Prognostic signature of interferon-γ and interleukin-17A in early rheumatoid arthritis

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Abstract

Objective

CD4+ T cells are crucial for the pathogenesis of rheumatoid arthritis (RA). Here, we evaluated gene expression in CD4+ T cells in early RA, and main purpose of present study was to seek the changes in CD4+ T-cell-related cytokines according to RA progression.

Methods

Early RA was defined as methotrexate (MTX)-naïve patients. Established RA was defined as patients with more than 6 months of DMARDs. Patients with osteoarthritis were evaluated as controls. Microarray analysis was used to identify overexpressed genes in CD4+ T cells, and RT-qPCR was used to validate. Plasma cytokine were measured in patients with early and established RA, and correlations with disease activity were assessed in patients with early RA, whereas clinical prognosis was assessed in established patients with RA.

Results

Thirty-four genes showed overexpression in CD4+ T cells from patients with early RA compared with OA controls. Nineteen were related to interferon (IFN)-γ, and eight were related to interleukin (IL)-17A. Plasma levels of IL-17A, IL-6, IL-12, and TNF-α correlated with IFN-γ, and correlation coefficient was highest between DAS28-ESR and plasma IFN-γ levels in patients with early RA (Rho=0.553, p=0.0025). In established RA with low disease activity, drug reduction group showed lower plasma IFN-γ and IL-17A than drug maintenance/relapse group (13.61±5.75 vs. 29.89±18.72, p<0.001; and 10.91±3.92 vs. 21.04±12.81 pg/mL, p<0.001, respectively).

Conclusion

The IFN-γ and IL-17 gene signature in CD4+ T cells was significantly increased in early RA. Patients with established RA with low levels of IFN-γ and IL-17A could be eligible for dose reduction.

Key words

early rheumatoid arthritis, helper T cell, microarray, interferon-γ, interleukin-17A

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Introduction

Rheumatoid arthritis (RA) is a type of destructive systemic arthritis with an autoimmune pathogenesis. RA can destroy normal joints and induce irreversible changes in articular structures, eventually causing disability and inability to work. Synovitis and pannus formation are the cornerstone pathologic components of RA pathogenesis, and these are mediated by various genetic / epigenetic / environmental factors (1). In inflamed RA synovium, cluster of differentiation (CD)4$^+$ T cells are predominant (2), suggesting that CD4$^+$ T cells may be cornerstone immune cells in RA pathogenesis. Moreover, T-cell responses to type II collagen are enhanced, particularly in early RA (3). Helper T cells (CD4$^+$ T cells) are important elements in adaptive immune systems and can be divided into several subtypes (4). Interferon (IFN)-γ-secreting type 1 helper T cells (Th1) are major pathologic cells in RA development (5); however, IFN-γ-blocking therapies fail to achieve the primary endpoint in phase II clinical trials of patients with RA. Thereafter, type 17 helper T cells (Th17 cells, mainly secreting interleukin [IL]-17A) have been identified as the main pathologic CD4$^+$ T cells in RA (4). Notably, IL-17 blocking agents show inferior therapeutic responses compared with other biologic disease-modifying antirheumatic drugs (DMARDs), including tumour necrosis factor (TNF)-α inhibitors, in established RA (6). Recent studies have shown that the *IFN* gene signature is increased in whole blood of patients with methotrexate (MTX)-naïve RA compared with that in patients with established RA, and these changes are correlated with RA disease activity in MTX-naïve RA (7), emphasizing the importance of IFN-γ in RA pathogenesis (8).

In this study, we investigated gene expression levels in CD4$^+$ T cells in MTX-naïve early RA and evaluated changes in CD4$^+$ T-cell-related cytokines during early RA. The main purpose was to evaluate the potential of up-regulated CD4$^+$ T-cell-related cytokines as biomarker of drug tapering in established RA.

