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T helper cell subpopulations repertoire in peripheral blood and its correlation with sex of newly diagnosed arthritis patients: A gender based study

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ABSTRACT

Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder. Autoreactive T cells play a very significant role in the pathogenesis of RA. However, the exact mechanisms of disease severity and pathogenesis are poorly understood. We attempted to correlate T-helper cell activities with sexes of newly diagnosed patients with RA. The patients were divided based on their sex and disease severity. Examination of the expression of various factors using quantitative real-time PCR and FACS analysis of peripheral blood mononuclear cells revealed that T-bet, ROR-γt, Foxp3, and the level of cytokines associated with Th1 cells were almost identical among male and female patients with RA.

Interestingly, there was a high correlation between Th17 expression and disease severity in female patients with RA. In general, there was no significant correlation between Th1 cell population and the disease severity in newly diagnosed patients with RA. In contrast, the frequency of both Th17 and Treg cells was higher in patients with more severe disease. The results suggested that, in patients with RA, the T-helper cell balance within peripheral blood was skewed towards the Th17 and Treg phenotypes. Besides Th17- and Treg-associated cytokines, elevated expression of IL-27/IL-23 cytokines might also be responsible for increased disease severity in female patients with RA.

1. Introduction

Rheumatoid arthritis (RA), an irreversible joint disorder, is characterized by synovial hyperplasia, angiogenesis, and degradation of bone and cartilage [1]. Disease onset involves infiltration of immune cells *viz*. mast cells, macrophages, and activated B and T cells in the synovial cavity, which leads to joint deformities. Loss of functional ability and increased mortality are among the long-term effects of RA. Multi-system damage is common in patients with RA, as vital tissues and organs are adversely affected by the inflammatory milieu, as well as oxidative stress-induced damage that occurs during disease progression [1,2]. Diagnostic tools, based on the rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPAs), revealed that around 1–2% of the world's population is affected by RA. Further, individuals succumb to the disease more readily with age and females are three times more affected than males [3,4].

The aetiology of the RA is complicated and difficult to understand. However, it is believed that several immune-modulators and multi signalling pathways are involved in its pathogenesis. Autoreactive T cells reportedly play a crucial role in the pathology of RA. In general, Th1 subclass of immunocompetent individuals show T-bet mediated expression of cytokines *viz*. interferon-gamma (IFN-γ), IL-2, etc. Meanwhile, Th2 cells secrete cytokines *viz*. interleukin-4 (IL-4), IL-5, IL-10, etc. [5,6]. Excessive accumulation of pro-inflammatory cytokines plays an important role in both the development and progression of RA [6]. The IL-12 family cytokines *viz*. IL-12 (IL-12p35/IL-12p40), IL-23 (IL-23p19/IL-12p40), and IL-27 (IL-27p28/Ebi3) have also been reported as important regulators of RA progression [7].

Traditionally, RA was believed to be a Th1 cell-mediated disease; however, with the discovery of other T-helper subsets such as Th17 cells and T regulatory cells, the focus has been shifted from Th1/Th2 skewing to Th17 and Treg cell population. In the current paradigm, both Th1 and Th17 cells are described as important mediators of RA pathogenesis [1,8]. Th17 cells express retinoic acid receptor-related

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orphan receptor-γt (ROR-γt) and secrete pro-inflammatory cytokines (such as IL-17, IL-21, and IL-22, etc.) involved in inflammation and tissue damage within the joints of patients with RA. The differentiation and activation of Th17 cells require participation of the pro-inflammatory IL-23 cytokine. The induced pro-inflammatory cytokines promote activation and expression of other cytokines (such as IL-6 and IL-8) that may produce joint inflammation [9]. T regulatory (Treg) cells, through the use of forkhead transcription factor (FoxP3), secrete IL-10 and TGF-β, which interact with various other immune cell types, thereby regulating inflammation in patients with RA [10].

In addition to the pro-inflammatory response, there may occur a compensatory anti-inflammatory responses mediated by IL-4, IL-5, IL-10, and IL-13 in patients with RA [11]. Considering that females are more prone to RA than males, we attempted to establish a correlation between induced T helper cells and sex in patients with RA residing in the north region of India. We found that an abundance of circulating Th1, Th17, Treg cells and levels of associated transcription factors and cytokines in patients with RA. Furthermore, we also studied the correlation of pro- and anti-inflammatory markers in modulating the severity of RA in inflicted patients.

2. Materials and methods

2.1. Patients

We followed a schedule approved by the Institutional Ethics and Research Advisory Committee, Faculty of Medicine, Jawaharlal Nehru Medical College, Aligarh Muslim University, India, to perform this study. We examined 50 patients who were newly diagnosed with RA (DMARD and corticosteroid naïve patients with symptom duration of fewer than 12 months) and who visited the Orthopaedic Out-Patient Department of Jawaharlal Nehru Medical College, Aligarh (INDIA). The patients enrolled in the study fulfilled the European League Against Rheumatism (EULAR) 2010 classification criteria for RA [12]. The study also included 30 age- and sex-matched healthy controls. Written informed consent was obtained from patients with RA and from healthy subjects who participated in the study. We ensured that both controls and patients with RA exhibited no chronic disease (other than RA) nor were they alcoholics or smokers. The characteristics of healthy controls and patients with RA are given in Table 1.

2.2. Disease severity measurement

Disease severity was assessed by calculating a 28-joint count disease activity score using C-reactive protein (DAS28 CRP). Disease severity was assessed through examination of swollen and tender joints, CRP level, and patient's health assessment as recorded on a visual analogue scale. DAS28 CRP of ≤ 3.2 indicated low severity, DAS28 CRP $> 3.2 <$ 5.1 indicated moderate severity, and DAS28 CRP > 5.2 indicated high disease severity [13].

Table 1
Demographic and clinical data of healthy controls and patients with RA included in the study.

Parameters	Controls	Patients with RA
Age	36.5 (29–46.5)	42.5 (32–54)
Gender (M/F)	12/18	22/28
Height (cm)	162 (158–164)	163 (159.8–165)
Weight (Kg)	57 (54–61)	59 (57.75–64)
BMI	22.65 (21.03–23.1)	22.75 (21.68–23.75)
ESR (mm/h)	13 (9–18.25)	28 (22–34)
CRP	16 (12–18.5)	38.5 (25.75–49.5)
DAS28-CRP	–	3.70 (3.09–4.13)

2.3. Blood samples

Blood from the cubital vein was collected in an EDTA-coated vial. We used ELISA to determine the levels of cytokines in plasma. PBMCs were isolated using histopaque and examined for the expression of cytokines and transcription factors at the mRNA level.

2.4. Enzyme-linked immune sorbent assay (ELISA)

The level of the cytokine IL-17 was estimated using a commercially available ELISA kit (R&D System, USA) following the manufacturer's protocol. ELISA kits from R&D Systems, USA were used to determine the levels of IL-23 and IL-27 in plasma. The absorbance was read at 450 nm on a microtiter plate reader (Genetix GMB-580). The level of cytokines was calculated by plotting the standard curve, and the concentration was expressed in pg/ml.

2.5. Quantitative real-time polymerase chain reaction

We used the PureLink@ RNA mini kit (Invitrogen, USA) to isolate mRNA from the PBMCs of patients with RA. Isolated RNA was reverse-transcribed into cDNA by Thermal Cycler (BioRad) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Amplification of the cDNA was achieved using an Applied Biosystems 7500 Real-Time PCR System. Forward and reverse primer sequences are presented in Table 2. RT conditions were maintained as follows: 10 min @ 25 °C, 120 min @ 37 °C and 5 s @ 85 °C, while PCR conditions were as follows: 2 min @ 50 °C, 10 min at 95 °C, and 40 cycles at 15 s @ 95 °C followed by 1 min @ 60 °C. The cycle threshold (Ct) values were used to calculate the relative amount of mRNA expression using the $2^{-\Delta\Delta Ct}$ method.

2.6. Fluorescence-activated cell sorting (FACS) analysis

Harvested PBMCs from healthy subjects and patients newly diagnosed with RA were washed using a FACS staining buffer (PBS with 1% BSA and 0.1% azide). Non-specific binding was blocked by Fc-gamma receptor-specific antibodies or by PE/FITC/APC for 30 min at 4 °C. A total of 2×10^6 cells were further stained in duplicate with anti-CD4, CD25, IFN-γ, IL-17, and Foxp3 antibodies using a BD Cytofix/Cytoperm kit according to the manufacturer's instructions. The live lymphocytes were gated on CD4+ cells, and the numbers of various subtypes viz. CD4+ IFN-γ+, CD4 + IL17+, and CD4+ CD25+ Foxp3+ T cells were calculated. Flow cytometric analysis was performed using FACS Aria-II or LSR-II (BD Biosciences) platforms, and a minimum of 10,000 events were recorded for each sample. Data were analysed using SPICE 4.0 and FlowJo software (Tree Star).

