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Specific Follicular Helper T Cell Signature in Takayasu Arteritis

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Objective. Our aim was to compare transcriptome and phenotype profiles of CD4+ T cells and CD19+ B cells in patients with Takayasu arteritis (TAK), patients with giant cell arteritis (GCA), and healthy donors.

Methods. Gene expression analyses, flow cytometry immunophenotyping, T cell receptor (TCR) gene sequencing, and functional assessments of cells from peripheral blood and arterial lesions from TAK patients, GCA patients, and healthy donors were performed.

Results. Among the most significantly dysregulated genes in CD4+ T cells of TAK patients compared to GCA patients (n = 720 genes) and in CD4+ T cells of TAK patients compared to healthy donors (n = 1,447 genes), we identified a follicular helper T (Tfh) cell signature, which included CXCR5, CCR6, and CCL20 genes, that was transcriptionally up-regulated in TAK patients. Phenotypically, there was an increase in CD4+CXCR5+CCR6+CXCR3− Tfh17 cells in TAK patients that was associated with a significant enrichment of CD19+ B cell activation. Functionally, Tfh cells helped B cells to proliferate, differentiate into memory cells, and secrete IgG antibodies. Maturation of B cells was inhibited by JAK inhibitors. Locally, in areas of arterial inflammation, we found a higher proportion of tertiary lymphoid structures comprised CD4+, CXCR5+, programmed death 1+, and CD20+ cells in TAK patients compared to GCA patients. CD4+CXCR5+ T cells in the aortas of TAK patients had an oligoclonal α/β TCR repertoire.

Conclusion. We established the presence of a specific Tfh cell signature in both circulating and aorta-infiltrating CD4+ T cells from TAK patients. The cooperation of Tfh cells and B cells might be critical in the occurrence of vascular inflammation in patients with TAK.

No potential conflicts of interest relevant to this article were reported. Drs. Régnier and Quiniou contributed equally to this work.

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INTRODUCTION

Takayasu arteritis (TAK) and giant cell arteritis (GCA) are the two most common types of large vessel vasculitis (LVV). Historically, TAK and GCA have been considered as distinct diseases based on differences in age at disease onset, ethnic distribution, and clinical features including a predisposition for different arterial territories. All patients with TAK have disease involvement of the aorta or its primary branches. In contrast, GCA is traditionally considered as a disease of the cranial arteries. However, with the more frequent use of angiography by computed tomography (CT) or ^{18}F-fluorodeoxyglucose–positron emission tomography (FDG-PET), recent studies have estimated the presence of large vessel involvement in 30–70% of patients with GCA (1,2). Another older study has shown vascular changes in the large arteries of <80% of GCA patients (3).

Lesions in LVV are characterized by granulomatous inflammatory infiltrates of the media, the media–intima junction, and the adventitia, often affecting the vasa vasorum. The inner half of the aorta is more often affected than the outer half and the adventitia in GCA lesions (4). Intimal hyperplasia is frequently observed, whereas the adventitia is relatively spared in patients with GCA as compared to patients with TAK. Scarring can be seen in the later phase, with dense adventitial fibrosis and great fibrous thickening of the intima.

Pathologic mechanisms in LVV are not well understood. T cells have been shown to be critical in the process, as demonstrated in an earlier study in which the secretion of inflammatory cytokines was abolished when T cells were depleted in SCID mice grafted with inflamed temporal arteries from human subjects (5). Consistently, both diseases are driven by Th1 and Th17 unbalanced immune responses (5–7). Associations with specific major histocompatibility complex (MHC) class II molecules (HLA–B52 in TAK and HLA–DRB1*04 in GCA) have also been observed (8,9). Increasing evidence also supports a role for B cells in the pathogenesis of LVV. Immunohistochemical analyses of aortic wall samples from patients with GCA/TAK have shown the presence of B cells in inflamed arterial lesions (10,11), and some studies have pinpointed the presence of tertiary lymphoid organs (TLOs) in the aortas of LVV patients (10,11).

Altogether, these data suggest a role for T cell and B cell interaction in the pathogenesis of LVV. However, the immune activation pathways specifically involved in each disease are poorly understood. Herein, we compared microarray gene analysis of purified CD4+ T cells and CD19+ B cells from TAK patients and GCA patients and healthy donors.

PATIENTS AND METHODS

Patients. The study population consisted of 54 TAK patients (median age 32.4 years [range 27.2–53.2 years]), 52 GCA patients (median age 74.7 years [range 66.3–83.2 years]) (Table 1), and 104 age- and sex-matched healthy donors, 38 of whom were matched to GCA patients by age (mean ± SD age 77.4 ± 11.9 years) and sex (60.5% women) and 66 of whom were matched to TAK patients by age (mean ± SD age 35.2 ± 9.3 years) and sex (62.1% women). TAK patients fulfilled the American College of Rheumatology (ACR) criteria for TAK and/or the Ishikawa criteria modified by Sharma (12,13). GCA patients fulfilled the GCA international criteria (14). Disease activity was defined according to the presence of the following: 1) new ischemic vascular sign (claudication, ischemic thoracic or abdominal pain, bruit or asymmetry in pulses, pulse abolition), 2) cranial sign of GCA (headache, anterior ischemic optic neuropathy, jaw claudication), 3) new large vessel involvement lesion or worsening of preexisting lesions on imaging, 4) systemic clinical features (weight loss, fever), and 5) biologic activity of disease (increased erythrocyte sedimentation rate [ESR] and/or C-reactive protein [CRP] level). Disease was considered active if an individual fulfilled ≥2 of the 5 areas mentioned above (including biologic activity) and inactive in the remaining individuals. Blood samples were collected from patients prior to any treatment, except for 18.8% of GCA patients who received <10 mg/day of prednisone.

The present study was approved by our Institutional Ethics Review Board and was performed in accordance with the Declaration of Helsinki. Written informed consent was provided by each patient.

Table 1. Demographic and clinical characteristics of patients with TAK and patients with GCA*

	Patients with TAK (n = 54)	Patients with GCA (n = 52)
Demographic features		
Age, median (IQR) years	32.4 (27.2–53.2)	74.7 (66.3–83.2)
Female sex	43 (79.6)	35 (67.4)
Geographic origin		
White	20 (36.4)	50 (96.2)
African	15 (27.4)	0 (0)
Northern African	15 (27.4)	2 (3.8)
Other	5 (9)	0 (0)
Clinical features		
Numano classification of TAK		
I	7 (14.3)	NA
II	8 (16.3)	NA
III	5 (10.2)	NA
IV	1 (2)	NA
V	27 (55)	
Stroke	12 (22.2)	7 (13.4)
Aortic aneurysms	18 (33.3)	4 (7.7)
Aortitis	54 (100)	14 (26.9)
Optic neuritis	–	5 (5.6)
CRP level, mean ± SD mg/liter	21.4 ± 28	45.3 (48.3)

* Except where indicated, values are the number (%) of patients. TAK = Takayasu arteritis; GCA = giant cell arteritis; IQR = interquartile range; NA = not applicable; CRP = C-reactive protein.