Patients and methods

Patients

Patients were recruited from Konkuk University Hospital and Ho-Youn Kim's Clinic for Arthritis and Rheumatism. Inclusion criteria for MTX-naïve, early RA were as follows: 1) fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA (9); 2) anti-citrullinated peptide antibody (ACPA) positive; 3) over 18 years old; 4) never taken DMARDs prior to enrolment; 5) symptom duration less than 1 year; and 6) presence of synovitis confirmed by ultrasonography or magnetic resonance imaging. Exclusion criteria were as follows: 1) presence of another autoimmune disease; 2) presence of infection; and 3) presence of malignancy. Patients with established RA who achieved low disease activity (disease activity score [DAS] 28-ESR ≤3.2) were also recruited and were all positive for either ACPA or rheumatoid factor. Patients with osteoarthritis (OA) were recruited as a control group. In patients with MTX-naïve early RA, cytokine levels were analysed before DMARDs were started. Disease activities were divided into three categories in early RA patients according to DAS28-ESR as follows; 1) low disease activity (DAS28-ESR ≤ .2), 2) moderate disease activity (3.2< DAS28-ESR ≤5.1), 3) high disease activity (DAS28-ESR >5.1). In patients with established RA with low disease activity, cytokines were measured at the time when DAS28-ESR less than 3.2 was achieved. Patients with RA who were treated at least 6 months of DMARDs were defined as established RA. The disease course in patients with established RA with low disease activity was followed for 6 months, and patients were divided into the drug reduction group, and drug maintenance/relapse after drug reduction group. The demographics of the enrolled patients are summarized in Table I. Finally, 24 patients for MTX-naïve early RA (among them only 14 were included in microarray analysis), 16 patients for OA (among them only 9 were included in microarray analysis), and 34 for established RA patients

Table I. Characteristics of enrolled early RA, established RA, and OA patients.

	Patient cohorts			
	Early RA	Established RA patients with	OA patients	*p*-value
DAS28-ESR ≤3.2				
Individuals (n)	24	34	16	
Females (n), (%)	21 (87.5)	30 (88.2)	11 (68.8)	0.183*
Median ages of samples (IQR)	48.5 (41.5-55.3)	56.5 (47.25-63.0)	61.5 (54.0-64.5)	0.071*
RF positive (n), (%)	18 (75.0)	19 (55.9)	-	0.224
ACPA positive (n), (%)	24 (100)	34 (100)	-	1
ESR (mm/hr, median and IQR)	25.0 (15.0-35.75)[a]	14.0 (10.0-18.75)[b]	9.5 (6.75-13.5)[c]	<0.001*
CRP (mg/dL, median and IQR)	0.24 (0.12-0.47)	0.12 (0.07-0.26)	0.07 (0.05-0.14)	0.069*
Disease duration (months, median and IQR)	3 (1-6)	36 (24-60)	-	<0.001
DAS28-ESR (0-10, median and IQR)	4.70 (4.22-5.28)	2.82 (2.53-3.08)	-	<0.001

The positive range for ACPA is more than 5.0; the positive range for RF is more than 8.0.
ACPA: anticitrullinated peptide antibodies; CRP: C-reactive protein; DAS28: disease activity score 28; ESR: erythrocyte sedimentation rate; IQR, interquartile range; OA, osteoarthritis; RA, rheumatoid arthritis.
*These were compared with ANOVA, and *post-hoc* analysis were performed by Bonferroni's method.

were included. The present study was planned as pilot study. Informed consent was obtained from all patients, and the study was approved by the Human Research Ethics Committee of the Konkuk University Medical Center (approval no. KUH1010511) and the Clinics for Arthritis and Rheumatism (approval no. 0301202005BR00101).

Sample size calculation

As the difference of baseline plasma cytokine levels between drug reduction group *versus* drug maintenance/relapse after drug reduction group was expected as 10 pg/mL with standard deviation (SD) 9 pg/mL (10), and alpha error 5%, statistic power 80%, and drop-out rate 10%. Finally, at least 14 patients were needed in each group.

Cytokine measurement via multiplex cytokine assay (MCA)

Millipore's MILLIPLEX MAP High Sensitivity Human Cytokine multiplex kit (cat. no. HSTCMAG-28SK; Merck, Billerica, MA, USA) was used to measure plasma concentrations of IFN-γ, IL-17A, IL-6, IL-12, and TNF-α, according to the manufacturer's instructions.