2.7. Data analysis

Results are expressed as medians and interquartile ranges (IQRs). The Shapiro-Wilk test was performed to check the data distributions. Spearman's rank correlation test was used to determine the correlation of the percentage of T-cell subsets with DAS28 CRP in male and female patients with RA. Statistical analyses were performed using Kruskal Wallis followed by Dunn's multiple comparison test. *P*-values < 0.05 (p-value < 0.05) were considered statistically significant.

3. Results

3.1. Flow cytometry analysis of Th1, Th17, and Treg cell population in patients with RA

The frequency of $CD4^+$ IFN-γ^+ cells in peripheral blood was down-regulated in both newly diagnosed male and female patients with RA compared to that in their respective control groups (Fig. 1). A non-

Table 2
Forward and reverse primers of different genes.

Gene	Forward primer	Reverse primer
IFN-γ	5′-TCGGTAACTGACTTGAATGTCCA-3′	5′-TCCTTTTTCGCTTCCCTGTTTT-3′
IL-10	5′-GATGCCTTCAGCAGAGTGAA-3′	5′-GCAACCCAGGTAACCCTTAAA-3′
TGF-β	5′-GCGTGCTAATGGTGGAAAC-3′	5′-CGGTGACATCAAAAGATAACCAC-3′
T-bet	5′-CGGCTGCATATCGTTGAGGT-3′	5′-GTCCCCATTGGCATTCCTC-3′
ROR-γt	5′-GTAACGCGGCCTACTCCTG-3′	5′-GTCTTGACCACTGGTTCCTGT-3′
Foxp3	5′-CTGCCCCTAGTCATGGTGG-3′	5′-CTGGAGGAGTGCCTGTAAGTG-3′
β-actin	5′-GCCAACCGCGAGAAGATGA-3′	5′-CATCACGATGCCAGTGGTA-3′

significant difference was observed in the frequency of $CD4^+$ IFN-γ^+ cells in newly diagnosed male and female patients with RA. Although the Th1 cell population was down-regulated in newly diagnosed patients with RA as compared to the healthy control subjects, the percentage of Th1 cells was almost equal in both male and female patients with low and moderate/high disease severity. Further, we observed a non-significant difference in the frequency of $CD4^+$ IFN-γ^+ cells in male and female patients.

The percentage of $CD4^+$ IL-17^+ cells was elevated in both male and female patients with RA as compared to that in their respective healthy control groups. We observed a more pronounced increment in $CD4^+$ IL-17^+ cells in male patients with RA ($p < 0.001$) (Fig. 2). A significant difference in the percentage of $CD4^+$ IL-17^+ cells was observed in patients with low and moderate severity RA ($p < 0.05$).

There was a substantial increase in the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in both male and female patients with RA as compared to the respective healthy control groups (Fig. 3). No significant difference was observed in the percentages of $CD4^+$ $CD25^+$ $Foxp3^+$ cells between male versus female patients with RA. The $CD4^+$ $CD25^+$ $Foxp3^+$ cells were found to be significantly higher in patients with moderate disease severity as compared to those with low disease severity ($p < 0.05$).

We conducted a correlation analysis to determine the association of T-cell subsets with disease severity in both male and female patients with RA. Generally, the expression of Th1 cells in RA patients was downregulated; however, we could not correlate the disease activity score in male and female patients with RA ($P > 0.05$). A positive correlation was found between the Th17 cell populations and disease activity scores in male ($p < 0.001$) and female ($p < 0.05$) patients with RA. Similarly, Treg cells were correlated with DAS28 CRP in male ($p < 0.001$) and female ($p < 0.001$) patients with RA (Fig. 4).

3.2. Assessment of T-bet, ROR-γt, and Foxp3 expression in PBMCs of patients with RA

The mRNA expression of T-bet was significantly lower in both male (0.61; 0.45–0.73) and female patients with RA (0.59; 0.44–0.76) than in respective healthy control groups (1.26; 0.95–1.44 in male controls and 0.89; 0.71–1.21 in female controls). There was a significant change in the T-bet expression in male versus female patients with RA ($p < 0.05$). The expression of T-bet was independent of disease severity in patients with both low (0.63; 0.51–0.75), and moderate severity RA (0.59; 0.41–0.75) who displayed an almost similar expression of T-bet transcription factor. There was upregulated expression of ROR-γt in both male (2.70; 2.05–3.95) and female patients with RA (1.84; 1.14–2.34) as compared to healthy control subjects, with more prominent increase in male patients ($p < 0.001$) (1.26; 0.92–1.37 in male controls and 1.06; 0.67–1.35 in female controls). ROR-γt expression was elevated in patients with DAS28 CRP > 3.2 (2.43; 1.84–3.30) as compared to those with DAS28 CRP ≤ 3.2 (1.72; 1.02–2.52) ($p < 0.01$). Foxp3 expression was significantly higher in female patients with RA (2.33; 2.03–2.87) than in male patients (1.90; 1.44–2.50). In addition, patients with DAS28 CRP > 3.2 (2.50; 2.18–2.87) showed significantly increased Foxp3 expression as compared to patients with low severity RA (1.65; 1.39–1.96) ($p < 0.05$) (Fig. 5).

3.3. Expression of IFN-γ, TGF-ß, and IL-10 in newly diagnosed patients with RA

The mRNA expression of IFN-γ was down-regulated in both male (0.69; 0.51–0.86) and female patients with RA (0.52; 0.44–0.74) as compared to their respective control groups [(male 1.04; 0.86–1.74); (female 1.04; 0.78–1.31). Although significantly down-regulated as compared to the healthy control group, no significant difference was observed in the expression of IFN-γ when we compared levels between male and female patients with RA. The expression of IFN-γ was also independent of disease severity, as its expression was not significantly different in patients with low (0.69; 0.522–0.76) and moderate severity disease (0.52; 0.45–0.82). In contrast, significantly higher TGF-β expression was observed in female (2.74; 1.97–3.36) than in male patients with RA (2.64; 1.50–3.20) ($p < 0.05$). However, the expression of TGF-ß in both male and female patients was higher than levels observed in the respective healthy control groups. The expression of TGF-ß was higher in patients with DAS28 CRP > 3.2 (3.14; 2.30–3.86) as compared to those with DAS28 CRP ≤ 3.2 (1.68; 1.23–2.07) ($p < 0.01$). The expression of IL-10 was upregulated in both male (2.81; 2.29–3.34) and female patients with RA (3.01; 2.71–3.77) as compared to that in healthy controls (0.81; 0.61–1.14 in male controls and 1.27; 0.69–1.62). Although non-significant, there was a higher expression of IL-10 in female patients than in male patients with RA. Nevertheless, the expression of IL-10 was significantly upregulated in patients with moderate disease severity (3.12; 2.71–3.84) as compared to those with low disease severity (2.53; 2.06–3.23) ($p < 0.05$) (Fig. 6).

3.4. Determination of the levels of IL-17, IL-23 and IL-27 cytokines in plasma of newly diagnosed patients with RA

The plasma levels of the cytokine IL-17 were higher in both male and female patients with RA than in their respective healthy controls. Interestingly, significantly higher IL-17 levels were found in female patients with RA than in male patients ($P < 0.01$). Furthermore, IL-17 levels were also elevated in patients with moderate disease severity as compared to that in healthy controls ($p < 0.001$). Similarly, elevated IL-23 levels were observed in both male (70.05; 61.93–78.68) and female patients with RA (80.95; 67.33–96.43) as compared to the healthy controls (41.85; 31.08–48.13 in male control groups and 44.35; 37.15–49.65) in female control. IL-23 was also elevated in patients with DAS28 CRP > 3.2 (82.10; 72.60–90.90) as compared to those with DAS28 CRP ≤ 3.2 (64.30; 54.75–71.2) ($p < 0.001$). Interestingly, the level of IL-27 was higher in both male (180.0; 143.1–196.9) and female patients with RA (202.4; 179.1–223.0) than in healthy controls (103.2; 96.2–116.0 in male controls and 108.80; 99.95–118.6 in female controls). However, when we analysed these findings relative to sex, the level of IL-27 levels were not found to be significantly different in male and female patients with RA. There were significantly higher IL-27 levels in patients with DAS28 CRP > 3.2 (204.3; 183.90–220.30) than in those with DAS28 CRP ≤ 3.2 (154.6; 139.6–184.3) ($p < 0.05$) (Fig. 7).

