• Basic Research •

IgG4 and IgE co-positive group found in idiopathic orbital inflammatory disease

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Abstract

• AIM: To reveal the cytokines involved in idiopathic orbital inflammatory disease (IOID) and the relationship between Th17 cells, IgE and IOID pathogenesis.

• METHODS: Whole blood samples were processed immediately after collection and serological IgG4, IgG, and IgE antibodies were tested using ELISA. IOID and orbital cavernous hemangioma (CH) tissue samples underwent Bio-Plex multiplex cytokine detection. Hematoxylin-Eosin (HE) staining of all paraffin samples suggested the histological features of IOIDs, and expressions of IgG4 and IL-17A in affected tissues were detected by immunohistochemistry.

• RESULTS: Among 40 IOID plasma samples, 52.5% (21/40) were positive for IgG4 and 25% (10/40) were positive for IgE. Overlapped IgG4 or IgE positive samples accounted for 22.5% (9/40). Therefore, IOID samples were separated into three groups. The IgE+/IgG4+ group had a relevantly lower level of pro-inflammatory cytokine expression. IL-4 (Th2 cell related), IL-10 and TGF-β1 (Treg cell immunity related) were elevated in all three groups. Some of the Th17 cell related cytokines (*i.e.* IL-17A/F, IL-25, IL-23, and IL-33) displayed higher expression levels in the IgE-/IgG4- group compared to the other two groups.

• CONCLUSION: We discovered an IgG4-IgE co-positive group as well as Th17 cell immune involvement in IgG4-IgE co-negative subgtroup in IOID for the first time. The pathogenesis of IOID could differ from different subgroups according to the IgG4 and IgE detection. Therefore, we recommend that, Treatment stratagy should be made according to the clinical assessment of IgG4-IgE and Th17 profile detection.

• KEYWORDS: idiopathic orbital inflammatory disease; IgE; IgG4; Th17

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INTRODUCTION

IgG4-related disease (IgG4-RD) has been recognized as a multiorgan fibro-inflammatory disease[1]. The typical clinical manifestations of patients with IgG4-RD are increased serum IgG4 levels, tissue infiltration with abundant IgG4-positive plasma cells in a background of dense storiform fibrosis[2-3], and multiple organ involvement, such as lacrimal gland (dacryoadenitis), extraocular muscles (orbital myositis), the uveal tract (uveitis), optic nerve sheath, superior orbital fissure and cavernous sinus, and the orbital fatty tissues[1,4-7], and response to corticosteroid treatment. However, both the pathogenesis and the role of IgG4 in the natural history of the disease remain unclear and are subject of many studies.

Idiopathic orbital inflammatory disease (IOID) is an inflammatory localized or diffused soft tissue orbital tumor of unknown etiology, which could affect extraocular muscle or lacrimal gland. IOID accounts for 7.1%-12.3% of eye diseases with pathological changes of fibrosis[8-9]. Recently, Inflammatory diseases of the lung, liver, orbit, and various other organs have been reported frequently in association with IgG4-RD. They share histopathological and immunohistochemical staining features. Some of the researchers believe IOID should be classified as an IgG4-RD[3], which is regarded as a systemic disease characterized by extensive IgG4-positive plasma cells and T-lymphocyte

infiltration of various organs. While no clear conclusion about the identity of IOID as an IgG4-RD because the frequently unmatched clinical manifestations of IOID with IgG4-RD.

Multiple immune pathways contribute to the fibro-inflammatory process of IgG4-RD. It is generally believed that autoimmunity and infectious agents are immunological triggers in IgG4-RD. Th2-cell mediated immune responses are predominantly activated at the affected sites, followed by activation of regulatory T cells[1]. Years ago, a third subset of T effector cells that produce IL-17, termed Th17 cells, have been discovered[10-11]. Th17 cells are distinct from the traditional Th1, Th2 and Treg cells, because they are highly pro-inflammatory and may induce severe autoimmunity[5].

The production of IgG4 and IgE antibodies were all controlled mainly by Th2 cells. IgE is manifested as a high frequency type I hypersensitivity[12]. In autoimmune pancreatitis (AIP), which is an IgG4-RD disease, a report showed a correlation between elevated IgG4 and IgE antibodies, in which 12 of 48 AIP patients showed serological IgE positivity[13].

Previously, IOID had not been related to any allergic disease or Th17 immunity. We tried to build a relationship between the Th17 cell immune cytokines, pro-inflammatory cytokines, IgE and IOID pathogenesis based on our findings.

