Clinical Impact of Interferon-γ in Anti-Citrullinated Protein Antibody-Positive Rheumatoid Arthritis

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Research

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Abstract

Background: $CD4^+$ T cells are crucial for the pathogenesis of rheumatoid arthritis (RA). The roles of gene overexpression in $CD4^+$ T cells and the predictive roles of Th1- and Th17-related cytokines have not been clearly defined in patients with RA. Here, we investigated gene expression levels in $CD4^+$ T cells in methotrexate (MTX)-naïve early RA (eRA) and evaluated changes in $CD4^+$ T-cell-related cytokines during eRA.

Methods: Patients with anti-citrullinated protein antibody (ACPA)-positive MTX-naïve eRA were recruited. Patients with osteoarthritis were evaluated as controls. Microarray analysis was used to identify overexpressed genes in $CD4^+$ T cells, and reverse transcription quantitative polymerase chain reaction was used to validate the identified genes. Plasma cytokine levels were measured, and correlations with disease activity were assessed.

Results: Thirty-four genes showed overexpression in $CD4^+$ T cells from patients with ACPA-positive MTX-naïve eRA compared with controls. Nineteen were related to interferon (IFN)-γ, and nine were related to interleukin (IL)-17A; five were related to both IFN-γ and IL-17A. Plasma levels of five cytokines were elevated in the ACPA-positive MTX-naïve eRA group compared with those in the control group, and C-X-C motif chemokine ligand 10 was significantly elevated in the ACPA-positive MTX-naïve eRA group compared with that in the established active RA and OA groups. In established RA with low disease activity, the drug-free remission group/drug reduction group showed significantly lower IFN-γ and IL-17A levels than the drug maintenance group and relapse after drug reduction group.

Conclusion: Our T2 research of IFN-γ emphasized that, not only the Th17 immune response, but the Th1 immune response is also very important for the RA pathogenesis. Concurrent increase in IFN-γ and IL-17 were observed in active stage of ACPA-positive MTX-naïve eRA, and the plasma levels of them could be useful for new clinical biomarkers before and/or after treatment of RA.

Background

Rheumatoid arthritis (RA) is a 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. Reducing arthritis and preventing additional structural damage in joints are the most important goals in RA treatment, and various agents have been develop to achieve these goals [1].

Synovitis and pannus formation are the cornerstone pathologic components of RA pathogenesis. Inflamed synovium from patients with RA shows increased fibroblast-like synoviocyte (FLS) and immune cell numbers [2]. Moreover, T-cell responses to type II collagen are enhanced, particularly in early RA (eRA) [3]. These immune responses can be explained by post-translational modifications (PTM) [4], which modify the architecture of peptides to generate autoantigens. The most well-known PTM in RA is citrullination, and autoantibodies against citrullinated peptide (ACPAs) are crucial in the diagnosis and

pathogenesis of RA [5]. These modified antigens activate B cells to produce autoantibodies and stimulate antigen-presenting cells (APCs), FLSs, and T cells.

Helper T cells (cluster of differentiation [CD] 4^+ T cells) are important elements in adaptive immune systems and can be divided into several subtypes [6]. In inflamed RA synovium, $CD4^+$ T cells are predominant [7], suggesting that $CD4^+$ T cells may be cornerstone immune cells in RA pathogenesis. Type 17 helper T cells (Th17 cells) have been identified in RA [8, 9], and before identifying Th17 cells, interferon (IFN)-γ-secreting Th1 cells have been thought as major pathologic cells in RA development [10]. IFN-γ is the main cytokine produced by Th1 cells [6], and autoreactive Th1 cells are increased in the peripheral blood and synovial fluid of patients with RA [11, 12]. However, the effects of IFN-γ in animal models are unclear [13–15]. Furthermore, trials of IFN-γ and IFN-γ-blocking therapies have shown conflicting therapeutic potentials in patients with RA [16, 17]. Thus, IFN-γ may have various effects in RA, and further studies are required.

