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ORIGINAL RESEARCH

Novel Th17 Lymphocyte Populations, Th17.1 and PD1+Th17, are Increased in Takayasu Arteritis, and Both Th17 and Th17.1 Sub-Populations Associate with Active Disease

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Video abstract

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Purpose: We evaluated T helper lymphocyte profile, including novel Th17 subsets Th17.1 (secrete IFN-γ, associate with corticosteroid resistance) and PD1+Th17 (secrete TGF-β1, implicated in fibrosis), and related cytokines in peripheral blood of Takayasu arteritis (TAK).
Materials and Methods: We evaluated circulating Th1, Th2, Th17, Th17.1, PD1+CD4+ T lymphocytes, PD1+Th17, and Treg lymphocytes, inflammatory (IFN-γ, IL-4, IL-6, IL-17A, IL-23, IL-1β, TNF-α) and regulatory (IL-10, TGF-β1) cytokines in peripheral blood of TAK (n = 57; median age 35 (interquartile range 26–45) years; 40 females) in a cross-sectional design. We studied inflammatory and regulatory cytokines in culture supernatant of peripheral blood mononuclear cells (PBMCs) from TAK following stimulation with anti-CD3/anti-CD28 and their modulation by tacrolimus (immunosuppressive) with/without tadalafil (anti-fibrotic). Furthermore, we followed up immunosuppressive-naïve active TAK (n = 16) and compared T helper lymphocyte populations and cytokines before and after immunosuppressive therapy. Healthy controls (HC, n = 21) and sarcoidosis (disease control, n = 11) were compared against TAK.
Results: TAK had higher Th17, Th17.1 and PD1+Th17 lymphocytes than HC (p < 0.001), and higher PD1+CD4+ T lymphocytes than sarcoidosis (p < 0.001). Th17 lymphocytes associated with active TAK after multivariable-adjusted logistic regression (p = 0.008). TAK had greater cytokine secretion from PBMCs (IFN-γ, IL-17A, IL-10 versus HC; IL-6, TNF-α, IL-1β versus HC or sarcoidosis) (p < 0.05). In-vitro, PBMCs from TAK showed reduced secretion of all inflammatory cytokines with tacrolimus, with synergistic reduction in IL-17A, IL-6, IL-1β and IL-10 following addition of tadalafil to tacrolimus. Serial follow-up of immunosuppressive-naïve TAK (n = 16) showed reduction in serum IL-6 and TGF-β1 (p < 0.05) and IL-6 in culture supernatant (p < 0.05) following immunosuppressive therapy.
Conclusion: Novel Th17 sub-populations (Th17.1 and PD1+Th17) are elevated in TAK. Th17 lymphocytes associate with active TAK. In-vitro experiments on cultured PBMCs suggest promise for further evaluation of a combination of immunosuppressive tacrolimus with anti-fibrotic tadalafil (or other anti-fibrotic therapies) in clinical trials of TAK.
Keywords: arteritis, aortic arch syndromes, PD-1 protein, drug resistance, corticosteroid, fibrosis

Introduction

Takayasu arteritis (TAK), a large vessel vasculitis affecting aorta and its major branches, associates with considerable morbidity and increased mortality risk.[1–5] Pathology is characterized by granulomatous vascular inflammation resulting in intimal hyperplasia, thickening of the media with inflammatory cell infiltrate and adventitial fibrosis.[6–8] Vascular obstruction might result from either inflammatory wall thickening or fibrosis of the hitherto inflamed arterial wall. Less commonly, aneurysms develop in affected vessels.[1,9,10] Assessment of disease activity and evaluation of biomarkers is challenging due to difficulty in accessing affected vessels other than during vascular bypass surgeries, therefore, relies on surrogate biomarkers, peripheral blood populations and cytokines.[11]

Vascular inflammation in TAK results from aberrant immune activation. The adaptive immune system, particularly T lymphocytes, are implicated in the pathogenesis of TAK as well as Giant Cell Arteritis (GCA).[6,12,13] Gene signature of T lymphocyte activation associates with active TAK.[14] Classically, T helper 1 (Th1) lymphocytes secreting interferon gamma (IFN-γ) have been implicated in vascular inflammation and granuloma formation in TAK.[15] An increase in circulating Th17 lymphocytes as well as IL-17A in aortic biopsies from patients with TAK undergoing surgical bypass procedures have been reported recently.[15,16] However, these Th17 lymphocytes from TAK were not corticosteroid responsive.[15,16]

The signature cytokine IL-17A secreted from Th17 lymphocytes attracts neutrophils to the site of inflammation.[17,18] Over the past decade, multifarious functions of Th17 lymphocytes have been recognized. Ramesh et al described a sub-population of Th17 lymphocytes which also secreted IFN-γ (signature cytokine of Th1 lymphocytes) along with Th17 cytokines such as IL-17 and expressed the chemokine marker CCR6 (called as Th17.1 or "pathogenic Th17" lymphocytes).[19,20] These Th17.1 lymphocytes were resistant to corticosteroids since they expressed the drug efflux protein p-glycoprotein [p-gp, also known as Multidrug Resistant Protein 1 (MRP-1), implicated in resistance to corticosteroid and other immunosuppressive therapy in myriad inflammatory diseases such as rheumatoid arthritis or systemic lupus erythematosus].[19–23] Th17 lymphocytes are now also recognized to play a role in granuloma formation.[24] Th17.1 lymphocytes are implicated in the pathogenesis of sarcoidosis.[25,26] However, Th17.1 lymphocytes have not been reported previously in TAK.

Another recently described population of Th17 lymphocytes express programmed cell death 1 (PD1, classically considered as a marker of T lymphocyte exhaustion).[27] However, it is now recognized that PD1+ CD4+ T lymphocytes as well as PD1+ Th17 lymphocytes secrete the regulatory, pro-fibrotic cytokine TGF-β1, and associate with lung fibrosis in sarcoidosis and idiopathic Pulmonary fibrosis (IPF). Celada et al demonstrated higher circulating PD1+ lymphocytes in the peripheral blood and lung tissue of patients with IPF than healthy controls. Peripheral circulating T lymphocytes secreted more TGF-β1 in IPF and sarcoidosis than healthy controls. Such T lymphocytes secreting TGF-β1 also expressed higher levels of PD1. Th17 lymphocytes expressing PD1 were identified as the major source of TGF-β1.[28] PD1+ CD4+ T lymphocytes and PD1+ Th17 lymphocytes have not been reported in TAK. Recent literature also suggests that circulating markers of fibrosis associate with vascular damage in TAK.[29] It is reasonable to hypothesize that PD1+ CD4+ T lymphocytes and PD1+ Th17 lymphocytes might drive vascular fibrosis in TAK. Other T lymphocyte populations that have been recently implicated in the pathogenesis of TAK include cytotoxic T lymphocytes as well as dysfunctional regulatory T lymphocytes (Treg).[30,31]

It is logical to hypothesize that the corticosteroid resistance demonstrated by Th17 lymphocytes in TAK might be due to Th17.1 lymphocytes since Th17.1 lymphocytes also express p-gp.[32,33] Additionally, PD1+ Th17 lymphocytes might drive vascular fibrosis in TAK. In this context, we aimed to evaluate T helper lymphocyte profile and related cytokines in the peripheral blood of patients with TAK.

Materials and Methods
Ethics Approval and Participant Recruitment

The present study complies with the Declaration of Helsinki. The study was approved by the Institute Ethics Committee of Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow [document submission numbers PGI/BE/497/2017 (22nd June 2017) and PGI/BE/310/2019 (16th April 2019)]. Fifty-seven patients with TAK, 21 healthy

controls and 11 patients with sarcoidosis (disease control group, where the role of Th17, PD1+ CD4+ T lymphocytes and PD1+ Th17 lymphocytes had been described before) were recruited between September 2017 and October 2021 into the study after obtaining written informed consent for study participation.[28,34] Patients were recruited from the out-patient departments or wards of the Departments of Clinical Immunology and Rheumatology, Cardiology, Nephrology, Surgical Gastroenterology, Medical Gastroenterology and Pulmonary Medicine of SGPGIMS, Lucknow. Healthy controls were frequency matched for age and sex with TAK.

Objectives

The primary objective of the study was to compare Th17 lymphocyte populations between patients with TAK and healthy controls. Secondary objectives included comparison of the circulating T helper lymphocyte profile (Th1, Th2, Th17, Th17.1, PD1+ CD4+, PD1+ Th17 and Treg lymphocytes, individually as well as normalized in ratio to Treg lymphocytes), serum cytokines (IFN-γ, IL-4, IL-6, IL-17A, IL-23, IL-1β, TNF-α, IL-10, TGF-β1) and cytokines secreted from culture supernatant of PBMCs between TAK and healthy controls as well as between TAK and sarcoidosis. Other secondary objectives involved culture of PBMCs from participants followed by analysis of the cytokines IFN-γ, IL-4, IL-6, IL-17A, IL-23, IL-1β, TNF-α, IL-10 and TGF-β1 in the culture supernatant following stimulation of the T-cell receptor with anti-CD3/CD28 and its modulation by the addition of immunosuppressive medication tacrolimus or anti-fibrotic agent tadalafil singly or in combination. Sub-group comparisons were performed between patients with TAK with active or inactive disease, and between patients with TAK on immunosuppressive medication at study enrollment with those that were immunosuppressive medication-naïve. A subset of patients with active TAK who were immunosuppressive medication-naïve were longitudinally followed up to compare T helper lymphocyte profile and cytokines before and after immunosuppression.

Inclusion and Exclusion Criteria

Adult patients (≥18 years of age) with TAK fulfilling the American College of Rheumatology 1990 classification criteria for TAK or the 2012 Chapel Hill Consensus Conference definition of TAK were recruited into the study.[9,10] Those patients who had active disease and were not on immunosuppressive medications were longitudinally followed up and sampled after initiation of immunosuppressive therapy. Consenting patients were recruited consecutively. Healthy controls were frequency-matched for age and sex with the patients of TAK. A further disease control group of sarcoidosis diagnosed by clinical presentation, supporting radiology with or without histopathological confirmation of granulomatous inflammation were also included. Patients who were critically ill and therefore unable to provide informed consent or those belonging to vulnerable populations such as pregnant women or children were excluded from the study.

