Interaction Between CCR6+ Th17 Cells and CD34+ Fibrocytes Promotes Inflammation: Implications in Graves' Orbitopathy in Chinese Population

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PURPOSE. Recent reports suggest that Th17 immunity and bone marrow–derived CD34+ fibrocytes contribute to the pathogenesis of Graves' orbitopathy (GO). This study investigated interactions between Th17 cells and fibrocytes in GO inflammation in Chinese subjects.

METHODS. Th17 cells and fibrocytes were derived from blood samples from Chinese GO patients and healthy controls. Proportions and phenotypes of Th17 cells, regulatory T cells (Tregs), and fibrocytes were examined by flow cytometry. Exogenous IL-17A was used to study inflammatory activity of fibrocytes from GO patients and control subjects. Coculture, quantitative RT-PCR, Luminex, and transwell assays were performed to investigate the relationship between Th17 cells and fibrocytes.

RESULTS. CC-chemokine receptor 6 (CCR6+) Th17 cells were increased in both active ($P <$ 0.001) and inactive ($P < 0.05$) GO patients, compared with healthy controls. There was a positive correlation between number of CCR6+ Th17 cells and GO clinical activity score ($P <$ 0.0001, $r = 0.8176$). Further, CD34+ fibrocytes were increased in GO patients, with increased expression of IL-17RA ($P < 0.05$), CD80 ($P < 0.05$), and CD86 ($P < 0.05$). A decreased population of effector Treg cells ($P < 0.01$) and increased CTLA-4 expression on naïve Treg cells ($P < 0.05$) were observed in GO patients. IL-17A stimulated cytokine production in fibrocytes; GO fibrocytes exhibited more robust production than normal fibrocytes. Autologous Th17 cells promoted inflammatory and antigen-presenting functions of GO fibrocytes; conversely, fibrocytes enhanced Th17 cell-function and recruited Th17 cells in a macrophage inflammatory protein 3 (MIP-3)/CCR6-dependent manner.

CONCLUSIONS. The crosstalk between CCR6+ Th17 cells and fibrocytes plays a role in the pathogenesis of GO. Suppressing these interactions may be a candidate molecular target for therapeutic approaches of GO.

Keywords: CCR6, Th17 cell, fibrocyte, inflammation, Graves' orbitopathy

Over the past decade, there has been extraordinary progress in understanding the pathogenesis of Graves' orbitopathy (GO), a disease that presents with visual impairment and the appearance of craniofacial injury.[1-3] GO originates from a combination of environmental, genetic, and immunological factors; autoimmunity is the key factor that precipitates the onset of disease.[1,2]

Early studies of GO focused primarily on levels of Th1 and Th2 cytokine expression and their pathogenic effects on GO patients.[1,3] Recent reports suggest that Th17-type immunity is

involved in the development of both Graves' disease (GD) and GO.[4–7] First isolated in 1993, IL-17A is a Th17-cell cytokine that participates in a wide variety of human autoimmune diseases.[8] Accumulation of IL-17A–producing T cells has been observed in the cerebrospinal fluid of multiple sclerosis patients, in the synovial fluid of rheumatoid arthritis (RA) patients, and in the skin lesions of psoriasis patients.[8,9] Notably, Th17 cells expressing CC-chemokine receptor 6 (CCR6) exhibit pathogenic activity in RA patients.[10] In GD and GO, high levels of IL-17A have been detected in patients' peripheral blood.[6,11] In studies of GO patients, we previously found an increase in the peripheral blood population of Th17 cells, and an increase in the orbital expression of IL-17A.[12,13] IL-17A promotes orbital inflammation by enhancing inflammatory activities of orbital fibroblasts (OFs), the target cell type in GO autoimmunity.[12,13] However, there has not been a careful examination of a potential relationship among CCR6 expression, IL-17A production, and GO pathogenesis.

Recent studies have revealed that a rare subset of circulating CD34+ fibrocytes participates in GO autoimmunity and tissue remodeling.[14] Importantly, these fibrocytes may infiltrate orbital tissues under inflammatory conditions and differentiate into CD34+ fibroblasts that cause pathophysiological changes in orbital tissues.[2,14] There is evidence that interplay between circulating fibrocytes and activated T cells may promote disease activity in RA patients.[15,16] Although the mechanism remains uncertain, there may be a possible interaction between IL-17A–producing T cells and fibrocytes that contributes to pathology in GO patients.

This present study was conducted in Chinese cohorts of patients with GO and healthy controls. We examined the incidence of CCR6+ Th17 cells during the course of GO development and found a positive trend between the incidence of CCR6+ IL-17A–producing T cells and GO clinical activity score (CAS). Further, we observed an increased number of CD34+ fibrocytes with higher levels of IL-17RA, CD80, and CD86 in GO patients, compared with healthy controls. We also found a decrease in the population of effector regulatory T cells (Tregs) in GO patients, compared with healthy controls; this was concurrent with CTLA-4 dysfunction in the GO patient group. Our experiments revealed that IL-17A and Th17 cells stimulated expression of inflammatory cytokines and costimulatory molecules in fibrocytes; these activated fibrocytes produced macrophage inflammatory protein 3 (MIP-3) to recruit CCR6+ Th17 cells to the site of inflammation, as well as to support the Th17 phenotype. Taken together, our data suggest that Th17-cell could promote fibrocyte inflammation, thereby activating T-cell function and migration. The crosstalk between CCR6+ Th17 cells and fibrocytes may play a role in the pathogenesis of GO.

MATERIALS AND METHODS

Patients and Controls

Procurement of human blood samples was conducted after obtaining informed consent from all study participants ($n = 30$, GO patients; $n = 20$, healthy controls) of Chinese ethnicity as approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai JiaoTong University School of Medicine. All studies were performed according to the tenets of the Declaration of Helsinki. Two patients were hyperthyroid and two were hypothyroid on initial diagnosis of GO. All GO patients were euthyroid at the time of study participation as assessed by clinical examination and serum-free T4, and not currently or recently (within past 6 months) undergoing immunosuppressive or corticosteroid therapy. Control samples

were obtained from donors who did not exhibit inflammatory orbital or thyroid disease; these donors underwent surgery for cosmetic reasons or routine medical care. Detailed information on GO patients and controls is provided in the Table.

Fibrocyte Separation and Cultivation

Human fibrocytes were generated from venous blood that was collected from individuals with GO, and from healthy control donors, according to previous studies.[17,18] Briefly, venous blood was mixed with an equal volume of PBS solution, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (GE Healthcare, Little Chalfont, UK) density gradient centrifugation. Following two washes with PBS, PBMCs were seeded into six-well plates (1×10^7 cells/well) and cultured in Dulbecco's modified Eagle's medium (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Medium was changed every 3 to 4 days. Cells were rinsed with PBS after 7 days of cultivation and nonadherent cells were discarded. The remaining monolayers were cultured for another 7 to 10 days; the resulting adherent fibrocytes were washed and removed from the substratum via trypsinization for future use.

