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OCULOPLASTICS AND ORBIT

Increased serum interleukin-17 in Graves' ophthalmopathy

Sung Eun Kim · Jin Sook Yoon · Keun Ho Kim · Sang Yeul Lee

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Abstract
Background Interleukin (IL)-17 and T helper 17 (Th17) cells are reported to be involved in many autoimmune diseases. The aim of this study is to investigate the involvement of IL-17 in the pathogenesis and disease activity of Graves' ophthalmopathy (GO).
Methods Sixty-two GO patients and 19 healthy controls were recruited. Serum concentrations of cytokines, IL-17, IL-6, IL-23, and IL-16, were measured using multiplexed microsphere-based flow cytometric immunoassays. GO hormonal parameters, clinical activity score (CAS), exophthalmometry, and extraocular muscle involvement were evaluated, and relationships with cytokine concentrations were analyzed.
Results The concentration and positive detection rates of serum IL-17 were significantly higher in patients with GO than in controls. The serum levels of IL-17 in active GO patients were higher than that of inactive GO patients. Serum IL-17 concentration had significant correlation with CAS ($p<0.001$).
Conclusion The increased serum level of IL-17 and related cytokines in GO patients and the correlation of IL-17 concentration with the clinical activity scores suggest that IL-17 may play a pathophysiological role in GO.

Keywords Interleukin-17 · Graves' ophthalmopathy · Cytokine

S. E. Kim · K. H. Kim
Department of Ophthalmology, Myongji Hospital, Kwandong University College of Medicine,
Koyang 412-270, Korea

J. S. Yoon · S. Y. Lee (✉)
Institute of Vision Research, Department of Ophthalmology, Yonsei University College of Medicine,
Seoul 120-752, Korea
e-mail: sylee@yuhs.ac

S. Y. Lee
Department of Ophthalmology, Yonsei University College of Medicine,
50 Yonsei-Ro, Seodaemun-Gu,
Seoul 120-752, Korea

Introduction

Graves' ophthalmopathy (GO), also known as thyroid associated ophthalmopathy (TAO), manifests as orbital inflammation and expansion of fat and extraocular muscles. The pathophysiology of GO remains uncertain, but lymphocytes and other mononuclear cells infiltrate the orbit, and are thought to drive tissue remodeling, possibly as a consequence of cytokine production and action on fibroblasts. GO is probably initiated by autoreactive T lymphocytes reacting with one or more antigens shared by the thyroid and orbit. T cells trigger a cascade of events, including secretion of cytokines. These cytokines stimulate the proliferation of orbital fibroblasts, expansion of adipose tissue, and secretion of hydrophilic glycosaminoglycans from fibroblasts. B cells are also involved as antigen-presenting and autoantibody-producing cells [1]. Orbital fibroblasts also participate in immune responses by virtue of their capacity to both produce cytokines and respond to them. Interleukin (IL)-1, IL-6, IL-16, CXCL-10, and RANTES (regulated upon activation normal T cell expressed and secreted) are synthesized by thyrocytes and orbital fibroblasts [2]. Of these, IL-16 and RANTES are known to be potent T lymphocyte chemoattractant molecules.

CD4+ T helper lymphocytes play a key role in the pathogenesis of inflammatory and autoimmune diseases. On the basis of their pattern of cytokine synthesis, CD4+ T helper cells were originally classified as Th1 and Th2 lymphocytes, which are involved in the cellular and humoral immune

Table 1 Characteristics of GO patients and control participants

	Active GO (n=34)	Inactive GO (n=28)	Normal control (n=19)	P-value
Sex (female:male)	27:7	21:7	13:6	0.68
Age (years)	40.7±13.5	38.8±15.5	39.6±16.5	0.89
TSH (μIU/ml)	0.85±2.46	1.48±2.67	NA	0.34
Free T4 (ng/dl)	1.62±0.93	1.53±0.88	NA	0.72
TSHR-Ab (U/l)	14.46±12.52	7.22±9.24	NA	0.01
CAS	4.1±1.3	0.6±0.8	NA	<0.001
Exophthalmos (mm)	19.0±2.8	17.1±2.5	NA	0.03
EOM involvement: n, (%)	14/34 (41.2 %)	8/28 (28.6 %)	NA	0.30