Disease Activity Assessment

Disease activity assessment utilized a composite of Indian Takayasu Clinical Activity Score (ITAS2010) of one or more, active extravascular disease such as uveitis or erythema nodosum, evidence of metabolic activity to suggest active large vessel vasculitis on positron emission tomography computerized tomography or evidence of clinical progression and angiographic worsening of disease extent on serial imaging.[1,11,35,36]

Antibodies Used for T-Lymphocyte Stimulation and Intracellular Staining

CD3 Monoclonal Antibody (Cat No.16–0037-85, eBioSciences[TM]), Purified NA/LE Mouse Anti-human CD28 (Cat No. 555725), anti-human CD4 BV510 (Cat. No. 563094), anti-human CD279 (PD1) BV421 (Cat. No. 562516), anti-Human IFN-γ PE-Cy7 (Cat. No. 557844), anti-human IL-4 APC (Cat. No. 554486), anti-human IL-17A Alexa Fluor 488 (Cat. No. 560488), anti-human CD25 PE-Cy7 (Cat. No. 557741), anti-human FoxP3 Alexa Fluor 647 (Cat. No. 560045), anti-human CD4 FITC (Cat. No. 555346) were all purchased from Pharmingen BD Biosciences.

Activation of CD4+ Lymphocytes and in-vitro Culture

Cytotoxicity and viability of the cells were assessed as per previously standardized protocol.[37] Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation. The cells were counted by using haemocytometer and then cells were re-suspended (10^6 cells/mL) in complete RPMI medium (Roswell Park Memorial

Institute; Cat No-R6504), containing 10% fetal bovine serum (FBS; Cat No-10270106) with 1% antibiotic. The cells were cultured in 6-well plates which were pre-coated (for 24 hr at 37°C with 5% CO_2 in incubator) with anti-CD3 (2.5 µg/mL final concentration in 1mL) monoclonal antibody. Subsequently mouse anti-human CD28 mAb was added (5 µg/ mL). Thereafter, cells were treated with tacrolimus (10mM; Cat. No. F4679), tadalafil (PDE-5 inhibitor-10Mm; Cat.No. SML1877) or combination of both tacrolimus and tadalafil for 48 hours. After completion of incubation, the cultures were transferred to micro centrifuge tubes and centrifuged to collect the culture supernatant for cytokine analysis.

Flow Cytometry Analysis

Intracellular staining of T helper lymphocyte subsets was done after 48-hour incubation of whole blood in complete RPMI culture media by the activation of CD3 and CD28 monoclonal antibodies. After that, red blood corpuscles (RBC) were lysed by using 1X RBC Lysis Buffer for 10 minutes at room temperature. Cells were incubated with CD4 (FITC for T regulatory, BV510 for other T lymphocyte populations), anti-human CD279 (PD1) and anti human CD25 (PE-Cy7) for surface stain and subsequently cells were fixed and permeabilized by using Cytofix/Cytoperm W/Golgi Stop (554715, BD Biosciences). For intracellular T helper lymphocyte subset staining, cells were incubated with anti-human IL-4 (APC), anti-human IFN-γ (PE-Cy7), anti-human IL-17A (Alexa Fluor 488) and anti-human FoxP3 (Alexa Fluor 647) for T-regulatory lymphocytes. Cells were finally resuspended in 500 µL of 1X PBS to acquisition on a BD FACS Canto II (BD BioSciences, USA) or BD FACSLyric™ (BD Biosciences,USA) flow cytometer. Th1 lymphocytes were identified as CD4+ IFN-γ+, Th2 lymphocytes were CD4+ IL-4+, Th17 lymphocytes were CD4+ IL-17A+, Th17.1 lymphocytes were CD4+ IL17A+ IFN-γ+, PD1+ T helper lymphocytes were CD4+ PD1+, PD1+Th17 lymphocytes were CD4+ IL-17A+ PD1+ and T regulatory lymphocytes were CD4+ CD25+ FoxP3+. Figure 1 provides representative flow cytometry plots for each of the T helper lymphocyte populations.

Cytokine Profile

The levels of IFN-γ [Catalogue number (Cat. No.) DY285B], TNF-α (Cat. no. DY210), IL-17 (Cat. no. DY317), IL-6 (Cat. no. DY203), IL-4 (Cat. no. DY204), IL-10 (Cat. no. DY417), IL-1β (Cat. no. DY401), IL-23 (Cat. no. DY1290) and TGF-β1 (Cat. no. DY240) in serum and culture supernatant of TAK patients, disease control group and in healthy donors were determined using commercially available enzyme-linked immunosorbent assays (ELISA, from R&D Systems, USA and Canada) according to the manufacturer's instructions. Biotek ELISA reader ELX800 (Biotek, VT, USA) was used to obtain the optical density of each sample at wavelength of 450 nm.

Sample Size Calculation and Statistical Analysis

Sample size calculation was based on the primary objective of comparison of Th17 lymphocyte population between patients with TAK and healthy control using an online sample size calculator.[38] In a previous study conducted by us, mean Th17 lymphocytes (as a percentage of CD4+ T lymphocytes) in patients with TAK was 2.87±2.07% and 0.87 ±0.61% in healthy controls.[16] The pooled effect size was 2% and pooled standard deviation was 1.65%. Assuming a ratio of 8:3 between patients with TAK and healthy controls with α error (level of significance) of 0.05 and study power of 80%, the minimum sample size calculated as 22 patients with TAK and 8 healthy controls. In our study, we recruited patients with TAK and healthy controls in the same proportion as above (57 TAK and 21 healthy controls).

Statistical analyses were conducted using Prism 9 for macOS (version 9.3.1). The presented data was summarized using medians with interquartile range (25th quartile – 75th quartile). Proportions were compared using Chi-square test or Fisher's exact test. Non-parametric tests were used for comparison viz. Mann–Whitney test for unpaired samples and Wilcoxon matched-pairs signed rank test for paired samples. A p value of ≤0.05 was considered as statistically significant. Whenever multiple comparisons were performed for a set of related variables, the p values were further corrected for multiple testing using the Bonferroni-Sidak method. These analyses were conducted using Prism 9 for macOS (version 9.3.1). Logistic regression analyses were conducted using Stata/IC 16.1 for Mac to identify multi-variable-adjusted associations for disease activity in TAK taking into account C-reactive protein and either T lymphocyte populations or cytokines in different regression models.
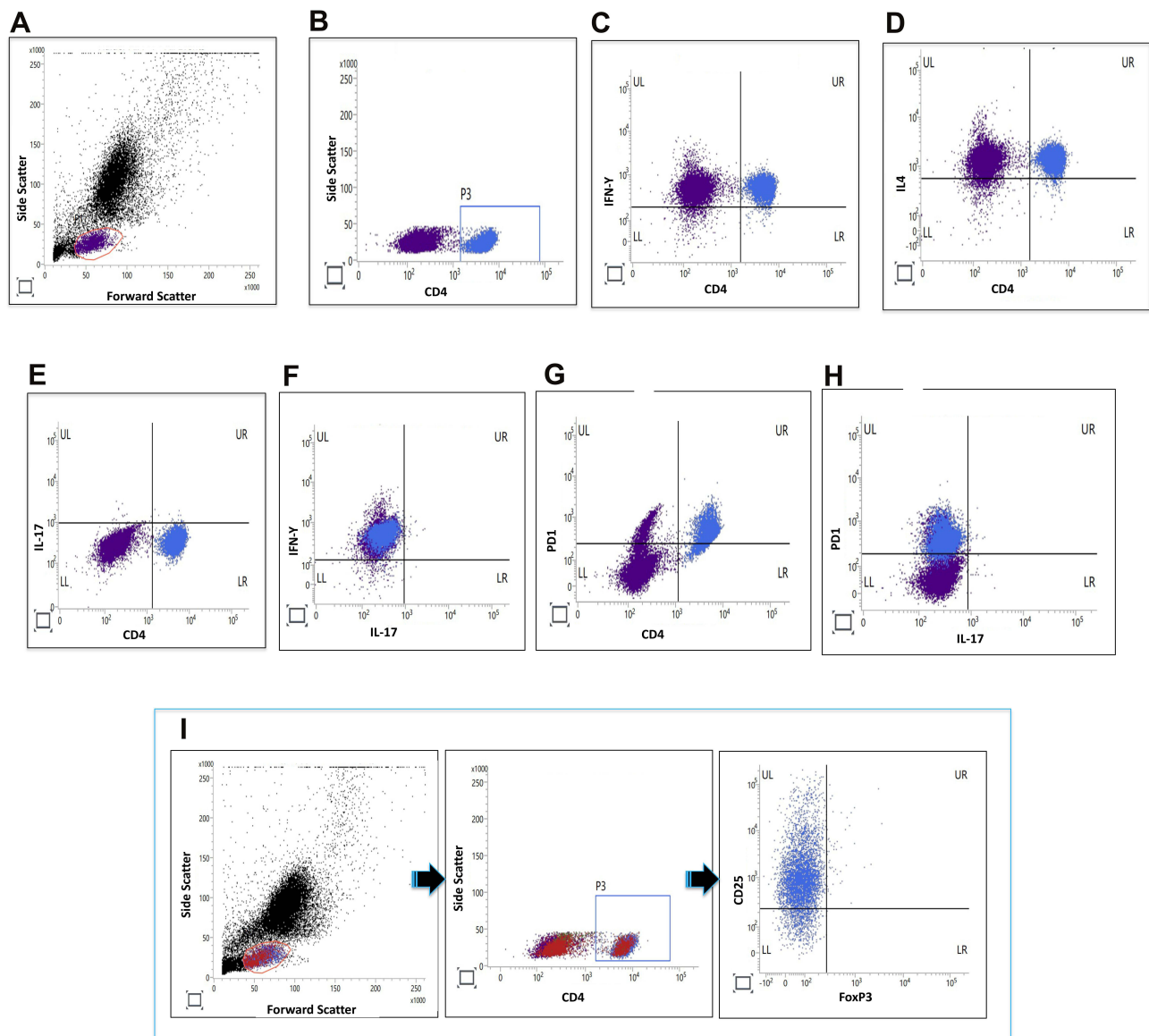
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Figure 1 Representative flow cytometry plots. The presented plots denote gating for lymphocytes (**A**), gating for CD4+ lymphocytes (**B**), Th1 lymphocytes (**C**), Th2 lymphocytes (**D**), Th17 lymphocytes (**E**), Th17.1 lymphocytes (**F**), PD1+ CD4+ T lymphocytes (**G**), PD1+ Th17 lymphocytes (**H**) and T regulatory lymphocytes (**I**). The red markings indicate gating for lymphocytes on forward scatter vs side scatter plot. The blue boxes indicate gating for CD4+ lymphocytes within the lymphocyte gate.
Abbreviations: CD, Cluster of differentiation, FoxP3, Forkhead box P3, IFN-γ, Interferon gamma, IL, Interleukin; PD1, Programmed cell death 1, Th, T helper; UL, Upper left quadrant; UR, Upper right quadrant, LL, Lower left quadrant, LR, Lower right quadrant.