SUBJECTS AND METHODS

Plasma and Tissue Samples A total of 41 IOID patients, along with 59 controls [including 40 healthy donors and 19 patients with orbital cavernous hemangioma (CH)] were recruited from Beijing Tongren Hospital with approval of the local ethical committee. IOID clinical samples were randomly chosen, with affected tissue in extraocular muscle and/or lacrimal gland. The diagnosis of IOID was provided by Beijing Tongren Hospital through histological detection, magnetic resonance imaging (MRI) scanning (swelling and thickening eyelid and soft tissue on cheekbones), as well as other clinical outcomes. The control groups of plasma and tissue were aimed for the comparison of IgG4 detection and cytokine profile with normal population (healthy donors) or non-inflammatory diseases (CH patients). Plasma samples from all IOID patients and controls were assayed by serologic tests. Tissue samples from affected location (extraocular muscle and/or lacrimal gland) were all collected immediately after surgical resection from 2011 to 2013 and then analyzed in pathological examination. Tables 1 and 2 showed patient information for all samples that underwent serological and tissue detection. Samples involved in biopsies detection were from diffused tissue type, in which extraocular muscle and lacrimal glands were included. Among the cell types, lymphocytic deformity (LD) referred to the compressional deformation into different shapes of lymphocytes.

Pretreatments Whole blood was centrifuged at 2000 rpm for 10min; the upper layer was then carefully transferred into a clean 1.5 mL tube and stored at -20℃.

Immunohistochemistry Detection Tissue sections were dewaxed at 70℃ for 30min, and then washed in xylene for 30min. Slides were rehydrated with sequential ethanol washes for 1min each, starting with 100%, followed by 80% and 70% ethanol washes, and finishing with a distilled water wash. An additional treatment was performed by incubating slides in 3% H_2O_2 for 15min to remove endogenous peroxidase. Microwave antigen retrieval in citrate buffer was performed for immunostaining following the steps of microwaving on high for 10min, low for 5min, then chilling to room temperature. Tissue sections were washed afterwards for 3min with phosphate buffer solution (PBS), and then blocked in goat serum for 60min at 37℃. Primary antibodies were applied and diluted in PBS (rabbit anti-human IgG4, 1:1000; rabbit anti-human IL-17A, 1:500) at 4℃ overnight. After extensive washing, slides were incubated for 20min at 37℃ with horseradish peroxidase (HRP)-conjugated detection antibodies (goat anti-rabbit-HRP, 1:1000). Diaminobenzidine (DAB) was then added to the slides, followed by washing 3 times in distilled water and counterstaining for 3min with Gill's hematoxylin. Slides were then dehydrated in ascending grades of ethanol (*i.e.* 70%, 80%, and 100%) and finally cleared in xylene and mounted with a cover slip.

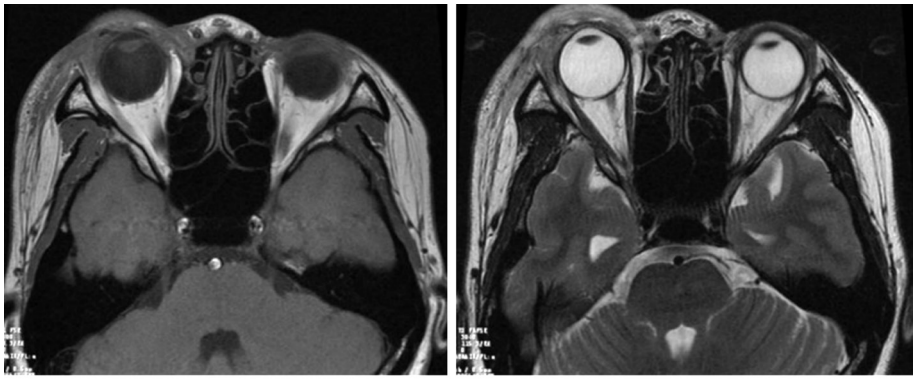
Immunohistochemistry (IHC) related reagents were obtained from Zhongshan Jinqiao Company (Beijing, China). Antibodies used in IHC were purchased from the Abcam Company (rabbit anti-IgG4, ab109493; anti-IL17A, ab136668). IHC procedures followed common experimental steps.