Flow Cytometry

Routinely-isolated PBMCs were inoculated into 24-well plates covered with X-VIVO 15 medium (Lonza, Basel, Switzerland) supplemented with 10% human AB serum (Gibco), 1% L-glutamine (Gibco), 1% sodium pyruvate (Gibco), 1% nonessential amino acids (Gibco), and 1% penicillin/streptomycin. For T-cell staining, PBMCs were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) and ionomycin (1 μg/mL; Sigma-Aldrich Corp.) at 37°C for 6 hours in the presence of GolgiPlug (1 μL/mL; BD Biosciences, San Jose, CA, USA). Dead cells were eliminated with APC-Cy7-Fixable Viability Dye (eBioscience, San Diego, CA, USA) before cell surface marker staining. Then, the remaining cells were stained with FITC-CD3, Alexa Fluor 700-CD8, and BV711-CCR6 (to identify Th17 cells); or FITC-CD3, Alexa Fluor 700-CD8, PE-CD25, PerCP-Cy5.5-CD45RA, and PE-Cy7-CTLA-4 (to identify Treg cells) (all from BD Biosciences), for 30 minutes on ice. After fixation and permeabilization with fixation/permeabilization reagents (eBioscience), cells were stained with PE-IL-17A (intracellular cytokine; eBioscience) or APC-FoxP3 (transcription factor; eBioscience) for 45 minutes on ice.

For circulating fibrocyte staining, isolated PBMCs were incubated with surface marker antibodies including PE-IL-17RA (R&D Systems, Minneapolis, MN, USA), PE-Cy5-CD34, PerCP-Cy5.5-CD45, APC-CXCR4, BV650-CD80, and PE-Cy7-CD86 (all from BD Biosciences). Fibrocytes were fixed and permeabilized, then stained for intracellular FITC-Collagen I (COLI) (Millipore, Bedford, MA, USA).

All flow cytometry studies were performed within 24 hours of venous blood collection and data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Immunofluorescence Staining

Immunofluorescence staining was performed using antibodies directed against CD34 (1:100, Abcam, Cambridge, UK) and IL-17RA (1:100, Abcam). Frozen specimens were fixed in ice-cold acetone and were incubated with primary antibodies at 4°C overnight. They were then incubated with Alexa Fluor 546-conjugated donkey anti-rabbit IgG (H+L) antibody and Alexa Fluor 488-conjugated donkey anti-mouse IgG (H+L) antibody (all from Life Technologies, Carlsbad, CA, USA) at 4°C for 1

TABLE. Clinical Features of GO Patients and Control Subjects

	Active GO, $n = 16$	Inactive GO, $n = 14$	Control, $n = 20$	*P* Value
Mean age	48.94 ± 15.57	38.93 ± 10.62	37.60 ± 12.80	0.033
Sex, M/F	6/10	3/11	9/11	0.366
Smoking history	5/16	8/14	0/20	0.001
Thyroid status				
Euthyroid	12/16	14/14	20/20	
Hyperthyroid	2/16	0/14	0/20	
Hypothyroid	2/16	0/14	0/20	
Thyroid function				
TSH, μIU/mL (0.34–5.6)	3.39 ± 3.47	2.00 ± 1.24	–	0.329
TRAb, IU/L (0–1.75)	6.64 ± 2.46	4.33 ± 4.84	–	0.454
TPOAb, IU/mL (0–9)	109.44 ± 211.73	90.98 ± 126.69	–	0.805
TgAb, IU/mL (0–115)	480.57 ± 1119.60	83.00 ± 119.54	–	0.505
Treatment				
Radioactive iodine	2/16	1/14	–	0.626
Thyroidectomy	3/16	1/14	–	0.351
Antithyroid drug	14/16	11/14	–	0.513
Corticosteroid	5/16	3/14	–	0.544
CAS	4.50 ± 0.89	1.00 ± 0.78	–	0.000
Disease duration, mo	3.78 ± 5.33	7.25 ± 5.40	–	0.088

TSH, thyroid-stimulating hormone; TRAb, thyrotropin receptor antibody; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody.

hour. Specimens were examined by fluorescence microscope (Olympus, Tokyo, Japan).

Fibrocyte Stimulation and In Situ Study of Proinflammatory Cytokine Expression

PBMCs were treated for 24 hours with IL-17A (100 ng/mL; R&D Systems) and examined for gene expression of proinflammatory cytokines by quantitative RT-PCR (qRT-PCR). Previously, our group has demonstrated that this condition provides maximal cellular response to IL-17A.[12,13] In some experiments, PBMCs were treated for 24 hours with IL-17A (100 ng/mL) in the presence or absence of human α-IL-17A antibody (0.25 μg/mL; R&D Systems); GolgiPlug (1 μL/mL) was added in the final 6 hours of these experiments. The resulting $CD34^+CD45^+CXCR4^+COLI^+$ fibrocytes were further examined for proinflammatory cytokine production (APC-IL-6, PE-MCP-1, or BV421-IL-8; all from BD Biosciences) using flow cytometry.

Human Naïve T-Cell Purification and Th17 Cell Differentiation

Human $CD4^+$ T cells were enriched from prepared PBMCs (isolated from GO patients) using magnetic cell selection (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The $CD4^+$ T cells were further sorted as $CD4^+CD127^{hi}CD25^{lo}CD45RA^{hi}$ using a BD FACS ARIA II sorter (BD Biosciences) to obtain purified naïve T cells. Before experimental manipulations, Th17 cells were differentiated for 10 days by subjecting 1 to 2×10^5 naïve T cells to culture conditions consisting of anti-CD3/CD28 Dynabeads (cell-to-bead ratio of 1:1) (Miltenyi Biotec), 1 ng/mL TGF-β, 50 ng/mL IL-6, 10 ng/mL IL-1β, and 50 ng/mL IL-23 (all from R&D Systems) in X-VIVO 15 medium supplemented with 1% penicillin/streptomycin.

Th17/Fibrocyte Coculture

Fibrocytes were seeded overnight in 24-well plates at 5×10^4 cells/well in X-VIVO 15 medium containing 1% penicillin/streptomycin. Differentiated autologous Th17 cells were inoculated into the cultures at a 20:1 ratio (Th17 cells to fibrocytes) for either 24 or 48 hours; suspended Th17 cells were then removed by aspiration. The remaining adherent fibrocytes and the suspended Th17 cells (those removed by aspiration) were further studied by qRT-PCR or flow cytometry. Coculture supernatants were also collected, and their cytokine profiles were analyzed using the Luminex assay.

Quantitative Real-Time RT-PCR

Total RNA was isolated and reverse-transcribed (PrimeScript RT Reagent Kit; Takara, Kusatsu, Japan). Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Predesigned primers are listed in Supplementary Table S1. The $2^{-\Delta Ct}$ method was used for quantification of gene expression and glyceraldehyde-3-phosphate dehydrogenase was set for normalization.