Results
Characteristics of Study Participants
Fifty-seven patients with TAK [median age 35 (26–45) years; 40 females and 17 males], 21 healthy controls [median age 32 (29.5–35) years; 15 females and 6 males], and 11 granulomatous disease control patients with sarcoidosis [median age 54 (46–56) years; 7 females and 4 males] were recruited. The age and gender of patients with TAK and healthy controls were comparable (p=0.616 for Mann–Whitney test for age, p=0.914 for Chi-square test for sex). Patients with sarcoidosis were older although of similar distribution of sex when compared with TAK (p<0.001 for Mann–Whitney test for age, p=0.727 for Fisher's exact test for sex).

Vascular bruits, hypertension, pulse loss, pulse inequality and constitutional symptoms were the most prevalent clinical features. The commonest angiographic subtype (Numano's) was Type V (58%) followed by Type I (19%).[39] Twenty-four patients (42%) were treatment-naïve, whereas 30 (53%) had active disease (Table 1). Sixteen

Table 1 Characteristics of Patients with Takayasu Arteritis

Characteristic	Number (%) (n=57)
Clinical features at presentation	
Systemic features	22 (39)
Hypertension	44 (77)
Stroke	8 (14)
Dizziness or syncope	11 (19)
Vascular bruits	46 (81)
Pulse inequality	34 (60)
Pulse loss	41 (72)
Limb claudication	18 (32)
Carotidodynia	7 (12)
Aortic regurgitation	3 (5)
Heart failure	3 (5)
Angiographic subtype (Numano's)[41]	
I	11 (19)
IIa	1 (2)
IIb	5 (9)
III	0 (0)
IV	7 (12)
V	33 (58)
Treatment-naive	24 (42)
Active disease	30 (53)
C-reactive protein (mg/dL) [median (interquartile range)]	0.69 (0.32–1.79)
ITAS2010 [median (interquartile range)]	0 (0–6)
ITAS-A CRP [median (interquartile range)]	2 (0–6)
Immunosuppressive medications at study recruitment	Corticosteroids (n=27) Methotrexate (n=14) Tacrolimus (n=5) Mycophenolate mofetil (n=5) Azathioprine (n=3) Hydroxychloroquine (n=1)

Abbreviations: ITAS2010, Indian Takayasu Clinical Activity Score; ITAS-A CRP, Indian Takayasu Clinical Activity Score adjusted to include C-reactive protein.

immunosuppressive treatment-naïve TAK with active disease were serially followed up to analyze T lymphocyte populations and cytokines before and after immunosuppressive therapy. The median duration of follow-up for these patients was 0.21 (0.13–2.52) years. All these patients had been treated with corticosteroids (corticosteroids were ongoing in 15/16 at the time of follow-up sample collection). Nine of the patients were on tacrolimus alone, two were on a combination of tacrolimus and methotrexate, whereas one was on methotrexate alone. At the time of follow-up sample collection, TAK was clinically inactive in 14/16 patients.

Cross-Sectional Analyses

T Lymphocyte Populations

Th1, Th2, Th17, PD1+CD4+ and Treg populations were increased in TAK when compared with healthy controls (individually as well as normalized to Treg lymphocytes). Novel Th17 populations viz. Th17.1 and PD1+Th17 lymphocytes were also elevated in TAK when compared with healthy controls (individually as well as normalized to Treg lymphocytes). Compared to the disease control group of sarcoidosis, patients with TAK had higher Th2, PD1+CD4+ and Treg populations (individually as well as normalized to Treg lymphocytes). Th17 and Th1 lymphocytes were higher in TAK than in sarcoidosis individually but not when normalized against Treg lymphocytes (Figure 2).

Cytokines

The cytokines IFN-γ, IL-4, IL-17A, IL-23, IL-1β and IL-10 were undetectable in serum in most patients and controls. IL-6 was higher in serum of patients with TAK when compared with healthy controls or sarcoidosis. TNF-α was higher in serum of TAK patients than healthy controls, but was similar to sarcoidosis. TGF-β1 was similar in patients with TAK when compared with sarcoidosis or healthy controls (Figure 3).

Previous studies have reported similar circulating serum cytokine levels between patients with TAK and healthy controls, possibly because serum cytokines do not accurately represent vascular wall inflammation in TAK.[40] Therefore, we also compared the level of cytokines in culture supernatant of PBMCs from patients with TAK, healthy controls and sarcoidosis after stimulating them with anti-CD3/anti-CD28. In the culture supernatant, IFN-γ, IL-17A, IL-10, IL-6, TNF-α and IL-1β were elevated in TAK when compared with healthy controls. However, only IL-6, TNF-α and IL-1β were elevated in TAK when compared with sarcoidosis (Figure 3).

Comparison Between Active and Inactive TAK

Patients with active TAK had higher circulating Th17 lymphocytes (individually as well as normalized to Treg lymphocytes) when compared with those with inactive disease. When taken alone, Th17.1 lymphocytes were increased in active TAK than in inactive TAK but were not significantly different when normalized against Treg lymphocytes. None of the cytokines in serum or in culture supernatant were significantly different between active and inactive TAK (Table 2).

Logistic regression analyses were undertaken to identify multivariable-adjusted associations of disease activity. ITAS2010 was excluded from logistic regression model as it demonstrated a perfect correlation with disease activity (it was a part of the disease activity assessment). In a logistic regression model adjusted for C-reactive protein, Th1, Th2, Th17 and Treg lymphocyte populations, only Th17 lymphocytes remained significantly associated with disease activity (p=0.008) (Table 3). The other logistic regression models using Th17.1 or PD1+Th17 lymphocytes in place of Th17 lymphocytes did not show any significant adjusted associations with disease activity for any of the covariates in the model. The logistic regression models using serum cytokines included only C-reactive protein with IL-6, TNF-α and TGF-β1 as covariates, since the other cytokines were mostly undetectable in serum of TAK. This model showed no significant adjusted associations with disease activity (Table 3). It was not possible to construct an adequately powered logistic regression model for C-reactive protein and cytokines in culture supernatant of PBMCs due to data sparsity.[41]

Comparison Between TAK on Immunosuppressive Treatment with Those Not on Such Treatment