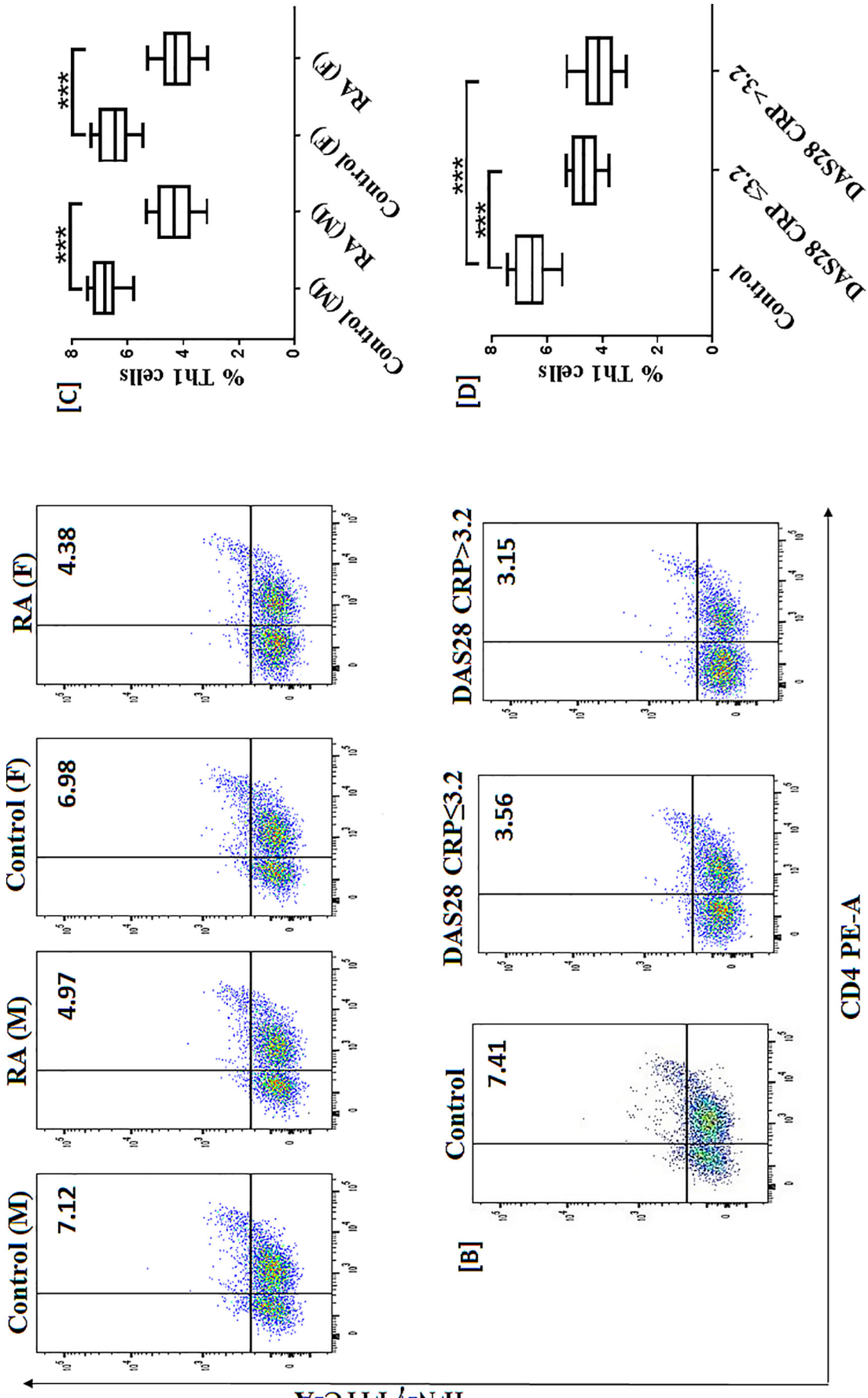

Fig. 1. Flow cytometry analysis of CD4+, IFN-γ Th1 cell population in patients with RA. This is a representative flow cytometry image depicting the frequency of IFN-γ producing CD4+ cells in PBMCs obtained from healthy controls and patients with RA sub-grouped according to [A] sex and [B] disease severity. The [C] and [D] boxplots represent the percentage of Th1 cells in healthy controls and patients with RA, respectively. ***$p < 0.001$.

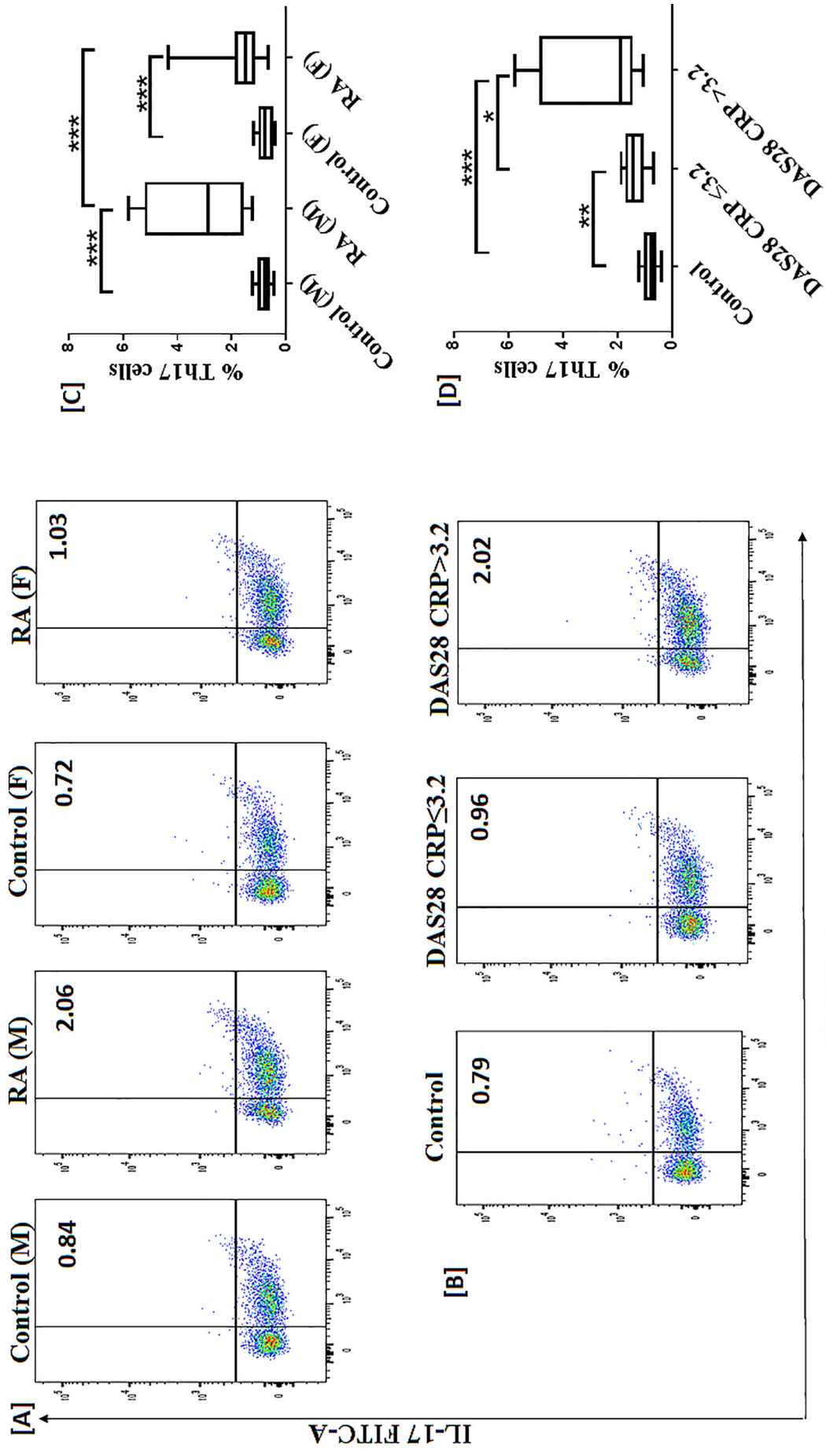

Fig. 2. Flow cytometry analysis of Th17 cells in newly diagnosed patients with RA. This is a representative flow cytometry image depicting the frequency of IL-17 cells in PBMCs of healthy controls and patients with RA sub-grouped according to [A] sex and [B] disease severity. [C] and [D] Boxplots represent the percentage of Th17 cells in healthy controls versus patients with RA, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