Recent genetic and epigenetic studies have revealed genes associated with the pathogenesis of RA [18]. For example, human leukocyte antigen (HLA)-DRB1 was the first genetic risk factor shown to be associated with RA development [19], and such genetic predisposition facilitates autoimmunity of PTM autoantigens [20]. Based on a genome-wide association study, approximately 100 genetic susceptibility single nucleotide polymorphisms (SNPs) for RA were found [21]. However, most of these SNPs were not resided in protein-coding regions, and further studies of the functions and effects of the identified genes are required. Relevantly, signal transducer and activator of transcription 3 (STAT3)-inducible genes are overexpressed in $CD4^+$ T cells from patients with undifferentiated arthritis who eventually developed seronegative RA [22]. Expression quantitative trait loci (eQTLs) are useful tools for identifying SNPs affecting disease-specific gene expression. Notably, RA-related eQTLs have been shown to be enriched in $CD4^+$ T cells [23, 24]. Moreover, the IFN gene signature has shown to be increased in the whole blood of patients with methotrexate (MTX)-naïve RA compared with that in patients with established RA, and these changes were correlated with RA disease activity in MTX-naïve RA [25]. However, gene expression levels in $CD4^+$ T cells in patients with MTX-naïve seropositive RA have not been clarified.

Accordingly, in this study, we investigated gene expression levels in $CD4^+$ T cells in ACPA-positive MTX-naïve eRA and evaluated changes in $CD4^+$ T-cell-related cytokines during eRA. The goal of this study is present to translate research from animal models (T1 research) to real patients and to identify novel biomarkers for future T2 research.

Methods

Patients

Patients were recruited from Konkuk University Hospital and Ho-Youn Kim's Clinic for Arthritis and Rheumatism. Inclusion criteria for MTX-naïve, eRA were as follows: (1) fulfilled the 1987 American College of Rheumatology (ACR) revised classification criteria for RA or 2010 ACR/European League

Against Rheumatism classification criteria for RA [26, 27]; (2) ACPA positive; (3) over 18 years old; (4) never taken disease-modifying antirheumatic drugs (DMARDs) prior to enrolment; (5) symptom duration of arthralgia 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 were also recruited and were all positive for either ACPA or rheumatoid factor. Patients with established RA were divided into low disease activity or moderate to high disease activity; patients who achieved a disease activity score (DAS) 28-erythrocyte sedimentation rate (ESR) less than 3.2 were defined as having low disease activity, whereas those with DAS28-ESR of greater than 3.2 were defined as having moderate to high disease activity. Patients with osteoarthritis (OA) were recruited as a control group. Synovial fluid was obtained from patients with RA or OA when performing arthrocentesis for therapeutic purposes. In patients with ACPA-positive, MTX-naïve eRA, cytokine levels were analysed before DMARDs were started. In patients with established RA with low disease activity, cytokines were measured at the time when DAS28-ESR less than 3.2 was achieved. The disease course in patients with established RA with low disease activity was followed for 6 months, and patients were divided into the drug-free remission group, drug reduction group, drug maintenance group, and relapse after drug reduction group. Detailed information for each group is summarized in Supplementary Table 1. 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).

Enzyme-linked immunosorbent assay (ELISA)

We measured C-X-C motif chemokine ligand 10 (CXCL10) protein concentrations in plasma samples isolated from the whole blood of patients by ELISA (Abcam, Cambridge, MA, USA), according to the manufacturer’s instructions. Absorbance at 450 nm was read on a VersaMax microplate reader with SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA).

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, interleukin (IL)-17A, IL-6, IL-12, and tumour necrosis factor (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

All data were expressed as means ± standard deviations or medians with interquartile ranges. Continuous variables were compared using Student's t tests or one-way analysis of variance and Bonferroni's multiple comparisons test. Correlation analysis was performed using Pearson's correlation coefficients. In all analyses, differences with *P* values of less than 0.05 were considered significant, and analyses were performed using SPSS version.20.0 (SPSS, Chicago, IL, USA).