Transcriptome of CD4+ T cells and CD19+ B cells. We performed a microarray gene analysis of purified CD4+ T cells from 25 patients with active TAK, 27 patients with active GCA, and 37 healthy donors (including 25 "younger" healthy donors [range 27.2–53.2 years] age-matched to TAK patients and 12 "older" healthy donors [range 55–91 years] age-matched to GCA patients). No patients received steroid doses higher than 10 mg/day or immunosuppressants. In peripheral blood mononuclear cells (PBMCs) obtained from patients with active TAK, patients with active GCA, and healthy donors, CD3+ T cells were isolated by negative isolation with Dynabeads using an untouched Human T Cells kit (ThermoFisher) according to the manufacturer's instructions. CD4+ cells were then isolated by positive selection using Dynabeads CD4 Isolation kit (ThermoFisher) according to the manufacturer's instructions. In PBMCs from 8 patients with active TAK, 6 patients with active GCA, and 17 healthy donors (including 13 younger individuals age-matched to TAK patients and 4 older individuals age-matched to GCA patients), CD19+ B cells were isolated by Dynabeads using a CD19 Positive Isolation kit (ThermoFisher) according to the manufacturer's instructions. Once isolated, total RNA from CD4+ cells or CD19+ cells was extracted using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. Total RNA was quantified by a NanoDrop ND-1000 spectrophotometer. Samples with an RNA concentration of <20 ng/µl were excluded.

For quality control, RNA dilution was performed using an Agilent RNA 6000 Nano kit, with 1 µl of the sample run on the nanochip using an Agilent 2100 electrophoresis bioanalyzer. The quality of total RNA was assessed by the profile of the electropherogram and by the RNA integrity number (RIN). All samples included in the study had an RIN of 7.3–9.3. For Illumina BeadArrays, complementary RNA samples were prepared using an Illumina TotalPre-96 RNA Amp kit (LifeTechnologies) and hybridized to Illumina Human HT-12 v4 BeadArrays. Raw data were then analyzed using the R Limma software package (Supplementary Methods, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract). Enrichment analysis of different gene sets within samples and groups was assessed using GSVA and R Limma software packages according to the authors' instructions (15). Gene lists and signatures used throughout this study, including significantly dysregulated genes of CD4+ T cells between TAK patients, GCA patients, and healthy donors and follicular helper T (Tfh) cell, Tfh17 cell, and B cell signatures, are listed in Supplementary Table 1 (http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract).

Immunohistochemical analysis. Detection of CXCR5, programmed death 1 (PD-1), CD20, CD38, CD27, BAFF, CXCL13, interleukin-21 (IL-21), and CD4 was performed on fixed paraffin-embedded samples of the inflamed aorta obtained from 7 patients with TAK and 7 patients with GCA as well as samples obtained from 3 noninflamed aortas (Supplementary Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]).

Cultures of B cells and T cells. CXCR5+CD4+ T cells or CXCR5–CD4+ T cells (50,000 cells each per well) from 8 patients with active TAK were cultured with 20,000 naive B cells (defined as CD27–IgD+CD19+ cells) in the presence of human SAg (CytoStim; Miltenyi Biotec) (2 µl per million cells) in RPMI 1640 complete medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) in the presence or absence of the JAK inhibitor ruxolitinib (Supplementary Methods [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]).

T cell receptor (TCR) repertoire. Aorta samples were dissociated with a gentleMacs instrument, and magnetic enrichment of CD4+ T cells was performed with Dynabeads using a CD4 Positive Isolation kit. Isolated cells were further stained with the following monoclonal antibodies: Alexa Fluor 700 (AF700)–conjugated CD3, fluorescein isothiocyanate (FITC)–conjugated CD4, and phycoerythrin (PE)/Dazzle–conjugated CXCR5. Cell sorting was performed with FACSAria II on CD3+CD4+CXCR5– cells and CD3+CD4+CXCR5+ cells. RNA was subsequently extracted with the Ambion RNAqueous kit according to the manufacturer's recommendations. Before RNA extraction, harvested cells were biobanked in lysis solution at a temperature of –80°C. Then, the lysate was diluted with an ethanol solution. This solution was passed through the filter pad, and the filter cartridge was washed 3 times to remove contaminants. RNA was eluted in elution buffer. Library for next-generation sequencing was made using the SMARTer Human TCR α/β Profiling kit (Takarabio). Two hundred fifty single-end base paired reads were obtained using the Illumina sequencing platform HiSeq 2500 on rapid run mode. MiXCR software was used for the alignment of raw data and the extraction of human TCR sequences.

Statistical analysis. Continuous variables are presented with the median and range or with the mean ± SEM. Categorical variables are shown as counts and proportions. Statistical comparisons were performed using Student's *t*-test or Mann-Whitney test for quantitative unpaired data and Wilcoxon's matched pairs signed rank test for quantitative paired data. All statistical tests were 2-tailed with a significance threshold of 0.05. Statistical significance was evaluated using GraphPad Prism version 5 for Windows (GraphPad Software).

RESULTS

Specific CXCR5, CCR6, and PD-1 CD4+ T cell gene signatures in TAK. A microarray gene analysis of purified CD4+ T cells from 25 patients with active TAK, 27 patients with active GCA, and 37 healthy donors (including 25 younger donors age-matched to TAK patients and 12 older donors age-matched to patients with GCA) was performed. Among the 720 genes that were the most significantly dysregulated between TAK patients and GCA patients (with an adjusted *P* threshold of 0.05), 364 genes were up-regulated and 356 genes were down-regulated in TAK patients compared to GCA patients, respectively. Among the 1,447 genes that were the most significantly dysregulated between TAK patients and total healthy donors, 471 genes were up-regulated and 976 genes were down-regulated, respectively, in TAK patients compared to total healthy donors. Interestingly, we identified *CXCR5*, *CCR6*, and *CCL20* as among the most up-regulated genes in TAK patients as compared to either GCA patients or total healthy donors (Figure 1A). Of note, none of the genes presented in the previous heatmap were significantly dysregulated between older healthy donors and younger healthy donors (data not shown). *CXCR5* and *CCR6* genes were expressed by Tfh cells in the peripheral blood, particularly by Tfh17 cells.