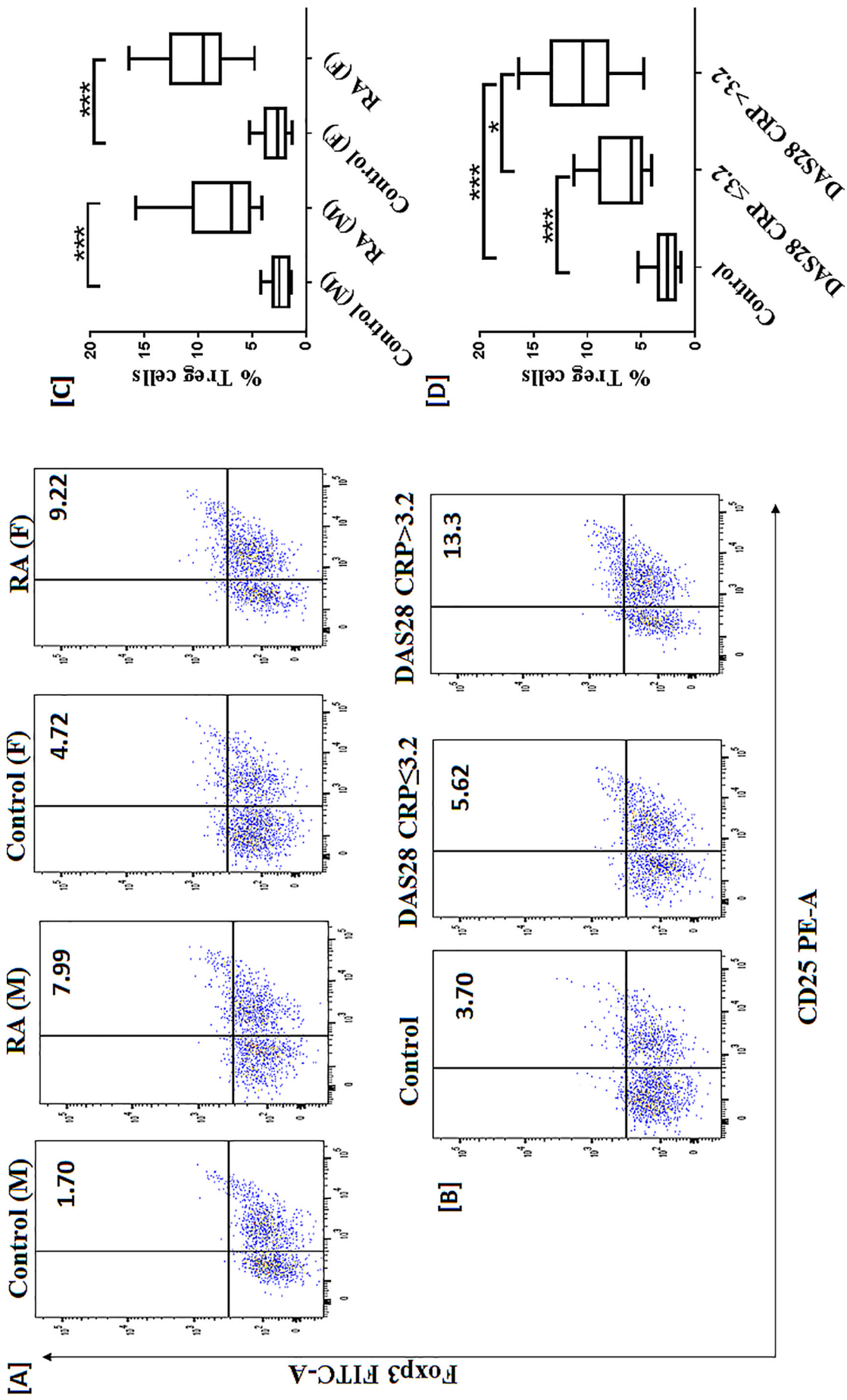

Fig. 3. Flow cytometry analysis of CD4+ CD25+, Foxp3+ Treg cells in patients with RA. This is a representative flow cytometry image depicting the abundance of Foxp3 cells in PBMCs of healthy control and patients with RA sub-grouped according to [A] sex and [B] disease severity, [C] and [D] a boxplot diagram represent the percentage of Treg cells in healthy controls and patients with RA. $^{*}p < 0.05$, $^{***}p < 0.001$.

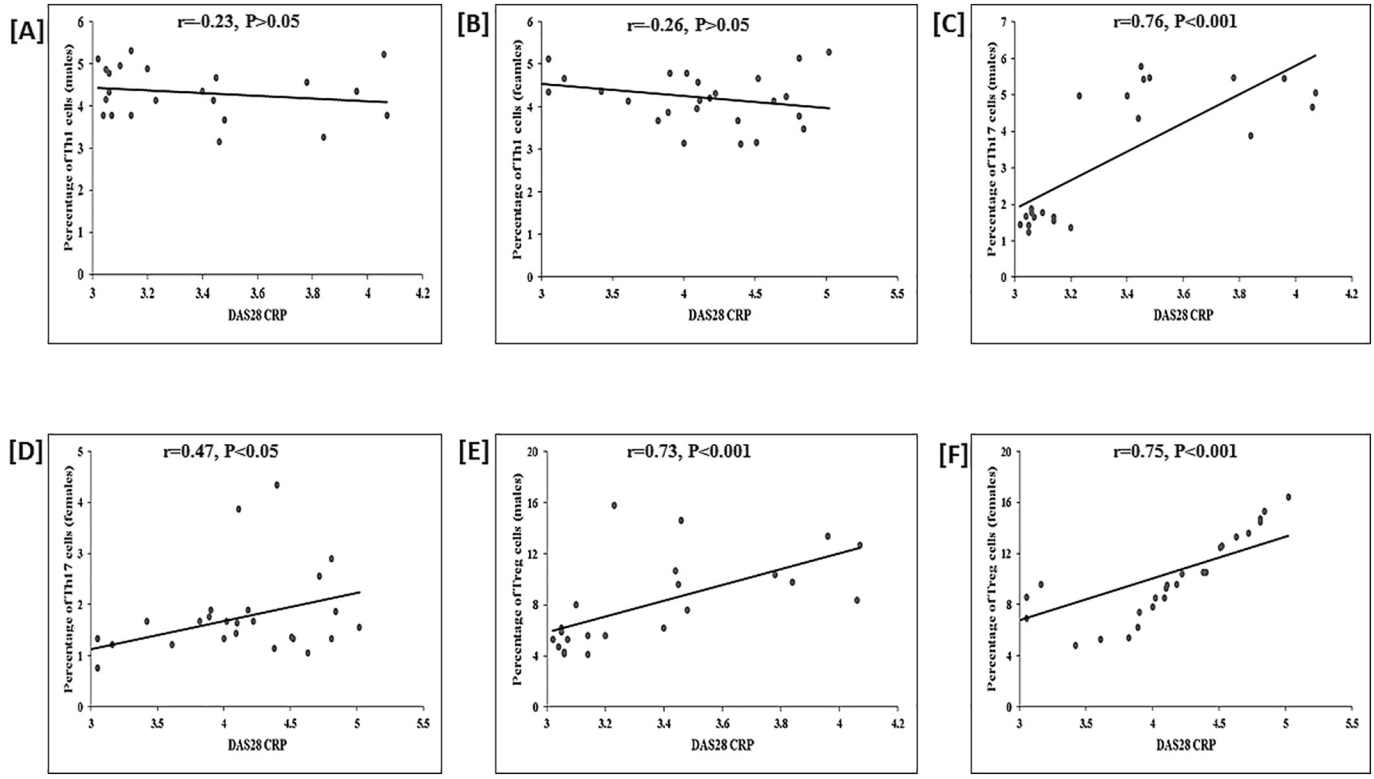

Fig. 4. Correlation analysis of T-cell subsets with DAS28 CRP in patients with RA. Correlation of DAS28 CRP with the percentage of Th1 cells [A, B], Th17 cells [C, D] and Treg cells [E, F] in male and female patients with RA.

4. Discussion

In general, female patients with RA exhibit more severe disease progression, including greater loss of function in both early and established phases of the disease [14]. To the best of our knowledge, this is the first study that correlates sex with the induction of CD4+ Th1, Th17, and Treg cells in the patients with RA. We observed down-regulation of Th1 cell-associated cytokines and increase in Th17 and Treg cell-associated cytokines in newly diagnosed patients with RA. Earlier, Aldridge et al. reported an association between Th17 cells and disease severity only in male patients [15]. Interestingly, we observed a sex-based positive correlation of Th17 and Treg cells subpopulations with disease severity in both male and female patients with RA.

In general, females possess increased RA specific immunoglobulin (cf. anti-citrullinated peptide and rheumatoid factor) levels compared to males. Further, the absolute number of CD4+ lymphocytes, Th1 mediated cellular immune responses, and cytokine profiles are higher in females [16,17]. Various components of the CD4+ T-helper cells analysed in the present study might explain disease progression in females as compared to males [18].

The transcription factor T-bet promotes the expression of Th1 cytokines, specially IFN-γ and IL-2, etc. [19]. It also suppresses the commitment of CD4+ T cells to Th2 and Th17 lineage [20]. The protective role of IFN-γ has been widely discussed in the literature. This suggests that genetic disruption of IFN-γ or its receptor results in increased RA severity in patients [21,22]. Our study found decreased Th1 cell populations in conjunction with downregulation of the mRNA of both T-bet and IFN-γ in newly diagnosed patients with RA. This corresponds with an overall decrease in peripheral Th1 responses in patients with RA. No prior studies have found that T cell migration had a high percentage of Th1 cells with elevated IFN-γ levels in the bone joints, as compared to PBMCs, in patients with RA [23,24]. Surprisingly, children with inflammatory arthritis also reportedly possess elevated Th1 cell populations [25]. Pandya et al. reported a decrease in the Th1 subset in early untreated patients with RA [26]. We have not observed any significant association of Th1 cells and other related mediators with sex or disease activity scores, in contrast with earlier findings that indicated decreased cell-mediated immunity in patients with active RA [27].