None of the T lymphocyte populations studied, serum cytokines or cytokines in culture supernatant were significantly different between patients of TAK receiving or not receiving immunosuppressive treatment (Table 4).
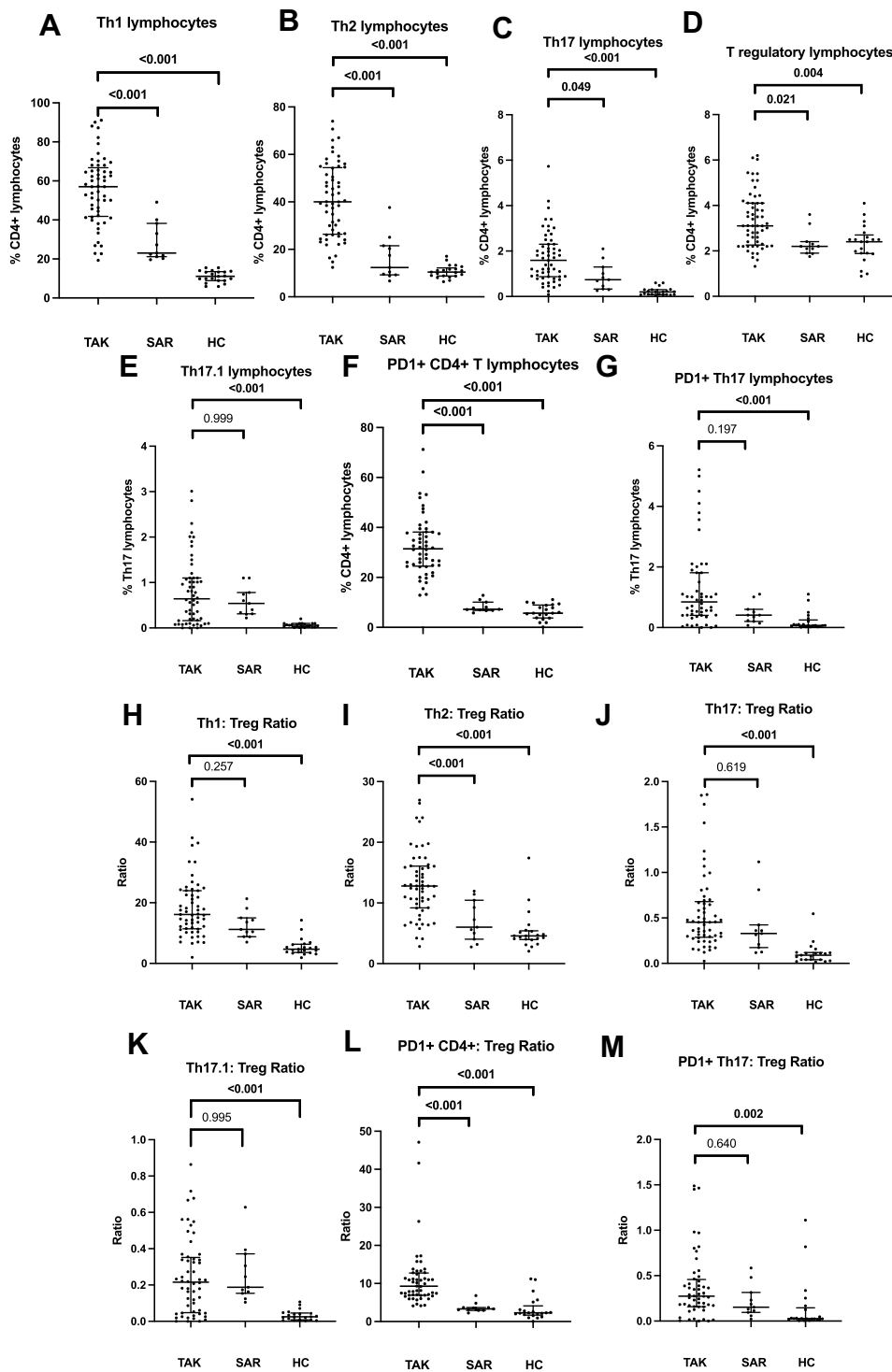
Figure 2 Comparison of T helper lymphocyte populations between patients with Takayasu arteritis (TAK), healthy controls (HC) and disease control group of sarcoidosis (SAR). (**A**) Th1 lymphocytes (**B**) Th2 lymphocytes (**C**) Th17 lymphocytes (**D**) T regulatory lymphocytes (Treg) (**E**) Th17.1 lymphocytes (**F**) PD1+CD4+ T lymphocytes (**G**) PD1+Th17 lymphocytes (**H**) Th1 lymphocytes normalised to Tregs (**I**) Th2 lymphocytes normalised to Tregs (**J**) Th17 lymphocytes normalised to Tregs (**K**) Th17.1 lymphocytes normalised to Tregs (**L**) PD1+CD4+ T lymphocytes normalised to Tregs (**M**) PD1+Th17 lymphocytes normalised to Tregs. Data on T helper lymphocyte populations are presented for 57 patients with TAK [except for Th17.1 lymphocytes (n= 56) and PD1+CD4+ and PD1+Th17 T lymphocytes (n=52)], 21 healthy controls and 11 patients with sarcoidosis. Comparisons are presented between TAK and HC, and between TAK and SAR (p values presented for Mann–Whitney test after Bonferroni-Sidak correction). p values <0.05 are highlighted in bold.
Abbreviations: CD, Cluster of differentiation; PD1, Programmed cell death 1; Th, T helper; Treg, T regulatory.
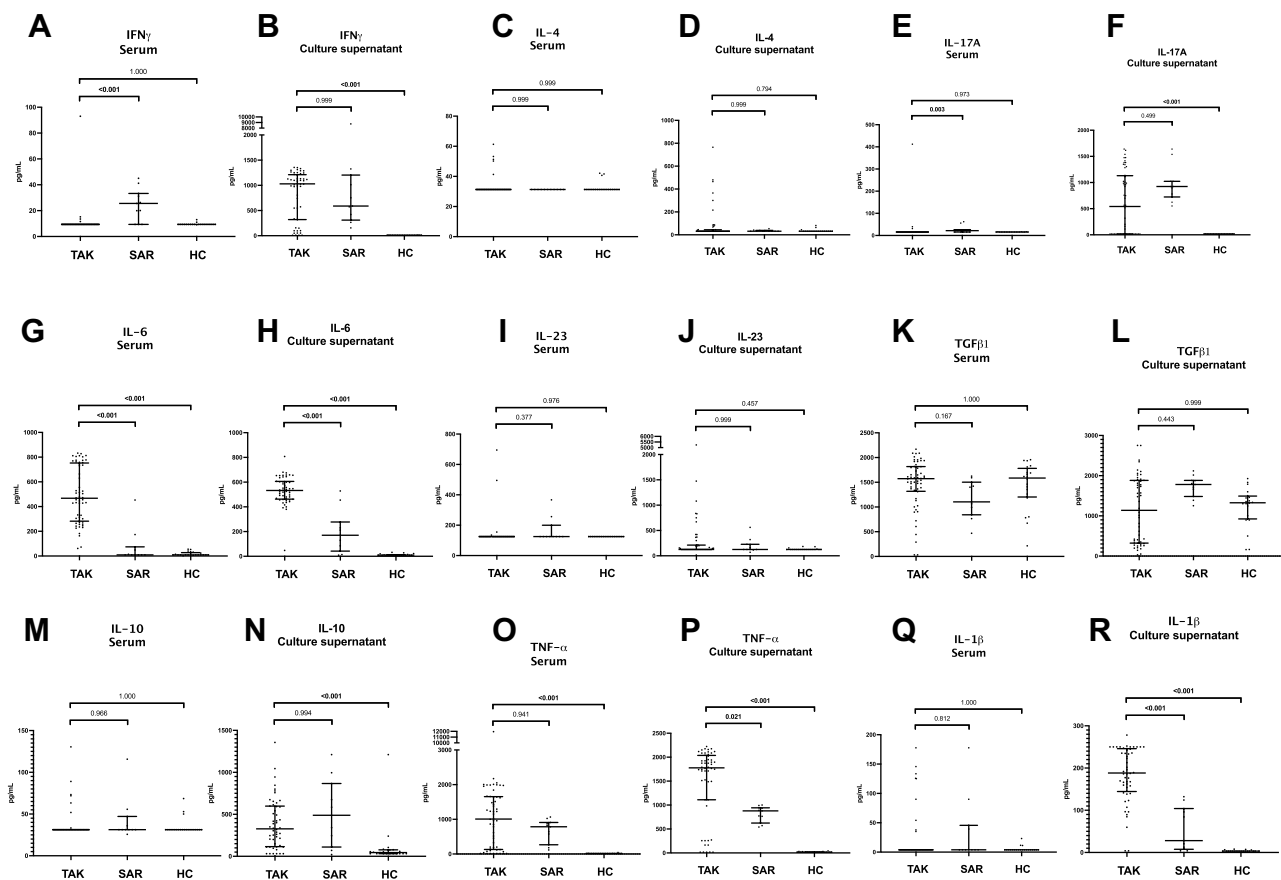
Figure 3 Comparison of cytokines in serum and in culture supernatant of peripheral blood mononuclear cells following stimulation with anti-CD3/ anti-CD28 between patients with Takayasu arteritis (TAK), healthy controls (HC) and disease control group of sarcoidosis (SAR). (**A**) IFN-γ in serum (**B**) IFN-γ in culture supernatant (**C**) IL-4 in serum (**D**) IL-4 in culture supernatant (**E**) IL-17A in serum (**F**) IL-17A in culture supernatant (**G**) IL-6 in serum (**H**) IL-6 in culture supernatant (**I**) IL-23 in serum (**J**) IL-23 in culture supernatant (**K**) TGF-β1 in serum (**L**) TGF-β1 in culture supernatant (**M**) IL-10 in serum (**N**) IL-10 in culture supernatant) (**O**) TNF-α in serum (**P**) TNF-α in culture supernatant (**Q**) IL-1β in serum (**R**) IL-1β in culture supernatant The cytokines IFN-γ, IL-4, IL-17A, IL-23, IL-1β and IL-10 were undetectable in serum in most patients and controls. In such instances, the lower limit of the detectable range for the ELISA kit was used for graphical representation of data. Data on serum cytokines presented for 56 patients with TAK, 21 healthy controls and 11 patients with sarcoidosis. Data on cytokines in culture supernatant are presented for 52 patients with TAK, 21 healthy controls and 11 patients with sarcoidosis. Comparisons are presented between TAK and HC, and between TAK and SAR (p values presented for Mann–Whitney test after Bonferroni-Sidak correction). p values <0.05 are highlighted in bold.
Abbreviations: IFNγ, Interferon gamma; IL, Interleukin; TGF-β1, Transforming growth factor beta 1; TNF-α, Tumor necrosis factor alpha.

In-vitro Stimulation Experiments on PBMCs from TAK and Modulation by Tacrolimus and Tadalafil

In-vitro stimulation experiments were performed on isolated PBMCs from 52 patients with TAK. All the cytokines studied were significantly increased following stimulation with anti-CD3/anti-CD28 when compared with baseline. Further treatment with tacrolimus significantly reduced the levels of all cytokines except TGF-β1 when compared with the stimulated state. Tadalafil alone significantly reduced the levels of IL-6, IL-1β and IL-10 when compared with the stimulated state. A combination of tacrolimus and tadalafil demonstrated a synergistic effect to further significantly reduce the levels of IL-17A, IL-6, IL-1β and IL-10 from the stimulated state when compared with tacrolimus alone (Figure 4).

Longitudinal Analyses
Comparison Between Patients with TAK Before and After Immunosuppressive Therapy

Th1, Th2, Th17, PD1+ CD4+ T lymphocytes, Th17.1 and PD1+ Th17 lymphocytes all reduced significantly following immunosuppressive therapy individually but not when normalized against Treg lymphocytes. Normalised Th17, Th17.1 and PD1+Th17 lymphocyte populations tended to reduce following immunosuppressive therapy but did not achieve

Table 2 Comparison of T Helper Lymphocyte Profiles and Cytokines Between Patients with Takayasu Arteritis with Active and Inactive Disease