Magnetic Resonance Imaging Scaning The patient underwent MRI detection was among one of the 40 patients mentioned above. The MRI images were kindly provided by Beijing Tongren Hospital, operated following the standard protocol of clinical IOID detection.

Enzyme-linked Immuno Sorbent Assay and Bio-plex Multiple Cytokine Test

Enzyme-linked immuno sorbent assay Human IgG4, total IgG and IgE enzyme-linked immuno sorbent assay (ELISA) kits were purchased from eBioscience (USA) and a human macrophage migration inhibitory factor (MIF) ELISA kit was obtained from RayBio. The 96-well microplates were coated with captured antibodies. Plasma samples and standards were then added and incubated for 2h. After washing, HRP-conjugated detection antibodies were added into each well. Plates were washed three times, followed by stopping the enzyme reaction with stop solution. The optical densities of each well were read after 30min at 450 nm using a micro-plate reader (PerkinElmer, USA).

Human transforming growth factor-β1 (TGF-β1) kits were purchased from 4aBio (Beijing, China, CHE0029). The protocol steps were similar to the protocol above except for one additional step of cytokine activation; *i.e.* adding 10 μL



T1WI T2WI

Figure 1 MRI scanning result for one case of IOID patient T1WI and T2WI revealed the thickening eyelid and soft tissue on cheekbones. Lesions involved rectus muscle of the right orbit and the extraconal orbital compartment. The right eyeball showed deformation due to the high pressure, and the right lacrimal gland enlarged.

Table 1 Case information for IOID, CH and control group plasma and/or tissue samples that underwent IgG4, IgE and/or Bio-plex detection

Groups	Case No.	Age (a)	Male/female	IgG4 and IgE detection	Bio-plex detection
IgE+/IgG4+	9	35-82	5/4	9	4
IgE-/IgG4+	14	17-72	7/7	14	11
IgE-/IgG4-	6	22-54	3/3	6	5
IgE-/IgG4-	11	24-78	1/10	11	10
Total IOID	40	17-82	16/24	40	30
Orbital CH	19	37-72	4/15	19	8
Normal control	40	29-83	20/20	40	0

1 mol/L HCl into 80 μL of the plasma sample, incubating 60min on ice; and then adding another 1 mol/L NaOH into the mixture above, in order to neutralize the HCl.

Bio-plex A total of 0.1 g of each tissue sample was transferred in liquid nitrogen and cystoplasmatic and nuclear protein were extracted following the protocol for the kit (nuclear and cytoplasmic extraction reagents, Thermo). A 15-factor Bio-plex kit was obtained from Bio-rad (CA, USA, #171-AA001M). Procedures were strictly in accordance with the protocols.

Statistical Analysis Statistical analysis was performed using Graphpad prism 5 (GraphPad Software Inc., La Jolla, CA, USA). The difference in IgG4 and IgE positive rates between IOIDs and the control group was assessed with a Chi-square test (P<0.001). Comparison analyses among IgE+/IgG4+, IgE-/IgG4+, IgE-/IgG4-, and CH groups were determined by a one-way ANOVA followed by Tukey's test. Data are shown as the mean±SEM. Statistical significance was set at P<0.05. Differences in cytokine profiles between IOIDs and the control group were analyzed with an unpaired t-test. All the differences were considered statistically significant at P<0.05.

RESULTS

Histological Observation According to the diagnostic report, almost all the IOID patients had a clinical manifestation of swelling and thickening eyelid and soft tissue on cheekbones (Figure 1). While histological staining of IOID paraffin slides showed that, not all of the IOID patients have the manifestations of elevated serological IgG4, IgG4-positive plasma cells or obliterative phlebitis in histological observation (Figures 2 and 3; Table 1). Most of the IOID patients have the affected tissue in orbital soft tissue and lacrimal glands. IHC of IL-17A showed no significant difference among all the 3 subgroups of IOID (Figure 3).

IgG4 & IgE Serological Detection and Tissue Cytokine Profiles Of the 60 orbital disease samples (Table 1), IOIDs (n=40; 16 males, 24 females) consisted of one or more cell types [*i.e.* lacrimal gland epithelium cells (LGEC), lymphocytes infiltration (LI), and LD]; orbital CHs (n=19; 4 males, 15 females) all showed different degrees of fibrosis. Corresponding blood samples were also collected for the 60 patients mentioned above, in which 40/41 IOID and 19/19 orbital CH plasma samples were used for IgG4, total IgG and IgE ELISA detection; 31/40 IOID and 9/19 CH plasma were analyzed using a Bio-plex test.