IL-27 is a member of the IL-12 family of cytokines. It possesses stimulatory effects on both Th1 and Treg cells, while exerting inhibitory effects on Th2 and Th17 cells, thus help in evoking both pro- and anti-inflammatory responses in the host [28]. In concordance with our findings, Wong et al. reported elevated levels of IL-27 in the blood of patients with RA. They found that blocking of the cytokine IL-27 lessened disease severity [29]. Moreover, elevated IL-27 mRNA expression was found in the synovial fluid macrophages of patients with RA [30]. The cytokine IL-27 downregulates differentiation of Th17 cells by inhibiting ROR-γT and STAT3 [31]. Recent studies have found that IL-27 inhibits the early differentiation of Th17 cells; however, it does not affect the differentiation of mature Th17 cells [32]. It also upregulates the expression of IL-10 by increasing the circulatory Treg cells in vivo [31]. The protective role of IL-27 has also been reported in animal models of arthritis. The IL-27 cytokine has been shown to ameliorate the severity of arthritis by decreasing monocyte recruitment and angiogenesis in a collagen-induced arthritis model [33]. Contrary to our observations, Lai et al. reported significantly higher IL-27 levels in female patients with chronic RA [32]. One can speculate that elevated IL-27 was a natural response of the host to counter increases in RA severity.

Th17 cells secrete effector molecules that act on many cellular targets and ultimately damage cartilage along with bones [34]. Like Th1 cells, Th17 cells can also induce secretion of pro-inflammatory cytokines (IL-6 and IL-8). They also induce the expression of tissue-destructive proteases (MMP-1 and MMP-3) by synovial fibroblasts [35]. IL-17 is a pleiotropic cytokine which is involved in the inflammation and tissue destruction by stimulating the secretion of other pro-inflammatory cytokines and MMPs. IL-17 shows synergism with TNF-α

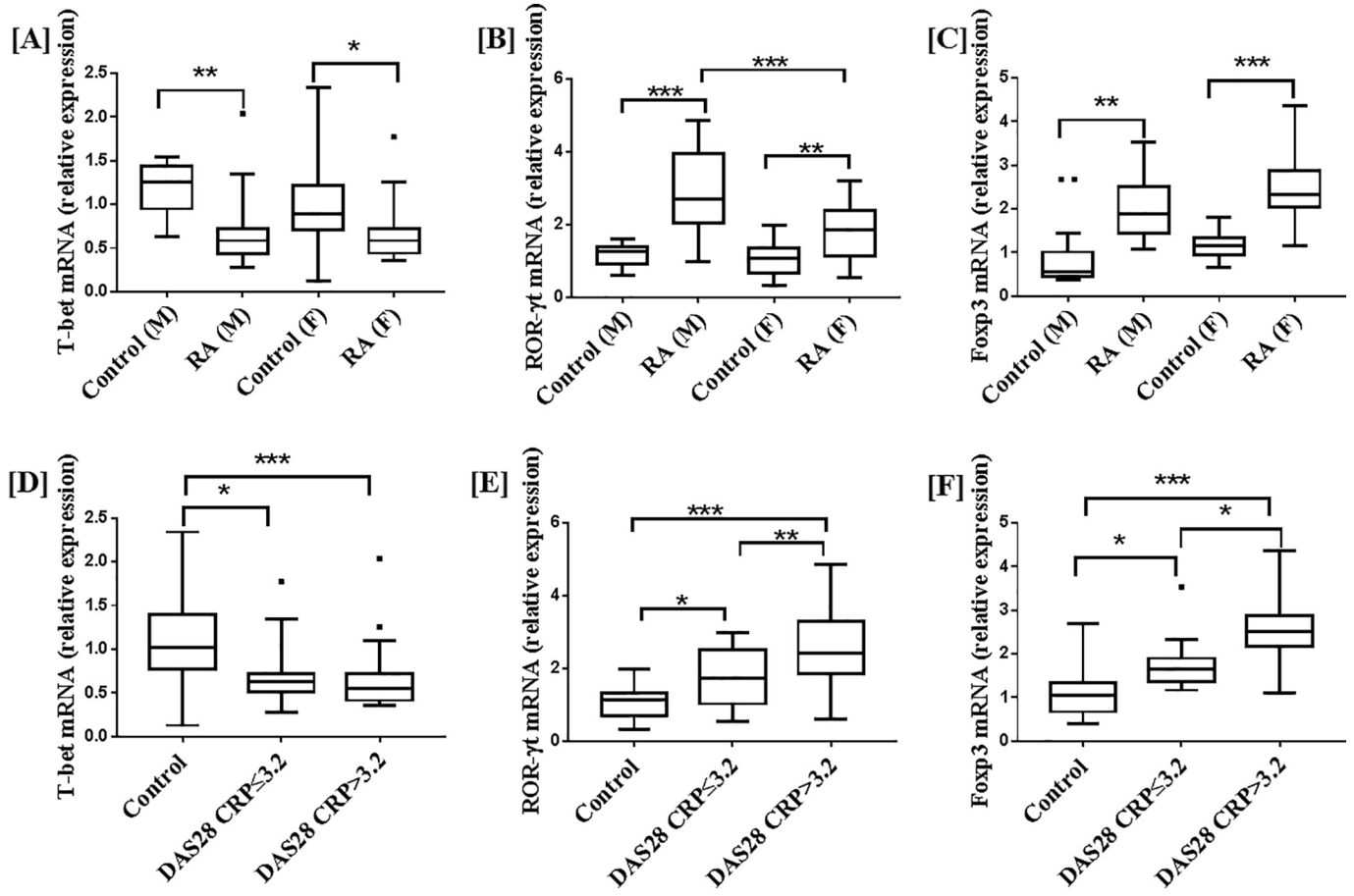

Fig. 5. Modulation in the expression of various regulating factors in patients with RA at the mRNA level. The mRNA expression of [A] and [D] T-bet, [B] and [E] ROR-γt, [C] and [F] Foxp3 in the PBMCs of healthy controls and patients with RA sub-grouped according to sex and disease severity. *p < 0.05, **$p < 0.01$, ***$p < 0.001$.

and induces loss of cartilage while promoting osteoclastogenesis. IL-17 is involved in the synovitis and tissue destruction symptoms observed in patients with RA [36]. The IL-23/IL-17 praxis has been reported to play an important role in joint inflammation, activation of leukocytes and osteoclastogenesis, etc. [37]. The two cytokines cumulatively induce the differentiation of osteoclasts by upregulating RANKL in synovial fibroblasts [38]. Mice, deficient in the IL-23p19 subunit, develop less-severe arthritis [39]. Our observations of elevated Th17 cell populations in patients with RA corresponds to the findings of earlier studies [24]. The cytokine IL-17 is reportedly increased in the PBMCs and synovial fluid of patients with RA as compared to the healthy controls [4]. IL-23 has been positively correlated with functional impairment and knee joint erosion in patients with RA [40]. Other reports also showed a direct correlation between disease severity and IL-17 and IL-23 expression [40,41]. We observed a relatively smaller Th17 cell population (p-value < 0.05) and ROR-γt mRNA expression in female patients, as compared to male patients. Surprisingly, the plasma levels of IL-17 and IL-23 were significantly elevated in female patients as compared to male patients ($p < 0.001$). On the basis of the total CD4+ cell population, lower Th17 cell abundance has been reported in females compared to males [42].

However, in unfractionated PBMCs, increased populations of IL-17A positive cells were found in females as compared to males [43]. This indirectly suggests that non-CD4 cells are the main contributors to IL-17A in females. Mast cells and other immune cell populations are major contributors to IL-17A in patients with RA [44,45]. A decline in Th17 cell populations and T-bet mRNA expression, along with the increase in plasma levels of IL-17 and IL-23 in female patients with RA, strongly correlates with the role of non-CD4 cells in the secretion of pro-inflammatory cytokines. This, in turn, plays a crucial destructive role in the pathogenesis of RA.

T regulatory cells are a thymically produced subpopulation of T cells which suppress the deleterious autoreactive activities of effector T cells. Data pertaining to T regulatory cells in patients with RA are conflicting [26]. Treg cells are enriched in the synovial fluid of patients with RA; however, results gleaned from peripheral blood are conflicting [46,47]. An earlier report by F. Ponchel et al. suggested that Foxp3+ Treg cells were downregulated in patients with RA [48]. In contrast, we found incremental upregulation in Treg populations and Foxp3 mRNA expression in patients newly diagnosed with RA. We attribute our observation to the host immune system-mediated suppression of the inflammatory response propagated by Th17 cells [49].