	Active Takayasu Arteritis [Median (Interquartile Range)]	Inactive Takayasu Arteritis [Median (Interquartile Range)]	p value (Mann Whitney Test)	p value Corrected for Multiple Testing
C-reactive protein (mg/dL)	0.83 (0.34–4.45) (n=30)	0.63 (0.15–1.44) (n=27)	0.162	0.162
T helper lymphocyte populations	n=30	n=27		
Th1 (% CD4+)	62.30 (53.48–68.01)	50.02 (39.80–62.30)	**0.034**	0.214
Th2 (% CD4+)	45.95 (26.10–56.05)	38.44 (26.64–46.65)	0.241	0.854
Th17 (% CD4+)	2.06 (1.10–2.95)	1.10 (0.60–1.71)	**<0.001**	**0.006**
Th17.1 (% CD4+ IL17+)	0.96 (0.38–1.45) (n=29)	0.21 (0.08–0.78)	**0.004**	**0.030**
PD1+CD4+ (% CD4+)	34.00 (26.01–44.26) (n=25)	27.40 (24.27–37.80)	0.148	0.673
PD1+Th17 (% CD4+ IL17+)	1.10 (0.38–2.83) (n=25)	0.64 (0.41–1.02)	0.086	0.467
Treg (% CD4+)	3.10 (2.21–4.41)	3.11 (2.30–4.10)	0.902	1.000
Th1: Treg	16.94 (13.26–24.07)	14.65 (8.88–22.58)	0.189	0.715
Th2: Treg	12.59 (9.10–19.36)	12.91 (9.15–15.66)	0.531	0.989
Th17: Treg	0.49 (0.35–0.97)	0.31 (0.18–0.52)	**0.002**	**0.012**
Th17.1: Treg	0.27 (0.17–0.36) (n=29)	0.10 (0.03–0.33)	**0.016**	0.091
PD1+CD4+: Treg	9.90 (6.77–13.06) (n=25)	9.22 (6.91–11.36)	0.856	1.000
PD1+Th17: Treg	0.28 (0.18–0.81) (n=25)	0.23 (0.16–0.39)	0.143	0.603
Serum cytokines	n=29	n=27		
IFNγ (pg/mL)	9.35 (9.35–9.35)	9.35 (9.35–9.35)	0.612	0.999
IL-4 (pg/mL)	31.30 (31.30–31.30)	31.30 (31.30–31.30)	0.052	0.383
IL-17 (pg/mL)	15.60 (15.60–15.60)	15.60 (15.60–15.60)	0.360	0.982
IL-6 (pg/mL)	455.50 (270.00–697.25)	478.50 (304.50–769.50)	0.476	0.997
IL-23 (pg/mL)	125.00 (125.00–125.00)	125.00 (125.00–125.00)	0.612	0.999
TGF-β1 (pg/mL)	1680.00 (1439.50–1929.29)	1478.57 (1024.33–1610.00)	**0.018**	0.151
IL-10 (pg/mL)	31.25 (31.35–31.25)	959.00 (67.00–1644.00)	0.582	0.999
TNF-α (pg/mL)	1016.00 (421.50–1806.50)	67.00 (15.62–959)	0.354	0.980
IL-1β (pg/mL)	3.91 (3.91–3.91)	3.91 (3.91–3.91)	0.817	1.000
Cytokines in culture supernatant after stimulation with anti-CD3/anti-CD28	n=25	n=27		
IFNγ (pg/mL)	1104.50 (236.75–1277.25)	987.00 (334.50–1126.00)	0.121	0.687
IL-4 (pg/mL)	31.30 (31.30–150.50)	31.30 (31.30–31.30)	**0.016**	0.138
IL-17 (pg/mL)	163.00 (15.60–1226.00)	701.00 (15.60–1137.00)	0.641	0.999

(*Continued*)

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Table 2 (Continued).

	Active Takayasu Arteritis [Median (Interquartile Range)]	Inactive Takayasu Arteritis [Median (Interquartile Range)]	p value (Mann Whitney Test)	p value Corrected for Multiple Testing
IL-6 (pg/mL)	556.50 (472.00–648.50)	521.50 (459.50–586.00)	0.234	0.909
IL-23 (pg/mL)	165.00 (125.00–545.75)	125.00 (125.00–145.00)	**0.012**	0.102
TGF-β1 (pg/mL)	1732.85 (366.43–1932.07)	1012.24 (254.29–1702.85)	0.165	0.803
IL-10 (pg/mL)	319.00 (174.71–516.86)	417.57 (110.43–613.29)	0.824	1.000
TNF-α (pg/mL)	1745.00 (637.50–1910.00)	1874.00 (1110.00–2087.00)	0.184	0.840
IL-1β (pg/mL)	186.33 (158.67–250.00)	196.00 (139.67–244.83)	0.831	1.000

Notes: p values <0.05 are highlighted in bold.
Abbreviations: CD, Cluster of differentiation; IFNγ, Interferon gamma; IL, Interleukin; PD1, Programmed cell death 1; TGF-β1, Transforming growth factor beta 1; Th, T helper; Treg, T regulatory; TNF-α, Tumor necrosis factor alpha.

statistical significance when p values were corrected for multiple testing [p value unadjusted (adjusted) for Wilcoxon matched-pairs signed rank test 0.074 (0.369), 0.055 (0.290) and 0.034 (0.188), respectively] (Figure 5).

Serum cytokines IL-6 and TGF-β1 significantly reduced following immunosuppressive therapy. In culture supernatant, IL-6 significantly reduced following immunosuppressive therapy (Figure 6).

Discussion

Our study identified higher circulating overall Th17 lymphocytes and novel Th17 lymphocyte populations viz. Th17.1 and PD1+Th17 lymphocytes in TAK when compared with healthy controls. PD1+CD4+ T lymphocytes were elevated in TAK when compared with disease control of sarcoidosis.[28] Cytokines secreted by adaptive [IFN-γ (by Th1 and Th17.1), IL-17A (by Th17), IL-10 (by Treg), when compared with healthy controls] and innate immune cells (IL-6, TNF-α and IL-1β, when compared with healthy controls or sarcoidosis) were elevated in TAK. Th17 and Th17.1 lymphocyte populations associated with active TAK on univariable analyses. However, only Th17 lymphocytes remained associated with active TAK after multivariable-adjusted logistic regression analyses. In-vitro experiments on isolated PBMCs from TAK identified a reduction in all the inflammatory cytokines following treatment with tacrolimus. While the reduction of cytokines associated with inflammatory T lymphocyte populations with tacrolimus treatment can be expected, the reduction in the regulatory cytokine IL-10 (predominantly secreted by T regulatory lymphocytes) was intriguing, possibly related to pan-T lymphocyte suppressant effect of tacrolimus by reducing IL-2 secretion.[42] Interestingly, a synergistic effect of further significant reduction in IL-17A, IL-6, IL-1β and IL-10 was noted when tadalafil was added to tacrolimus. Serial follow-up samples from immunosuppressive-naïve TAK identified a reduction in cytokines involved in Th17 polarization ie IL-6 and TGF-β1 in serum, and IL-6 in culture supernatant following immunosuppressive therapy.

Saadoun et al reported increased circulating Th17 lymphocytes and greater secretion of Th17-related cytokines from cultured PBMCs of TAK when compared with healthy controls. Moreover, treatment of in-vitro cultured PBMCs from healthy controls with sera from active TAK induced greater Th17 polarization than sera from inactive TAK or from healthy controls. TAK had higher IL-17A expression in three aortic tissue samples.[15] Misra et al reported increased circulating Th17 lymphocytes, serum IL-17A and IL-23 in TAK versus healthy controls, however, these did not associate with active disease.[16] Goel et al reported similar levels of IL-17 and IL-23 in patients with TAK with and without active disease.[40] Our findings affirm the elevation of circulating Th17 lymphocytes in TAK. To the best of our knowledge, we have reported for the first time elevation of recently identified Th17 populations viz. Th17.1 and PD1+ Th17 lymphocytes, as well as overall PD1+CD4+ T lymphocytes in TAK.

Table 3 Logistic Regression Analysis for Associations of Disease Activity in Takayasu Arteritis

Covariates	Odds Ratio (95% Confidence Intervals)	p value
Model 1 (including Th17 lymphocyte population) (n=57)		
CRP	1.43 (0.96–2.12)	0.077
Th1	1.01 (0.98–1.05)	0.446
Th2	0.99 (0.94–1.04)	0.785
Th17	2.89 (1.32–6.36)	**0.008**
Treg	0.87 (0.51–1.47)	0.604
Model 2 (including Th17.1 lymphocyte population) (n=56)		
CRP	1.47 (0.95–2.26)	0.081
Th1	1.01 (0.98–1.05)	0.425
Th2	1.00 (0.96–1.05)	0.860
Th17.1	1.97 (0.82–4.74)	0.130
Treg	0.91 (0.55–1.48)	0.689
Model 3 (including PD1+ Th17 lymphocyte population) (n=52)		
CRP	1.50 (0.97–2.33)	0.071
Th1	1.02 (0.98–1.05)	0.335
Th2	0.99 (0.94–1.04)	0.693
PD1+ Th17	1.71 (0.91–3.24)	0.098
Treg	1.03 (0.60–1.75)	0.922
Model 4 (including cytokines in serum) (n=56)		
CRP	1.56 (0.96–2.51)	0.072
TGF-β1	1.00 (0.99–1.00)	0.212
TNF-α	1.00 (0.99–1.00)	0.362
IL-6	0.99 (0.99–1.00)	0.852

Notes: p values <0.05 are highlighted in bold.
Abbreviations: CRP, C-reactive protein; IL, Interleukin; PD1, Programmed cell death 1; TGF-β1, Transforming growth factor beta 1; Th, T helper; TNF-α, Tumor necrosis factor alpha; Treg, T regulatory.

Both Saadoun et al and Misra et al observed the Th17 lymphocytes and cytokines failed to reduce following glucocorticoid therapy in TAK, in contrast to corticosteroid-responsive Th17 lymphocytes in GCA.[15,16,43] Increased circulating Th17.1 lymphocytes might explain corticosteroid resistance of Th17 lymphocytes in TAK. Literature suggests that Th17.1 lymphocytes secrete IFN-γ in addition to IL-17A and express the efflux protein p-glycoprotein, which might be responsible for corticosteroid resistance.[19] In line with previous observations by Saadoun et al and contrary to those of Misra et al, Th17 lymphocyte population associated with active TAK.[15,16] This discrepancy with Misra et al might relate to the use of only clinical disease activity scales in the former study, whereas, the present study also used imaging modalities including positron emission tomography computerized tomography.[16] Such discrepancy might also relate to the considerably larger sample size of the present study when compared with Misra et al.[16] In the present study, Th17, Th17.1 and PD1+ Th17 lymphocytes appeared to reduce following immunosuppressive therapy, contrary to prior

Table 4 Comparison of T Helper Lymphocyte Profiles and Cytokines Between Patients with Takayasu Arteritis Who Were Immunosuppressive Treatment-Naïve and Those on Immunosuppressive Therapy