NA: not applicable

responses, respectively. IL-17-producing Th17 cells are another CD4+ T-cell lineage recently identified. Recent studies have revealed that Th17 immune responses play a major role in numerous autoimmune diseases, such as rheumatoid arthritis [3], psoriasis [4], and systemic lupus erythematosus (SLE) [5]. These cells secrete a variety of cytokines, including IL-17, IL-21, and IL-22, with proinflammatory functions [6]. It has been suggested that Th17 cells can promote humoral autoimmunity. Some cytokines play important roles in the development of Th17 cells; a combination of IL-6 and transforming growth factor-beta (TGF-β) is essential for the initial differentiation of Th17 cells [6]. IL-23 plays a role in stabilization and survival of the Th17 cells [7, 8]. A recent publication has also reported elevation of IL-23 p19 in human autoimmune diseases such as multiple sclerosis [9].

Recent reports have demonstrated that Th17 cells have critical roles in the development of autoimmune thyroiditis and Graves' hyperthyroidism [10, 11]. Thus, we aimed at investigating whether the secretion of IL-17 and related cytokines is associated with the presence of GO in its active or inactive phase, and whether these cytokines are linked to thyroid status and GO clinical activity. Therefore, serum concentrations of IL-17, IL-6, IL-16, and IL-23 in GO patients were measured, to determine whether the concentrations of these cytokines are linked to GO hormonal parameters and clinical activity.

Materials and methods

GO patients, control subjects, and blood samples

A total of 62 patients with GO (48 females, 14 males; mean age, 39.8±14.8 years) were recruited from May 2010 to June 2011, from the Department of Ophthalmology at Severance Hospital, Seoul, Korea. Patients who had had prior treatment with steroids or radiation were excluded. Ophthalmopathy was diagnosed by performing a complete eye examination, and the disease activity was evaluated by the

Table 2 Serum IL-17, IL-23, IL-6, and IL-16 detection rate and concentrations of GO patients (active GO and inactive GO) and control group (NC)

Cytokine		Normal control (n=19)	Inactive GO (n=28)	Active GO (n=34)	P-value
IL-17	Positive detection rate: n, (%)	1/19 (5.3 %)	12/28 (42.9 %)	22/34 (64.7 %)	<0.001*
	Concentration: mean±SD (pg/ml) (range)	1.02±4.38 (0.02–19.11)	3.59±10.14 (0.02–47.40)	12.36±25.68 (0.02–99.62)	<0.001**
IL-23	Positive detection rate: n, (%)	6/19 (31.6 %)	19/28 (67.9 %)	25/34 (73.5 %)	0.01*
	Concentration: mean±SD (pg/ml) (range)	134.73±287.07 (0.8–955.32)	305.20±825.51 (0.85–4,197.09)	860.18±1,965.54 (0.85–8,819.15)	0.03**
IL-6	Positive detection rate: n, (%)	0/19 (0.0 %)	4/28 (14.3 %)	7/34 (20.6 %)	0.09*
	Concentration: mean±SD (pg/ml) (range)	0.01±0.00 (0.01~0.01)	0.05±0.16 (0.01~0.69)	2.29±8.91 (0.01~49.91)	0.05**
IL-16	Positive detection rate: n, (%)	10/19 (52.6 %)	20/28 (71.4 %)	26/34 (76.5 %)	0.19*
	Concentration: mean±SD (pg/ml) (range)	32.04±46.68 (4.42–195.45)	133.24±336.74 (4.42–1,099.59)	138.53±316.75 (4.42–1,444.35)	0.19**

SD, standard deviation

* Chi-square test

** Kruskal–Wallis test, followed by Dunn's multiple comparisons post test

clinical activity score (CAS). Active GO was defined as CAS≥3/7, and inactive GO was defined as CAS≤2/7 [12]. The extraocular muscle (EOM) involvement was evaluated as positive when the patient had diplopia on a binocular single vision test and corresponding extraocular muscle enlargement on the computerized tomography (CT) scan. Serum concentrations of thyroid-stimulating hormone (TSH) and free T4 were measured to determine thyroid function. TSH receptor antibody (TSHR-Ab) was also measured. Nineteen age- and sex-matched healthy volunteers (13 females, six males; mean age, 39.6±16.5 years) were included as normal controls; none had any autoimmune-related disease.

After informed consents were obtained, 10 ml of EDTA-treated venous peripheral blood was collected from each patient and control subject. Blood samples were collected at the time of each subject's outpatient visit during the daytime [13, 14]. Samples were immediately centrifuged for 15 min at 3,000 rpm and stored at −70 °C until assay.