The onset of RA is generally prompted by the Th1 cell subpopulation. The activation of Th1 cells is cross-regulated by IL-10. IL-10 has been reported to act on monocytes and macrophages by interfering with their ability to activate Th1 cells. IL-10 is one of the key anti-inflammatory cytokines that inhibits the production of a variety of pro-inflammatory cytokines and proteases by monocytes/macrophages [50]. The activation of Th1 cell subpopulations is counter-regulated by elevated plasma levels of IL-10 in patients with RA. Interestingly, there were no significant differences in the Treg cell populations and mRNA expressions of Foxp3 and IL-10 in both female and male patients with RA. This suggests that the sex of patients with RA does not influence Treg cell populations, on the one hand, and IL-10 levels on the other. The role of TGF-β in RA progression is controversial. Many studies have found TGF- β in the synovial fluid and synovial tissue of patients with

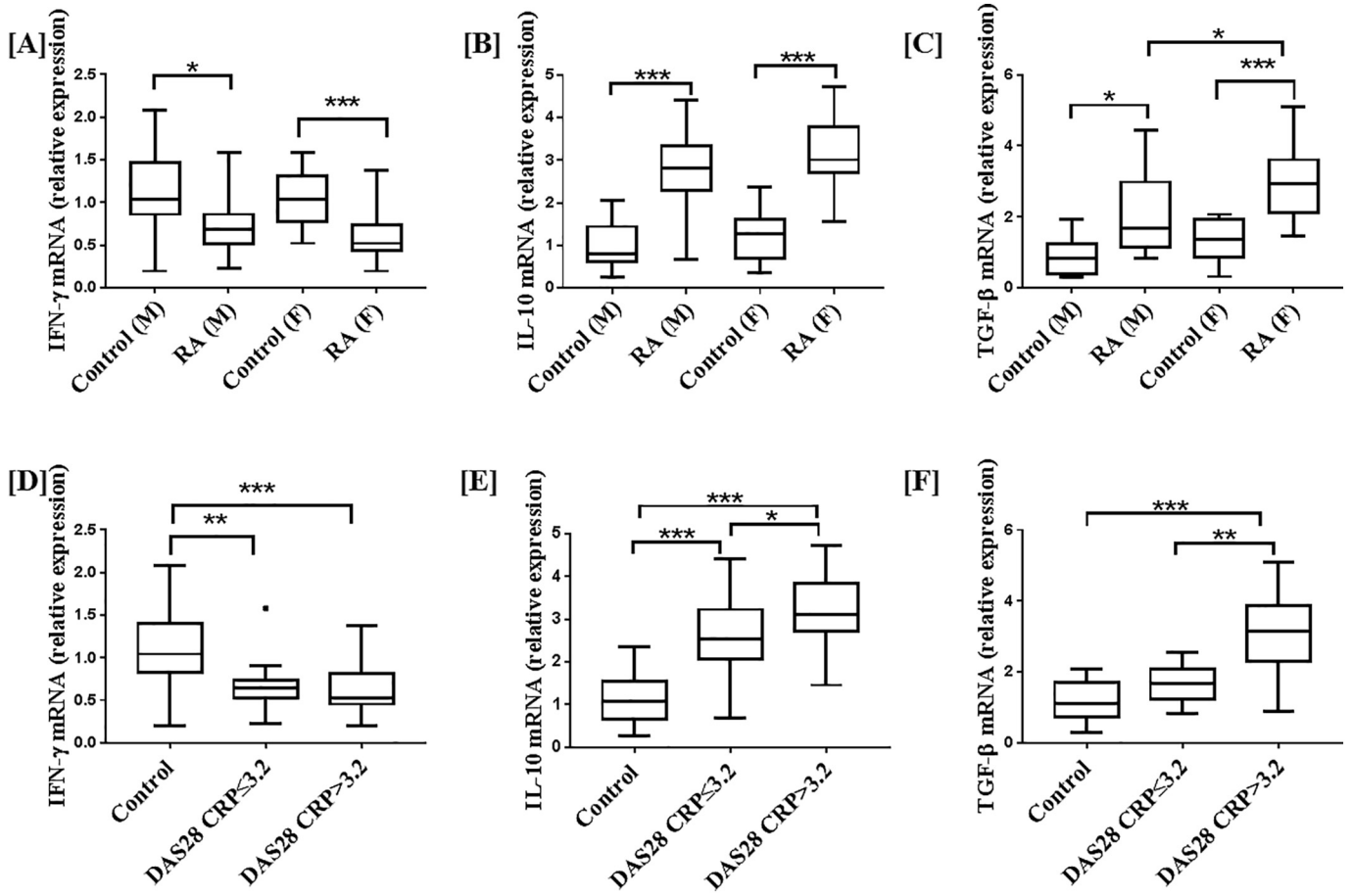

Fig. 6. Expression of various classes of cytokines in patients with RA as revealed by mRNA analysis. mRNA expression of [A] and [D] IFN-γ, [B] and [E] IL-10, [C] and [F] TGF-ß in the PBMCs of healthy controls and patients with RA, sub-grouped according to sex and disease severity. *p < 0.05, **p < 0.01, ***p < 0.001.

RA [51]. TGF- β has also been correlated with inflammation in the host. Further, a positive correlation between TGF- β and clinical markers of the disease has been found in the rheumatoid synovial fibroblasts [52].

In contrast, the expression of TGF- β isoforms in the joints of arthritic mice has been correlated with the disease remission. It can be concluded that TGF- β is induced in the joints at the time of onset, and its level increases with progression of arthritis [53]. TGF- β plays an important role in the differentiation and activation of Treg cells by inducing the expression of Foxp3 [54]. The expressed TGF-β suppresses immune responses in different target cells [55]. Apart from the anti-inflammatory effect, TGF-β also induces differentiation of Th17 cells in the presence of IL-6 or IL-21 cytokines [56,57]. The observed increase in the TGF-β mRNA expression in female patients with RA indicates the involvement of other CD4 cells with relatively fewer copies of CD4+ molecules (such as macrophages) in its expression [58]. Overall, an increased population of Treg cells, mRNA expressions of Foxp3, IL-10, and TGF-β might be well-correlated with induced inflammation in newly diagnosed patients with RA.

5. Conclusion

The data of the present study demonstrate a skewed T helper cell population in favour of both the Th17 and Treg cells phenotype in the peripheral blood of patients newly diagnosed with RA. Furthermore, we observed that inflammatory markers associated with Th1 and Treg cells (except TGF-ß) could not be correlated with the sex of patients with RA. In contrast, Th17 cell populations are different in male versus female patients with RA. Interestingly, we observed elevated expression of both IL-23 and IL-27 cytokines in plasma. It seems that the two subpopulations are at play in the establishing phase; these populations counter each other in patients that are newly inflicted with RA. The IL-12 family of cytokines (cf. IL-23 and IL-27) might be responsible for increased disease severity in female patients with RA.

Ethics statement

This study schedule was approved by the Institutional Ethics and Research Advisory Committee, Faculty of Medicine, Jawaharlal Nehru Medical College, Aligarh Muslim University, India. The patients with RA fulfilled the criteria of the European League Against Rheumatism (EULAR) 2010 classification.

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Declaration of Competing Interest

The authors declared that they have no competing financial or non-financial competing interests, as described by the journal.