	Takayasu Arteritis – Treatment-Naïve [Median (Interquartile Range)]	Takayasu Arteritis – on Treatment [Median (Interquartile Range)]	p value (Mann Whitney Test)	p value Corrected for Multiple Testing
C-reactive protein (mg/dL)	0.66 (0.33–3.49) (n=24)	0.75 (0.27–1.78) (n=33)	0.764	0.764
T helper lymphocyte populations	n=24	n=33		
Th1 (% CD4+)	57.03 (42.75–64.65)	60.10 (41.11–70.61)	0.605	0.999
Th2 (% CD4+)	42.45 (26.28–55.76)	40.00 (26.37–51.70)	0.583	0.998
Th17 (% CD4+)	1.60 (0.80–2.85)	1.59 (0.89–2.07)	0.668	0.999
Th17.1 (% CD4+ IL17+)	0.90 (0.31–1.40) (n=23)	0.62 (0.09–1.04)	0.244	0.859
PD1+CD4+ (% CD4+)	28.38 (24.27–37.73) (n=20)	32.33 (24.80–39.18) (n=32)	0.592	0.998
PD1+Th17 (% CD4+ IL17+)	0.68 (0.40–2.10) (n=20)	0.91 (0.44–1.11) (n=32)	0.618	0.999
Treg (% CD4+)	2.75 (2.20–4.11)	3.20 (2.55–4.15)	0.251	0.868
Th1: Treg	16.68 (13.11–24.21)	14.66 (10.30–23.29)	0.408	0.957
Th2: Treg	12.81 (10.44–16.25)	12.30 (8.02–15.76)	0.271	0.849
Th17: Treg	0.42 (0.30–0.79)	0.46 (0.26–0.65)	0.680	0.999
Th17.1: Treg	0.23 (0.12–0.34) (n=23)	0.18 (0.03–0.36)	0.225	0.783
PD1+CD4+: Treg	9.69 (6.88–12.17) (n=20)	8.73 (6.90–12.84) (n=32)	0.918	1.000
PD1+Th17: Treg	0.29 (0.19–0.55) (n=20)	0.26 (0.13–0.40) (n=32)	0.280	0.861
Serum cytokines	n=24	n=32		
IFNγ (pg/mL)	9.35 (9.35–9.35)	9.35 (9.35–9.35)	>0.999	1.000
IL-4 (pg/mL)	31.30 (31.30–31.30)	31.30 (31.30–31.30)	0.484	0.997
IL-17 (pg/mL)	15.60 (15.60–15.60)	15.60 (15.60–15.60)	0.714	1.000
IL-6 (pg/mL)	450.75 (259.63–611.38)	491.50 (317.13–772.88)	0.143	0.750
IL-23 (pg/mL)	125.00 (125.00–125.00)	125.00 (125.00–125.00)	0.171	0.815
TGF-β1 (pg/mL)	1550.94 (1050.75–1813.54)	1577.86 (1343.93–1893.93)	0.684	1.000
IL-10 (pg/mL)	31.25 (31.25–31.25)	31.25 (31.25–31.25)	>0.999	1.000
TNF-α (pg/mL)	819.00 (82.75–1594.00)	1228.50 (157.75–1798.75)	0.202	0.869
IL-1β (pg/mL)	3.91 (3.91–3.91)	3.91 (3.91–3.91)	0.300	0.959
Cytokines in culture supernatant after stimulation with anti-CD3/CD28	n=20	n=32		
IFNγ (pg/mL)	1058.00 (114.25–1245.88)	1029.50 (548.00–1181.63)	0.952	1.000
IL-4 (pg/mL)	31.30 (31.30–62.25)	31.30 (31.30–37.83)	0.425	0.993
IL-17 (pg/mL)	136.50 (15.60–929.50)	836.00 (15.60–1299.00)	0.173	0.819
IL-6 (pg/mL)	533.25 (440.13–643.50)	530.25 (463.38–602.63)	0.720	1.000

(Continued)

Table 4 (Continued).

	Takayasu Arteritis – Treatment-Naïve [Median (Interquartile Range)]	Takayasu Arteritis – on Treatment [Median (Interquartile Range)]	p value (Mann Whitney Test)	p value Corrected for Multiple Testing
IL-23 (pg/mL)	135.00 (125.00–408.63)	125.00 (125.00–193.75)	0.363	0.983
TGF-β1 (pg/mL)	1116.64 (332.86–1990.29)	1137.14 (278.57–1803.20)	0.439	0.995
IL-10 (pg/mL)	334.71 (132.93–580.43)	319.71 (110.79–609.71)	0.834	1.000
TNF-α (pg/mL)	1810.00 (1206.00–2028.75)	1777.00 (1014.00–2036.25)	0.967	1.000
IL-1β (pg/mL)	204.50 (158.25–250.00)	188.25 (139.04–235.46)	0.408	0.991

Abbreviations: CD, Cluster of differentiation; IFNγ, Interferon gamma; IL, Interleukin; PD1, Programmed cell death 1; TGF-β1, Transforming growth factor beta 1; Th, T helper; Treg, T regulatory; TNF-α, Tumor necrosis factor alpha.

observations of Saadoun et al and Misra et al[15,16] Eleven of our sixteen patients were on tacrolimus along with corticosteroids. Tacrolimus inhibits the expression and function of p-glycoprotein in other treatment-refractory immune-mediated inflammatory diseases such as rheumatoid arthritis.[44] The reduction in Th17 as well as Th17.1 lymphocytes in our patients following treatment with corticosteroids and tacrolimus supports our hypothesis that expression of p-glyco-protein might underlie corticosteroid resistance of Th17 lymphocytes in TAK. These findings also highlight the potential for the evaluation of tacrolimus as an immunosuppressive agent in TAK in future clinical trials. As a proof of concept, tacrolimus reduced the levels of inflammatory cytokines (including those involved in the Th17-IL-17 axis viz. IL-6 and IL-17A) from cultured, in-vitro stimulated PBMCs of TAK in our study.

Vascular wall inflammation in TAK resolves by fibrosis resulting in vascular stenosis.[6] It is possible that PD1+CD4+ and PD1+ Th17 lymphocytes might drive vascular fibrosis in TAK. Our novel observation of an increased population of PD1+CD4+ and PD1+ Th17 lymphocytes in TAK when compared with healthy controls or with disease control group of sarcoidosis might have relevance from a therapeutic viewpoint. Interestingly, in-vitro experiments on cultured PBMCs from TAK identified a synergistic effect of reduction of IL-17A, IL-6, IL-1β and IL-10 when tadalafil was added to tacrolimus. Our previous experiments had identified the anti-fibrotic effect of tadalafil on cultured human adult dermal fibroblasts from scleroderma (a disease which causes systemic fibrosis in skin, lung and other internal organs).[45] At present, no immunosuppressive drug has been proven to be effective in clinical trials of TAK.[46] Tocilizumab (IL-6 receptor blocker) and abatacept (T lymphocyte co-stimulation blocker via agonism of the cytotoxic T lymphocyte antigen 4) failed to meet primary endpoints in randomized controlled trials.[47,48] Based on observational studies, anti-tumor necrosis factor-alpha (anti-TNF) agents and tocilizumab achieve clinical response in nearly three-fourth patients, although their effect on angiographic stabilization is less defined.[49] Sub-optimal angiographic response with currently used immunosuppressive therapies in TAK lends credence to the hypothesis that adding an anti-fibrotic therapy to immunosuppressive therapy might favorably modulate vascular remodeling by reducing fibrosis consequent to inflam-mation-associated vascular injury. Costly biological therapies are inaccessible in vast swathes of the globe including India. The use of tacrolimus in patients with TAK was based on our favourable experience with the use of this drug in other diseases associated with corticosteroid resistance such as steroid-resistant nephrotic syndrome.[50] Results from the in-vitro experiments conducted on PBMCs in our study demonstrate the potential for the combination of tacrolimus (or other immunosuppressants) with anti-fibrotic tadalafil (or with other anti-fibrotic agents such as pirfenidone or nintedanib, or other immunosuppressive agents with anti-fibrotic potential such as tocilizumab).[51–53] These require further exploration as a therapeutic regimen in TAK in clinical trials before translation into clinical practice. Figure 7 depicts potential roles played by Th17 lymphocytes in driving vascular inflammation of TAK, including granuloma formation by secreting IFN-γ from Th17.1 lymphocytes.[19,54]
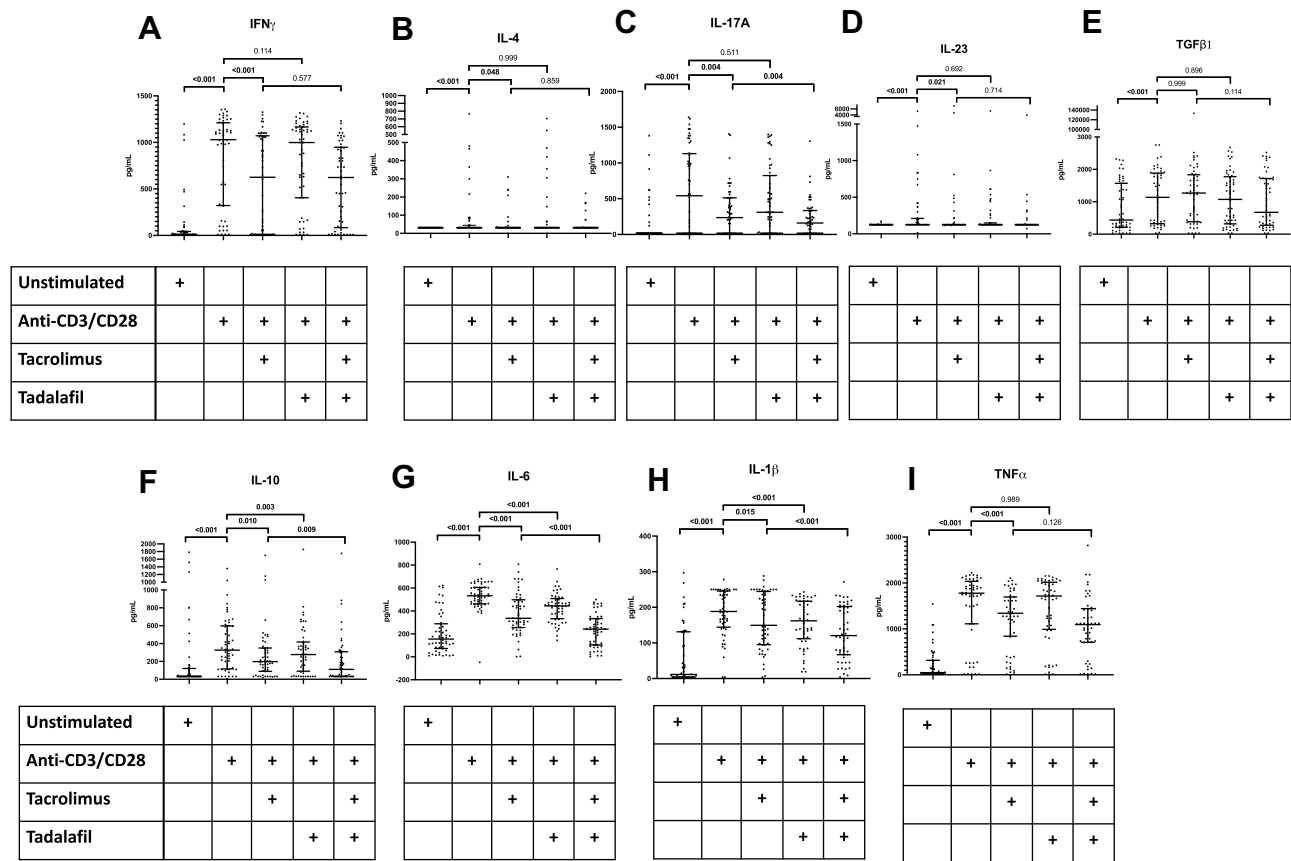
Figure 4 Cytokine production following in-vitro stimulation of peripheral blood mononuclear cells from patients with Takayasu arteritis with anti-CD3/anti-CD28 alone, with tacrolimus, with tadalafil or with a combination of tacrolimus and tadalafil. Cytokines presented in each graph (**A**) IFN-γ (**B**) IL-4 (**C**) IL-17A (**D**) IL-23 (**E**) TGF-β1 (**F**) IL-10 (**G**) IL-6 (**H**) IL-1β (**I**) TNF-α Wherever cytokines were undetectable, the lower limit of the detectable range for the ELISA kit was used for graphical representation of data. Data on cytokines in culture supernatant are presented for 52 patients with TAK. Comparisons are presented between unstimulated and post-stimulation with anti-CD3/anti-CD28, post-stimulation with anti-CD3/anti-CD28 alone or along with tacrolimus, post-stimulation with anti-CD3/anti-CD28 alone or along with tadalafil, and post-stimulation with anti-CD3/anti-CD28 with tacrolimus or with tacrolimus and tadalafil (p values presented for Wilcoxon matched-pairs signed rank test after Bonferroni-Sidak correction). p values <0.05 are highlighted in bold.
Abbreviations: CD, Cluster of differentiation; IFNγ, Interferon gamma; IL, Interleukin; TGF-β1, Transforming growth factor beta 1; TNF-α, Tumor necrosis factor alpha.