Multiplexed microsphere-based flow cytometric immunoassay (MFCA)

We measured the levels of IL-17, IL-6, IL-16, and IL-23 using a commercially available LINCOplex Cytokine/Chemokine kit (LINCO Research, Billerica, MA, USA), which uses fluorescently labeled microsphere beads. Measurement was with a Luminex 200 system (Luminex, Austin, TX, USA). All samples were measured in duplicate and analyzed simultaneously. Lower detection limits for IL-17, IL-6, IL-16, and IL-23 were 0.02, 0.01, 4.42, and 0.85 pg/ml respectively.

Statistical analysis

Data are expressed as mean±standard deviation or median (range). Data were analyzed using both parametric and non-parametric tests. The comparisons between groups were made using the Mann–Whitney U test, Kruskal–Wallis test, and Chi-square test (Fisher's exact test). A two-tailed *P*-value less than 0.05 was considered to indicate statistical significance.

Results

GO patients and control subjects

Thirty-four active GO patients, 28 inactive GO patients, and 19 healthy control subjects were studied. Their age, sex, thyroid hormonal assay results, CAS scores, exophthalmometry, and EOM involvement are summarized in Table 1. There was no statistical difference in age or sex between the groups.

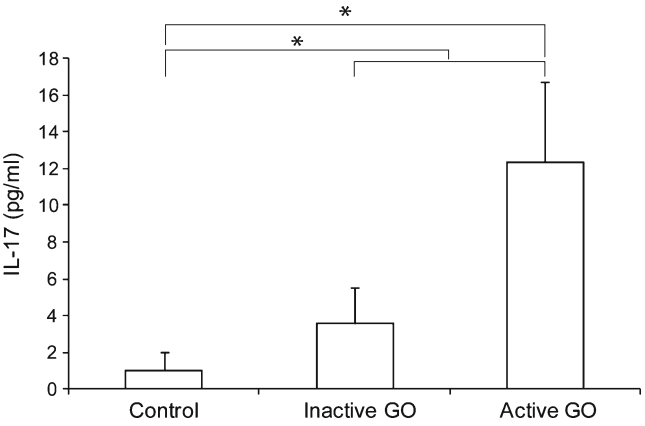

Fig. 1 Serum IL-17 concentrations in the control, inactive Graves' ophthalmopathy (GO), and active GO groups (mean±standard error, * $p<0.05$)

Increased serum concentration of IL-17 and IL-23 in GO

We first analyzed the serum levels of IL-17 in all participants. Among the normal controls, only one person (5.3 %) had detectable IL-17 in serum. However, 12 patients among 28 inactive GO patients (42.9 %) and 22 patients among 34 active GO patients (64.7 %) had detectable serum IL-17 (Chi-square test, $p<0.001$) (Table 2). The concentrations of serum IL-17 were significantly higher in patients with GO groups than in controls (Kruskal–Wallis test, $p<0.001$). The serum levels of IL-17 in active GO patients were higher than those in inactive GO patients (Fig. 1). The positive detection rate of serum IL-23 was also significantly higher in GO groups than in controls (Chi-square test, $p=0.008$). Serum IL-23 concentrations were also significantly higher in GO groups than in controls (Kruskal–Wallis test, $p=0.032$) (Fig. 2). However, the serum concentrations of IL-6 and IL-16 were not significantly different between GO patients and controls.

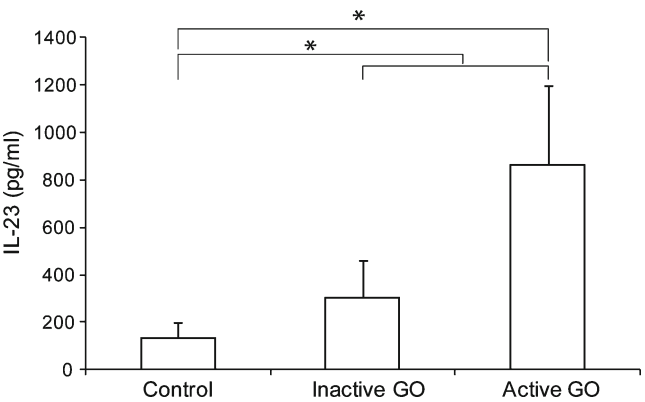

Fig. 2 Serum IL-23 concentrations in the control, inactive Graves' ophthalmopathy (GO), and active GO groups (mean±standard error, * $p<0.05$)