Results

Upregulated genes in ACPA-positive, MTX-naïve eRA

We performed microarray analysis of peripheral blood $CD4^+$ T cells from ACPA-positive, MTX-naïve eRA (14 patients) and OA (9 patients); expression data for 103 genes were obtained. A heatmap of total gene expression is shown in Fig. 1A. In total, 101 genes were upregulated by more than 3-fold in ACPA-positive, MTX-naïve eRA 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; Table 1). 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 (Tables 2 and 3). Interestingly, five genes (*IFIT3*, *RSAD2*, *GBP1*, *CCL20*, and *OASL*) were related to both IFN-γ and IL-17A.

Validation of the overexpressed genes in ACPA-positive, MTX-naïve eRA and measurement of CXCL10 in peripheral blood

Ten genes (*CXCL10*, *IFIT3*, *IFIT1*, *RSAD2*, *TNFAIP6*, *SLAMF7*, *IFI44*, *IFIH1*, *OASL*, *HERC5*) were selected for validation by RT-qPCR. Except *SLAMF7* and *IFI44*, all genes showed overexpression in ACPA-positive, MTX-naïve eRA (n = 5) compared with that in OA (n = 5; Fig. 1C). Next, we measured protein levels of the most prominently overexpressed gene, *CXCL10*, in plasma samples from patients with ACPA-positive, MTX-naïve eRA, established RA, or OA. CXCL10 levels were significantly higher in ACPA-positive, MTX-naïve eRA than in established RA or OA (Fig. 1D). However, plasma CXCL10 levels did not correlated with clinical disease activity, DAS28-ESR, or other laboratory parameters (data not shown).

Comparison of IFN-γ levels between MCAs and sandwich ELISA in ACPA-positive, MTX-naïve eRA

To compare the sensitivity of ELISA and MCA, IFN-γ levels in the plasma of the same ACPA-positive, MTX-naïve eRA patient (N = 20) were measured. Mean plasma IFN-γ levels were higher in MCA than ELISA (39.2 ± 19.6 vs 7.5 ± 4.6 pg/ml, $P < 0{\cdot}0001$). The detectable level of IFN-γ was wider in MCA than in ELISA (0.61–2500 vs 15.6–1000 pg/ml, respectively).

Multiplex cytokine measurement in ACPA-positive, MTX-naïve eRA and OA

We selected five cytokines representing Th1 cells (IFN-γ), Th17 cells (IL-17A), APCs (IL-12), or pro-inflammatory cytokines (IL-6 and TNF-α) and evaluated expression levels using MCA. All five cytokines were significantly elevated in ACPA-positive, MTX-naïve eRA (N = 28) compared with OA (N = 16; Fig. 2A). Significant correlations were observed between IFN-γ and IL-17A/IL-6/IL-12/TNF-α (Fig. 2B). DAS28-ESR were correlated with IFN-γ, IL-6, and IL-12 (Fig. 2C). Moreover, patients with a relatively low inflammatory status in eRA (high sensitivity C-reactive protein [hs-CRP] < 0.5 mg/dL; N = 20) exhibited higher levels of

these five cytokines than patients with OA (N = 16; Fig. 2D). $CD4^+$ T cell-related cytokines, including IFN-γ and IL-17A, were dramatically upregulated in the peripheral blood of patients with ACPA-positive, MTX-naïve eRA.