RNA preparation

Total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified using RNeasy columns (Qiagen, Valencia, CA, USA). RNA samples were then quantified, aliquoted, and stored at -80°C until use. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis and analysis of the optical density at 260/280 using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA (500 ng), extracted as described above, was reverse transcribed into cDNA with a RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RT-qPCR was conducted in a 20-μL total volume containing 7.2 μL PCR-grade distilled water, 0.4 μL forward primers and reverse primers, and 10 μL SYBR Green I Master mix (Roche Diagnostics, Mannheim, Germany). PCR conditions were as follows: 95°C for 10 min; followed by 35 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s. All primers were synthesised by Bioneer Corp. (Daejeon, Republic of South Korea). The relative mRNA expression levels were normalised to the levels of β-actin mRNA.

Isolation of peripheral blood mononuclear cells (PBMCs) and CD4⁺ T cells

Whole blood was collected using standard blood draw procedures. PBMCs were isolated from whole blood using Ficoll-Paque density medium (GE Healthcare, Chicago, IL, USA). The heparinised blood was diluted 1:1 in phosphate-buffered saline (PBS) and layered over the density gradient. The tube was centrifuged for 30 min at 1,800 rpm at room temperature. The whitish cell layer was collected and washed in PBS. To purify $CD4^+$ T cells from PBMCs, the cells were incubated with anti-human CD4 microbeads (Miltenyi Biotec, Auburn, CA, USA). $CD4^+$ T cells were then separated magnetically using a MACS column.

Gene expression profiling and data analysis

Gene expression analyses were performed in $CD4^+$ T cells isolated from PBMCs using Affymetrix GeneChip Human Gene 2.0 ST oligonucleotide arrays. RNA (300 ng) was applied to the Affymetrix GeneChip as recommended by the manufacturer (http://www.affymetrix.com). The Affymetrix GeneChip Human Gene 2.0 ST oligonucleotide array was then scanned using an Affymetrix Model 3000 G7 scanner, and image data were extracted and analysed using Affymetrix Command Console software 1.1. For normalisation, we used the robust multi-average algorithm implemented in Affymetrix Expression Console software.

Statistical analysis

Normal distribution of continuous variables was assessed by the Kolmogorov-Smirnov test. Then continuous variables were expressed as means ± standard deviations or median with inter-