Th17 Cell Transwell Assay

Transwell chambers (24-well, 5-μm pore size; Corning Costar Corp., Corning, NY, USA) were loaded with supernatants from 24- and 48-hour Th17/fibrocyte cocultures. Supernatants from fibrocytes alone served as a negative control; 500 pg/mL MIP-3 (R&D Systems) served as a positive control according to a previous study.[19] For some experiments, human α-MIP-3 antibody (0.2 μg/mL; R&D Systems) was used to neutralize the effect of MIP-3. Approximately 5×10^5 differentiated Th17 cells were seeded into the chambers. The number of transmigrated Th17 cells was evaluated after 6 hours of incubation, using the CCK-8 assay. Transmigrated Th17 cells were then collected to examine CCR6 expression by flow cytometry.
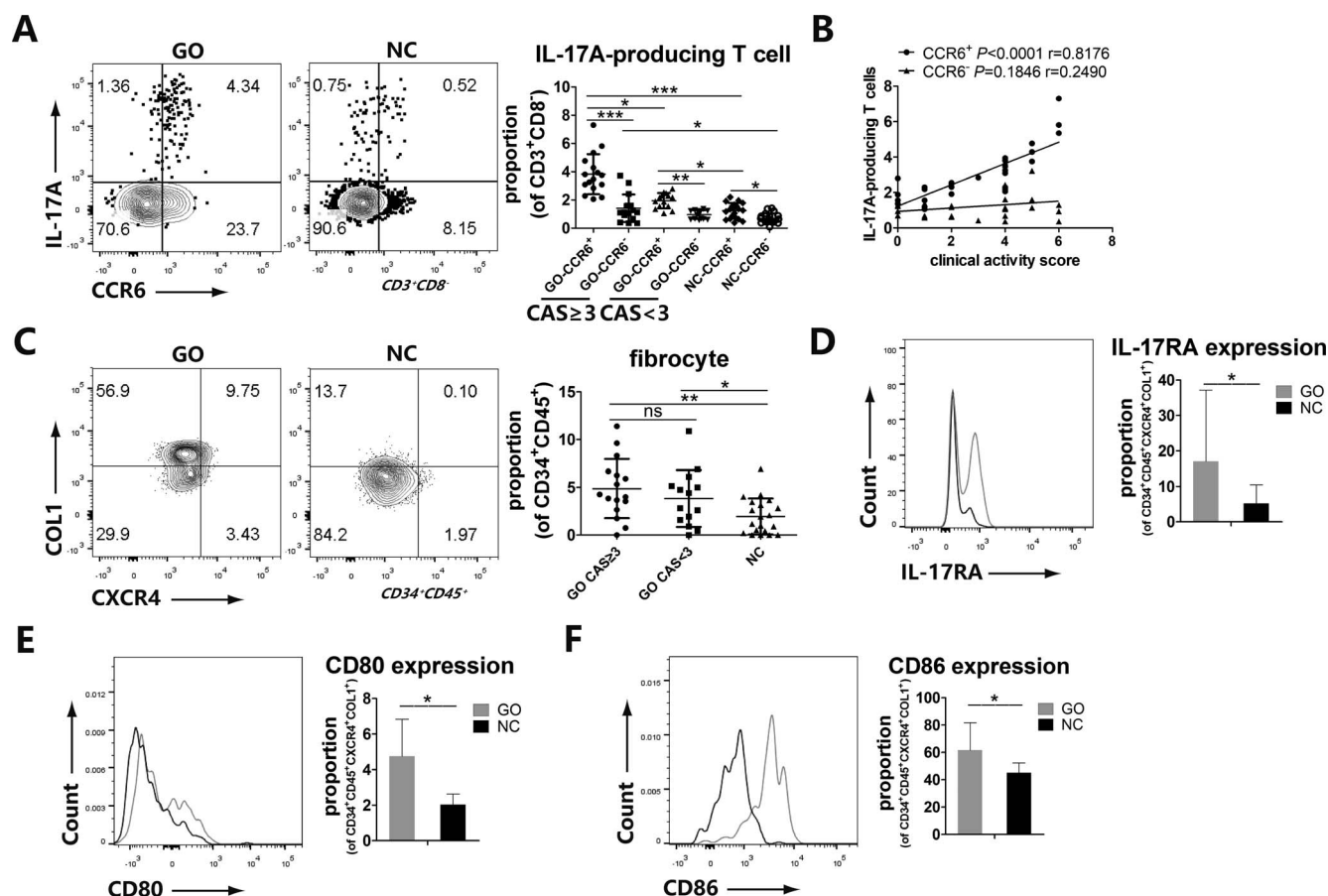
FIGURE 1. Phenotype studies of Th17 cells and fibrocytes from Chinese GO patients and healthy controls. (**A**) The proportions of CD3$^+$CD8$^-$CCR6$^+$ IL-17A-producing T cells and CD3$^+$CD8$^-$CCR6$^-$ IL-17A-producing T cells in active GO patients, inactive GO patients, and healthy controls. (**B**) Relationship between the GO CAS and the proportions of CD3$^+$CD8$^-$CCR6$^+$ IL-17A-producing T cells and CD3$^+$CD8$^-$CCR6$^-$ IL-17A-producing T cells. (**C**) The proportion of circulating fibrocytes in active GO patients, inactive GO patients, and healthy controls. Fibrocytes from isolated PBMCs were first gated on CD34$^+$CD45$^+$ cells, then on CXCR4$^+$COL1$^+$ cells (shown). (**D–F**) Increased expression of IL-17RA (**D**), CD80 (**E**), and CD86 (**F**) on CD34$^+$CD45$^+$CXCR4$^+$COL1$^+$ fibrocytes generated from GO patient PBMCs, compared with fibrocytes generated from healthy control PBMCs. Data are presented as mean ± SD ($n = 30$ for GO patients and $n = 20$ for healthy controls). ns, nonsignificant, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Luminex Assay

Expression levels of IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), MIP-3, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-X-C motif) ligand (CXCL)9, and CXCL10 in coculture supernatants were tested by Luminex assay (eBioscience) according to the manufacturer's instructions.

Statistical Analysis

Comparisons among groups were evaluated using either a two-tailed Student's *t*-test, ANOVA, or the nonparametric Kruskal-Wallis test. Data were analyzed using SPSS version 19.0 (SPSS Inc., IBM Corp., Chicago, IL, USA). *P* values < 0.05 were regarded as significant.

RESULTS

GO Patients Exhibit a Higher Frequency of CCR6$^+$ IL-17A–Producing T Cells

To determine the potential role of Th17 cells in GO patients, we first examined the relationship between IL-17A production and cell surface expression of CCR6. We found an increase in the number of CD3$^+$CD8$^-$CCR6$^+$ IL-17A-producing T cells in GO patients, compared with healthy controls (Fig. 1A). The proportion of CCR6$^+$IL-17A$^+$ T cells was significantly greater in both active GO (CAS ≥ 3; 38306 ± 14178 CCR6$^+$ IL-17A-producing T cells per 10^6 PBMCs [mean ± SD, $n = 16$]) and inactive GO (CAS < 3; 19507 ± 5366 CCR6$^+$ IL-17A–producing T cells per 10^6 PBMCs [$n = 14$]) patients than in healthy controls (12425 ± 5464 CCR6$^+$ IL-17A-producing T cells per 10^6 PBMCs [$n = 20$]; $P < 0.001$ versus active GO, $P < 0.05$ versus inactive GO) (Fig. 1A). Additionally, active GO patients had more CCR6$^+$IL-17A$^+$ T cells compared with patients with inactive GO ($P < 0.01$) (Fig. 1A). Intriguingly, the number of CCR6$^+$IL-17A$^+$ T cells was greater than the number of CCR6$^-$IL-17A$^+$ T cells among all three study cohorts (Fig. 1A). Further, the proportion of CD3$^+$CD8$^-$CCR6$^-$ IL-17A-producing T cells was significantly elevated in active GO patients, but not in inactive GO patients, compared with healthy controls (Fig. 1A). Our study also demonstrated a significant positive correlation between proportion of CD3$^+$CD8$^-$CCR6$^+$ IL-17A-producing T cells and CAS of GO patients ($P < 0.0001$, $r = 0.8176$); there was no correlation between proportion of CD3$^+$CD8$^-$CCR6$^-$ IL-17A-producing T cells and CAS of GO patients ($P = 0.1846$, $r = 0.2490$) (Fig. 1B). Taken together, these data indicate that an increase in the number of CCR6$^+$ IL-17A-producing T cells is associated with GO development and disease activity.