Qiagen Ingenuity Pathway Analysis software was then used to construct a network of the most up-regulated genes in TAK patients versus GCA patients and total healthy donors (Figure 1B). *CXCR5*, *CCL20*, *CCR6,* and *PDCD1* genes (strongly associated with Tfh cells) were tightly connected to each other in the gene interaction network. We also confirmed by quantitative polymerase chain reaction the overexpression of *CXCR5* messenger RNA in CD4+ T cells of TAK patients as compared to that in the CD4+ T cells of GCA patients ($P = 0.02$) (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract).

Next, using both the literature (16–22) and general knowledge, we established and manually curated a Tfh cell–specific gene signature and computed a signature score using an R GSVA software package that estimated the enrichment of a gene signature in a given patient group. We found that this Tfh cell–specific gene signature was significantly more present in CD4+ T cells of TAK patients as compared to GCA patients (even after adjustment of *P* values for age with an R Limma software package) and younger age-matched healthy donors (Figures 1C and D). Of note, this Tfh cell signature score was not significantly different between younger and older healthy donors. Furthermore, none of the genes comprising this Tfh cell signature were significantly dysregulated between older and younger healthy donors (data not shown). As TAK patients and GCA patients are characterized by different demographic features, we confirmed that none of the transcriptomic differences at both the Tfh gene and signature levels were related to the age, sex, or geographic origin of the TAK patients and GCA patients or total healthy donors (Supplementary Table 2 [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]). We observed that the following cell functions and genes were the most up-regulated in GCA patients as compared to healthy donors: cell metabolism/homeostasis, cytokine/chemokine secretion and associated cell response, chemotaxis of T cells (*TNFSF8*, *TNFSF9*, *IL2RA*, *CD70*, *IL2RB*, *LTA*, *CXCR3*, *CXCR1*, *CXCL11*, and *CXCL6*), and innate immunity (*LY96*, *IRAK2*, and *IRAK3*).

We next confirmed by flow cytometry the overexpression of CXCR5 by CD4+ cells in TAK patients. The proportion of CXCR5+CD4+ cells (defined as circulating Tfh cells) among total CD4+ T cells was dramatically higher in TAK patients compared to that in total CD4+ T cells of GCA patients or younger healthy donors. In TAK patients, the median proportion of CXCR5+CD4+ T cells was 15.4% (range 10–30.8%) whereas in GCA patients and healthy donors, the median proportion of CXCR5+CD4+ T cells was 5.3% (range 1.4–12.2%) and 9.7% (range 5.6–12.5%), respectively ($P < 0.0001$ and $P = 0.0001$, respectively) (Figure 1E). Levels of CXCR5+ cells were also higher in patients with active TAK as compared to patients with inactive TAK (Figure 1F). We concluded that circulating Tfh cell frequency is specifically increased in the peripheral blood of TAK patients as compared to GCA patients and healthy donors.

