IL-17A Promotes RANTES Expression, But Not IL-16, in Orbital Fibroblasts Via CD40-CD40L Combination in Thyroid-Associated Ophthalmopathy

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PURPOSE. This present study aims to investigate the phenotype of IL-17A–producing T cells in thyroid-associated ophthalmopathy (TAO) and the role of IL-17A on RANTES and IL-16 expression in orbital fibroblasts (OFs) from TAO patients.

METHODS. Blood samples were obtained from TAO patients and healthy controls and were subjected to ELISA and flow cytometry analysis. Primary human OFs cultured from surgical wastes were stimulated with IL-17A in the presence or absence of CD40L and were examined by qRT-PCR, ELISA, Western blotting, and apoptosis assays.

RESULTS. We reported upregulated IL-17A, IFN-γ, RANTES, and IL-16 serum levels and increased frequency of IL-17A– and IFN-γ–producing T cells in peripheral blood mononuclear cells from patients with TAO compared with healthy controls. In addition, TAO orbital tissues were rich in T lymphocytes, expressing more IL-17A, IFN-γ, RANTES, and IL-16. Moreover, IL-17A could enhance the expression of RANTES, but not IL-16, in cultured primary OFs in cooperation with CD40L. We further validated that MAPK signaling was largely responsible for RANTES production in IL-17A–treated OFs. Finally, we demonstrated that IL-17A could not promote apparent apoptosis in OFs from TAO patients and healthy controls.

CONCLUSIONS. Our results indicate the potent effect of IL-17A–induced RANTES expression on OFs and elaborate a possible mechanism in understanding Th17 cells in the pathology of TAO and its potential as a target to immunotherapy of TAO and other autoimmune disorders.

Keywords: thyroid-associated ophthalmopathy, IL-17A, RANTES, CD40L

Thyroid-associated ophthalmopathy (TAO) is a common disorder that occurs in approximately 25% to 50% of patients with Graves’ disease (GD). Because of the poorly understood pathophysiological mechanism of TAO, it has puzzled physicians and scientists for centuries and few improvements have been made in the etiologic treatment of TAO. A multitude of previous studies have already unraveled that TAO is an autoimmune-mediated inflammatory reaction, in which different lymphocytes recruit and infiltrate orbital connective tissues. In the early phase of TAO, type 1 T helper (Th1) cells dominate the immune responses in the orbits, whereas in the late stage, it tends to be a Th2 bias reaction. Both Th1 and Th2 cells produce various cytokines, such as interferon-γ (IFN-γ) and IL-4, respectively. Meanwhile, the orbital fibroblasts (OFs) recognized as the target cells are activated with these orbit-infiltrated immune cells and their products, leading to OF proliferation and differentiation marked by orbital tissue remodeling and enlargement. Also, activated OFs secrete inflammatory mediators and growth factors, resulting in further chemotaxis of more lymphocytes into the orbits and uncontrolled inflammatory responses. However, the detailed and exact interplay among these cells in this progress still remains unclear and the regulation of each component in this sophisticated circuit contributes greatly to the therapeutic strategies for TAO patients. Thelper 17 cells, characterized by producing IL-17A, IL-17F, and IL-22, are vital effector cells in host defense against certain pathogens and can also induce tissue inflammation and autoimmunity. Currently, autoimmune disorders are considered to be closely interrelated with the abnormality of Th17 cells. In GD, the proportion of Th17 cells and IL-17A protein level are increased in the circulation, and the polymorphism of IL-17A is associated with GD susceptibility. Although in TAO the serum IL-17A level is upregulated, yet, it still remains unclear about the possible role of those IL-17A–producing T cells.

Regulated upon activation, normal T-cell expressed and secreted (RANTES) belongs to the C-C chemokine subfamily and is a feature potentially relevant in a range of inflammatory disorders. Interleukin-16 acts as a chemoattractant, based on the initial observation for the induction of CD4+ T-cell...
chemotaxis. In the early phase of TAO, activated OFs release IL-16 and RANTES, which initiate T-cell migration into the orbit, leading to uncontrolled inflammatory responses. As Th17 cells are proinflammatory and involved in the attack of many autoimmune diseases, it is of great interest to dive into the interaction between Th17 cells and those chemokines in TAO.