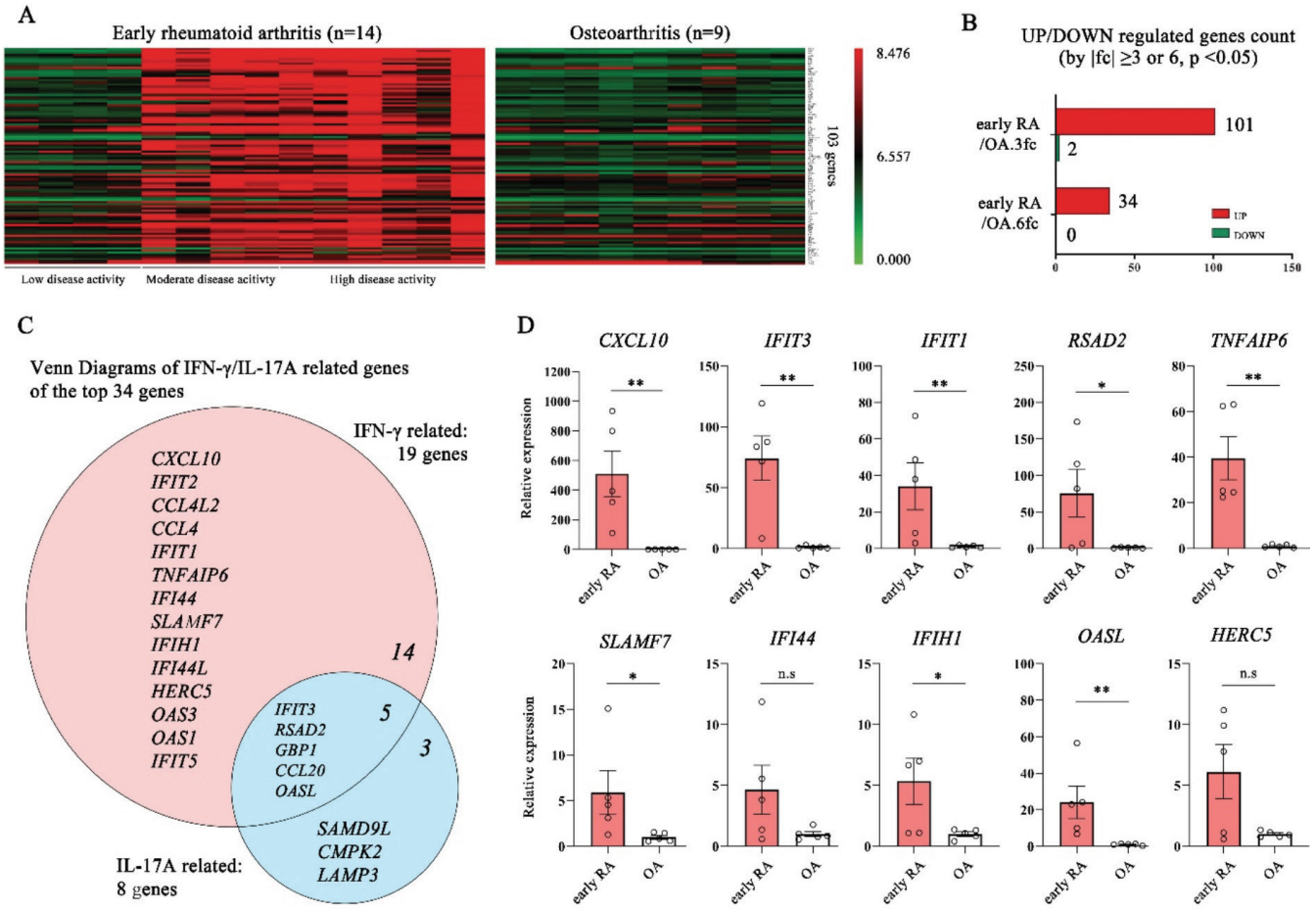

Fig. 1. Microarray analysis and RT-qPCR of MTX-naïve early RA. **A**: Total microarray analysis data of CD4⁺ T cells from patients with MTX-naïve early RA or OA. **B**: Three-fold and six-fold upregulated genes in MTX-naïve early RA. **C**: List of IFN-γ- and IL-17A-signalling related genes. **D**: Validation of 10 upregulated genes in MTX-naïve early RA by RT-qPCR. *p<0.05, **p<0.01.

quartile range according to the results of normality test. Continuous variables were compared using Student's t-tests or Mann-Whitney test or one-way analysis of variance and Bonferroni's multiple comparisons test. Correlation analysis was performed using Spearman correlation coefficients. In all analyses, differences with p-values less than 0.05 were considered significant, and analyses were performed using SPSS v. 20.0 (SPSS, Chicago, IL, USA).

Results

Upregulated genes in MTX-naïve early RA

We performed microarray analysis of peripheral blood CD4⁺ T cells from MTX-naïve early RA (14 patients) and OA (9 patients); expression data for 103 genes were obtained. A heatmap of total gene expression is shown in Figure 1A. The gene expression patterns were differed between early RA patients with low disease activity and moderate to high disease activity (data not shown). In total, 101 genes were upregulated by more than 3-fold in MTX-naïve early RA compared with those in OA; two genes were downregulated (Fig. 1B). We further selected genes that were overexpressed by at least 6-fold (34 genes; Fig. 1B). Among the 34 genes, we clustered gene sets according to associations with IFN-γ or IL-17A. Nineteen genes were related to IFN-γ signalling, and eight were related to IL-17A signalling (Fig. 1C). Interestingly, five genes (*IFIT3*, *RSAD2*, *GBP1*, *CCL20*, and *OASL*) were related to both IFN-γ and IL-17A. Ten genes (*CXCL10*, *IFIT3*, *IFIT1*, *RSAD2*, *TNFAIP6*, *SLAMF7*, *IFI44*, *IFIH1*, *OASL*, *HERC5*) were selected for validation by RT-qPCR. All genes except for *SLAMF7* and *IFI44* showed overexpression in MTX-naïve early RA (n=5) compared with that in OA (n=5; Fig. 1D).