There were limitations to our study. Peripheral blood populations of immune cells and circulating cytokines need not necessarily reflect ongoing vascular wall inflammation. This is an inherent limitation of TAK studies, since the site of inflammation viz. large vessels are largely inaccessible for direct histopathological examination. We attempted to address this issue by culturing in-vitro PBMCs from patients with TAK and stimulating the T-cell receptor with anti-CD3/ anti-CD28 to study the behavior of such activated cells. We also evaluated potential therapeutic modalities in vitro by using tacrolimus or tadalafil alone or in combination in the PBMC cultures. Another limitation was that only sixteen patients with active TAK who were immunosuppressive-medication naïve were followed up longitudinally. However, TAK is a rare disease, and the ongoing coronavirus disease 19 pandemic further hampered recruitment of treatment-naïve TAK. A third limitation was that we have not isolated Th17.1 and Th17 PD1+ T lymphocytes from peripheral blood and examined their expression of p-gp (Th17.1) and TGF-β1 (Th17 PD1+), since these experiments were beyond the scope of the present study. The disease control group included sarcoidosis of older age than TAK, however, sarcoidosis inherently affects older age groups.[55] Another suitable control group might have been GCA (which is a large vessel vasculitis predominantly affecting older individuals). This was not feasible as GCA is uncommon in India.[56] A strength of our study was disease activity assessment using imaging modalities including positron emission tomography computerized tomography in addition to clinical scales. Discordance between traditionally used inflammatory markers (erythrocyte sedimentation rate and C-reactive protein) and vascular inflammation in TAK necessitates the use of multiple modalities
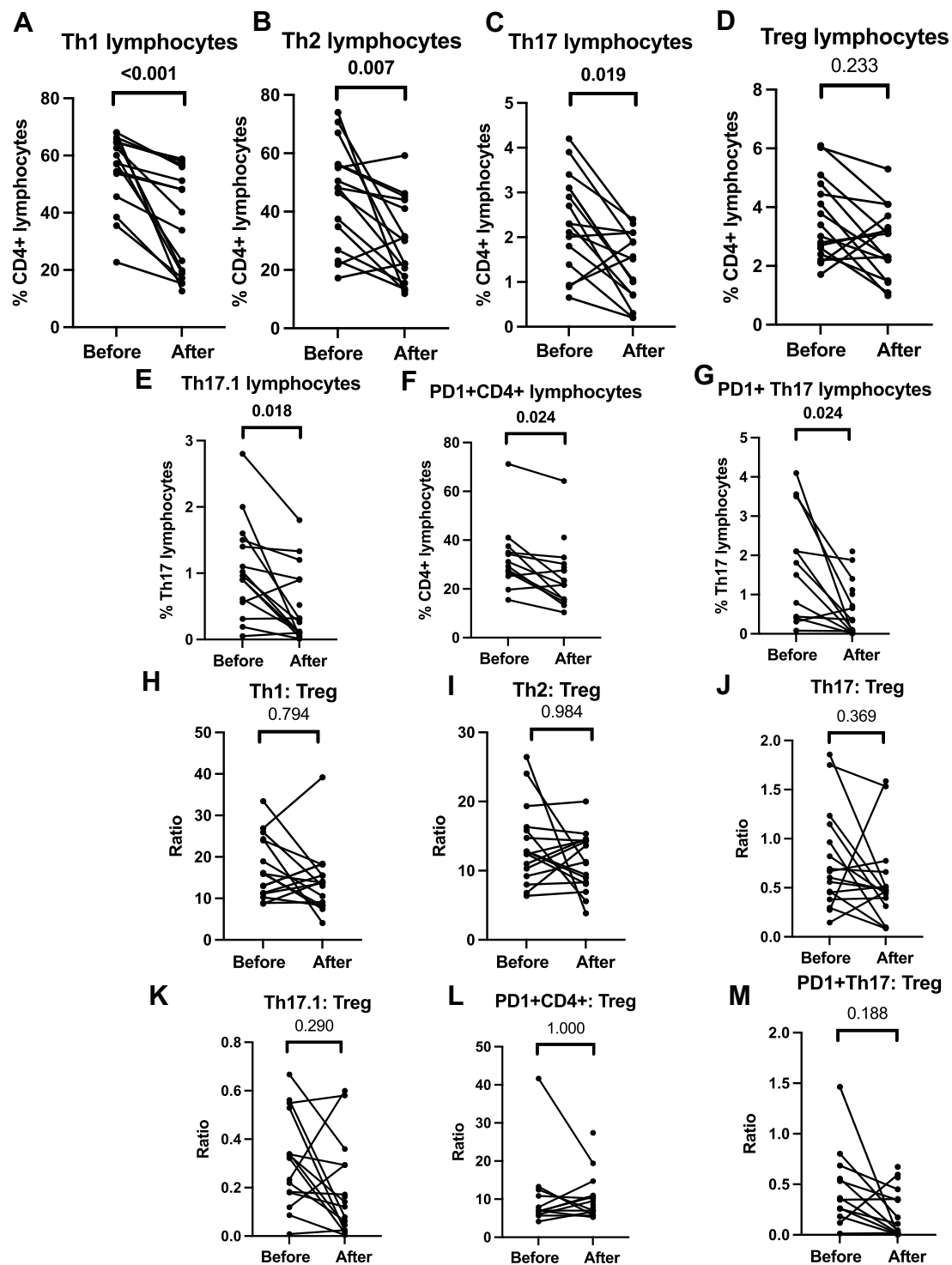
Figure 5 T helper lymphocyte profiles in patients with TAK before and after immunosuppressive therapy. (**A**) Th1 lymphocytes (**B**) Th2 lymphocytes (**C**) Th17 lymphocytes (**D**) T regulatory lymphocytes (Treg) (**E**) Th17.1 lymphocytes (**F**) PD1+CD4+ T lymphocytes (**G**) PD1+Th17 lymphocytes (**H**) Th1 lymphocytes normalised to Tregs (**I**) Th2 lymphocytes normalised to Tregs (**J**) Th17 lymphocytes normalised to Tregs (**K**) Th17.1 lymphocytes normalised to Tregs (**L**) PD1+CD4+ T lymphocytes normalised to Tregs (**M**) PD1+Th17 lymphocytes normalised to Tregs. Data on Th1, Th2, Th17 and Treg are presented for 16 pairs, Th17.1 for 15 pairs, PD1+CD4+ T lymphocytes and PD1+Th17 lymphocytes for 12 pairs. Comparisons are present before and after treatment (p values presented for Wilcoxon matched pairs signed rank test after Bonferroni-Sidak correction). p values <0.05 are highlighted in bold. Median duration of follow-up 0.21 (0.13–2.52) years. All patients had been treated with corticosteroids (ongoing in 15/16 at follow-up sample collection). 9 were on tacrolimus alone, 2 were on tacrolimus and methotrexate, one was on methotrexate alone. At the time of follow-up sample collection, disease activity had ceased clinically in 14/16 patients.
Abbreviations: CD, Cluster of differentiation; PD1, Programmed cell death 1; Th, T helper; Treg, T regulatory.
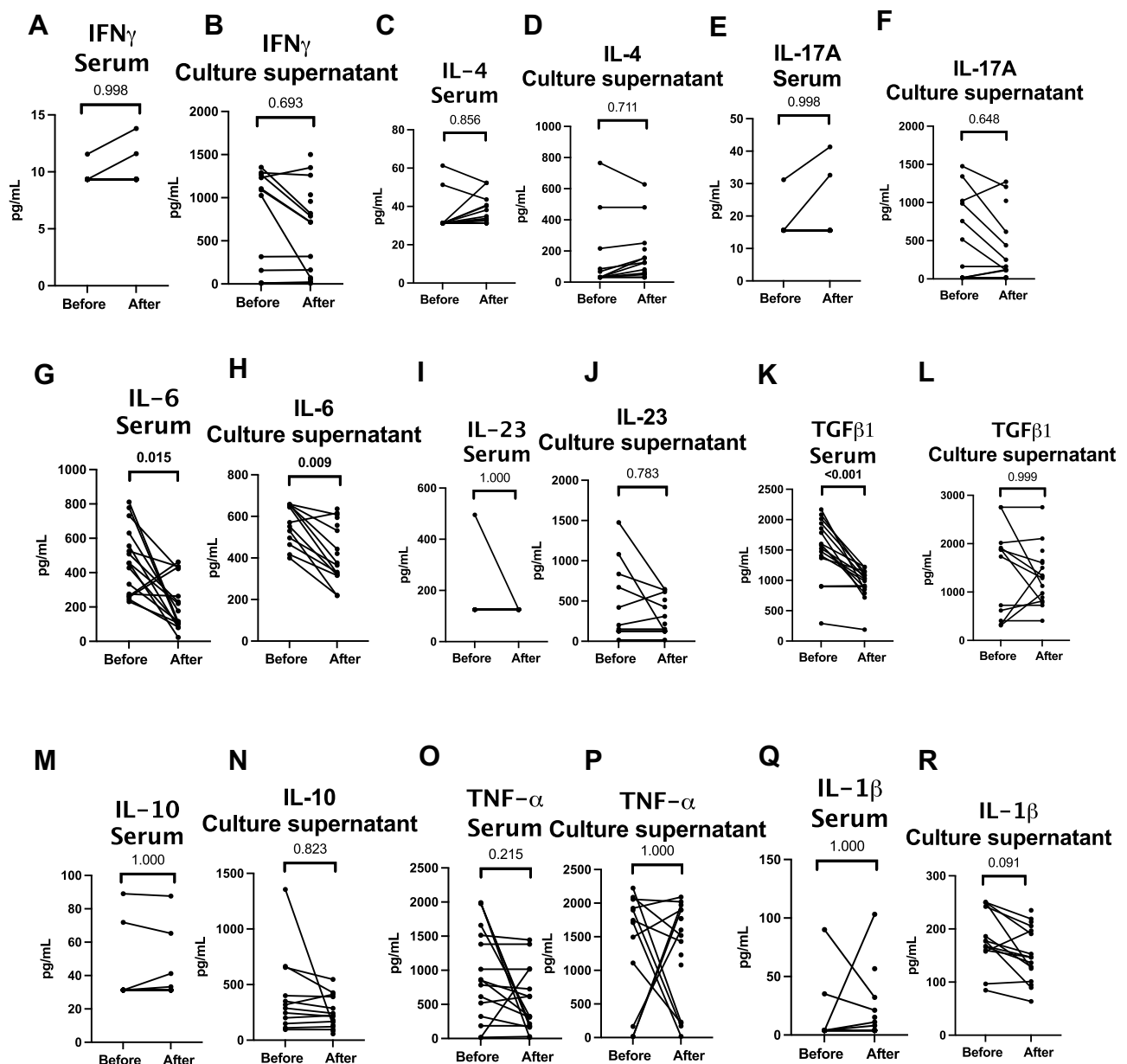
Figure 6 Cytokine profiles in patients with TAK before and after immunosuppressive therapy. (**A**) IFN-γ in serum (**B**) IFN-γ in culture supernatant (**C**) IL-4 in serum (**D**) IL-4 in culture supernatant (**E**) IL-17A in serum (**F**) IL-17A in culture supernatant (**G**) IL-6 in serum (**H**) IL-6 in culture supernatant (**I**) IL-23 in serum (**J**) IL-23 in culture supernatant (**K**) TGF-β1 in serum (**L**) TGF-β1 in culture supernatant (**M**) IL-10 in serum (**N**) IL-10 in culture supernatant) (**O**) TNF-α in serum (**P**) TNF-α in culture supernatant (**Q**) IL-1β in serum (**R**) IL-1β in culture supernatant. Wherever cytokines were undetectable, the lower limit of the detectable range for the ELISA kit was used for graphical representation of data. Data are presented for 16 pairs for serum cytokines and 12 pairs for culture supernatant for IFN-γ, IL-4, IL-17A, IL-6, IL-23, TGF-β1, IL-10, TNF-α; for 13 pairs for serum cytokines and 13 pairs for culture supernatant for IL-1β. Comparisons are present before and after treatment (p values presented for Wilcoxon matched pairs signed rank test after Bonferroni-Sidak correction). p values <0.05 are highlighted in bold. Median duration of follow-up 0.21 (0.13–2.52) years. All patients had been treated with corticosteroids (ongoing in 15/16 at follow-up sample collection). 9 were on tacrolimus alone, 2 were on tacrolimus and methotrexate, one was on methotrexate alone. At the time of follow-up sample collection, disease activity had ceased clinically in 14/16 patients.
Abbreviations: IFNγ, Interferon gamma; IL, Interleukin; TGF-β1, Transforming growth factor beta 1; TNF-α, Tumor necrosis factor alpha.