Plasma cytokine measurement via MCA in patients with established RA and OA controls

We measured the plasma levels of 5 cytokines in patients with established RA (N = 58) and OA (N = 16) group. Among established RA, 34 of 58 patients achieved low disease activity (DAS28-ESR ≤ 3.2) after DMARDs treatment. Both IFN-γ and IL-17A levels were elevated in patients with established RA with low disease activity compared with those in OA group (Supplementary Fig. 1A). In contrast, 25 of 58 patients remained with moderate to high disease activity (DAS28-ESR > 3.2), all five cytokines were significantly increased, compared with the OA group (Supplementary Fig. 2A). In patients with established RA with low disease activity, plasma IFN-γ levels were correlated with plasma IL-17A, IL-6, IL-12, and TNF-α levels (Supplementary Fig. 1B). Disease activity was not significantly correlation with plasma IFN-γ, IL-6, IL12, or TNF-α (Supplementary Fig. 1C). In patients with established RA with moderate to high disease activity, plasma IFN-γ levels were correlated with IL-17A, IL-12, and TNF-α (Supplementary Fig. 2B). DAS28-ESR levels were significantly correlated with all five cytokine plasma levels (Supplementary Fig. 2C).

Notably, in comparison of OA, the mean levels of IFN-γ was significantly higher in early stage of RA as well as in established RA with low disease activity. Therefore, in addition to IL-17, plasma IFN-γ levels could be useful for diagnostic biomarker in early stage of RA, as well as responsible for chronic inflammatory status of patients with DMRDs treated established RA.

Evaluation of clinical outcome according to plasma IFN-γ in patients with low disease activity

After 6 months follow up, RA patients with LDA (N = 34) were able to divide into 3 group of clinical outcome. To evaluate the impact of IFN-γ on clinical outcome, the plasma levels of IFN-γ and IL-17A were measured at time of achieving low disease activity and compared with 3 different clinical outcomes after approximately 6 months. The plasma levels of IFN-γ in drug free / drug reduction group were lower than drug maintenance and relapse group (13.61 ± 5.54 vs 28.26 ± 17.69 vs 39.15 ± 18.59 pg/mL, respectively, Fig. 3A). The plasma level of IL-17A showed similar results with IFN-γ between drug free / drug reduction, drug maintenance, and relapse group (10.91 ± 3.78 vs 21.33 ± 13.49 vs 19.42 ± 2.12 pg/mL, respectively, Fig. 3B).

Discussion

In this study, we evaluated gene expression patterns in ACPA-positive, MTX-naïve eRA and assessed the roles of cytokines as biomarkers of clinical treatment response. ACPA-positive, MTX-naïve eRA was associated with upregulation of IFN-γ and IL-17A gene signatures. Moreover, among patients with ACPA-positive, MTX-naïve eRA or established RA with moderate to high disease activity, plasma IFN-γ levels

correlated well with other inflammatory cytokines, including IL-12 and IL-17A, and with clinical disease activity. Notably, in patients with RA with low disease activity, plasma IFN-γ and IL-17A levels may enable to discriminate the patients who can tolerate drug tapering or achieve drug-free remission.

Over 100 genes have shown to be associated with RA [28], and many RA-associated risk factor genes are dominantly expressed in $CD4^+$ T cells [23]. Additionally, the gene enhancer region (H3K4me1) is uniformly overlapped with most effector and memory $CD4^+$ T cells [24], 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 these patients, suggesting the potential roles of Th1 and Th17 cells in early stage RA pathogenesis. Interestingly, five upregulated genes in patients with ACPA-positive, MTX-naïve eRA were related to both IFN-γ and IL-17A signalling, and these genes may provide important insights into molecular targeting in RA.