In a recent, large retrospective study[14], serum IgG4 >135 mg/dL and IgG4/IgG ratios >8% demonstrated high sensitivity and specificity, and these values were also utilized as diagnostic criteria for IgG4-RD in our study. An IgE serological concentration cutoff value of >900 ng/mL was used as the positive IgE criterion[15]. Table 2 showed the IgG4-related pathologic and immunostaining test in IOID clinical samples.

As seen in Table 3, among the 40 IOID plasma samples, up to 52.5% (21/40) were positive for IgG4 and 25% (10/40) were

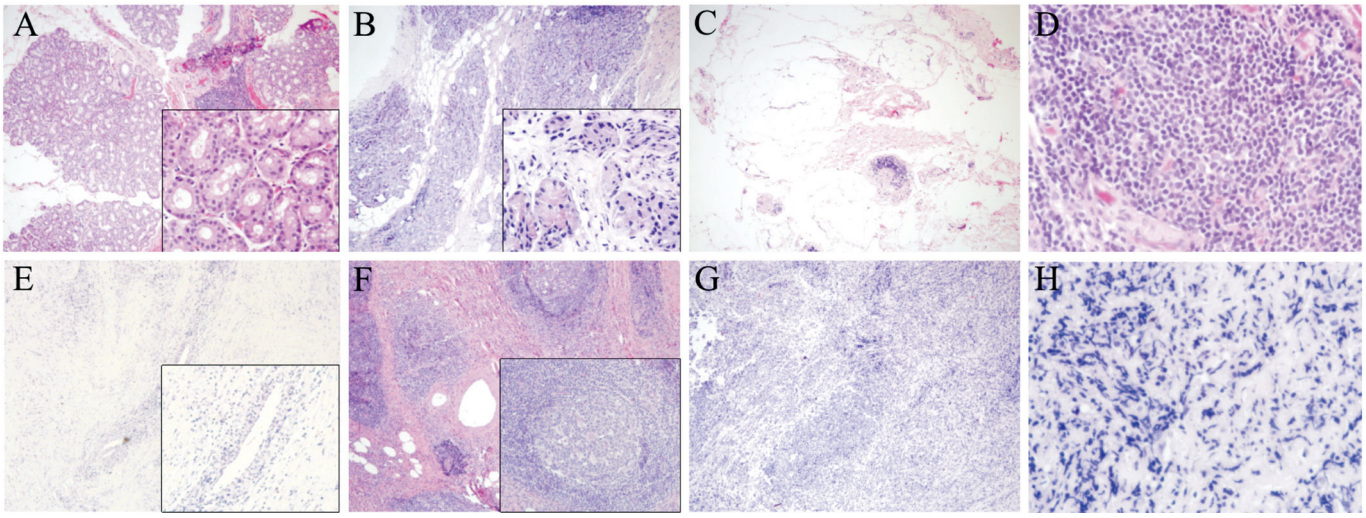


Figure 2 Hematoxylin-Eosin staining for IOID pathological sections A: A low-magnification microscopic section of the common lacrimal gland which reveals a partial lymphoplasmacytic infiltrate associated with fibrosis (original magnification ×100), the inlet demonstrates proliferated lacrimal gland cells (original magnification ×400); B: Atrophic lacrimal gland cells are frequently noted together with mild fibrosis which surrounded lacrimal glands (original magnification ×100), the inner figure shows morphology of atrophic gland cells and fibrosis (original magnification ×400); C: Dense adipose tissue with few lymphocytes infiltration in the middle. (original magnification ×100); D: High-magnification view of the mixed inflammatory infiltrate (original magnification ×400); E: This photomicrograph displays low degrees of lymphocytic infiltration and fibrosis in connective tissue area (original magnification ×100), the inlet demonstrates obliterative phlebitis and surrounded inflammation (original magnification ×400); F: Storiform fibrosis (top left and right of figure) together with dense lymphocytes infiltration (original magnification ×100), the inner figure shows high-magnification view of lymphoplasmacytic infiltration in a follicle area (original magnification ×400); G: Dense fibrosis and lymphocytes infiltration (original magnification ×100); H: Deformed lymphocytes and fibrocytes (original magnification ×400).