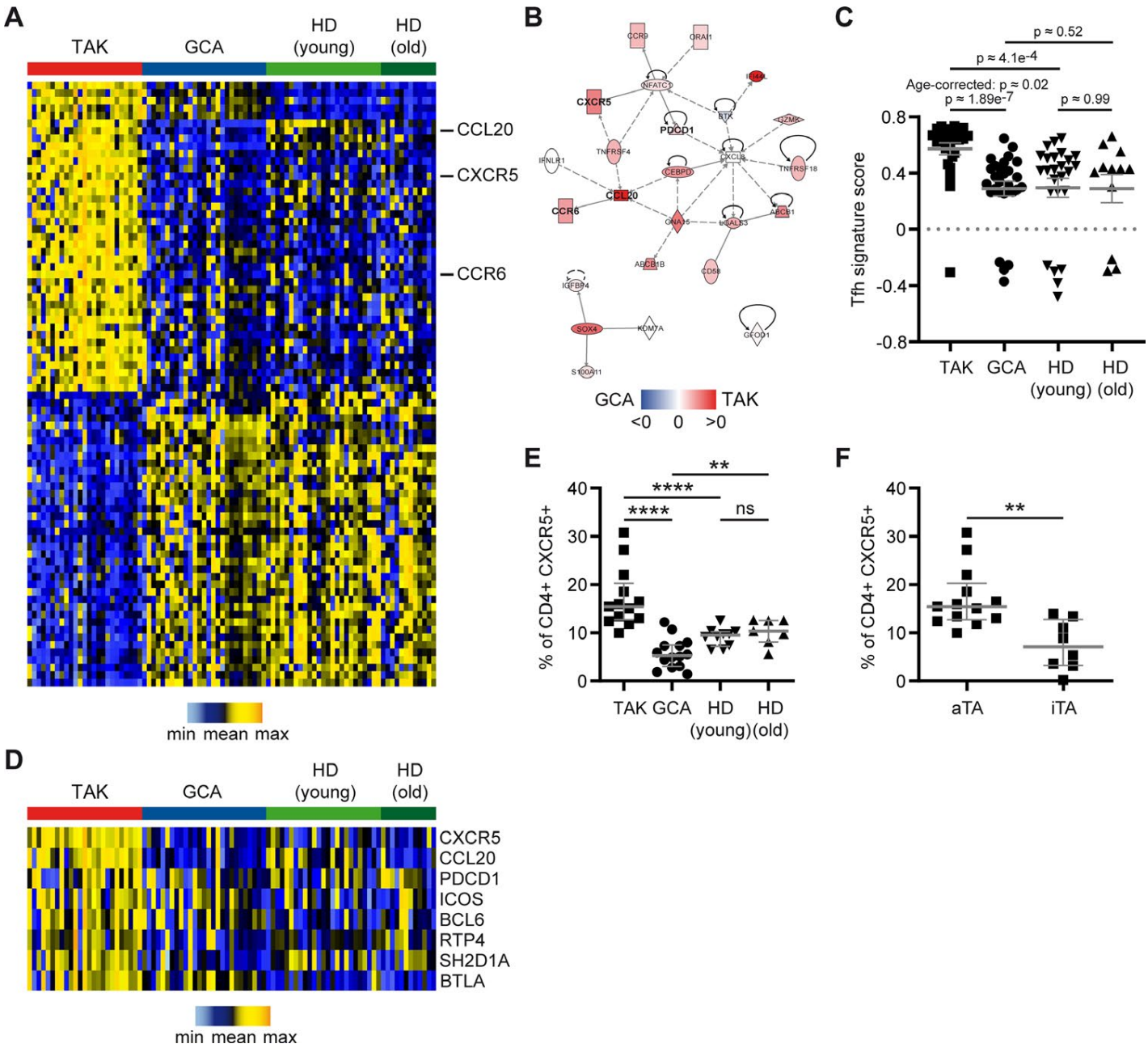

Figure 1. Specific *CXCR5*, *CCR6*, and *PDCD1* CD4+ T cell gene signatures in patients with Takayasu arteritis (TAK). **A**, Purified CD4+ T cells from 25 patients with active TAK, 27 patients with giant cell arteritis (GCA), and 37 healthy donors (HDs) (who were age-matched to 25 "younger" patients with TAK and 12 "older" patients with GCA) were analyzed by microarray gene analysis. No patients included in the analysis received steroid doses higher than 10 mg/day or immunosuppressants. CD4+ T cells of TAK patients and GCA patients and age-matched healthy donors exhibited distinct mRNA signatures. *CXCR5*, *CCR6*, and *CCL20* were significantly up-regulated in patients with TAK as compared to patients with GCA and age-matched healthy donors. **B**, Qiagen Ingenuity Pathway Analysis revealed the most dysregulated genes between TAK patients, GCA patients, and healthy donors. **C** and **D**, A follicular helper T (Tfh) cell–specific gene signature that is significantly enriched in the CD4+ T cells of TAK patients versus GCA patients and age-matched healthy donors was constructed based on profiles reported in the literature (16,17,21). **E**, Increased frequency of CXCR5+ cells in CD4+ T cells of patients with TAK compared to patients with GCA and healthy donors was confirmed by flow cytometry. **F**, Frequency of CXCR5+ cells was higher in TAK patients with active disease (aTA) (n = 13) compared to those with inactive disease (iTA) (n = 8). Symbols represent individual subjects; values are the mean ± SEM (**C**) or median (interquartile range) (**E** and **F**). ** = $P < 0.01$; **** = $P < 0.0001$ by Student's *t*-test (Limma-moderated *t*-test) with Benjamini-Hochberg correction (**C**) and standard Mann-Whitney test with no *P* value correction (**E** and **F**). NS = not significant.

Circulating CD4+CXCR5+CCR6+CXCR3− Tfh17 cells significantly increased in patients with TAK. We next studied, in depth, Tfh cell differentiation in patients with LVV and healthy donors. The same procedure described earlier (Figure 1C) was performed with a Tfh17 cell–specific gene signature based on the literature (15,16,20,21). Results showed that this Tfh17 cell signature was also very significantly enriched in the CD4+ T cells of TAK patients as compared to those in GCA patients (even after adjustment of *P* values for age with an R Limma software package) and younger age-matched healthy donors (Figure 2A). Additionally, this Tfh17 cell signature score was not significantly different between younger and older healthy donors. Moreover, the raw expression levels of the genes comprising this Tfh17 cell–specific signature were highly enriched in CD4+ T cells of TAK patients versus that in the CD4+ T cells of GCA patients and total healthy donors (Figure 2A), and, importantly, none of these genes were significantly dysregulated between older and younger healthy donors. Additionally, the Tfh17 signature score and the expression of each gene composing this signature were not correlated with age, sex, or geographic origin in TAK patients, GCA patients, and total healthy donors (Supplementary Table 2 [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]).

Furthermore, flow cytometry analysis of PBMCs from different patient groups confirmed a higher frequency of total CXCR6+ cells in CD4+ T cells from TAK patients as compared to that in CD4+ T cells from GCA patients or healthy donors. The proportion of CXCR5+CCR6+ cells in CD4+ cells was increased in TAK patients (median 4.52% [range 1.07–13.35%]) as compared to the proportion of CXCR5+CCR6+ cells in CD4+ T cells in GCA patients (median 0.69% [range 0.13–2.16%]) and younger healthy donors (median 2.3% [range 0.3–4.2%]) (P = 0.0001 and P = 0.02, respectively, for GCA patients and healthy donors) (Figure 2B and Supplementary Figures 2A and B [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]). Circulating CXCR5+ T cells in patients with TAK predominantly comprised Tfh17 cells (Figure 2C). We also found that levels of IL-17 and IL-6 were increased in culture supernatants of patients with TAK as compared to patients with GCA and younger healthy donors (for IL-17, mean ± SEM 134.7 ± 147.9 pg/ml in patients with TAK versus mean ± SD 9.6 ± 9.2 pg/ml in GCA patients [P = 0.02]; for IL-6, mean ± SEM 101.4 ± 121.6 pg/ml in patients with TAK and mean ± SEM 27.6 ± 40.1 pg/ml in patients with GCA [P = 0.06]) (Figures 2D and E). We concluded that Tfh17 frequency is specifically increased in the peripheral blood of patients with TAK compared to patients with GCA and healthy donors.