IFN-γ plays crucial roles in primary immune defence against microbial infection and is considered a pro-inflammatory cytokine. However, IFN-γ has been shown to have both pro-inflammatory and anti-inflammatory activities under different biological contexts [29]. Importantly, RA synovium exhibits increased IFN-γ expression [30], and IFN-γ^+ $CD4^+$ T cells are dominant in RA synovial fluid [31]. 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 [12]. However, animal models of RA have shown conflicting results; some have suggested that IFN-γ induces arthritis [15], whereas others have demonstrated that IFN-γ has protective effects against arthritis [13, 14]. Furthermore, IFN-γ reduces osteoclast formation, which is essential for joint destruction in RA [32]. After the discovery of Th17 cells, IFN-γ was found to suppress arthritis by regulating Th17 cell development [33, 34]. 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 [25]. In the current study, *OAS1* and *IFI44L* were also significantly increased in ACPA-positive, MTX-naïve eRA. Furthermore, increased CXCL10 levels in MTX-naïve eRA have been found to be correlated with disease activity [35]. Similarly, we showed that plasma CXCL10 levels were significantly elevated in MTX-naïve eRA compared with that in established RA. Our findings 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-17 among all $CD4^+$ T cells [8]. Additionally, Th17 cells are now considered the main pathological cells in RA pathogenesis [36, 37]. IL-17 induces osteoclastogenesis [38], and Th17 levels are correlated with RA disease activity [39]. Moreover, IL-17 is involved in the pathological processes of early or preclinical RA; IL-17 blocking agents show inferior therapeutic responses compared with other biologic DMARDs, including TNF-α inhibitors, in established RA [40]. 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 response [41]. Discontinuation or tapering of DMARD is a major goal of RA treatment; however, few predictors of this strategy have been identified [42]. In the current study, IFN-γ- and IL-17A-associated genes and *CXCL10* were significantly elevated in ACPA-

positive, MTX-naïve eRA. Plasma IFN-γ and IL-17A levels were significantly elevated in ACPA positive MTX naïve eRA even in patients with a low inflammatory status. Additionally, plasma IFN-γ and IL-17A levels in established RA with low disease activity were associated with drug-free or dose reduction at 6 months. Thus, these cytokines may play crucial roles in early RA pathogenesis and may be useful for prediction of tapering or discontinuing 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 [43, 44]; however, in this study, both IFN-γ- and IL-17A-related genes were upregulated in ACPA-positive, MTX-naïve eRA. This result could be explained by several hypotheses. First, Th1 and Th17 signals could both be upregulated and both suppress the other, and this could be described as “sleeping with enemy”. *In vitro*, autoreactive PTM peptide promotes Th17 induction in RA [45, 46]. Moreover, autoreactive T-cell responses to type II collagen show skewing toward Th1 [12]. In animal models, collagen injection induces Th1 polarisation [47], and STAT3 inhibition ameliorates arthritis severity by reducing the Th17 population in a collagen-induced arthritis (CIA) model [48]. In addition, type II collagen-stimulated $CD4^+$ T cells from patients with RA exhibit increased IFN-γ and IL-17 production [49]. Theoretically, PTM autoreactive peptide can induce both Th1 and Th17 dominant responses. IFN-γ suppresses Th17 differentiation in CIA and experimental autoimmune encephalitis by inducing IDO in APCs or via suppressor of cytokine signalling (SOCS) [33, 50]. SOCS has several subtypes and acts as an intracellular regulator for the Janus kinase (JAK)/STAT pathway. SOCS1 is critical a negative regulator of IFN-γ/STAT1 signalling and suppresses Th1 differentiation [51]. Furthermore, SOCS1 simultaneously enhances Th17 differentiation via STAT3 signalling, and SOCS3 modulates Th1/Th17 responses with SOCS1 [51]. Interestingly, IFN-γ also accelerates $Foxp3^+$ regulatory T cell (Treg) induction and enhances the regulatory functions of Tregs [52]. The protective roles of Tregs on systemic autoimmunity have been extensively studied, and Tregs have been shown to reduce Th17-mediated autoimmune responses [53]. In contrast, IL-17 downregulates Th1 induction via IL-12Rβ2 suppresion [54], and the Th17-inducing transcription factor STAT3 blocks *IL-12/p35* gene expression, thereby suppressing the Th1 response [55]. Citrullinated peptide reacts with the HLA-DRβ shared epitope and stimulates $CD4^+$ T-cell responses; therefore, these PTM peptide-mediated $CD4^+$ T-cell responses may be only observed in ACPA-positive RA. We hypothesise that initially enhanced Th1 and Th17 responses induced by the PTM peptide could counteract each other, particularly during the early stages of ACPA-positive RA pathogenesis. Second, recent studies have shown that some Th17 cells can produce IFN-γ [56]. These IFN-γ-positive Th17 cells are increased in the synovial fluid of patients with juvenile inflammatory arthritis [57], suggesting the pathological roles of this Th17 subset in inflammatory arthritis. Our study demonstrated the importance of IFN-γ and IL-17A in ACPA-positive eRA and highlighted the roles of these cytokines in drug-free remission. However, further studies are needed to determine the specific mechanisms of ACPA-positive eRA pathogenesis.