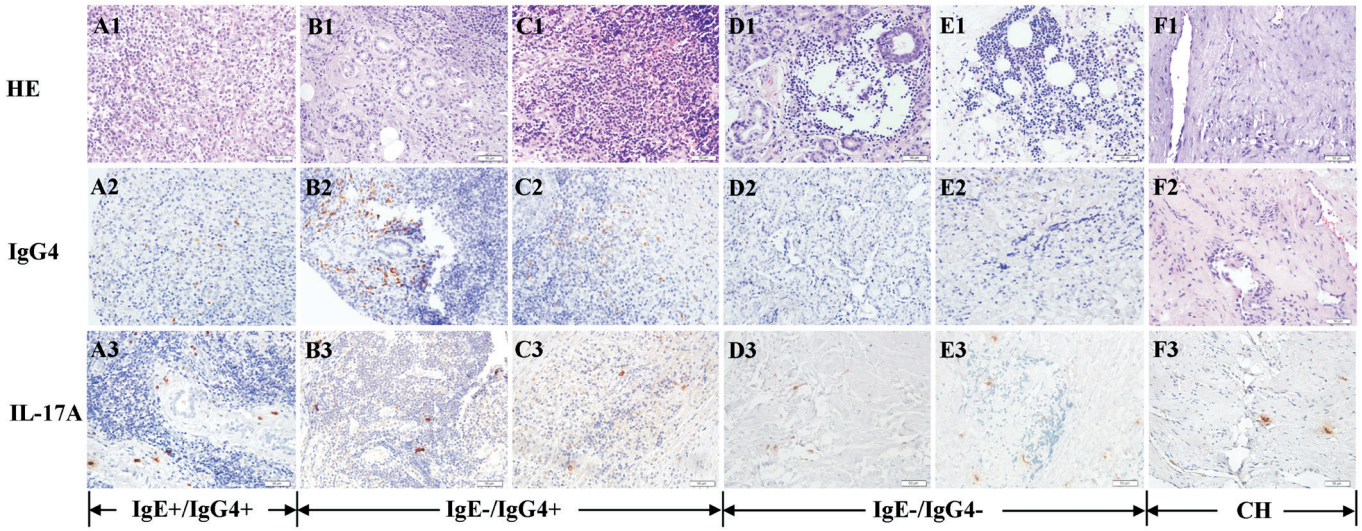


Figure 3 HE staining and histological detection in IOID samples HE staining and IHC detection of IgG4 and IL-17A for IgE+/IgG4+, IgE-/IgG4+, IgE-/IgG4-, and CH group paraffin slices. Line 1 shows HE staining results; lines 2 and 3 display IgG4 and IL-17A IHC detection results, respectively (original magnification ×400).

positive for IgE. Most of the IgE positive samples also showed IgG4 positivity (9/10), except for one outlier (the IgE+IgG4-, Bio-plex cytokine profile of this sample was excluded from the analysis). Thereby, IOIDs were divided into three groups: IgE and IgG4 co-positive (IgE+/IgG4+, *n*=9), IgG4 positive and IgE negative (IgE-/IgG4+, *n*=12) and IgG4 and IgE co-negative (IgE-/IgG4-, *n*=18) for further analysis. IgG4 IHC showed IgG4 positive plasma cells in two IgG4+ groups (Figure 4A2, 4B2 and 4C2). HE staining demonstrated that tissue sections in the IgE+/IgG4+ group were featured as LGEC with different degrees of LI (Figure 4A1); both IgE-/IgG4+ and IgE-/IgG4- groups consisted of the LGEC and massive LI (Figure 3B1-3E1). IL-17A IHC in the three groups displayed similarly positive levels (Figure 4B3-4E3).

Table 2 Pathologic and immunostaining test in IOID clinical samples

IgG4-RD clinical diagnostic criterias	Positive No./total No.	Positive ratio
Dense lymphoplasmacytic infiltrate	15/40	37.5%
Fibrosis, arranged at least focally in a storiform pattern	11/40	27.5%
Obliterative phlebitis	6/40	15%
Massive IgG4+ plasma cells	3/15	20%
Serological IgG4/IgG	21/40	52.5%

Table 3 IgG4 and IgE positive ratios in IOID and CH plasma samples *n* (%)

Detected items	IOID			CH		
	Total	IgG4-	IgG4+	Total	IgG4-	IgG4+
IgG4+	21/40 (52.5)			0/23 (0)		
IgG4-	19/40 (47.5)			0/23 (0)		
IgE+	10/40 (25)	1/10 (10)	9/10 (90)	0/23 (0)	0/23 (0)	0/0 (0)
IgE-	30/40 (75)	18/30 (60)	12/30 (40)	23/23 (100)	23/23 (100)	0/23 (0)