Here, we first reported increased proportions of both IL-17A- and IFN-γ-producing T cells in TAO peripheral blood. Besides, the expressions of IL-17A and IFN-γ were higher in TAO orbits with T-cell invasion. Thyroid-associated ophthalmopathy orbital connective tissues overexpressed RANTES and IL-16, and IL-17A was shown to promote the transcription and translation of RANTES in the presence of CD40L via MAPK signaling. Our current data unraveled the possible role of IL-17A and activated T cells in orchestrating the local inflammatory responses in TAO.

Materials and Methods

Patients and Controls

A total of 43 subjects (12 males and 31 females) were recruited from the patient population of Shanghai Ninth People’s Hospital. Informed consent was obtained from each subject as approved by the Ethics Committee of Shanghai Ninth People’s Hospital, Shanghai JiaoTong University School of Medicine. All research complies with the tenets of the Declaration of Helsinki. Blood samples and surgical explants of TAO patients were collected during orbital decompression surgery when all patients were in stable thyroid function. Control blood samples and tissues from 20 healthy subjects (10 males and 10 females) with no history of thyroid dysfunction, autoimmune diseases, and ocular disorders were obtained during blepharoplasty. Historical information and clinical characteristics for those patients and control subjects are presented in Table 1.

Cell Isolation, Culture, and Treatment

Orbital fibroblasts were cultivated from inactive TAO patients according to our previous study. Briefly, surgical wastes were minced and placed on six-well plates with Dulbecco’s modified Eagle’s medium ( Gibco, Waltham, MA, USA) containing 10% fetal bovine serum, allowing OFs to grow out and proliferate. Cell monolayers were serially passaged and fibroblasts were used between the third and eighth passages. Three independent strains from different donors were used for repeated experiments. When reaching 70% confluence, cells were used for flow cytometry.

Summary for the Clinical Characteristics of TAO Patients and Controls

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>Active TAO, n = 17</th>
<th>Inactive TAO, n = 26</th>
<th>Control, n = 20</th>
</tr>
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<tbody>
<tr>
<td>Age (range)</td>
<td>50.94 ± 10.51</td>
<td>34.08 ± 10.87</td>
<td>36.40 ± 10.91</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>7/10</td>
<td>5/21</td>
<td>10/10</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>4/17</td>
<td>4/26</td>
<td>0/20</td>
</tr>
<tr>
<td>Thyroid function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthyroid</td>
<td>7/17</td>
<td>25/26</td>
<td>20/20</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>8/17</td>
<td>0/26</td>
<td>0/20</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>2/17</td>
<td>1/26</td>
<td>0/20</td>
</tr>
<tr>
<td>Treatment for thyroid</td>
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<td></td>
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<tr>
<td>Antithyroid drug</td>
<td>15/17</td>
<td>16/26</td>
<td>0/20</td>
</tr>
<tr>
<td>Radio-iodine</td>
<td>0/17</td>
<td>5/26</td>
<td>0/20</td>
</tr>
<tr>
<td>Surgery</td>
<td>2/17</td>
<td>1/26</td>
<td>0/20</td>
</tr>
<tr>
<td>Clinical activity score (range)</td>
<td>4.59 ± 0.87</td>
<td>0.85 ± 0.92</td>
<td>-</td>
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</table>

Immunohistochemical Staining

Sections of 4 μm paraffin were deparaffinized, quenched of endogenous peroxidase, and blocked with 3% BSA. Sections were then stained with primary antibodies (anti-CD3, anti-CD4, anti-IL-17A, anti-IFN-γ, anti-RANTES, or anti-IL-16 antibody; all from Abcam, Cambridge, MA, USA) overnight at 4°C. A biotinylated secondary antibody was then used before sections were incubated with horseradish peroxidase streptavidin. Slides were examined with an Olympus BX51 (Olympus, Tokyo, Japan).