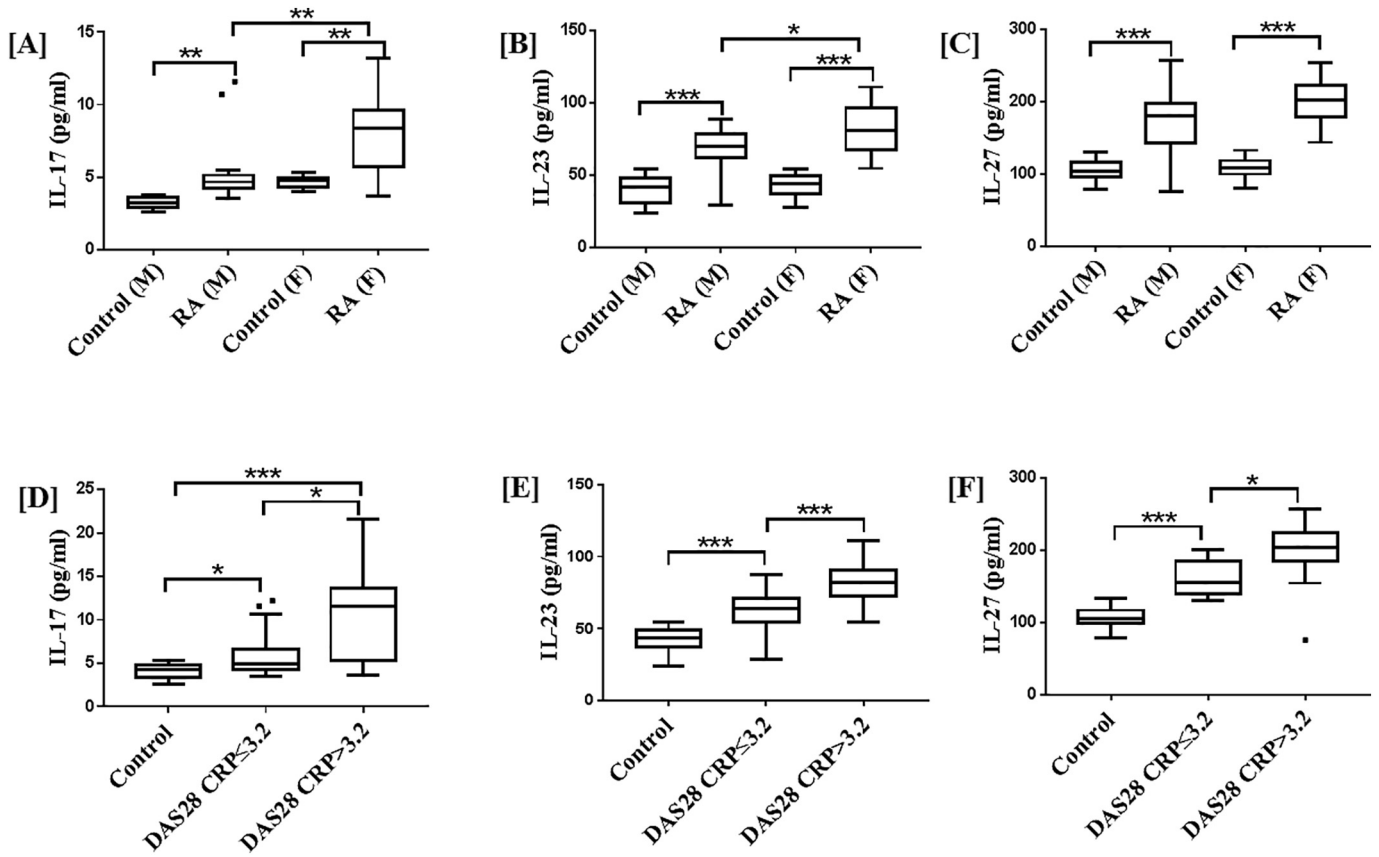

Fig. 7. Modulation of cytokine expression in newly diagnosed patients with RA. Plasma level of [A] and [D] IL-17, [B] and [E] IL-23, [C] and [F] IL-27 in the healthy controls and patients with RA sub-grouped according to sex and disease severity. *p < 0.05, **p < 0.01, ***p < 0.001.

References

[1] I.B. McInnes, G. Schett, The pathogenesis of rheumatoid arthritis, N. Engl. J. Med. 365 (23) (2011) 2205–2219.
[2] L.P. Blanco, Oxidative Stress in Rheumatoid Arthritis: New Insights, Role of Oxidative Stress in Chronic Diseases, 1 CRC Press, 2014, pp. 493–512.
[3] S. Mateen, S. Moin, A. Zafar, A.Q. Khan, Redox signaling in rheumatoid arthritis and the preventive role of polyphenols, Clin. Chim. Acta 463 (2016) 4–10.
[4] S. Mateen, A. Zafar, S. Moin, A.Q. Khan, S. Zubair, Understanding the role of cytokines in the pathogenesis of rheumatoid arthritis, Clin. Chim. Acta 455 (2016) 161–171.
[5] J.D. Cañete, S.E. Martínez, J. Farrés, R. Sanmartí, M. Blay, A. Gómez, G. Salvador, J. Muñoz-Gómez, Differential Th1/Th2 cytokine patterns in chronic arthritis: interferon γ is highly expressed in synovium of rheumatoid arthritis compared with seronegative spondyloarthropathies, Ann. Rheum. Dis. 59 (4) (2000) 263–268.
[6] J. Sharma, S. Bhar, C.S. Devi, A review on interleukins: the key manipulators in rheumatoid arthritis, Mod. Rheumatol. 27 (5) (2017) 723–746.
[7] D.A. Vignali, V.K. Kuchroo, IL-12 family cytokines: immunological playmakers, Nat. Immunol. 13 (8) (2012) 722.
[8] M. Mellado, L. Martínez-Muñoz, G. Cascio, P. Lucas, J. Pablos, J. Rodríguez-Frade, T cell migration in rheumatoid arthritis, Front. Immunol. 6 (384) (2015) (2015).
[9] S.L. Gaffen, The role of interleukin-17 in the pathogenesis of rheumatoid arthritis, Curr. Rheumatol. Rep. 11 (5) (2009) 365.
[10] D.S. Al-Zifzaf, S.A. El Bakry, R. Mamdouh, L.A. Shawarby, A.Y.A. Ghaffar, H.A. Amer, A.A. Alim, H.M. Sakr, R.A. Rahman, FoxP3+ T regulatory cells in rheumatoid arthritis and the imbalance of the Treg/TH17 cytokine axis, The Egyptian Rheumatologist 37 (1) (2015) 7–15.
[11] S.S. Iyer, G. Cheng, Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease, Crit. Rev. Immunol. 32 (1) (2012).
[12] D. Aletaha, T. Neogi, A.J. Silman, J. Funovits, D.T. Felson, C.O. Bingham III, N.S. Birnbaum, G.R. Burmester, V.P. Bykerk, M.D. Cohen, 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative, Arthritis & Rheumatism 62 (9) (2010) 2569–2581.
[13] J. Kirwan, Measures of rheumatoid arthritis disease activity, J. Rheumatol. 36 (2) (2009) 442.
[14] N. Lesuis, R. Befrits, F. Nyberg, R.F. van Vollenhoven, Gender and the treatment of immune-mediated chronic inflammatory diseases: rheumatoid arthritis, inflammatory bowel disease and psoriasis: an observational study, BMC Med. 10 (1) (2012) 82.
[15] J. Aldridge, J.M. Pandya, L. Meurs, K. Andersson, I. Nordström, E. Theander, A.-C. Lundell, A. Rudin, Sex-based differences in association between circulating T cell subsets and disease activity in untreated early rheumatoid arthritis patients, Arthritis research & therapy 20 (1) (2018) 150.
[16] L.S. Ackerman, Sex hormones and the genesis of autoimmunity, Arch. Dermatol. 142 (3) (2006) 371–376.
[17] C. Libert, L. Dejager, I. Pinheiro, The X chromosome in immune functions: when a chromosome makes the difference, Nat. Rev. Immunol. 10 (8) (2010) 594.
[18] A. Bouman, M. Schipper, M.J. Heineman, M.M. Faas, Gender difference in the non-specific and specific immune response in humans, Am. J. Reprod. Immunol. 52 (1) (2004) 19–26.
[19] R.G. Jenner, M.J. Townsend, I. Jackson, K. Sun, R.D. Bouwman, R.A. Young, L.H. Glimcher, G.M. Lord, The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes, Proc. Natl. Acad. Sci. 106 (42) (2009) 17876–17881 (pnas. 0909357106).
[20] V. Lazarevic, L.H. Glimcher, T-bet in disease, Nat. Immunol. 12 (7) (2011) 597.
[21] B. Manoury-Schwartz, G. Chiocchia, N. Bessis, O. Abehsira-Amar, F. Batteux, S. Muller, S. Huang, M.-C. Boissier, C. Fournier, High susceptibility to collagen-induced arthritis in mice lacking IFN-gamma receptors, J. Immunol. 158 (11) (1997) 5501–5506.
[22] C.E. Page, S. Smale, S.M. Carty, N. Amos, S.N. Lauder, R.M. Goodfellow, P.J. Richards, S.A. Jones, N. Topley, A.S. Williams, Interferon-gamma inhibits interleukin-1beta-induced matrix metalloproteinase production by synovial fibroblasts and protects articular cartilage in early arthritis, Arthritis research & therapy 12 (2) (2010) R49.
[23] J.A. van Roon, C.M. Verhoef, J.L. van Roy, F.H. Gmelig-Meyling, O. Huber-Bruning, F.P. Lafeber, J.W. Bijlsma, Decrease in peripheral type 1 over type 2 T cell cytokine production in patients with rheumatoid arthritis correlates with an increase in severity of disease, Ann. Rheum. Dis. 56 (11) (1997) 656–660.
[24] H. Yamada, Y. Nakashima, K. Okazaki, T. Mawatari, J. Fukushi, N. Kaibara, A. Hori, Y. Iwamoto, Y. Yoshikai, Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis, Ann. Rheum. Dis. 67 (9) (2008) 1299–1304.
[25] K. Nistala, S. Adams, H. Cambrook, S. Ursu, B. Olivito, W. de Jager, J.G. Evans, R. Cimaz, M. Bajaj-Elliott, L.R. Wedderburn, Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment, Proc. Natl. Acad. Sci. 107 (33) (2010) 14751–14756.
[26] J.M. Pandya, A.C. Lundell, M. Hallström, K. Andersson, I. Nordström, A. Rudin, Circulating T helper and T regulatory subsets in untreated early rheumatoid arthritis and healthy control subjects, J. Leukoc. Biol. 100 (4) (2016) 823–833.
[27] M. Kawashima, P. Miossec, mRNA quantification of T-bet, GATA-3, IFN-γ, and IL-4 shows a defective Th1 immune response in the peripheral blood from rheumatoid