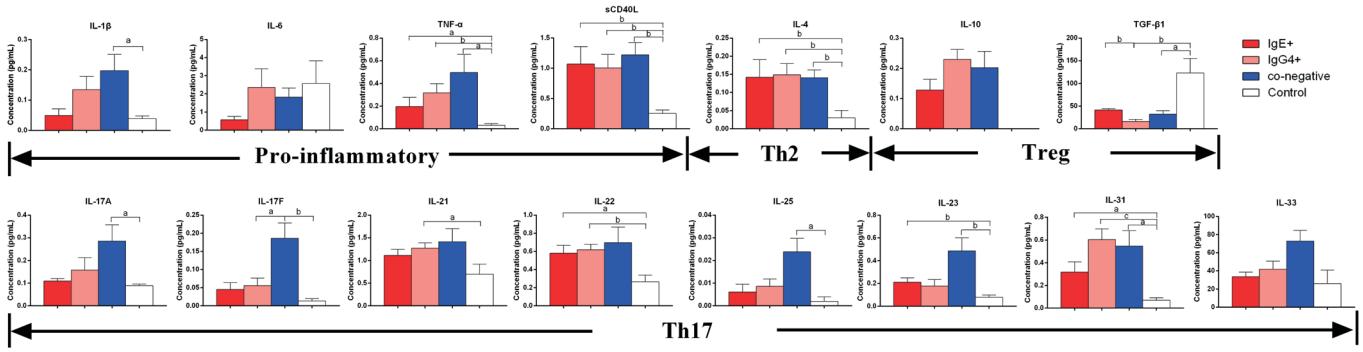


Figure 4 Cytokine profile of pro-inflammatory, Th2, Treg, and Th17 cell immunity in IOID samples Cytokine profiles related to typical pro-inflammatory (IL-β, IL-6, TNF-α, and sCD40L), Th2 cell (IL-4), Treg cell (IL-10 and TGF-β1) and Th17 cell immune factors (IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31 and IL-33). Each sample was analyzed in duplicate. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

Plasma IgG4 titer >135 mg/dL or serological IgG4/IgG >8% is a cutoff for IgG4 serological positive; and IgE titer >900 pg/mL is a cut-off for IgE serological positive. The difference between IOIDs and controls was statistically significant ($P<0.001$).

In order to investigate Th17 cells and other cytokine involvement in IOID pathogenesis and progression, 19 tissue samples were analyzed for the detection of up/down-stream cytokines (15 cytokines using Bio-plex and one cytokine of TGF-β using an ELISA). The cytokine profile is shown in Figure 4 except for interferon-γ (IFN-γ) (not detectable). All of the other 15 cytokines were classified and analyzed in the groups (*i.e.* IgE+/IgG4+, IgE-/IgG4+, IgE-/IgG4-, and the control group).

The IgE+/IgG4+ group had a relevantly lower level of pro-inflammatory cytokine expression than the IgE-/IgG4- group, especially for IL-1β (0.049 pg/mg tissue in IgE+/IgG4+ subgroup *vs* 0.197 pg/mg tissue in IgE-/IgG4- subgroup), TNF-α (0.196 pg/mg tissue in IgE+/IgG4+ subgroup *vs* 0.497 pg/mg tissue in IgE-/IgG4- subgroup), and IL-6 (0.565 pg/mg tissue in IgE+/IgG4+ subgroup *vs* 1.819 pg/mg tissue in IgE-/IgG4- subgroup), although no significant differences were found. However, sCD40L (a member of the TNF family) demonstrated equivalent expression levels in both groups discussed above. In addition, the IgE+/IgG4+ and IgE-/IgG4+ groups showed similar expression levels for most of the cytokines. Th2 cell (IL-4) and Treg cell immunity (IL-10 and TGF-β1) related factors were elevated in all three groups, in which TGF-β1 showed a high absolute value (12 pg/mL), which was lower than the CH group. Some of the Th17 cell related cytokines (*i.e.* IL-17A, IL-17F, IL-25, IL-23, and IL-33) showed higher expression levels in the IgE-/IgG4- group than the other two groups.