Flow Cytometry

Prepared PBMCs were collected and stained with APC-Cy7- Fixable Viability Dye (eBioscience, San Diego, CA, USA) to exclude dead cells. They were then incubated with surface markers (FITC-CD3, Alexa Fluor 700-CD8, or PerCP-Cy5.5- CD145RO; all from BD Biosciences). For cytokine staining, cells were fixed and permeabilized using fixation/permeabilization reagents (eBioscience) and stained with PE-IL-17A (eBioscience), anti-IFN-γ, and APC-CD4 (BD Biosciences). Flow cytometry data were collected using a BD LSRFortessa (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Enzyme-Linked Immunosorbent Assay

Interleukin-17A, IFN-γ, RANTES (all from eBioscience), and IL-16 (R&D Systems) were quantified in the serum from TAO patients and healthy subjects by ELISA assay according to the manufacturers’ instructions strictly.

Quantitative Real-Time RT-PCR

Total cellular RNA from fibroblast was extracted with TRIzol (Sigma-Aldrich) reagent and reversely transcribed into cDNA with PrimeScript RT Reagent Kit ( Takara, Tokyo, Japan). Quantitative RT PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara) on a 7900HT Fast Real-Time PCR.
IL-17A Promotes RANTES Expression in TAO

TABLE 2. Nucleotide Sequences of Primers Used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sequence, 5’ to 3’</th>
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<tr>
<td>RANTES</td>
<td>F: GAAAGAACCAGCCAACTGTGT</td>
</tr>
<tr>
<td></td>
<td>R: GCAAGCAGAAACAGGCAAAT</td>
</tr>
<tr>
<td>IL-16</td>
<td>F: CTTTGGCTCTCTCTCAACTG</td>
</tr>
<tr>
<td></td>
<td>R: CAGAAAACCCTCCTCTCTCA</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: GGACTCGAGCAAGAGATCC</td>
</tr>
<tr>
<td></td>
<td>R: AGCACATGTGGCGCTTACAG</td>
</tr>
</tbody>
</table>

Western Blotting

Approximately 1 × 10⁷ OFs were lysed on ice for 30 minutes in cell lysis buffer. Cell lysates were then centrifuged at 10,000g for 15 minutes and the supernatants were subject into SDS-PAGE, separated, and transferred electrophoretically onto nitrocellulose membranes (Millipore, Boston, MA, USA). The membranes were blocked in 5% skim milk for 1 hour and incubated with primary antibodies (anti-p-ERK, anti-ERK, anti-p-p38, anti-p38, anti-p-JNK, anti-JNK, anti-p-c-Jun, anti-c-Jun, anti-p-AKT, anti-AKT, anti-p-STAT3, or anti-STAT3 antibody; all from Cell Signaling Technology, Boston, MA, USA) overnight at 4°C. The membranes were then reincubated with secondary peroxidase-labeled antibodies (Jackson ImmunoResearch, West Grove, PA, USA) at room temperature for 1 hour. Blots were developed with the ECL Plus reagent (Millipore).

Apoptosis Detection Assay

For apoptosis detection assay, IL-17A–treated OFs were stained by using FITC Annexin V apoptosis detection kit 1 (BD Biosciences) following the manufacturer’s instructions. Samples were run on a BD LSRFortessa and results were analyzed using FlowJo software.

Statistical Analysis

Each experiment was repeated at least three times unless otherwise specified and data were analyzed by using the statistical software of SPSS version 19.0 (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA). Two-tailed Student’s t-test, ANOVA, or Mann-Whitney U test was used as appropriate. All values were presented as the mean ± SD and statistical significance was defined as a P value less than 0.05.

RESULTS

The Th17- and Th1-Related Cytokines Are Increased in the Serum of TAO Patients

To identify whether IL-17A and IFN-γ protein levels were increased in the peripheral blood, serum from active and inactive TAO patients or healthy subjects was examined first by ELISA assay. Our data revealed that the Th17 cytokine IL-17A was upregulated in TAO patients compared with healthy controls (Fig. 1A). Moreover, the expression level of IL-17A was much higher in active TAO than inactive TAO and healthy subjects (Fig. 1A). Simultaneously, the Th1 cytokine IFN-γ was also elevated in both active and inactive TAO patients compared with healthy controls (Fig. 1B). However, there was no significant difference of IFN-γ serum level between active and inactive TAO (Fig. 1B). Additionally, RANTES and IL-16, the two T-cell chemokines, were observed to be significantly increased in active, but not inactive, TAO patients compared with healthy subjects (Figs. 1C, 1D).