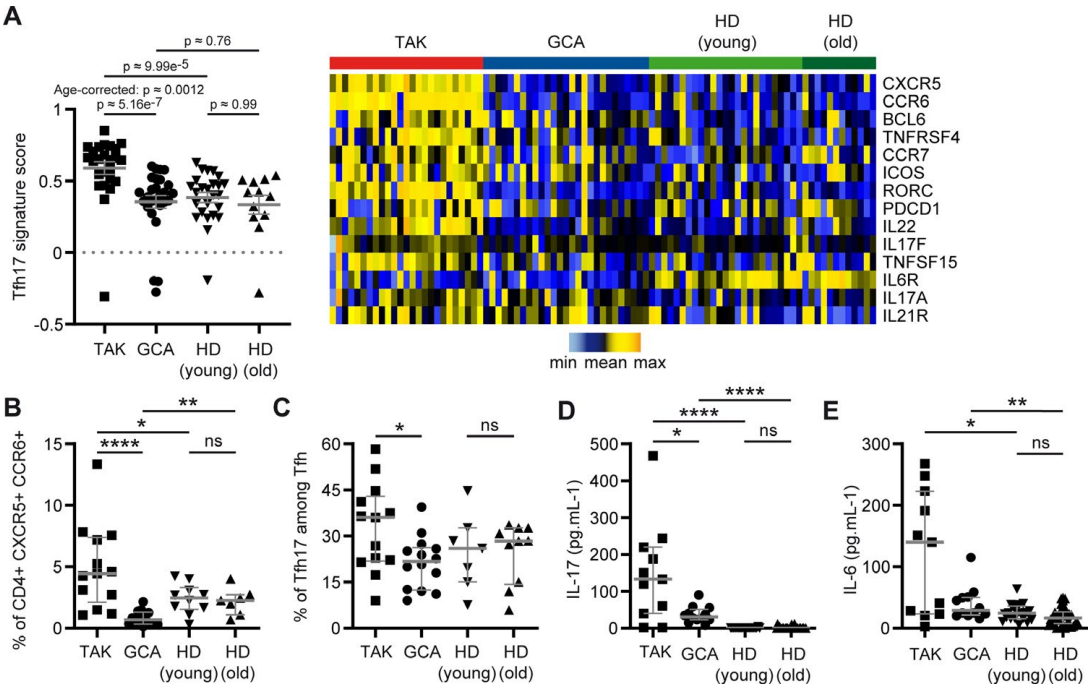

Figure 2. Significantly increased circulating CD4+CXCR5+CCR6+CXCR3− T cells (Tfh17) in patients with TAK. **A**, The Tfh17-specific gene signature was shown to be highly enriched in CD4+ T cells from 25 patients with TAK compared to 27 patients with GCA and 37 age-matched healthy donors (including 25 younger healthy donors age-matched to TAK patients and 12 healthy donors old age-matched to GCA patients). **B**, The frequency of CXCR5+CXCR6+CXCR3− cells was also higher in CD4+ T cells from 13 patients with TAK as compared to 14 patients with GCA or 17 total healthy donors. **C**, The proportion of Tfh17 cells (defined as CD4+CXCR5+CCR6+CXCR3− T cells) was higher in TAK patients compared to GCA patients and younger and older healthy donors. **D** and **E**, Peripheral blood mononuclear cells (PBMCs) from 11 patients with TAK, 12 patients with GCA, and 49 total healthy donors were stimulated for 4 hours with 0.05 μg/ml phorbol 12-myristate 13-acetate (PMA) and 1 m*M* (1 μg/ml) ionomycin. Interleukin-17 (IL-17) (**D**) and IL-6 (**E**) levels in culture supernatants were quantified. Levels of IL-17 were significantly higher in TAK patients compared to GCA patients and healthy donors, and levels of IL-6 were higher in TAK patients as compared to GCA patients and younger healthy donors, although the difference between TAK and GCA patients was not significant. Symbols represent individual subjects; values are the mean ± SEM (**A**) or the median (interquartile range) (**B–E**). * = $P < 0.05$; ** = $P < 0.01$; **** = $P < 0.0001$ by Student's *t*-test (Limma-moderated *t*-test) with Benjamini-Hochberg correction (**A**) or standard Mann-Whitney test (unadjusted *P*) (**B–E**). See Figure 1 for other definitions.

Specific B cell activation profile in TAK patients. As Tfh cells are known to stimulate B cells, another microarray gene analysis of purified CD19+ B cells of 8 patients with active TAK, 6 patients with active GCA, and 17 healthy donors (including 13 younger healthy donors age-matched to patients with TAK and 4 older healthy donors age-matched to patients with GCA) was performed. We generated from the literature (23) a gene signature specific for B cell activation/proliferation and tested its enrichment in patients with TAK, patients with GCA, and healthy donors using an R GSVA software package. We found that this signature is significantly enriched in B cells from patients with TAK as compared to patients with GCA, even after adjustment of *P* values for age with an R Limma software package (Figures 3A and B). Furthermore, the expression of the genes comprising the B cell signature was not correlated with age, sex, or geographic origin in TAK patients, GCA patients, and healthy donors (Supplementary Table 2). Consistent with this, histologic analysis of aorta samples from patients with TAK showed major infiltrates of CD20+ cells with nodular organization, whereas the presence of CD20+ cells was weaker in patients with GCA and had less nodular organization (Figure 3C). We also observed the expression of B cells differentiation and growth markers such as CD27, CD38, and BAFF (Supplementary Figure 3A, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract).

We next quantified the expression of CD20 in aorta samples from TAK patients and GCA patients and found that the surface area of CD20+ cells was significantly higher in the aortas of TAK patients as compared to those of GCA patients ($P = 0.01$) (Figure 3D). The frequency and the absolute number of circulating CD19+ cells among lymphocytes were also increased in patients with active and untreated TAK as compared to patients with active and untreated GCA and age-matched healthy donors (mean ± SEM 12.6 ± 4% for TAK patients versus mean ± SEM 8.1 ± 5% for GCA patients and mean ± SEM 9.8 ± 4.5% for healthy donors [$P = 0.0002$ and $P = 0.0032$, respectively, for GCA patients and healthy donors]) (Figure 3E and Supplementary Figure 3B). Importantly, both metrics were not significantly different between older and younger healthy donors and were not significantly correlated with age, sex, or geographic origin in TAK patients, GCA patients, and total healthy donors (Supplementary Table 3 [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]). Also, levels of BAFF cytokines measured by enzyme-linked immunosorbent assay in the sera of TAK patients tended to be higher compared to those observed in GCA patients (mean ± SEM 498.9 ± 141.3 pg/ml versus 424.9 ± 177.4 pg/ml; $P = 0.0571$] (Supplementary Figure 3C). We concluded that the peripheral blood and aortas of patients with TAK presented elevated levels of B cells with an activated/proliferating profile as compared to patients with GCA and healthy donors.