Because autoimmune diseases are more prone in females and smoking is a risk factor for GO,[2,3] we analyzed whether those elements affected Th17 cell distribution in our patients. Both sex and cigarette smoking did not appear to affect Th17 cell frequency. The abundance of CCR6$^+$ IL-17A–producing T cells was similar in 9 male patients and 21 female patients ($P = 0.160$) and so were CCR6$^-$ IL-17A–producing T cells ($P = 0.887$) (Supplementary Fig. S1A). The number of CCR6$^+$ IL-17A–producing T cells was greater than CCR6$^-$ ones in both sexes (Supplementary Fig. S1A). Similarly, the proportion of CCR6$^+$ IL-17A–producing T cells ($P = 0.813$) as well as CCR6$^-$ IL-17A–producing T cells ($P = 0.954$) was invariant with respect to smoking (Supplementary Fig. S1B).

GO Patients Exhibit an Increased Fraction of CD34$^+$ Fibrocytes

To validate fibrocyte involvement in GO pathogenesis, we studied the proportion of CD34$^+$ fibrocytes in circulating blood of both GO patients and healthy control subjects. As shown in Figure 1C, the fraction of CD34$^+$CD45$^+$CXCR4$^+$COLI$^+$ fibrocytes was significantly higher in PBMCs from GO patients than in PBMCs from healthy controls. However, GO disease activity did not appear to affect fibrocyte frequency (Fig. 1C); fibrocyte abundance was similar between active and inactive GO patients (Fig. 1C). In-depth analysis of fibrocyte phenotype revealed enhanced IL-17RA expression (Fig. 1D) in fibrocytes from GO patients, compared with fibrocytes from control donors. GO fibrocytes also demonstrated enhanced expression of costimulatory molecule CD80 (Fig. 1E) and CD86 (Fig. 1F).

GO Patients Exhibit Dysregulated Function of Treg Cells

Because there was an increase in the number of CCR6$^+$ IL-17A–producing T cells in GO patients, we investigated whether there was an imbalance between Th17 cells and Treg cells in GO patients. CD3$^+$CD8$^-$CD25$^+$ Treg cells were gated on the basis of CD45RA and Foxp3 expression and subdivided into three major groups, as previously reported.[20] Equivalent proportions of FoxP3^{lo}CD45RA$^+$ naïve Treg cells and FoxP3^{lo}CD45RA$^-$ cells were found in GO patients and healthy controls (Fig. 2A). In contrast, there was a decrease in the proportion of FoxP3^{hi}CD45RA$^-$ effector Tregs in GO patients, compared with healthy controls (Fig. 2A). Because we observed increased CD80 and CD86 expression on fibrocytes from our GO patients, we wondered whether CTLA-4 expression might also be altered on the surface of Treg cells. However, CTLA-4 expression was not changed on FoxP3^{lo}CD45RA$^-$ cells or on FoxP3^{hi}CD45RA$^-$ effector Tregs in both GO patients and healthy controls (Figs. 2B, 2C). Unexpectedly, CTLA-4 expression was significantly more frequent on FoxP3^{lo}CD45RA$^+$ naïve Tregs from GO patients than on naïve Tregs from healthy control subjects (Figs. 2B, 2C). Moreover, our results showed a higher number of FoxP3$^+$RORγt$^+$ cells in GO patients than in healthy controls (Fig. 2D). Taken together, these data indicate that dysregulated function of Tregs is present in GO patients; this dysregulated function is marked by Treg numerical abnormality and aberrant CTLA-4 expression.

IL-17A Promotes an Inflammatory Fibrocyte Phenotype In Situ

Fibrocytes are capable of producing abundant inflammatory cytokines[14]; thus, we hypothesized that in situ IL-17A exposure could promote secretion of proinflammatory cytokines by fibrocytes. To test this, gene expression of *Il8*, *Il6*, and *MCP1* was examined in GO and control PBMCs treated with IL-17A (100 ng/mL) for 24 hours. Increased levels of *Il8*, *Il6*, and *MCP1* genes were found in PBMCs from GO patients as well as healthy controls (Supplementary Fig. S2). In addition, we measured IL-8, IL-6, and MCP-1 protein expression in fibrocytes under IL-17A incubation in isolated PBMCs in situ. Our data demonstrated significant increase in the production of IL-8 (Fig. 3A, Supplementary Fig. S3A), IL-6 (Fig. 3B, Supplementary Fig. S3B), and MCP-1 (Fig. 3C, Supplementary Fig. S3C) by CD34$^+$CD45$^+$CXCR4$^+$COLI$^+$ fibrocytes from both GO patients and healthy control subjects, following incubation with IL-17A (100 ng/mL) for 24 hours. Basal IL-8 (Fig. 3A, Supplementary Fig. S1A), IL-6 (Fig. 3B, Supplementary Fig. S1B), and MCP-1 (Fig. 3C, Supplementary Fig. S1C) secretion was higher in fibrocytes from GO patients than in fibrocytes from health controls, which might arise from a difference in systemic inflammatory status. Furthermore, IL-17A was more effective in regulating cytokine production by fibrocytes from GO patients than by fibrocytes from healthy controls (Fig. 3, Supplementary Fig. S3). Treatment with 0.25 μg/mL α-IL-17A could block some effects of IL-17A on IL-8 (Fig. 3A, Supplementary Fig. S3A), IL-6 (Fig. 3B, Supplementary Fig. S3B), and MCP-1 (Fig. 3C, Supplementary Fig. S3C) synthesis by fibrocytes from GO patients and healthy controls.

Th17 Cells Induce Fibrocyte Inflammation in GO

Our immunofluorescence study demonstrated that fibrocytes express IL-17RA (Fig. 4A), which was consistent with our flow cytometry observation (Fig. 1D). However, IL-17RA expression on fibrocytes remained unchanged following addition of IL-17A to fibrocyte cultures for either 24 or 48 hours (Fig. 4B), suggesting that IL-17A may not affect the expression of its receptor on fibrocytes.

To better understand potential regulatory interactions between Th17 cells and fibrocytes in GO patients, we performed a direct Th17 cell/fibrocyte coculture experiment. Gene expression of *Il6*, *Il8*, *MCP1*, *MIP3*, *TNFA*, *Cxcl9*, and *Cxcl10* was observed to be upregulated in fibrocytes after 24-hour coculture treatment with autologous Th17 cells, which was decreased after 48 hours (Supplementary Fig. S4). Yet, the *GMCSF* gene expression level was slightly upregulated after 48-hour coculture treatment (Supplementary Fig. S4F). Additionally, we found that Th17 cell/fibrocyte coculture supernatants were highly enriched for those proinflammatory cytokines, such as IL-6 ($P < 0.05$, 24 hour; $P < 0.001$, 48 hour), IL-8 ($P < 0.001$, 24 hour; $P < 0.001$, 48 hour), MCP-1 ($P < 0.001$, 24 hour; $P < 0.001$, 48 hour), MIP-3 ($P < 0.01$, 24 hour; $P < 0.001$, 48 hour), TNF-α ($P = 0.172$, 24 hour; $P < 0.01$, 48 hour), CXCL9 ($P < 0.05$, 24 hour; $P < 0.001$, 48 hour), and CXCL10 ($P < 0.01$, 24 hour; $P < 0.001$, 48 hour), but not GM-CSF ($P = 0.382$, 24 hour; $P = 0.085$, 48 hour) (Fig. 4C), after 24 and 48 hours of coculture, which was consistent with our findings in elevated cytokine gene expression after coculture and in IL-17A–treated fibrocytes (Fig. 3).