Table 3 Serum IL-17, IL-23, IL-6, and IL-16 concentrations of GO patients hyperthyroid group and euthyroid group

Cytokine concentration	Euthyroid GO (n=41): mean±SD (pg/ml) (range)	Hyperthyroid GO (n=21): mean±SD (pg/ml) (range)	P-value*
IL-17	9.45±23.22 (0.02–99.62)	6.34±14.19 (0.02–62.26)	0.20
IL-23	733.89±1,738.70 (0.85–8819.15)	366.76±1,179.50 (0.85–5,405.21)	0.17
IL-6	0.43±2.34 (0.01–14.99)	2.94±10.93 (0.01–49.91)	0.43
IL-16	145.91±350.40 (4.42–1,484.14)	117.05±269.28 (4.42–1,198.28)	0.35

SD, standard deviation

* Mann–Whitney U test

Serum concentration of cytokines and thyroid function

We re-classified the GO patients into hyperthyroid and euthyroid groups, and the serum concentration of cytokines were compared between the two groups (Table 3). Among the active GO patients, 14 were in the hyperthyroid state and 20 were in the euthyroid state; and among the inactive GO patients, seven were in the hyperthyroid state and 21 were in the euthyroid state. However, serum concentrations of interleukins were not significantly different between the two groups (Mann–Whitney U test, $p>0.05$).

Correlations between serum IL-17, IL-6, IL-23, and IL-16

There were significant positive correlations of serum concentrations of IL-17 with IL-6 ($r=0.430$, $p<0.001$), and IL-23 ($r=0.256$, $p=0.021$) in all participants. There was no significant correlation of serum IL-16 with other cytokines (all values had $p>0.05$, data not shown).

Correlation between disease activity and serum cytokines

There were significant correlations of CAS with TSHR-Ab, exophthalmometry, and EOM involvement ($p<0.001$, $p=0.002$, and $p=0.034$ respectively) in active and inactive GO patients. The weak positive correlation of CAS with TSHR-Ab was shown in Figure 3. Serum IL-17 concentration had significant correlation with CAS ($p<0.001$) (Fig. 4), and serum IL-6 had significant correlation with TSHR-Ab in GO patients ($p=0.021$). We did not observe any other significant associations of thyroid function or GO activity with serum concentrations of IL-23 and IL-16 (data not shown).

Discussion

IL-17 produced by Th17 cells enhances the expression of chemokines and inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor alpha (TNF-α), cell adhesion factors, and other inflammatory factors, and promotes leukocyte migration to inflammatory sites [6, 15]. The increase of IL-17 has been discovered in various autoimmune diseases, particularly in SLE, where the IL-17 concentration has been reported to be associated with disease activity [16].

In a recent report, Th17 lymphocyte levels were found to be higher in patients with autoimmune thyroid disease compared with healthy controls, and those patients with intractable

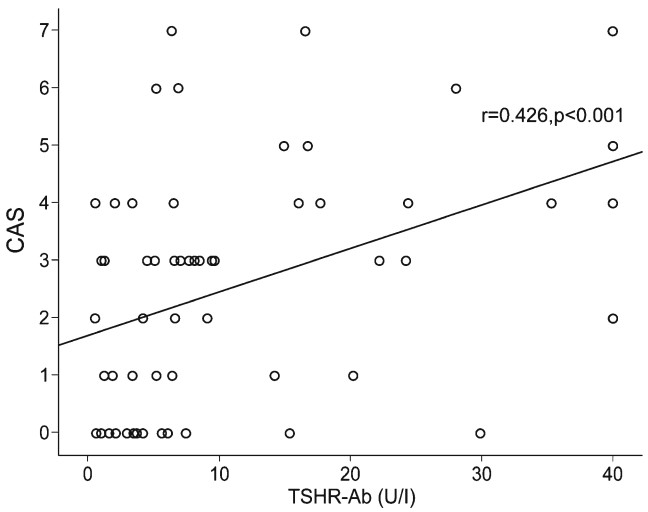

Fig. 3 Correlation between clinical activity score (CAS) and thyroid-stimulating hormone receptor antibody (TSHR-Ab)