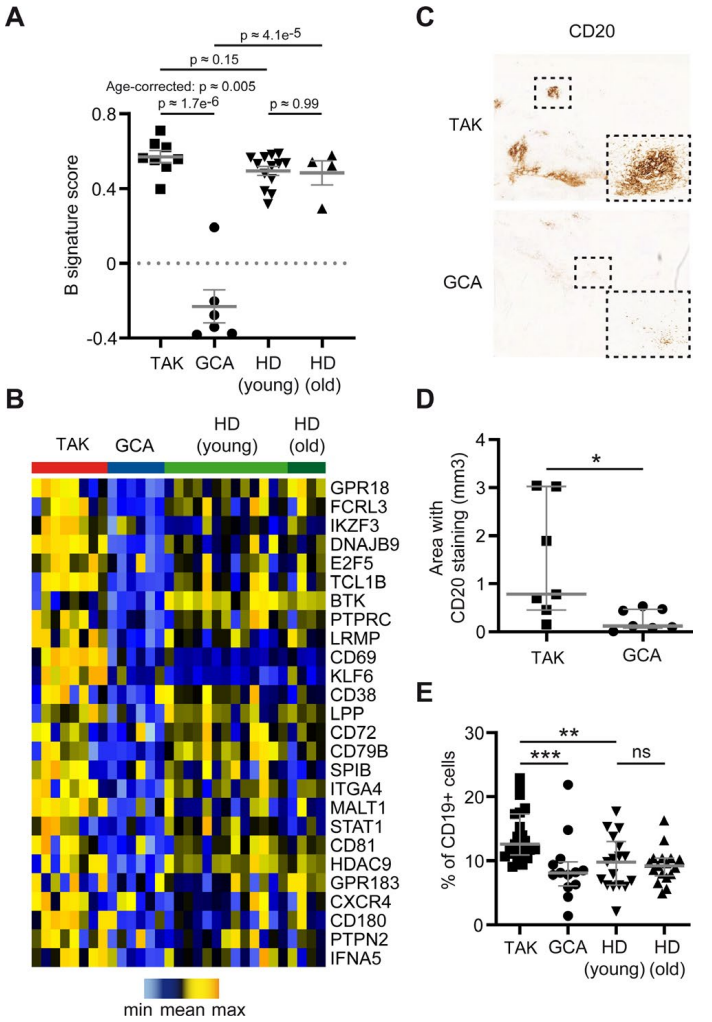

Figure 3. Specific B cell activation profile in patients with TAK. **A**, Specific gene signature for B cell activation/proliferation is highly enriched in the CD19+ B cells of 8 patients with TAK as compared to 6 patients with GCA and 17 age-matched healthy donors (including 13 younger healthy donors age-matched to TAK patients and 4 older healthy donors age-matched to GCA patients). **B**, Heatmap demonstrates the distinction in B cell activation/proliferation gene signatures between TAK patients and GCA patients and total healthy donors. **C**, Major infiltrates of CD20+ cells with nodular organization are evident in the aorta specimens from TAK patients compared to those from GCA patients. **D**, Results of surface area staining of CD20+ cells in aorta samples from 7 patients with TAK and 7 patients with GCA are shown. **E**, Frequency of CD19+ cells is increased in 23 patients with active and untreated TAK compared 13 patients with active and untreated GCA and 77 total healthy donors. Symbols represent individual subjects; values are the mean ± SEM (**A**) or the median (interquartile range) (**D** and **E**). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ by Student's *t*-test (Limma-moderated *t*-test) with Benjamini-Hochberg correction (**A**) or standard Mann-Whitney tests (unadjusted *P*) (**D** and **E**). See Figure 1 for definitions.

Assistance of naive B cells through the JAK/STAT pathway via circulating CD4+CXCR5+ Tfh cells from TAK patients. As we have shown a specific Tfh cell differentiation in patients with TAK, we next aimed to confirm their functionality. CD4+CXCR5+ or CD4+CXCR5– T cells from TAK patients were cultured with naive CD27–IgD+CD19+ B cells of the same patients in the presence of a superantigen (Cyto-Stim). CD4+CXCR5+ T cells induced a higher proliferation of B cells on day 3 as compared to CXCR5–CD4+ T cells (Figure 4A and Supplementary Figure 4A [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]). Consistently, the proportion of CD19+ cells on day 7 was significantly increased in cultures including CD4+CXCR5+ T cells as compared to those including CD4+CXCR5– T cells (mean ± SEM 33.8% ± 12% versus 24.9% ± 6%; $P = 0.008$) (Figure 4B). In TAK patients, CD4+CXCR5+ T cells were shown to enhance naive B cells into differentiating into CD27+ memory B cells compared to CD4+CXCR5– T cells (mean ± SEM 6.7% ± 3.4% versus mean ± SEM 3.7% ± 2.1%; $P = 0.008$) and were also shown to help B cells secrete IgG ($P = 0.04$) (Figures 4C and D).

We next tested whether the JAK/STAT cellular pathway was involved in the cooperation of CD4+CXCR+ T cells, with B cells ultimately leading to B cell differentiation. Inhibition of the JAK/STAT pathway with ruxolitinib (anti-JAK1/2) led to a significant decrease in B cell maturation as measured by the frequency of CD27+ B cells on day 7 (mean ± SEM 6.7% ± 3.4% versus 1.3% ± 0.52%; $P = 0.01$) (Figure 4C) and a decrease of the IgG/IgM ratio (mean ± SEM 3.17% ± 0.58% versus 1.5% ± 0.05%; $P < 0.0001$) (Figure 4D). Finally, we found that ruxolitinib significantly inhibited secretion of IL-6 in the B cells/CXCR5+ coculture supernatants (mean ± SEM 11.26 ± 10.26 pg/ml versus 0.63 ± 1.07 pg/ml; $P = 0.02$) (Figure 4E), although no difference in secretion of IL-6 was found between CD4+CXCR5+ and CD4+CXCR5– T cells in the absence of ruxolitinib. Moreover, we also performed the same experiments comparing fluorescence-activated cell sorted CD45RA–CXCR5+ T cells to CD45RA–CXCR5–CD4+ T cells for cocultures. Results of CD27 expression and IL-6 secretion were similar to those previously presented (Supplementary Figures 4B and C [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]).

Additionally, we performed the same experiments by pretreating either B cells or Tfh cells with ruxolitinib before cocultures. It was found that ruxolitinib also inhibited IL-6 secretion in both conditions (i.e., only B cells treated with ruxolitinib and only CXCR5+ T cells treated with ruxolitinib). However, ruxolitinib did not inhibit B cell maturation (as defined by CD27 expression) when B cells and Tfh cells were not treated together with ruxolitinib (i.e., ruxolitinib did not have any impact on B cell maturation when either B cells only or Tfh cells only were pretreated with the drug before cocultures) (Supplementary Figures 4B and C).

Altogether, these results showed that CD4+CXCR5+ T cells, which were known to be increased in patients with TAK, remained functional (with Tfh cell properties) and were able to help B cell proliferation and differentiation through the JAK/STAT pathway.