Fibrocytes Promote Th17 Migration Through MIP-3/CCR6 Interaction

To evaluate the role of cell contact in Th17 cell migration and the impact of MIP-3 on Th17 cell chemotaxis, in vitro–generated autologous Th17 cells were cultured in a 24-well plate transwell system, separated by a semipermeable membrane. Our migration assays demonstrated that in vitro–generated Th17 cells were not recruited by medium from cultivated fibrocytes alone, our negative control condition (Fig. 4D). However, in vitro–generated Th17 cells actively migrated
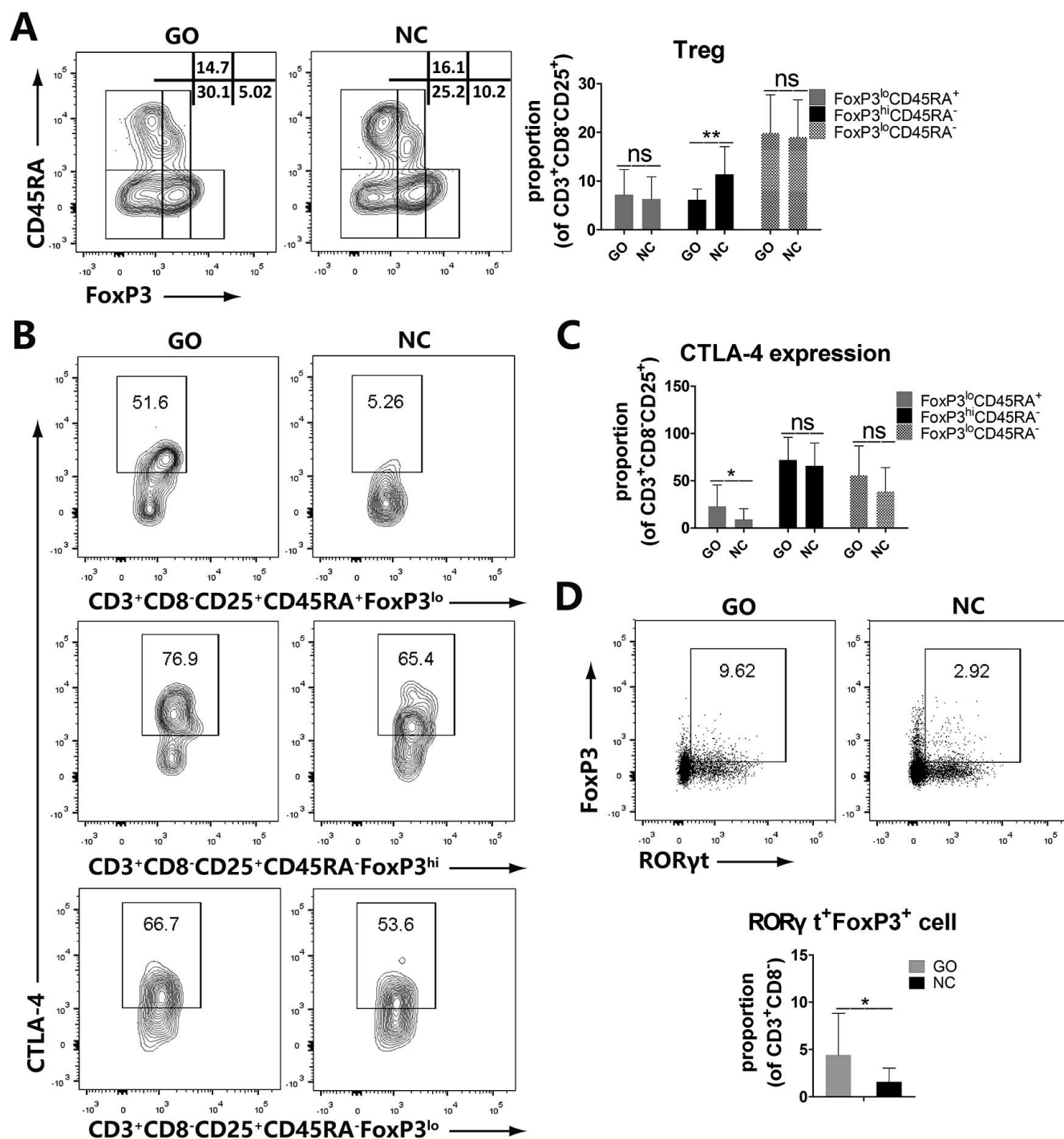
FIGURE 2. Quantities and phenotypes of Treg cell subsets in Chinese GO patients and healthy controls. (A) The proportions of FoxP3^{lo}CD45RA^{+} naïve Tregs, FoxP3^{lo}CD45RA^{-} cells, and FoxP3^{hi}CD45RA^{-} effector Tregs gated on CD3^{+}CD8^{-}CD25^{+} T cells from GO patients and healthy controls. (B, C) Expression of CTLA-4 by different subpopulations of Tregs from GO patients and healthy controls. (D) The number of FoxP3^{+}RORγt^{+} T cells gated on CD3^{+}CD8^{-} T cells from GO patients and healthy controls. Data are presented as mean ± SD ($n = 30$ for GO patients and $n = 20$ for healthy controls). ns, nonsignificant, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

toward the medium taken from Th17 cell/fibrocyte cocultures, as well as toward the medium that contained 500 pg/mL MIP-3 (Fig. 4D). Notably, migration of Th17 cells could be inhibited with α-MIP-3 treatment (Fig. 4D). Flow cytometry analysis indicated that these responsive Th17 cells were CCR6^{+} (Fig. 4D). Altogether, our results suggest that fibrocytes attract Th17 cells in a MIP-3/CCR6-dependent manner.