The Proportions of IL-17A– and IFN-γ-Producing T Cells Are Elevated in TAO Patients

To investigate the roles of Th17 and Th1 cells in the pathogenesis of TAO, we next analyzed the phenotypes of IL-17A–producing and IFN-γ–producing T cells in the peripheral blood from TAO patients and control subjects. Isolated PBMCs were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 6 hours with subsequent cell surface and intracellular staining for CD3+, CD8+, and IL-17A+ or IFN-γ+ cells. Our data displayed that the proportion of CD3+CD8+ IL-17A+ T cells was significantly higher in TAO patients, especially in active TAO (Fig. 2A). Similarly, the CD3+CD8+ IFN-γ+ T cells were also detected to be upregulated in TAO patients compared with healthy persons (Fig. 2B). It should be noted that, in inactive TAO, the Th1 and Th17 cells continue to
FIGURE 2. The percentages of IL-17A–producing and IFN-γ–producing T cells from active TAO patients, inactive TAO patients, and healthy controls (gated on CD3). (A) The proportions of CD3⁺CD8⁺ IL-17A⁺ T cells in isolated PBMCs from the three groups. (B) The proportions of CD3⁺CD8⁺ IFN-γ⁺ T cells in isolated PBMCs from the three groups. (C) The proportions of CD3⁺CD8⁺ IFN-γ⁺IL-17A⁺ T cells in isolated PBMCs from the three groups. (D) Interleukin-17A expression on CD3⁺CD8⁺CD45RO⁺ T cells in isolated PBMCs from the three groups. (E) Interferon-γ expression on CD3⁺CD8⁺CD45RO⁺ T cells in isolated PBMCs from the three groups. Data are presented as mean ± SD (n = 17 per active TAO, 26 per inactive TAO, and 20 per control). ns, nonsignificant; *P < .05, **P < .01, and ***P < .001.
experience high proportions, although slightly decreased, which means that abnormal cellular immunity function and inflammatory process still exist in the stable period of TAO (Figs. 2A, 2B). Intriguingly, among these CD3⁺CD8⁻ T cells, a small population of IL-17A⁺IFN-γ⁺ cells, namely pathogenic Th17 cells, was observed to be upregulated significantly in active TAO patients (Fig. 2C).

As the most common complication of GD, it could be safely speculated that self-reactive T lymphocytes have been exposed to specific antigens and been activated in the early stage of TAO (Figs. 2A, 2B). We, therefore, examined whether these CD3⁺CD8⁻ T cells were memory T cells by analyzing CD45RO expression. When compared with healthy controls, both active and inactive TAO groups showed significant elevation in the frequency of CD3⁺CD8⁻CD45RO⁺IFN-γ⁺ producing and CD3⁺CD8⁻CD45RO⁺IL-17A⁺producing T cells (Figs. 2D, 2E).

**Retrobulbar Connective/Adipose Tissues From Active TAO Patients Are Infiltrated by T Lymphocytes With Higher Expressions of RANTES and IL-16**

We next examined orbital connective tissues obtained from active and inactive TAO patients who underwent orbital decompression surgery. Immunohistochemistry staining revealed that CD3⁺ cells infiltrated the retrobulbar fatty connective tissues from active TAO patients, but not those from inactive TAO or healthy persons (Fig. 3A). Additionally, in active TAO patients, we observed CD4⁺ T lymphocytes in and around small blood vessels, invading fibrous septa and intrusion into adipocytes (Fig. 3A). Moreover, our results demonstrated enhanced expressions of IL-17A and IFN-γ in the fiber cords as well as microvessels of active TAO orbital tissues compared with inactive TAO and normal ones (Fig. 3B).

