)	Non-OT Uveitis Median (Q25,Q75)	Control Median (Q25,Q75)	P value (K_W test)	P value OT vs Control	P value OT vs Uveitis	P value Uveitis vs Control
	GM-CSF	6.54(4.46,10.87)	4.93 (2.38,7.69)	4.74 (4.08,5.51)	0.05			
	TGF- α	1.17(1.17,1.17)	1.17(1.17,1.17)	1.17(1.17,1.17)	0.294			
	FGF-2	24.66(19.36,33.72)	24.66 (19.36,44.87)	24.66 (24.66,31.56)	0.956			

The cytokines were broadly clustered into two groups, as seen on the top dendrogram in Fig. 1, based on the overall cytokine levels in all patients. The first cluster at left end of the dendrogram consisted of IL-5, IL-9, IL-10 and IL-13, the expression of which were markedly increased in OT eyes, compared with the non-OT uveitis ($P < 0.05$) and control ($P < 0.05$) groups. The second cluster at right, including IL-1 β , IL-2, IL-3 and TNF- β , had low levels of expression in all samples.

To analyze the trend changes, we depicted a Venn diagram (Fig. 2). From the 31 cytokines, 19 exhibited higher expression in the OT group than in the control group, while decreased expression was observed in 3 cytokines. Concentrations of 17 cytokines were increased in the OT group, compared with the non-OT uveitis or control group, including IL-1ra, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-15, sCD40L, TNF- α , IFN- α 2, EGF, FLT3l, GCSF, GM-CSF, PDGF-AA, PDGF-AB/BB. Levels of 3 cytokines (IL-7, VEGF, and TGF- α) were decreased in the OT group compared with the control group, and no cytokine level was decreased compared between the OT and non-OT uveitis groups.

Forest analysis was performed to explore the extent of expression changes among the three groups (Fig. 3). Among the cytokines that showed significant difference ($P < 0.05$), IL-5 showed the most obvious, 27-fold increase compared with that in controls. Furthermore, IL-6, IL-10 and IL-13 exhibited more than 3-fold increase in OT patients compared with that in controls. IL-5, IL-10 and IL-13 showed 3-fold or more significant elevations in OT than in non-OT uveitis patients.

Furthermore, to investigate the scattered distribution of each cytokine, IL-5, IL-6, IL-10, and IL-13 were analyzed (Fig. 4). Deviations were observed in all cytokine expression profiles; however, a number of significant differences were still detected based on current data. Results revealed that levels of IL-1 α , IL-6, IL-10, TNF- α , FLT3l, PDGF-AB/BB, and sCD40L were significantly increased in both OT and non-OT uveitis patients, compared with the control group levels. Moreover, expression of IL-5, IL-9, IL-10, IL-13, PDGF-AA, and GCSF, were significantly increased in the OT group alone, compared with the non-OT uveitis and control groups.

We also reviewed the cytokines based on their role in inflammation. Most Th1 cytokines, including IL-2, IL-7, IL-12p40, IL-12p70, IL-15, and TNF- β , were unchanged between the OT and control groups. The most significant increase was detected in Th2 cytokines, including IL-4, IL-5, IL-9, IL-10, and IL-13. There was no change in Th17 cytokine levels, represented by IL-17. Proinflammatory mediators showed significant changes in IL-1 α , IL-6, and sCD40L levels, but no difference in IL-1ra, IL-1 β , and IL-8. Growth factors, including PDGF-AA, PDGF-BB, GCSF, EGF and FLT3l, also showed significant level difference, but not in VEGF, GM-CSF, TGF- α and FGF-2.