Th17 Cells Upregulate Costimulatory Molecule Expression on Fibrocytes in GO

Fibrocytes are potent antigen-presenting cells that present costimulatory molecules, such as MHC II and CD40.[21,22] To study whether Th17 cells regulate expression of MHC II and CD40 on fibrocytes, we stimulated fibrocytes with 100 ng/mL
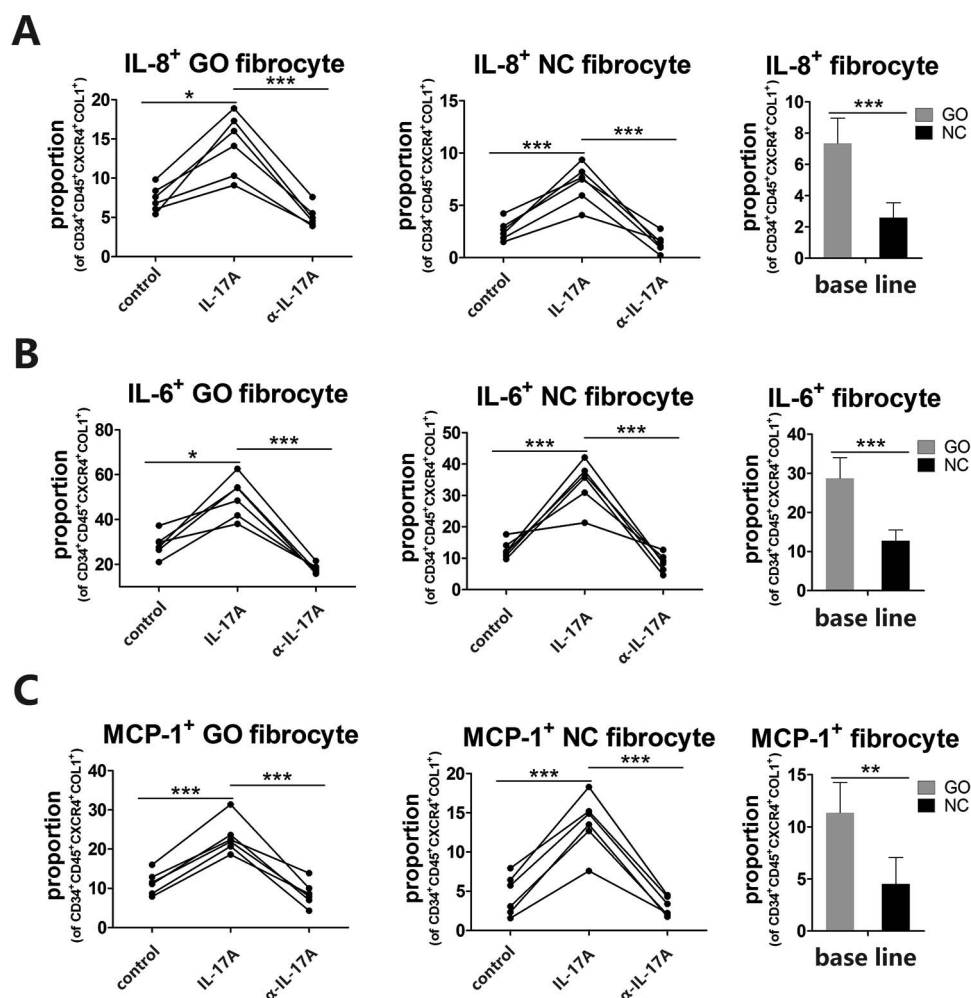
FIGURE 3. In situ regulation of proinflammatory function of fibrocytes by IL-17A. (**A**) The secretion of IL-8 was measured in CD34^{+}CD45^{+}CXCR4^{+}COL1^{+} fibrocytes generated from GO patient and healthy control PBMCs, with or without 100 ng/mL IL-17A treatment, in the presence or absence of α-IL-17A (0.25 μg/mL) for 24 hours. (**B**) The secretion of IL-6 was measured in CD34^{+}CD45^{+}CXCR4^{+}COL1^{+} fibrocytes generated from GO patient and healthy control PBMCs, with or without 100 ng/mL IL-17A treatment, in the presence or absence of α-IL-17A (0.25 μg/mL) for 24 hours. (**C**) The secretion of MCP-1 was measured in CD34^{+}CD45^{+}CXCR4^{+}COL1^{+} fibrocytes generated from GO patient and healthy control PBMCs, with or without 100 ng/mL IL-17A treatment, in the presence or absence of α-IL-17A (0.25 μg/mL) for 24 hours (PBMCs were obtained from six GO patients and six healthy controls). ns, nonsignificant, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

IL-17A for 24 or 48 hours. IL-17A significantly promoted MHC II and CD40 expression on fibrocytes from GO patients and healthy controls (Fig. 5A). Intriguingly, enhanced CCR6^{+} Th17-cell function was observed, as there was an increase in the number of IL-17A–producing CCR6^{+} T cells after 24 and 48 hours of coculture treatment with fibrocytes from GO patients (Fig. 5B). In addition, we observed elevated expression of MHC II and CD40 on fibrocytes from GO patients, following either 24 or 48 hours' incubation with in vitro–generated Th17 cells in the Th17 cell/fibrocyte coculture system (Fig. 5C).

DISCUSSION

Immune homeostasis of orbital connective tissues is precisely regulated by resident OFs and a variety of immune cells.[2,3] In GO, proinflammatory Th1 cell cytokines are present in the orbits, contributing to the transition from early orbital inflammation to a late chronic stage that is enriched for the Th2 cell cytokines.[1] However, this does not explain recent reports of increased IL-17A serum levels in GO patients.[6,11] As demonstrated here, most IL-17A–expressing T cells were

CCR6^{+} cells, both in GO patients and in healthy controls. Additionally, GO patients exhibited a significant increase in CCR6^{+} IL-17A–producing T cells. The gradual decrease of the CCR6^{+} IL-17A–producing T-cell fraction in GO groups, from active GO patients to inactive GO patients, suggests that CCR6^{+} Th17 cells might implicate disease activity and that their numbers may slowly decline with the remission of GO. This is consistent with studies of patients in the early stages of RA.[23,24] Increases in GO CAS were associated with concomitant increases in the number of CCR6^{+} Th17 cells. This suggests that CCR6^{+}IL-17A^{+} T cells may play a role in orchestrating the immune responses during bouts of GO, and may contribute to increased orbital inflammation. Importantly, GO patients comprised a clinically heterogeneous group in our study; their age imbalance is a notable limitation of this study. The average ages of active GO patients in this study were distinctly higher than the average ages of both inactive GO patients and healthy control subjects. Although there was no statistical difference in sex distribution among the three groups in this study, approximately 38% of the active GO patients were male. Thus, these elderly males in our study exhibited a higher CAS and presented with more severe GO, increasing the
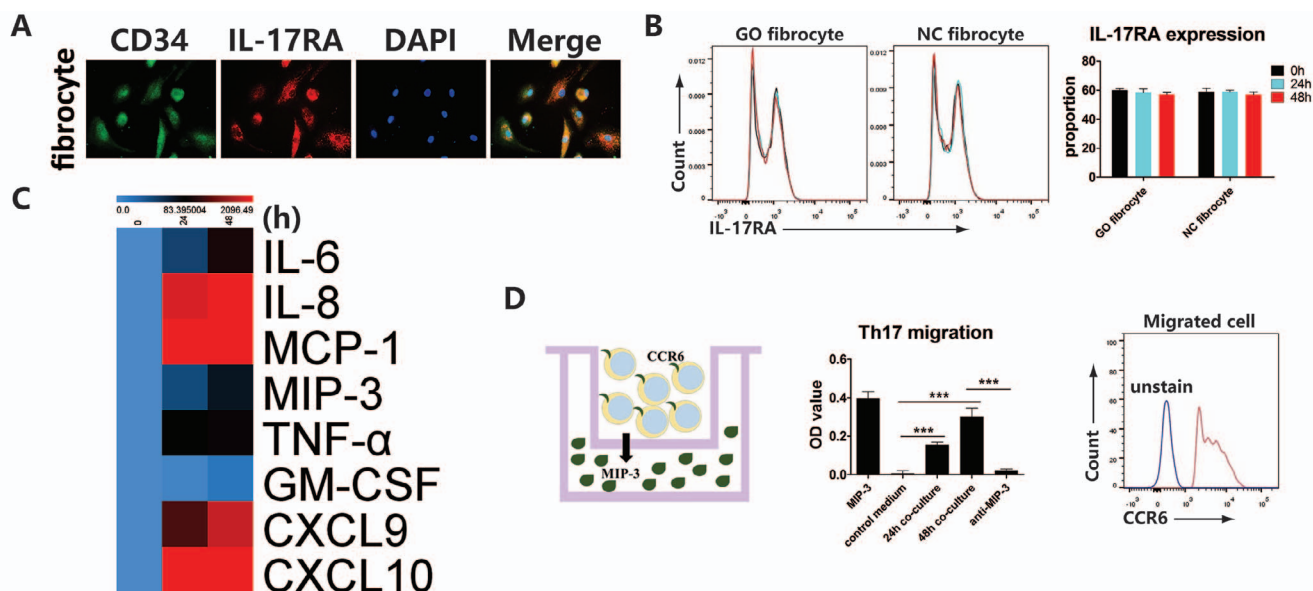
FIGURE 4. Th17 cells promote fibrocytic inflammation in GO. (**A**) Immunofluorescent staining for IL-17RA on CD34+ fibrocytes from GO patients (original magnification, ×400; representative data are shown; data are combined from at least three independent experiments). (**B**) IL-17RA expression on CD34+ fibrocytes from GO patients and healthy donors, with or without 100 ng/mL IL-17A treatment, at the indicated time points. (**C**) Proinflammatory cytokine production of fibrocytes from GO patients, detected in the coculture supernatants at the indicated time points. Medium from cultured fibrocytes alone was used as control. (**D**) Transwell assay using differentiated Th17 cells in media from Th17/fibrocyte cocultures from GO patients, with or without α-MIP-3 (0.2 μg/mL), at the indicated time points. Medium from cultured fibrocytes alone was used as a negative control; 500 pg/mL MIP-3 was used as a positive control. The migrated Th17 cells were further examined for CCR6 expression. Data are presented as mean ± SD and are combined from at least three independent experiments. ns, nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