Our further evaluation for RANTES and IL-16 staining showed that active TAO orbital connective tissues presented strong positive expressions of those two proinflammatory cytokines than inactive TAO and healthy subjects (Fig. 3C). Generally, they were expressed by OFs in TAO, but not control fibroblasts. It could be conjectured that these RANTES and IL-16–expressing fibroblast-like cells were induced and activated to produce proinflammatory cytokines through interaction with orbital infiltrating T cells, especially in the active stage.

**Interleukin-17A Promotes the Secretion of RANTES, But Not IL-16, in Primary OFs**

To evaluate the effect of IL-17A on OFs or control fibroblasts, primary fibroblasts isolated from orbital connective/adipose tissues in TAO patients and healthy subjects were treated with
different concentrations of IL-17A for different time points. The ability of OFs to express RANTES was first assessed by treating cell cultures with IL-17A alone for graded intervals. However, we did not detect any RANTES gene expression change or increased protein level in the supernatants of IL-17A–treated OFs (Figs. 4A, 4B). Intriguingly, using a range from 1 to 100 ng/mL for 48 hours, we observed a dose-dependent manner of IL-17A–treated OFs in the presence of CD40L for RANTES gene expression. To induce RANTES gene fold change, 20 ng/mL IL-17A combined with CD40L was sufficient enough, which was strengthened with the increase of IL-17A dosage (Fig. 4A). Moreover, the enhancement of RANTES gene expression was also observed to be time-dependent, as this positive performance was seen at 12 hours and reached its peak after 48 hours of incubation (Fig. 4A). We further tested whether CD40L alone could upregulate RANTES expression. Our data demonstrated that with a low dose of CD40L (1, 10, or 50 ng/mL) treatment, the expression level of RANTES was not enhanced in OFs (Supplementary Fig. S1A); yet, CD40L could promote the secretion of RANTES in OFs at high dose (Supplementary Fig. S1A). We therefore used 100 ng/mL CD40L to stimulate OFs and observed a time-dependent manner of RANTES secretion (Supplementary Fig. S1B). Additionally, RANTES protein level revealed time and dose dependence in the cooperation of IL-17A with CD40L as well (Fig. 4B), which scaled the peak at 100 ng/mL for 48 hours. The costimulation of IL-17A and low dose of CD40L showed an additive effect on RANTES expression compared with IL-17A or high dose of CD40L treatment, respectively. Furthermore, we stimulated fibroblasts derived from healthy persons using 100 ng/mL IL-17A with or without 50 ng/mL CD40L for 48 hours and found a significant increase both in RANTES gene and protein expressions, which was analogous to TAO OFs (Figs. 4C, 4D).

Notably, our data demonstrated that no change on the level of transcription for IL-16 was observed under IL-17A treatment even if CD40L was added to the system (Fig. 4A), and IL-16 protein also was not detectable in cultured OF supernatants in our study (data not shown). Altogether, IL-17A could trigger the production of RANTES, but not IL-16, in both transcriptional and translational levels, mainly in a CD40L dependent way.

**The Expression of RANTES in IL-17A–Treated Primary OFs Is Mainly Induced Via MAPK Signaling Activation**

To determine how IL-17A activates OFs to synthesize and secrete RANTES, we tested the MAPK pathways with Western blotting. Interleukin-17A could activate several MAPK signaling pathways.
pathways in OFs, including ERK1/2, p38, and JNK/c-Jun, as indicated by time-dependent modifications in their phosphorylation levels (Figs. 5A, 5C). Prominent increase in phospho-MAPK components was observed after stimulation with 100 ng/mL IL-17A from 15 minutes to 120 minutes (Figs. 5A, 5C). Meanwhile, IL-17A triggered STAT3-phosphorylation in OFs (Figs. 5A, 5C), which might also contribute to the proinflammatory cytokine production. In contrast, no obvious phosphorylation of AKT was detected in IL-17A–incubated OFs (data not shown).