Multiplex cytokine measurement in MTX-naïve early RA and OA

We selected five inflammatory cytokines (IFN-γ, IL-17A, IL-12, IL-6, and TNF-α) and evaluated expression levels using MCA. Significant correlations were observed between IFN-γ and IL-17A/IL-6/IL-12/TNF-α (Fig. 2A). DAS28-ESR was correlated with IFN-γ, IL-6, and IL-12, and plasma levels of IFN-γ showed the highest correlation coefficient with DAS28-ESR in patients with early RA (*Rho*=0.553, p=0.0025, Fig. 2B).

Evaluation of clinical outcomes according to plasma IFN-γ and IL-17A in patients with established RA with low disease activity

After 6 months follow up, patients with established RA with low disease activity (n=34) were divided into two groups, *i.e.* the drug reduction and drug maintenance/relapse after drug reduc-

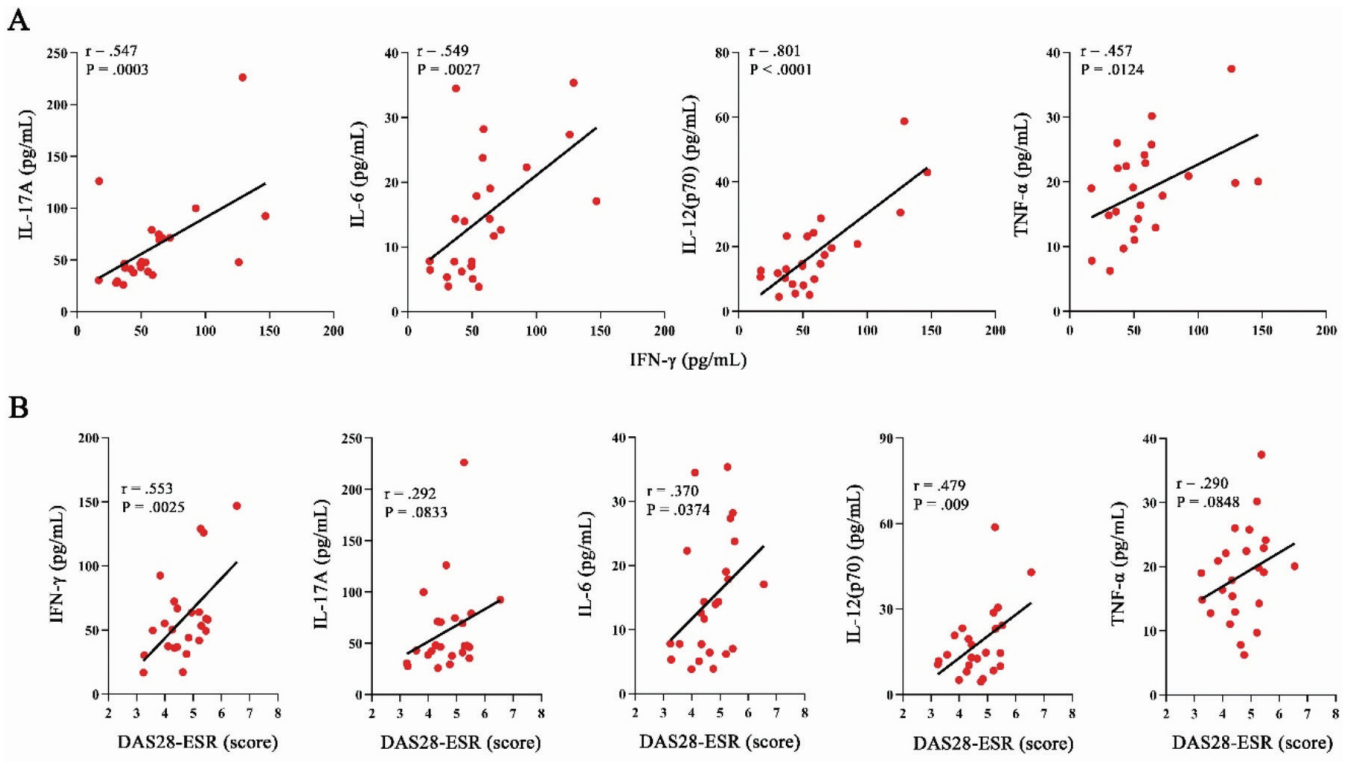

Fig. 2. Multiplex cytokine assay of MTX-naïve patients with early RA and baseline plasma IFN-γ and IL-17A levels in patients with established RA with low disease activity. **A**: Correlation between plasma IFN-γ and IL-17A/IL-6/IL-12/TNF-α. **B**: Correlation between DAS28-ESR and plasma cytokine levels.