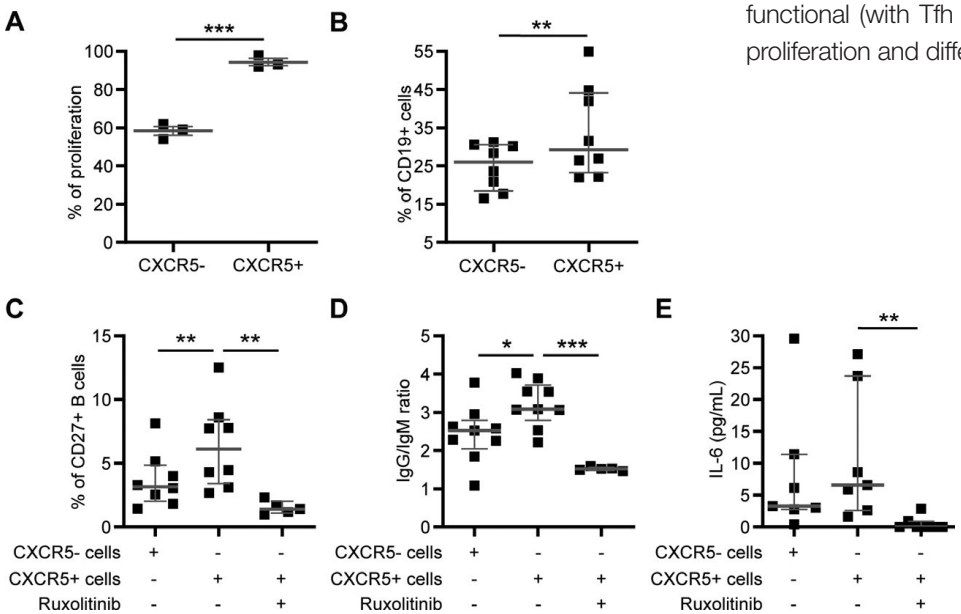

Figure 4. Assistance of naive B cells through the JAK/STAT pathway via circulating CD4+CXCR5+ Tfh cells from TAK patients. CD4+CXCR5+ or CD4+CXCR5– T cells from 8 patients with TAK were grouped by fluorescence-activated cell sorting and cultured with naive B cells (defined as CD27–IgD+CD19+ cells), followed by stimulation with a superantigen. Proliferation of B cells was assessed on day 3 by 5,6-carboxyfluorescein succinimidyl ester staining. **A**, Proportion of proliferative B cells was higher in the presence of CD4+CXCR5+ T cells compared to CD4+CXCR5– T cells. **B**, Proportion of CD19+ cells was significantly higher on day 7 in the presence of CXCR5+ T cells compared to CXCR5– T cells. **C**, Proportion of CD27+ memory B cells was significantly higher on day 7 in the presence of CXCR5+ T cells compared to CXCR5– T cells. Inhibition of the JAK/STAT pathway with ruxolitinib led to the suppression of differentiation into CD27+ B cells. Results from 8 patients with active TAK are shown in **B** and **C**. **D**, Measurement of IgG and IgM secretion was performed in supernatants (n = 9) of naive B cells cultured with CXCR5+ or CXCR5– T cells. The subsequent secretion of IgG (measured relative to IgM) was higher in B cells cultured with CXCR5+ T cells. Inhibition with ruxolitinib led to a decreased IgG/IgM ratio. **E**, Secretion of interleukin-6 (IL-6) was significantly inhibited when ruxolitinib was added to cocultures (n = 7). Symbols represent individual samples; values are the mean ± SEM (**A**) or median (interquartile range) (**B**–**E**). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ by Student's *t*-test (**A**) or Mann-Whitney test (for comparisons of CXCR5– and CXCR5+ T cells) or Wilcoxon's matched pairs test (for comparisons of CXCR5+ T cells in the absence or presence of ruxolitinib) (**B**–**E**). See Figure 1 for definitions.

Increased tertiary lymphoid structures comprising CXCR5+, CD4+, PD-1+, and CD20+ cells within inflamed aortas of TAK patients. The presence of TLOs has previously been shown in LVV (11,24). Thus, we compared the presence of TLOs in the aortic wall samples of 12 patients with TAK and 15 patients with GCA to further study the immune response within inflamed aortas. Interestingly, we confirmed that the presence of TLOs was more frequently observed in the aortas of patients with TAK as compared to patients with GCA ($P < 0.05$) (Supplementary Figures 5A–C [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]). TLOs exhibited high expression of CXCR5 within their periphery, as shown with CD4 staining in the aortas of patients with TAK (Supplementary Figure 5B). PD-1 and Bcl-6 were also highly expressed in these structures, unlike aortas of patients with GCA.

TCR sequencing of CD4+CXCR5+ T cells in the aortas of patients with TAK. TCR repertoire analysis of fluorescence-activated cell sorted CD4+CXCR5+ T cells and CD4+CXCR5– T cells was performed on peripheral blood and aorta samples from 2 patients with TAK (number of CD4+ T cells in the aortas and peripheral blood of TAK patients was 4,686 cells and 4,655,000 cells, respectively, for patient 1 and 1,534 cells and 3,558,000 cells for patient 2) (Figure 5A). We observed a very broad repertoire of peripheral CD4+ T cells in the peripheral blood of TAK patients, whereas CD4+ T cells originating from aorta lesions of TAK patients had a more oligoclonal profile (Figure 5B and Supplementary Figure 6A [http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract]). It was shown that the TCR repertoire in aortic cells was narrower for CD4+CXCR5+ T cells than in CXCR5–CD4+ T cells. In the first patient with TAK, one clonotype represented 97.6% of the TCRα repertoire in CD4+CXCR5+ aortic T cells. In the second patient with TAK, two major clonotypes represented 96.1% of the TCRα repertoire found in CD4+CXCR5+ aortic T cells. The most frequent clonotypes found in CD4+CXCR5+ aortic T cells are reported in Supplementary Figure 6B (http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract). Of note, some patterns of the major aortic CD4+CXCR5+ sequences have been previously reported in other autoimmune/inflammatory diseases (25), as well as in vascular diseases (26,27) (Supplementary Figure 6B).

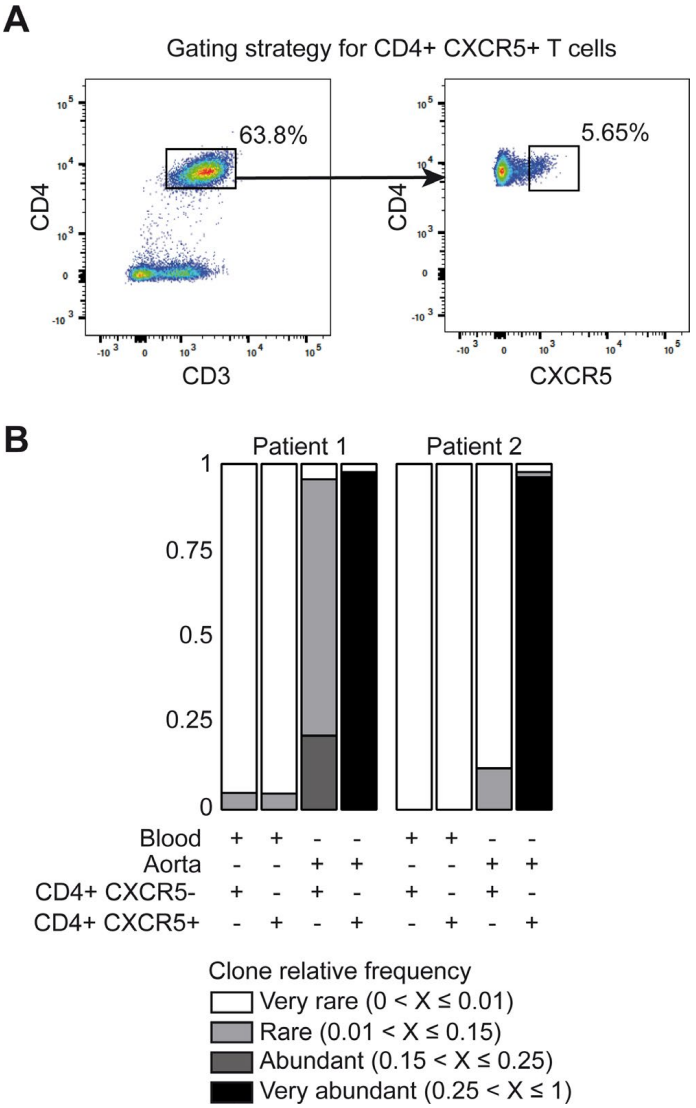

Figure 5. T cell receptor repertoire oligoclonal distribution in Tfh cells from the arteries of patients with TAK. **A**, Gating strategy used in the fluorescence-activated cell sorting of CXCR5– and CXCR5+ CD4+ T cells either from peripheral blood or aorta samples in 2 patients with TAK. **B**, Oligoclonal profile of CXCR5+ and CXCR5– CD4+ T cells in aorta and peripheral blood samples from 2 patients with TAK. See Figure 1 for definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41672/abstract.