The production of RANTES protein level in IL-17A–treated fibroblasts could be completely abolished by blocking IL-17 receptor A with neutralizing antibody (Fig. 5B), and was significantly downregulated by using different MAPK chemical inhibitors, including U0126 (MEK1/2 inhibitor, an upstream molecule of ERK1/2), SB203580 (p38 inhibitor), and SP600125 (JNK inhibitor) (Fig. 5B). Our data revealed that both U0126, SB203580, and SP600125 significantly suppressed the production of RANTES in OFs stimulated with IL-17A combined with CD40L (Fig. 5B). Collectively, our results demonstrated the involvement of multiple MAPK pathways in IL-17A–mediated inflammation in TAO.

**Interleukin-17A Does Not Induce Apoptosis in OFs**

As apoptosis and necrosis often accompanies with inflammation, we therefore examined the role of IL-17A in OF apoptosis to further understand the damaging effect of this cytokine on orbital tissue. In our study, IL-17A could not induce apoptosis in OFs as well as control fibroblasts with the dosage range from 1 to 100 ng/mL for 48 hours (Fig. 6). Additionally, we did not observe any convincing sign of necrosis in IL-17A-stimulated OFs and control fibroblasts as well (Fig. 6).

**DISCUSSION**

In this study, we showed that serum IL-17A and IFN-$\gamma$ levels were significantly higher in patients with TAO than in...
controls, especially in active TAO patients. This was in line with the studies of Kim et al.\textsuperscript{13} and Shen et al.\textsuperscript{14} Recently, our group demonstrated an upregulated proportion of IL-17A–producing T cells in TAO patients with higher serum expression of IL-6, IL-23, IL-1β, and TGF-β.\textsuperscript{20} Based on this evidence, here we further explored the frequency of circulating IL-17A–producing and IFN-γ–producing T cells with disease activity. Our data unraveled that both CD3\textsuperscript{+}CD8\textsuperscript{+}IL-17A\textsuperscript{+} and CD3\textsuperscript{+}CD8\textsuperscript{+}IFN-γ\textsuperscript{+} T cells were augmented in TAO patients, particularly during the active period, which was consistent with what we found in the serologic study. Additionally, it is noteworthy that a small subset of CD3\textsuperscript{+}CD8\textsuperscript{+}IL-17A\textsuperscript{+}IFN-γ\textsuperscript{+} T cells was also detected to raise significantly in active TAO patients, which was in conformity with the study of Peng et al.\textsuperscript{10} on GD. T-helper 17 cells that coexpress IL-17A and IFN-γ are defined as inflammatory or pathogenic Th17 cells.\textsuperscript{21} They are a rare population, but are increased in psoriasis,\textsuperscript{22} Behçet’s disease,\textsuperscript{23} rheumatoid

**Figure 6.** The apoptosis and necrosis of OFs from TAO and control subjects induced by IL-17A. (A, B) Thyroid-associated ophthalmopathy OFs were stimulated with IL-17A (0, 1, 10, 20, 40, 100 ng/mL) for 48 hours and the early apoptosis (Annexin V\textsuperscript{+}PI\textsuperscript{−} cells) and necrosis (Annexin V\textsuperscript{+}PI\textsuperscript{+} cells) were assayed by flow cytometry. (C, D) Control fibroblasts were stimulated with IL-17A (0, 1, 10, 20, 40, 100 ng/mL) for 48 hours and the early apoptosis (Annexin V\textsuperscript{+}PI\textsuperscript{−} cells) and necrosis (Annexin V\textsuperscript{+}PI\textsuperscript{+} cells) were assayed by flow cytometry. Data are presented as mean ± SD pooled from three experiments using three independent strains.
arthritis, inflammatory bowel disease, and multiple sclerosis, or murine experimental autoimmune encephalomyelitis models. It is tempting to speculate that those IL-17A–IFN-γ-producing T cells emerge to a higher extent in the peripheral blood as well as the orbits of TAO patients during inflammation, causing ocular tissue damage.