tion groups. The drug reduction group was defined as having low disease activity for at least 6 months after medications were modified, and the relapse after drug reduction was defined as having high disease activity after medications were modified. The choice as to whether medications were reduced or maintained was made after consultation between physician and patient. Plasma levels of IFN-γ and IL-17A were measured at the time of achieving low disease activity and compared between the drug reduction group (n=14) and the drug maintenance/relapse after drug reduction group (n=20). Plasma levels of IFN-γ and IL-17A in the drug reduction group were lower than those in the drug maintenance/relapse after drug reduction group (13.61±5.54 *vs*. 28.26±17.69 pg/mL, $p<0.001$, and 10.91±3.92 *vs*. 21.04±12.81 pg/mL, $p<0.001$, respectively; Fig. 3). Detailed information on drug modification and flow chart are described in Supplementary Table S1 and Supplementary Figure S1.

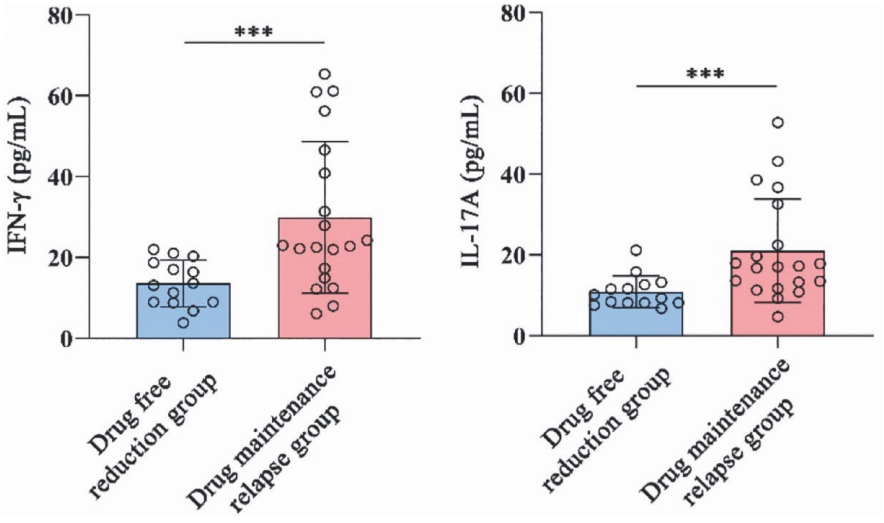

Fig. 3. Baseline plasma levels of IFN-γ and IL-17A in established RA patients with low disease activity. Comparison of baseline plasma levels of IFN-γ and IL-17A in the established RA with drug reduction group (n=14) and drug maintenance/relapse after dose reduction group (n=20). **$p<0.01$.

Discussion

In this study, we evaluated gene expression patterns of CD4⁺ T cells in MTX-naïve early RA and assessed the roles of cytokines as biomarkers of clinical treatment response. MTX-naïve early RA was associated with upregulation of IFN-γ and IL-17A gene signatures. Moreover, plasma IFN-γ levels correlated well with other inflammatory cytokines in MTX-naïve patients with early RA. Correlation coefficient of DAS-ESR was highest with plasma IFN-γ levels in these patients. Notably, in patients with established RA with low disease activity, patients with low plasma IFN-γ and IL-17A showed tolerability on drug reduction.