DISCUSSION

We showed for the first time a specific gene signature of circulating Tfh cells that discriminates TAK patients from GCA patients and age-matched healthy donors. Circulating CD4+CXCR5+ T cells have been shown in recent studies to have functional characteristics similar to Tfh cells. Indeed, circulating CD4+CXCR5+ T cells promote survival, proliferation, and differentiation of B cells into plasma cells as Tfh cells are present in germinal centers (28). However, the phenotype of circulating Tfh cells differs from "conventional" tissue-specific Tfh cells that express high levels of PD-1 and inducible costimulator (ICOS). In peripheral blood, only a few CD4+CXCR5+ T cells express ICOS or PD-1 (29). Thus, PD-1 and ICOS do not define circulating Tfh cells. Instead, these can be distinguished according to their membrane expression of CCR6 and CXCR3 (30).

In our study, we also demonstrated an increase of CD4+CXCR5+CCR6+CXCR3– T cells in patients with TAK, corresponding to the Tfh17 cell population, which was consistently confirmed by transcriptomic analysis using a Tfh17-specific gene signature that was greatly enriched in TAK patients as compared to GCA patients and age-matched healthy donors (but also other healthy donors that were not age-matched to TAK or GCA patients). Importantly, we demonstrated that the up-regulation of Tfh and Tfh17 signatures observed in TAK patients were not explained by differences in age, sex, or geographic origin, as the expression of the genes comprising these signatures (along with the global signatures enrichment scores) were not correlated with these demographic characteristics. Our findings are consistent with previous cytometric data on small cohorts of TAK and GCA patients that showed a higher proportion of Tfh cells in TAK as compared to GCA patients (7).

Our transcriptomic analysis of CD19+ B cells in LVV patients revealed a significant enrichment of a gene signature associated with B cell activation, proliferation, and differentiation in TAK patients as compared to GCA patients. Consistently, we identified an increase in the number of B cells in the peripheral blood and arterial lesions of TAK patients as compared to GCA patients. Serum levels of BAFF tended to be higher in patients with TAK. Only few studies have described B cell profiles in patients with TAK, but one study has shown an increased absolute number and frequency of peripheral blood CD19+CD20–CD27^{high} antibody–secreting B cells in patients with active TAK (31). The role of B cells in TAK has also been supported by the use of rituximab in case reports (31). In addition, studies have shown a decrease in the level of circulating B cells in patients with active GCA (32) that normalized rapidly with treatment.

Consistent with previous studies, we observed the presence of TLOs in inflamed aortas of patients with TAK and patients with GCA (11,24,33). However, we found significant differences between the two diseases. First, the proportion of TLOs in aorta lesions was clearly increased in TAK patients as compared to GCA patients. Moreover, their repartition was dramatically different, with a highly ordered nodular organization of the inflammatory infiltrates in TAK patients. We highlighted the marked expression of CXCR5, Bcl-6, PD-1, IL-21, BAFF, and CXCL13, which are critical molecules for the development and homeostasis of tertiary lymphoid structures. TLOs may be observed in tissues affected by unresolving inflammation as a result of infection, autoimmunity, or cancer. These highly ordered structures are composed of cells present in the lymphoid follicles typically associated with the spleen and lymph node compartments (34). The structural similarities between TLOs and B cell follicles found in secondary lymphoid organs suggest a local recruitment of naive cells via high endothelium venules, their activation, and the establishment of an immunologic humoral memory supported by Tfh cells. In the present study, TLOs were mainly detected in the adventitia aorta specimens of TAK patients. Analysis of immune adventitial cells showed a high percentage of memory- and antigen-experienced B cells and the presence of cells expressing canonical Tfh cell markers, such as CXCR5, Bcl-6, and PD-1. TCR sequencing of CD4+CXCR5+ T cells located within inflamed aortas of patients with TAK showed oligoclonal populations.

The restricted repertoire of CD4+CXCR5+ T cells within the aorta strongly suggests antigenic selection of Tfh cells. Interestingly, the TCR sequences found in our study have already been reported in diseases known to be linked to autoimmune mechanisms and B cell abnormalities, such as Sjögren's syndrome (35) and multiple sclerosis (25), and in coronary and cardiac injuries (26,36). Altogether, these results suggest an antigen-specific immune response based on cooperation of Tfh cells and B cells occurring in the adventitia of blood vessels in patients with TAK.

Cooperation between Tfh cells and B cells in TAK patients was further demonstrated by functional tests showing that CD4+CXCR5+ T cells helped B cells to proliferate and differentiate. The cooperation between Tfh cells and B cells in TAK was mediated by the JAK/STAT pathway. Zhang et al have previously shown that in GCA patients, tofacitinib effectively suppresses innate and adaptive immunity in the vessel wall, with reduced proliferation and minimal production of the effector molecules interferon-γ, IL-17, and IL-21 (37,38). Taken together, these data suggest that JAK inhibitors might be a promising therapeutic strategy in TAK (38).

In summary, we established specific Tfh cell and Tfh17 cell signatures that characterize TAK. We highlight the cooperation between Tfh cells and B cells through the JAK/STAT pathway in patients with TAK. Our results provide new therapeutic approaches toward LVV and important insight into its pathogenesis.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Saadoun and Desbois had full access to all the study data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Desbois, Régnier, Garrido, Cacoub, Klatzmann, Saadoun.

Acquisition of data. Desbois, Régnier, Quiniou, Lejoncour, Maciejewski-Duval, Comarmond, Vallet, Rosenzwajg, Darrasse-Jèze, Derian, Pouchot, Samson, Bienvenu, Fouret, Koskas, Garrido, Sène, Bruneval, Cacoub, Klatzmann, Saadoun.

Analysis and/or interpretation of data. Desbois, Régnier, Quiniou, Maciejewski-Duval, Bienvenu, Klatzmann, Saadoun.

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