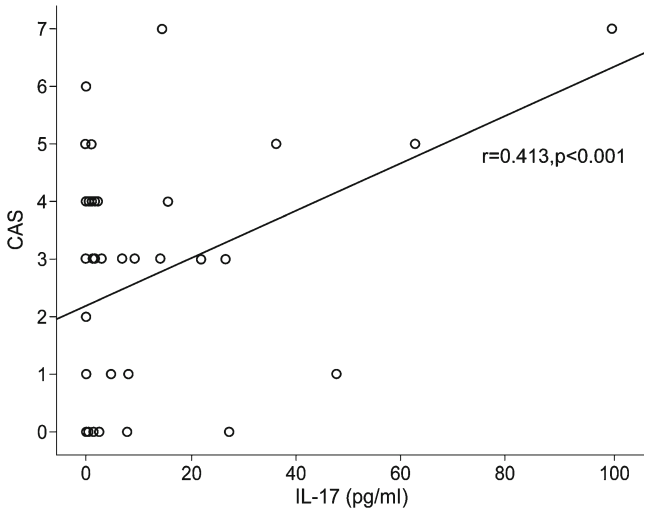

Fig. 4 Correlation between clinical activity score (CAS) and serum IL-17

Graves' disease showed a higher proportion of Th17 cells than patients with Graves' disease in remission [17].

We attempted to determine whether serum IL-17 increases in GO and correlates with disease activity. The sequential increase of IL-17 concentration and positive detection rate in the normal group, the inactive GO patient group, and the active GO patient group suggests that IL-17 may play a pathophysiological role in GO through the recruitment of the effector leukocytes to the inflamed tissue sites, thus orchestrating the immune response at the site of inflammation. Disease activity measured with the CAS significantly correlated with serum IL-17 concentration. Thus, our data suggest that, as in the cases of rheumatoid arthritis or systemic lupus erythematosus, IL-17 may have a relevant role in the inflammatory phenomenon seen in GO.

In addition to IL-17 levels, we also measured serum IL-6 and IL-23 levels. These cytokines are necessary for the development of Th17 cells, which have been shown to produce abundant IL-17. The important role of IL-6 in the induction of Th17 cell differentiation has been described thoroughly [18]. Once the Th17 cells develop, their survival and pathogenic phenotype can be maintained by IL-23 [19]. The accompanying increase of cytokine involved in Th17 cell differentiation in GO patients may suggest that the increase of Th17 cells is related to the occurrence of GO.

The data in this study demonstrating increased serum IL-17 levels in GO patients provide a new insight for a better understanding of GO. The interactions between the immune components should be further clarified.

There are some limitations in our study. First, we evaluated a limited number of patients and the positive detection rates of cytokines, especially IL-6, were relatively low. Second, we did not evaluate the number of circulating Th17 cells or the levels of IL-17 or other cytokines in the culture of peripheral blood mononuclear cells (PBMCs). Because it has been reported that different cell subsets can synthesize IL-17, if IL-17 had been measured by direct isolation and stimulation of Th17 cells, analysis might have been more accurate. This will be tried in further studies. Investigation of the immune response of the activated PBMCs and Th17 cells by evaluating their ex vivo production of IL-17 or IL-22 could also provide a better understanding of their interrelationships and immunopathological roles in GO. Third, since this study aimed to determine whether or not IL-17 is involved in immune responses induced by autoimmunity in GO, and to examine the correlation between the clinical aspects of ophthalmopathy and IL-17, we did not plan to include toxic multinodular goiter (TMG) or Graves' disease (GD) patients without ophthalmopathy. We think it may be necessary to include TMG and GD patients without ophthalmopathy and compare them through further studies in the future, to explain the relation between IL-17 and thyroid status more clearly.

The discovery of IL-17 producing T cells, and the realization that they play a critical role in the pathogenesis of autoimmune diseases, have provided the potential to develop new therapeutic approaches for autoimmune diseases [20]. Likewise, explaining the mechanism of action of Th17 cells and IL-17 in GO will play a crucial role in clarifying the pathologic mechanism of GO, and will increase the possibility of developing new drugs for GO.

Acknowledgments Approval to conduct this study was obtained from the Institutional Review Board of the Yonsei University College of Medicine, Seoul, Korea. The study was conducted in accordance with the tenets of the Declaration of Helsinki, and written informed consent was obtained from all the study participants. This study was supported by a grant from the Yonsei University College of Medicine (grant number 6-2010-0180).

Competing interests None

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