Over 100 genes are associated with RA, and many RA-associated risk factor genes are dominantly expressed in CD4⁺ T cells (11). Additionally, the gene enhancer region (H3K4me1) is

uniformly overlapped with most effector and memory CD4+ T cells (12), highlighting the roles of CD4+ T cell in RA pathogenesis. In this study, we showed that IFN-γ- and IL-17A-associated genes were significantly increased in patients with early RA, suggesting the potential roles of Th1 and Th17 cells in early-stage RA pathogenesis. Interestingly, five upregulated genes in MTX-naïve patients with early RA were related to both IFN-γ and IL-17A signalling, and these genes may provide important insights into molecular targeting in RA. Based on the Th1/Th2 hypothesis, IFN-γ (the main cytokine of Th1) is considered the main pathologic cytokine in RA pathogenesis. However, animal studies have shown conflicting results, and IFN-γ blocking agents failed to meet the primary endpoint in phase II studies. However, a recent study found that five IFN-related genes (*MxA*, *IFI6*, *OAS1*, *ISG15*, and *IFI44L*) were highly expressed in MTX-naïve RA compared with that in established RA, and this upregulation was correlated with disease activity and predicted treatment resistance in MTX-naïve RA (7). In the current study, *OAS1* and *IFI44L* were also significantly increased in CD4+ T cells of MTX-naïve patients with early RA. Thus, the roles of IFN-γ in RA pathogenesis have been reconfirmed, and several mechanisms have been elucidated as follows: 1) IFN-γ activates Janus kinase-signal transducer and activator of transcription 1 signalling, and 2) augmentation of ACPA production via inducing major histocompatibility complex class II in antigen-presenting cells (8). Our findings and previous study (7) suggested that IFN-γ-related genes could play important roles in RA pathogenesis, particularly during the early phase.

Th17 cells are the primary producers of IL-17A among all CD4+ T cells (13), and Th17 cells are now considered the main pathological cells in RA pathogenesis (4). In patients with RA, collagen type II reactive T cells produce more IFN-γ than IL-4, suggesting an autoreactive T-cell skew toward the Th1 phenotype (14). Patients with RA with higher proportions of circulating Th1 and Th17 cells show poor clinical responses, indicating the potential predictive roles of Th1 and Th17 in treatment responses (15). Discontinuation or tapering of DMARDs is a major goal of RA treatment; however, few predictors of this strategy have been identified. In the current study, established RA with low disease activity could tolerate drug reduction when the plasma IFN-γ and IL-17A levels were low. Thus, analysis of plasma levels of IFN-γ and IL-17A may be useful for prediction of tapering RA medication.

Dual overexpression of IFN-γ- and IL-17A-related gene signatures is an interesting phenomenon. Th1 and Th17 signals typically suppress each other (16); however, in this study, both IFN-γ- and IL-17A-related genes were upregulated in MTX-naïve early RA. This result could be explained by several hypotheses. First, Th1 and Th17 signals could both be upregulated to suppress each other. Type II collagen-stimulated CD4+ T cells from patients with RA exhibit increased IFN-γ and IL-17 production (17). Theoretically, post-translational modification (PTM)-induced autoreactive peptide can trigger both Th1 and Th17 dominant responses. We hypothesise that initially enhanced Th1 and Th17 responses induced by the PTM peptide could counteract each other, particularly during the early stages of RA pathogenesis. Second, recent studies have shown that some Th17 cells can produce IFN-γ. These IFN-γ-positive Th17 cells are increased in the synovial fluid of patients with juvenile inflammatory arthritis (18), suggesting the pathological roles of this Th17 subset in inflammatory arthritis. Our study demonstrated the importance of IFN-γ and IL-17A in early RA and highlighted the roles of these cytokines in drug reduction. However, further studies are needed to determine the specific mechanisms of these two cytokines in early RA pathogenesis.

There were several limitations to the current study. First, the microarray data for MTX-naïve early RA were obtained from a relatively small sample size. However, our results were consistent with a previous study of MTX-naïve early RA (7). Second, the follow-up duration for patients with established RA with low disease activity was only 6 months, and the number of patients was relatively low. Third, drug tapering was not decided in a double blinded manner. Although our study was not a double-blind randomised study, we believe our findings are still beneficial as a pilot study. Finally, all patients were seropositive for RA; therefore, the results from the current study cannot be generalised to patients who are seronegative for RA.

In conclusion, our findings showed that increased IFN-γ- and IL-17A-related gene signatures in CD4+ T cells were prominent in early RA. Furthermore, plasma IFN-γ levels could properly reflect disease activity in patients with early RA. Patients with low plasma levels of IFN-γ and IL-17A tended to show a fair clinical response, allowing for reduction of the dosage of DMARDs in established RA.

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