prominence of Th17-mediated inflammation. We suspect that those CCR6⁻ T cells may also account for increased IL-17A expression during the active phase of GO, and may help to sustain autoimmune inflammation in patients with established disease. This indicates that CCR6+ Th17 cell–specific therapies may provide a promising GO treatment in the future.

Recent publications report the observation of fibroblast-like cells expressing the marker CD45 and CD34. A growing body of evidence suggests a role for bone marrow–derived progenitor cells (also known as fibrocytes) in many human diseases, including the autoimmune disorder asthma, sclero-derma, RA, Crohn's disease, and idiopathic orbital inflammation.[25-28] PBMCs from GD and GO patients generate larger number of fibrocytes, relative to PBMCs from healthy controls[17]; these fibrocytes from GD and GO patients exhibit increased expression of thyroid-stimulating hormone receptor (TSHR).[18,29] In our study, we also detected an increase in CD34+CD45+CXCR4+COLI+ fibrocytes from GO patients, though these fibrocytes did not correlate with an increased GO CAS. Our findings are consistent with studies from the Douglas laboratory,[17,18,22] which reported no statistical difference in the number of fibrocytes between patients with active GO and patients with stable disease. This is probably because fibrocyte numbers were substantially increased in GD patients, regardless of whether they manifested clinically apparent GO.[14] During these studies, participating GO patients were euthyroid; some had experienced significant disease duration. This indicates that, in addition to changes in the number of fibrocytes, fibrocytic dysfunction and other environmental factors (such as IL-17A) may play important roles in GO autoimmunity.

Multiple studies (from our group and others) have reported increased serum IL-17A levels in GO patients.[6,11–13] In the current study, we found that baseline expression levels of IL-6, IL-8, and MCP-1 were higher in fibrocytes from GO patients than in fibrocytes from healthy controls. Increased IL-17A may enhance basal secretion levels of these proinflammatory cytokines by fibrocytes from GO patients, compared with fibrocytes from healthy controls. Although IL-17RA was expressed by fibrocytes from both GO patients and healthy controls, a detailed analysis of the immunophenotype of circulating fibrocytes revealed increased expression of IL-17RA in GO patients, suggesting a higher in vivo sensitivity to stimuli from Th17 cells. Therefore, addition of exogenous IL-17A was able to stimulate the in situ secretion of IL-6, IL-8, and MCP-1 by fibrocytes generated from PBMCs from both GO patients and healthy controls; more potent responses were observed in fibrocytes from GO patients. Addition of anti-IL-17A blocking antibody both inhibited IL-17A treatment effects and partly reduced baseline production of these cytokines from fibrocytes in our study. Thus, increased endogenous IL-17A expression by CCR6+ Th17 cells may contribute to autoimmune inflammation from fibrocytes exhibiting elevated IL-17RA expression in GO patients, leading to increased cytokine secretion in both basal and stimulated conditions. Moreover, we found enhanced expression of CD80 and CD86, two surface proteins that are necessary for antigen presentation,[21] on fibrocytes from GO patients, compared with fibrocytes from healthy controls. Taken together, these data indicate enhanced T-cell immunity, possibly through CCR6+ Th17 activity, in response to activated fibrocytes in GO patients, which implies potential changes in Treg-cell function.

Although previous studies indicated no difference in the frequencies of Treg cells between GO patients and healthy control subjects,[30,31] they did not examine subpopulations of FoxP3+ Tregs. Our current results showed that the number of effector Tregs declined in GO patients. Pawlowski et al.[32] reported enhanced *FoxP3* mRNA expression and diminished *CTLA-4* mRNA expression in orbital tissues of GO patients. In a clinical study of GO patients, Treg frequency increased on initiation of rituximab.[33] Furthermore, we found an increased number of FoxP3+RORγt+ T cells in GO patients in our current
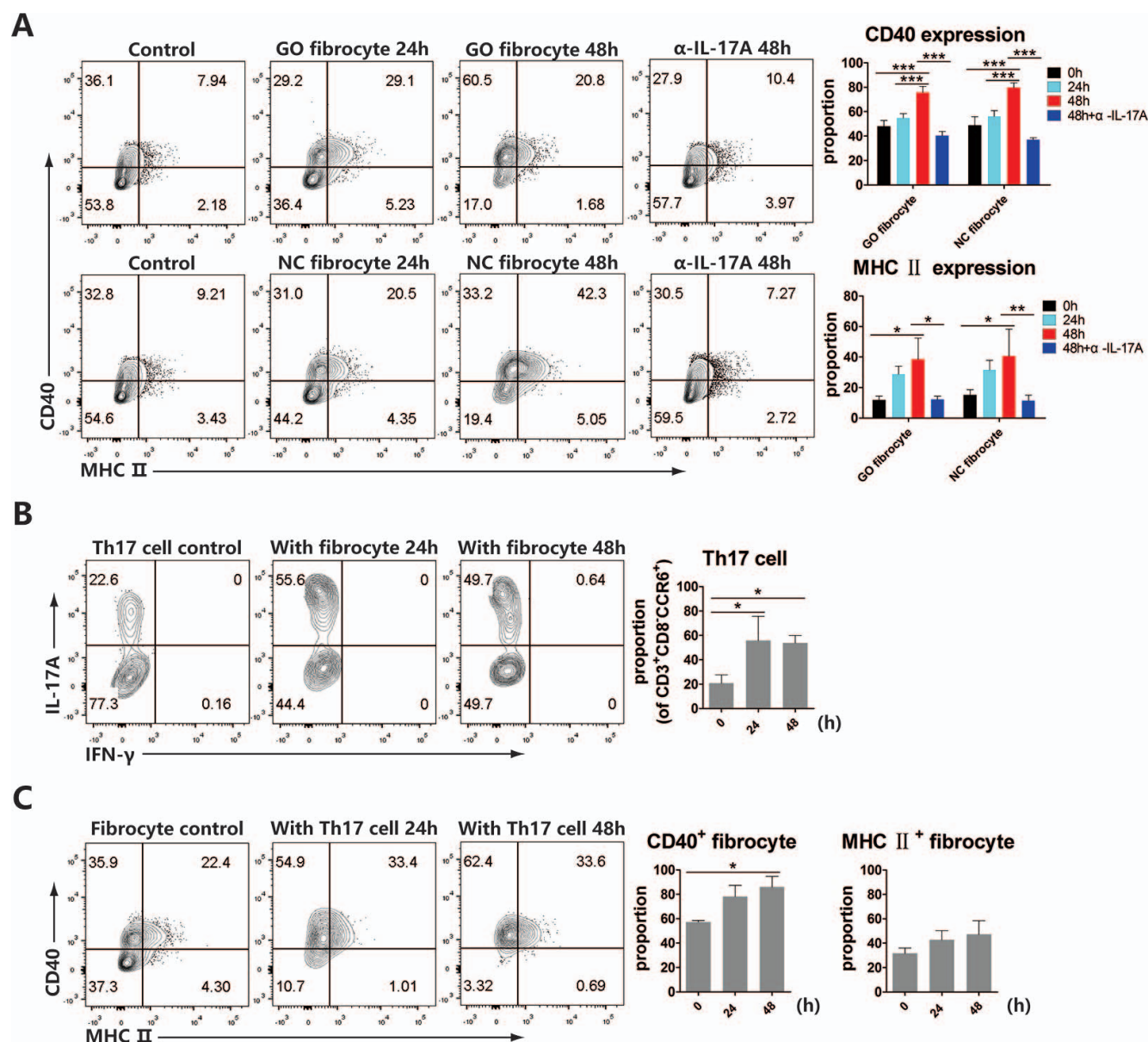
FIGURE 5. Th17 cells promote the antigen-presenting function of fibrocytes in GO. (A) CD40 and MHC II expression on CD34^{+} fibrocytes from GO patients and healthy donors, with or without 100 ng/mL IL-17A treatment, in the presence or absence of α-IL-17A (0.25 μg/mL) at the indicated time points. (B) An enhanced Th17-cell phenotype was observed after 24 and 48 hours of coculture with fibrocytes from GO patients. (C) The expression of CD40 and MHC II on fibrocytes from GO patients, cocultured with differentiated Th17 cells at the indicated time points. Data are presented as mean ± SD and are combined from at least three independent experiments. ns, nonsignificant, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