DISCUSSION

Relationships of IgG4, IgE and Th17 Immune IgG4-RD disease is recognized as an autoimmune disease, while Th17 cells are also known to trigger many severe autoimmune diseases. Different kinds of immune cells, such as Th1, Th2, and Treg, have a specific cytokine profile that can dictate cellular functions. Th17 cells could also influence some severe

immune functions and pathological processes through the coordinated expression of Th17-related cytokines (*e.g.* IL-17A, IL-17F, IL-21, IL-23 and IL-33).

IgE can mediate type I allergies and is a drug target for many allergic diseases. For example, omalizumab, which is an effective drug for many allergic diseases, is thought to have predominant anti-IgE mechanisms[16]. A high level of IgE in the IgE+/IgG4+ group indicated it was very likely associated with allergy. Pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, have been reported to contribute to Th17 cell activation and development[17]. Th17 cell immunity is highly pro-inflammatory and may induce severe autoimmunity[11,18]. High expression levels of these pro-inflammatory and Th17 cell related cytokines in two groups (*i.e.* IgE-/IgG4+ and IgE-/IgG4-) with IOID suggested they might undergo some autoimmune disorders.

IgE+/IgG4+ Subgroup and Its Cytokine Profile Among the three IOID subgroups separated by serological IgE and IgG4 detection, the IgE+/IgG4+ group was the most exceptional. Regarding an IgG4-IgE co-elevation mechanism, one hypothesis is that IgG4 inhibits mast cell triggering so that it blocks IgE pathways and results in a slow build-up of IgE to high levels[19]. This co-elevation/stimulation system was seen in features of the IgE+/IgG4+ group in our study. Considering the cytokine profile (Figure 4), except for elevated Th2 cell- and Treg cell-related cytokines, the IgE+/IgG4+ group had a relatively lower expression level of Th17 cell-related cytokines (*e.g.* IL-17A, IL17F and IL-25) as well as pro-inflammatory factors (*e.g.* IL-1β, TNF-α and IL-6). We identified this group as an allergy-related subgroup, in which inflammation was mild and autoimmunity-associated Th17 cell immunity was less activated.

IgE-/IgG4+ Subgroup and Its Cytokine Profile With only IgG4 elevation, the IgE-/IgG4+ group showed similar immunity outcomes as for the IgE+/IgG4+ group; *i.e.* there were elevated Th2 cell- and Treg cell-related cytokines in addition to the Th17 cell-related cytokines. Pro-inflammatory cytokines were expressed at slightly higher levels, which might be a subtle difference compared to the IgE+/IgG4+ group. The similar immunity outcomes suggested this group might also be related to allergy or autoimmune responses. Our results may indicate another stage for the IgE+/IgG4+ or IgE-/IgG4- groups. More investigation is required to determine the answer to this question. IL-4 displayed relevant expression levels in the two IgG4+ groups, while IL-10 showed a slightly different outcome (Figure 4). IL-10 has been reported to decrease IL-4-induced IgE differentiation but augments IL-4-induced IgG4 production[20]. Thus, IL-10 expression might provide a potential contribution to the formation of the two IgG4+ groups in IOID.

IgE-/IgG4- Subgroup and Its Features It is accepted that not all IgG4-RD patients have the clinical manifestation of elevated serological IgG4 antibodies. Th17 cell immunity has not been reported to be related to IgG4-RD, however, in our study, this IgE-/IgG4- group was shown to have stronger Th17 cell immunity involvement (cytokines and histological expression), in addition to Th2 cell, Treg cell, and pro-inflammatory factors. The Th17 cell involvement found in IOID provided a clue that some IgG4-RD diseases (for example, Miculicz's disease, which is another orbital disease) are potentially associated with Th17 cell activation, or IgG4 elevation might be a stage after Th17 cell immunity involvement.

In conclusion, we discovered an IgG4+/IgE+ co-positive subgroup as well as Th17 cell immune involvement in IOID. Pro-inflammatory and Th17 cell immune related cytokines could contribute to the pathogenesis of IgE-/IgG4+ and IgE-/IgG4- subgroups: for the IgG4+/IgE- subgroup of IOID patients, pro-inflammatory cytokines were dominant and Th17 cell immune responses were less active; a strong immune response was suggested of the IgG4-/IgE- subgroup in these patients. Therefore, we recommend that treatment strategy should be made according to the clinical assessment of IgG4 and IgE detection. For patients with elevated serological IgE levels, allergic pathogenesis and treatments, such as allergen screening and desensitization therapy, could be taken into consideration. More clinical trials on different treatment strategies are needed.

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