Before the identification of Th17 cells, previous studies corroborated the requirement of IFN-γ-producing Th1 cells for the development of several autoimmune diseases. Recently, evidence is accumulating that IL-17A–producing Th17 cells play a central role in autoimmunity. In murine models of proteoglycan-induced arthritis, colitis, encephalomyelitis, and experimental autoimmune uveitis, either a Th17 or a Th1 effector response can drive inflammatory disorders. This fully illustrates the complexity of disease-inducing T-cell subsets in autoimmunity. As a matter of fact, we could not elucidate any disease mechanism only by a single and absolute cytokine or cell subset. Our current study confirmed that IL-17A–producing T cells, together with IFN-γ-producing T cells, may contribute synergistically to orbital inflammation in TAO. Human T lymphocytes can be separated into CD45RA+ resting T cells or CD45RO+ antigen-experienced memory T cells. Interleukin-17A is synthesized and released from T lymphocytes of the CD45RO+ subset, not the CD45RA+ cell subset. In our current study, most of the IL-17A– and IFN-γ–producing T cells are CD45RO+ regardless of the disease activity. Thus, we can draw a safe conclusion that T effector cells are activated and recruited at the onset of TAO, or even earlier at GD attack. Meanwhile, we acknowledged the limitation in our study that it would be necessary to enroll GD patients without orbitopathy and compare them with TAO patients, which needs to be further investigated in the future to determine a more definite relevancy between IL-17A and thyroid status.

We further demonstrated infiltrating T lymphocytes in the fibrovascular septae of orbital connective tissues with strong and diffuse IL-17A and IFN-γ expressions in active TAO orbits compared with inactive TAO and periocular control tissues. This was in concert with upregulated IL-17A– and IFN-γ–producing T cells in peripheral blood of TAO patients, particularly in the active stage.

The CD40 and CD40L interaction is required for lymphocyte activation. Orbital fibroblasts constitutively express CD40, and the CD40L on T-cell binding to OF not only promotes T-cell proliferation and activation, but also enhances proinflammatory cytokine production in OFs, such as ICAM-1, IL-6, IL-8, and MCP-1, leading to a positive feedback loop in TAO autoimmunity. Intriguingly, IL-17A alone could promote the expression of IL-6, IL-8, and MCP-1 in OFs, which was in accordance with the research by Gillespie et al. on circulating fibrocytes in TAO, combined use of IL-17A and CD40L was more efficacious than the separate role of each one. Collectively, the inflammatory effect of T effector cells on OFs attributes to both soluble cytokines in the local microenvironment and direct cell-cell contact. As RANTES can induce in vitro migration and recruitment of T cells, dendritic cells, eosinophils, natural killer cells, mast cells, and basophils, it might be assumed that the regulatory loop among RANTES, IL-17A, and T cells results in massive amplification of TAO inflammatory process. Our further studies illustrated that IL-17A performed proinflammatory function on binding to its specific receptor as blockade of the IL-17 receptor A could markedly attenuate the induction of RANTES. Additionally, the MAPK pathways, including ERK1/2, p38, and JNK/c-Jun, were activated and RANTES induction in OFs with combined IL-17A and CD40L stimulation could be significantly restrained when specific MAPK inhibitors were used. Our results may be partially explained by the possible molecular mechanism involved in IL-17A-regulated orbital autoimmunity in TAO.

Cho et al. reported that IL-17A could induce the production of IL-16 in synovial fibroblasts. However, in our study, IL-16 could not be induced in OFs under IL-17A stimulation with or without CD40L. This discrepancy could be explained by differences in the experimental conditions and tissue specificity in OFs and synovial fibroblasts when activated with IL-17A.

Apoptosis is a normal process occurring in the maintenance of tissue homeostasis and during inflammation. In human acute coronary syndrome, IL-17A could induce vascular endothelial cell apoptosis. Here we showed the proinflammatory effect of IL-17A on OFs, but we did not observe any damage effect of IL-17A to induce OF injury and disorder, as no increase of apoptosis or necrosis was exhibited in IL-17A-stimulated OFs from TAO and healthy subjects.

In conclusion, our current findings shed new light on a potential molecular mechanism of IL-17A–mediated autoimmunity associated with TAO. They provide a possible positive feedback loop for OFs and activated T effector cells in promoting the uncontrolled inflammatory responses regulated by IL-17A and RANTES in the orbit (Fig. 7). Our results may develop a more in-depth understanding of Th17 pathogenesis in TAO and other autoimmune diseases and suggest a potential anti-IL17A method on TAO treatment.
Acknowledgments

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References