Discussion

Toxocariasis is one of a group of diseases known as neglected parasitic infections, targeted by the Centers for Disease Control and Prevention for public health action^{12,13}. This disease is classified as neglected because relatively little attention has been directed towards its surveillance, prevention and treatment⁴. In China, similar to other countries, the clinical awareness of the disease is insufficient. It is established that OT is caused by toxocara larvae invading the eye, however, the underlying mechanisms are still unclear. Clinically, OT shares certain clinical features with uveitis of other etiology^{14,15}. The diagnosis of OT is hindered by the atypical clinical signs and the limited knowledge of the physicians. Thus, in most cases, clinical characteristics cannot serve as a standard diagnostic tool. In this study, these expressions were analyzed in OT patients and compared with the non-OT uveitis and control groups. Significant increase was detected in Th2 cytokines mostly, as well as growth factors and proinflammatory mediators, but only a slight increase in Th1 cytokines. This profile is expected to help understand the pathogenesis of OT, the identification of cytokines that help differentiate OT from uveitis caused by other factors, and ultimately it may help identify diagnostic markers and develop treatment strategies of OT.

Our results showed that Th2 cells are the major effector T cells in OT. CD4 + T cells are differentiated into two functional subsets based on their profiles of cytokine production. Th1 cells produce IL-2, IL-7, IL-12p40, IL-15, and TNF- α . They are responsible for cell-mediated immunity, and they are also involved in the pathogenesis of organ-specific autoimmune disorders¹⁶. In contrast, Th2 cells, which produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, induce strong antibody responses by B cells, induce eosinophil activation, and are responsible for allergic reactions¹⁷. Our research revealed that IL-4, IL-5, IL-9, IL-10 and IL-13 were significantly increased in OT patients, indicating that Th2 cells are the major effector

T cells in OT. Among all 31 cytokines, IL-5 showed the highest fold change in the OT group in our study. IL-5 induces terminal differentiation of activated B cells into antibody-forming cells in mice. Another signature function of IL-5 is the promotion of proliferation and prolonging the survival of mature eosinophils¹⁸. Our data are generally an important extension of previous animal/experimental studies based on system toxocariasis. For instance, Del Prete GF et al reported a prominent peak pattern of IL-5 noted in cerebra of *Toxocara canis*-infected mice¹⁹. However, IL-5 and IL-13 were almost undetectable in non-OT uveitis in our study. These data are consistent with those of previous studies, which have shown that IL-5 is undetectable or mildly increased in noninfectious anterior uveitis and appears to be absent in panuveitis where there is a greater uveal tract involvement^{9,20}. Thus, a significant increase of IL-5 and IL-13 in the AH of OT patients may be specific for OT patients. Further investigation of the potential therapeutic benefits of IL-5 and IL-13 would be of great interest.

On the other hand, our results revealed Th1 and Th17 cells played mild role in OT pathogenesis. Out of 9 Th1 associated cytokines detected in our study, 3 of them were undetectable among all three groups, including IL-2, IL-12p70 and TNF- β , while 4 of them were unchanged, including IL-7, IL-12p40, IL-15 and IFN γ . Although TNF- α and IFN- α 2 showed significant level increases, low expressions and multiples at 2.4 and 1.3 times respectively were observed between OT and control group. Meanwhile, no Th1 cytokine showed significant difference between OT and non-OT uveitis group. These results suggest that Th1 cells play mild role in OT pathogenesis. Additionally, IL-17 was undetected among all groups. IL-17 is an essential proinflammatory cytokine for the host's defense against bacteria and fungi, and its important role in autoimmune disease has only recently been discovered²¹. Interestingly, our study demonstrated the absence of Th17 cells in OT inflammation.