to accurately assess disease activity in TAK.[36,57,58] A disease control group of sarcoidosis (which causes lung and major organ fibrosis) was particularly relevant due to the previous description of PD1+CD4+ and PD1+Th17 lymphocytes as drivers of fibrosis in sarcoidosis.[28] Our study utilized multiple approaches to study pathophysiological processes, viz. T lymphocyte populations, serum cytokines and cytokines in culture supernatant from PBMCs as well as in-vitro evaluation of therapeutic modalities on PBMC cultures.
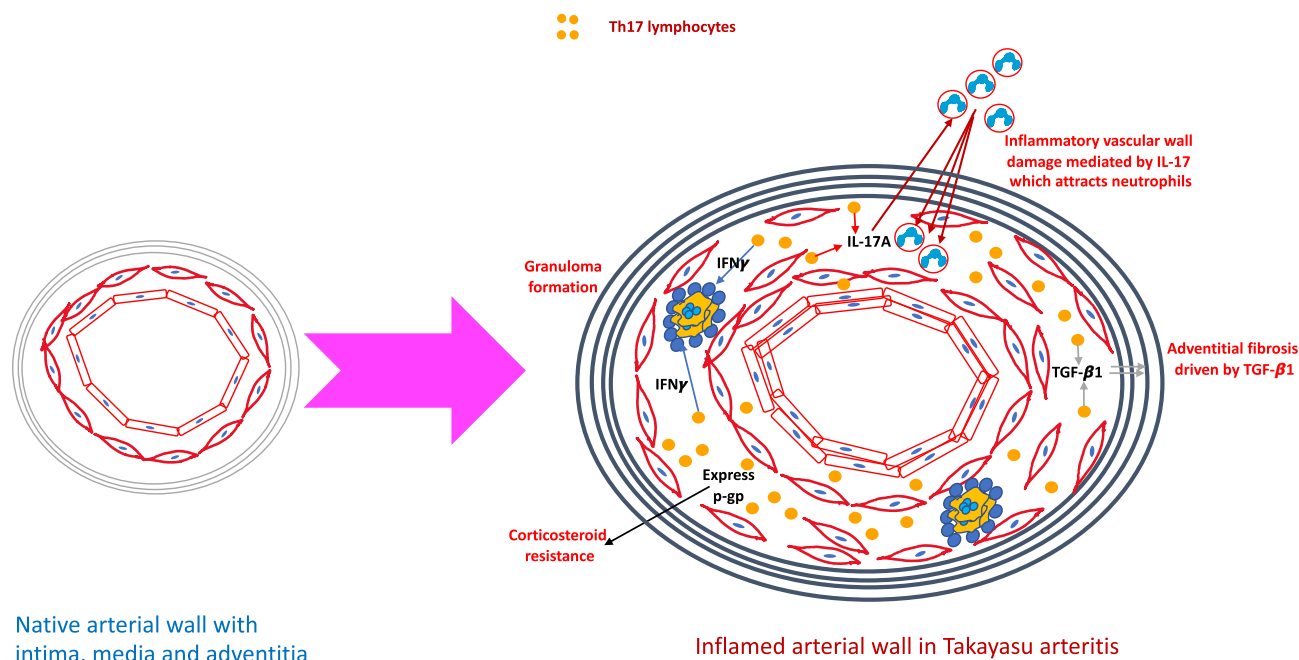
Figure 7 Putative mechanisms by which Th17 lymphocytes play a role in the pathogenesis of Takayasu arteritis. When compared with the native arterial wall, the arterial wall in large vessel vasculitis is thickened, with intimal proliferation, thickening of the media due to inflammatory cell infiltrate and adventitial fibrosis. T helper 17 (Th17) lymphocytes secrete the cytokine interleukin 17A (IL-17A) which attracts neutrophils to the media where they degranulate and release enzymes resulting in tissue destruction. Th17.1 lymphocytes secrete interferon gamma (IFN-γ) which contributes towards granuloma formation. Th17.1 lymphocytes also express p-glycoprotein and are resistant to corticosteroids. Programmed cell death 1 (PD1) positive Th17 lymphocytes secrete transforming growth factor beta 1 (TGF-β1) which in turn drives adventitial fibrosis.

The multifarious roles played by Th17 lymphocytes in human disease merit their further evaluation in TAK. Isolating T lymphocytes from peripheral blood of TAK and studying their gene expression profile using transcriptomics could provide further clues to pathological processes operational in TAK. Such an approach has been successfully used to characterize T regulatory dysfunction in GCA.[59] In the light of in-vitro experiments supporting the potential use of tacrolimus and tadalafil in TAK, future clinical trials in TAK might consider evaluating tacrolimus along with tadalafil or other anti-fibrotic therapies (nintedanib, pirfenidone).

Conclusion

Th17 lymphocytes and their sub-populations of Th17.1 and PD1+ Th17 lymphocytes are elevated in TAK. Th17 lymphocytes also associate with disease activity in TAK. In-vitro experiments on cultured PBMCs from TAK suggest the need to further evaluate a combination of immunosuppressive medication with tadalafil or other anti-fibrotic agents in clinical trials of TAK.

Prior Conference Presentations

Part of the work in this paper was presented as an oral presentation at the Indian Rheumatology Association (IRACON) Online Virtual conference in December 2021.

Ethics Approval and Informed Consent

The present study complies with the Declaration of Helsinki. The study was approved by the Institute Ethics Committee of Sanjay Gandhi Postgraduate Institute of Medical Sciences (Sgpgims), Lucknow [document submission numbers PGI/BE/497/2017 (22nd June 2017) and PGI/BE/310/2019 (16th April 2019)]. All subjects were included into the study after obtaining written informed consent for participation.

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