study. These results suggest that, in the context of orbital inflammation, Treg instability and the presence of a T-effector-cell–like phenotype[34] ultimately led to Treg transformation to Th17 cells. However, it remains unclear how Treg cells are reprogrammed toward the Th17 cell lineage, or how to normalize Th17 cell responses to mediate GO autoimmunity.

Importantly, approximately 27% of the GO patients in this study were previously treated with steroids, indicating that these patients had progressed from the inflammatory stage of GO to the "burnt out," or stable, stage. This may have affected our observations of the Th17/Treg imbalance and Th17-fibrocyte interactions. Our current study aimed to investigate Th17-fibrocyte interactions in relation to the clinical aspects of orbitopathy. In future studies, we suspect that it is necessary to characterize GO patients with different thyroid statuses and GO patients who have not undergone treatment, and to

compare these patients with the patients from our current study, to further elucidate distribution and function of Th17 cells, Treg cells, and fibrocytes in GO and GD autoimmunity.

In our coculture experiments, autologous Th17 cells stimulated gene expression of proinflammatory cytokines in 24 hours and further promoted those cytokine production, especially IL-8, MCP-1, CXCL9, and CXCL10, after 24 and 48 hours. An increased level of MIP-3 was also observed in coculture supernatants. To investigate the role of MIP-3 in mediating Th17 cell recruitment, we performed a transwell assay, which demonstrated that supernatants from the Th17/fibrocyte cocultures attracted Th17 cells expressing CCR6 (in a manner similar to MIP-3 stimulation). This suggests a potential MIP-3/CCR6 dynamic during Th17 cell migration in GO, and is consistent with a study from Dohlman et al.[35] showing that the
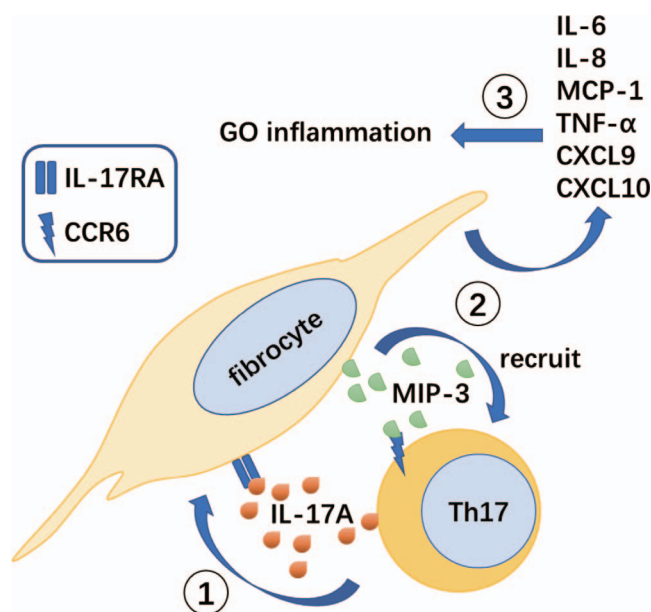
FIGURE 6. Hypothetical model for Th17 cell and fibrocyte interplay in GO. (1) In the development of GO, CCR6$^+$ Th17 cells produce an excessive level of IL-17A that stimulates fibrocytes. (2) Activated fibrocytes release inflammatory cytokines and MIP-3. Increased MIP-3 expression may recruit more Th17 cells by synergy with CCR6. (3) The increase in cytokines, such as IL-6, IL-8, MCP-1, CXCL9, CXCL10, and TNF-α, eventually leads to GO inflammation.

MIP-3/CCR6 axis mediates Th17 cell migration in dry eye disease.

OFs express CD40, and are characterized by low levels of MHC II[36,37] that can be upregulated by exposure to the Th1 cytokine IFN-γ, or by a total T-cell coculture treatment.[36-38] The constitutive CD40 and MHC II expression on fibrocytes is more potent than on OFs[21,22] and fibrocytes can efficiently present antigens to T cells in GO.[2] We found that both IL-17A treatment and Th17 cell coculture could upregulate CD40 and MHC II expression on fibrocytes. Similar to Th1-type immunity, Th17-type immunity could also increase the costimulatory molecules required for specific engagement and activation of T lymphocytes. Furthermore, an expanded number of CCR6$^+$ IL-17A–producing T cells was observed in the coculture system, suggesting a greater Th17 cell activity resulting from the communication with activated fibrocytes. It should be noticed that a murine model or in vivo study is of great importance to confirm our current hypothesis. However, no convincing evidence of GO was reported in any animal models.[39,40] Although encouraging findings of preclinical murine models for GO have appeared, there exist inconsistent results in different studies and a potentially confounding deviation from GO patients.[39,40]

In conclusion, we discovered an elevated level of CCR6$^+$ Th17 cells in Chinese GO patients, which likely contribute to disease activity. Increased surface expression of IL-17RA, CD80, and CD86 on fibrocytes implies an imbalance in Th17-cell and Treg-cell function during GO. The interaction between fibrocytes and Th17 cells promotes an inflammatory response in fibrocytes; it also attracts Th17 cells and maintains their phenotype, which may contribute to the exacerbation of GO (Fig. 6). We propose that developing novel agents to target the disruption of the Th17-fibrocyte interaction may provide powerful therapeutic strategies for GO and other autoimmune disorders.

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