Interestingly, our results showed that IL-10 might be a crucial differentiating cytokine in OT. IL-6 and IL-10 were both detected in our study. IL-6 was considered a general inflammation marker in OT. In proinflammatory cytokines, IL-6 increases approximately 13-fold in OT compared with control group, however, our study showed almost the same expression in OT and non-OT uveitis groups. Elevated intraocular levels of IL-6 were found repeatedly in uveitis of diverse etiology (including ocular toxoplasmosis, viral uveitis, Fuchs heterochromic uveitis syndrome, and Behcet's uveitis), as well as in ocular fluids of children with uveitis^{22,23}. Thus, IL-6 is considered a general marker of active uveitis and is not specific for particular uveitis entities^{24,25}. This helps explain the similar expression level of IL-6 in both OT and uveitis patients in our study. In contrast, IL-10 is known as an anti-inflammatory cytokine that suppresses the expression of proinflammatory cytokines, including TNF- α , IFN- γ , and IL-1 β ²⁶. The intraocular level of IL-10 reported in uveitis is still debatable²⁷⁻²⁹. Elevated IL-10 level was associated with uveitis, but lower or no different IL-10 expressions, were also documented in uveitis. However, in our study, IL-10 showed significant difference in pairwise comparison between the three groups. It increased by 20-fold in OT, compared with controls, and 8-fold compared with the level in AH in non-OT uveitis eyes. Furthermore, TNF- α , IFN- γ , and IL-1 β , whose expressions could be suppressed by elevated IL-10, were low-leveled or absent in our study (2.04, 1.39 and 1.34 ng/ml, respectively). These data suggest that IL-10 can be used as a unique biomarker to differentiate OT from non-OT uveitis and healthy subjects.

Our study did have some limitations. First, the study was conducted on a restricted small number of participants. Since OT is a rare disease and has been defined as a neglected parasitic infection, we are working on gathering more cases. Second, healthy people are actually the best control group; however, it is inappropriate to collect their AH by undertaking invasive procedures. Third, the mean age of the patients in uveitis or cataract groups was higher than that of the OT group. However, no significant correlation was found between the AH cytokine levels and age, based on most previous studies³⁰. Lastly, the etiology of non-OT uveitis was diverse. Investigating uveitis and its various etiologies should prove useful in future, larger cohort studies, in order to substantiate the findings of our study.

In conclusion, our study was the first to investigate the cytokine profile expression in OT, and it revealed that Th2-associated cytokines, including IL-5, IL-10 and IL-13, present a significantly higher concentration in OT compared with concentrations in the non-OT uveitis and control groups. These cytokines may be important for the pathogenesis of OT and may help identify diagnostic markers and develop treatment strategies for OT patients in the near future.

Declarations

Availability of data and materials

The datasets used for the current study are available on reasonable request. Please contact the corresponding author, Xiaoyan Ding, for data requests.

Ethics approval and consent to participate

All procedures adhered to the tenets of the Declaration of Helsinki, and local approval was received from the Investigational Review Board of Zhongshan Ophthalmic Center, Sun Yat-sen University. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DXY conceived and designed the study. JZX, SLM, and ZT participated in data collection, laboratory analysis and interpretation. JZX and DXH analyzed the data and wrote the first draft of the manuscript. DXY, WWQ and LSS critically reviewed the manuscript. All authors approved the submitted version.

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Figures

Figure 2

Venn diagram showing the numbers and overlap of cytokines analyzed in the 3 groups. Expressions of 31 cytokines in total, were grouped as: increased or decreased expression in OT patients when compared with control subjects ($O/C > 1$, $O/C < 1$); increased or decreased expression in OT patients when compared with non-OT patients ($O/U > 1$ or $O/U < 1$). Expressions of 19 cytokines were increased in OT group when compared with controls, while 3 were decreased, but no cytokine was decreased in OT when compared with non-OT uveitis group. Of these 31 cytokines, 17 were increased in OT group, when compared with either uveitis or control groups.

Figure 3

Bar chart displaying cytokines with significant concentration difference between OT and control groups. Individual cytokine expression levels between OT and control group, as shown on the horizontal bars. The vertical axis shows the individual cytokines with significant difference. IL-5 showed the most noticeable expression increase, followed by IL-10, IL-13, and IL-6, which showed 3-folds or more difference between the groups.

Figure 4

Representative cytokines in AH detected by multiplex bead immunoassay. Between 31 cytokines, expressions of IL-5, IL-10, IL-13, IL-9, PDGF-AA and GCSF were significantly increased in OT group alone, when compared with uveitis and control group (a-f). IL-6, FLT3L and PDGF-BB also showed significant differences between OT and control groups (g-i), but there were no significant differences between OT and uveitis groups.