arthritis patients: link with disease activity, J. Clin. Immunol. 25 (3) (2005) 209–214.
[28] O. Ali, A. Khalifa, H. Atia, Levels of interleukin 33 and interleukin 27 in rheumatoid arthritis and osteoarthritis in Egyptian patients, Int. J. Adv. Biotechnol. Res. 5 (2014) 465–473.
[29] C.K. Wong, D.P. Chen, L.S. Tam, E.K. Li, Y.B. Yin, C.W. Lam, Effects of inflammatory cytokine IL-27 on the activation of fibroblast-like synoviocytes in rheumatoid arthritis, Arthritis research & therapy 12 (4) (2010) R129.
[30] S. Shahrara, Q. Huang, A.M. Mandelin, R.M. Pope, TH-17 cells in rheumatoid arthritis, Arthritis research & therapy 10 (4) (2008) R93.
[31] S.-J. Moon, J.-S. Park, Y.-J. Heo, C.-M. Kang, E.-K. Kim, M.-A. Lim, J.-G. Ryu, S.J. Park, K.S. Park, Y.-C. Sung, In vivo action of IL-27: reciprocal regulation of Th17 and Treg cells in collagen-induced arthritis, Exp. Mol. Med. 45 (10) (2013) e46.
[32] X. Lai, H. Wang, J. Cao, Y. Li, Y. Dai, Y. Xiang, L. Zhang, Circulating IL-27 is elevated in rheumatoid arthritis patients, Molecules 21 (11) (2016) 1565.
[33] F. Larousserie, P. Charlot, E. Bardel, J. Froger, R.A. Kastelein, O. Devergne, Differential effects of IL-27 on human B cell subsets, J. Immunol. 176 (10) (2006) 5890–5897.
[34] P. Miossec, J.K. Kolls, Targeting IL-17 and T H 17 cells in chronic inflammation, Nat. Rev. Drug Discov. 11 (10) (2012) 763.
[35] J.P. van Hamburg, P.S. Asmawidjaja, N. Davelaar, A.M. Mus, E.M. Colin, J.M. Hazes, R.J. Dolhain, E. Lubberts, Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production, Arthritis Rheum. 63 (1) (2011) 73–83.
[36] H.M. Al-Saadany, M.S. Hussein, R.A. Gaber, H.A. Zaytoun, Th-17 cells and serum IL-17 in rheumatoid arthritis patients: correlation with disease activity and severity, The Egyptian Rheumatologist 38 (1) (2016) 1–7.
[37] E. Lubberts, The IL-23-IL-17 axis in inflammatory arthritis, Nat. Rev. Rheumatol. 11 (10) (2015) 562.
[38] X. Li, K.W. Kim, M.L. Cho, J.H. Ju, C.M. Kang, H.J. Oh, J.K. Min, S.H. Lee, S.H. Park, H.Y. Kim, IL-23 induces receptor activator of NF-kappaB ligand expression in fibroblast-like synoviocytes via STAT3 and NF-kappaB signal pathways, Immunol. Lett. 127 (2) (2010) 100–107.
[39] C.A. Murphy, C.L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R.A. Kastelein, J.D. Sedgwick, D.J. Cua, Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation, J. Exp. Med. 198 (12) (2003) 1951–1957.
[40] A.S. Dalila, M.S. Mohd Said, S.S. Shaharir, A.W. Asrul, S.F. Low, A.S. Shamsul, R. Sakthiswary, Interleukin-23 and its correlation with disease activity, joint damage, and functional disability in rheumatoid arthritis, Kaohsiung J. Med. Sci. 30 (7) (2014) 337–342.
[41] E.M. Abu Al Fadl, M. Fattouh, A.A. Allam, High IL-23 level is a marker of disease activity in rheumatoid arthritis, The Egyptian journal of immunology 20 (2) (2013) 85–92.
[42] M. Rodriguez-Garcia, F.D. Barr, S.G. Crist, J.V. Fahey, C.R. Wira, Phenotype and susceptibility to HIV infection of CD4+ Th17 cells in the human female reproductive tract, Mucosal Immunol. 7 (6) (2014) 1375.
[43] L.P. Blanco, M. Plegue, W.P. Fung-Leung, J. Holoshitz, Gender-biased regulation of human IL-17-producing cells in vitro by peptides corresponding to distinct HLA-DRB1 allele-coded sequences, Journal of immune based therapies, vaccines and antimicrobials 2 (3) (2013) 29–38.
[44] A.J. Hueber, D.L. Asquith, A.M. Miller, J. Reilly, S. Kerr, J. Leipe, A.J. Melendez, I.B. McInnes, Mast cells express IL-17A in rheumatoid arthritis synovium, J. Immunol. (Baltimore, Md. : 1950) 184 (7) (2010) 3336–3340.
[45] E.M. Moran, R. Heydrich, C.T. Ng, T.P. Saber, J. McCormick, J. Sieper, H. Appel, U. Fearon, D.J. Veale, IL-17A expression is localised to both mononuclear and polymorphonuclear synovial cell infiltrates, PLoS One 6 (8) (2011) e24048.
[46] A. Alunno, M. Manetti, Altered Immunoregulation in Rheumatoid Arthritis: The Role of Regulatory T Cells and Proinflammatory Th17 Cells and Therapeutic Implications, 2015 (2015), p. 751793.
[47] D. Cao, R. van Vollenhoven, L. Klareskog, C. Trollmo, V. Malmstrom, CD25brightCD4+ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease, Arthritis Research & Therapy 6 (4) (2004) R335–R346.
[48] F. Ponchel, V. Goeb, R. Parmar, Y. El-Sherbiny, M. Boissinot, J. El Jawhari, A. Burska, E.M. Vital, S. Harrison, P.G. Conaghan, E. Hensor, P. Emery, An immunological biomarker to predict MTX response in early RA, Ann. Rheum. Dis. 73 (11) (2014) 2047–2053.
[49] P.D. Katsikis, C.Q. Chu, F.M. Brennan, R.N. Maini, M. Feldmann, Immunoregulatory role of interleukin 10 in rheumatoid arthritis, J. Exp. Med. 179 (5) (1994) 1517–1527.
[50] L. Ding, P.S. Linsley, L.Y. Huang, R.N. Germain, E.M. Shevach, IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression, Journal of Immunology (Baltimore, Md. : 1950) 151 (3) (1993) 1224–1234.
[51] F.M. Brennan, D. Chantry, M. Turner, B. Foxwell, R. Maini, M. Feldmann, Detection of transforming growth factor-beta in rheumatoid arthritis synovial tissue: lack of effect on spontaneous cytokine production in joint cell cultures, Clin. Exp. Immunol. 81 (2) (1990) 278–285.
[52] D. Pohlers, A. Beyer, D. Koczan, T. Wilhelm, H.J. Thiesen, R.W. Kinne, Constitutive upregulation of the transforming growth factor-beta pathway in rheumatoid arthritis synovial fibroblasts, Arthritis research & therapy 9 (3) (2007) R59.
[53] L. Marinova-Mutafchieva, C. Gabay, K. Funa, R.O. Williams, Remission of collagen-induced arthritis is associated with high levels of transforming growth factor-beta expression in the joint, Clin. Exp. Immunol. 146 (2) (2006) 287–293.
[54] Y. Liu, P. Zhang, J. Li, A.B. Kulkarni, S. Perruche, W. Chen, A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells, Nat. Immunol. 9 (6) (2008) 632–640.
[55] K. Nakamura, A. Kitani, W. Strober, Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta, J. Exp. Med. 194 (5) (2001) 629–644.
[56] E. Bettelli, Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, V.K. Kuchroo, Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells, Nature 441 (7090) (2006) 235–238.
[57] L. Yang, D.E. Anderson, C. Baecher-Allan, W.D. Hastings, E. Bettelli, M. Oukka, V.K. Kuchroo, D.A. Hafler, IL-21 and TGF-beta are required for differentiation of human T(H)17 cells, Nature 454 (7202) (2008) 350–352.
[58] E. Gonzalo-Gil, G. Criado, B. Santiago, J. Dotor, J.L. Pablos, M. Galindo, Transforming growth factor (TGF)-beta signalling is increased in rheumatoid synovium but TGF-beta blockade does not modify experimental arthritis, Clin. Exp. Immunol. 174 (2) (2013) 245–255.