MCAs have several advantages compared with conventional ELISA. Because the minimal detectable range of MCA is lower than that of ELISA, MCA can be used to identify relatively low levels of cytokines in blood, and detect multiple cytokines at the same time using the same sample. MCA using antibody

capture with magnetic beads and razor detection can be used to measure multiple cytokines simultaneously and can detect these cytokines at levels close to the physiological concentration [58]. In our study, combinations of 5 cytokines (IFN-γ, IL-6, IL-17, TNF-α, IL-12) can detect the stable reference ranges of the same analytes and enable to determine the precise concentrations of these cytokines in patients with ACPA-positive, MTX-naïve eRA.

There were several limitations to the current study. First, the microarray data for ACPA-positive, MTX-naïve eRA were obtained from a relatively small sample size. However, our results were consistent with a previous study of MTX-naïve eRA [25]. Second, the follow-up duration for patients with established RA with low disease activity was only 6 months. Third, all patients were seropositive RA; therefore, the results from the current study cannot be generalised to seronegative RA.

Conclusions

In conclusion, our findings showed that increased IFN-related gene signalling in $CD4^+$ T cells was critical in ACPA-positive eRA. Simultaneous increases in the concentrations of IFN-γ and IL-17 in the blood could affects disease onset and cause associated with clinical disease activity after achieving low disease activity in established RA. Patients with low circulating levels of IFN-γ and IL-17A tended to show a fair clinical response, allowing reduction of the dosage of DMARDs over 6 months in established RA. In particular, the continued decrease in blood levels of IFN-γ suggested the possibility of achieving complete remission in the future (the concept of complete cytokine remission). Our current analysis of gene expression and plasma IFN-γ levels provides important insights into the discovery of new biomarkers of disease onset and the chronic pathological process in patients with RA. The potential roles of IFN-γ in RA were confirmed by our results, and these findings could have implications in translation research from bench to bedside.

Abbreviations

RA: rheumatoid arthritis; FLS: fibroblast-like synoviocyte; eRA: early rheumatoid arthritis; PTM: post-translational modification; ACPA: anti-citrullinated protein antibody; APC: antigen-presenting cell; CD: cluster of differentiation; Th17: type 17 helper T cell; IFN: interferon; HLA: human leukocyte antigen; SNP: single nucleotide polymorphism; STAT: signal transducer and activator of transcription; eQTL: expression quantitative trait loci; MTX: methotrexate; ACR: American college of rheumatology; DMARD: disease-modifying antirheumatic drug; DAS: disease activity score; ESR: erythrocyte sedimentation rate; OA: osteoarthritis; CXCL: C-X-C motif chemokine ligand; ELISA: Enzyme-linked immunosorbent assay; MCA: multiplex cytokine assay; IL: interleukin; TNF: tumor necrosis factor; RT-qPCR: reverse transcription quantitative polymerase chain reaction; PBMC: peripheral blood mononuclear cell; PBS: phosphate-buffered saline; hs-CRP: high sensitivity C-reative protein; CIA: collagen-induced arthritis; SOCS: suppressor of cytokine signaling; JAK: janus kinase; Treg: regulatory T cell

Declarations

Ethics approval and consent to participate

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).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HYK and JHJ designed the study and achieved clinical data. HYK developed the theory. KL performed the experiments, statistical analysis, and formed the figures. SHK performed the multiplex cytokine measuring experiment. HRK and SHL interpreted the data and recruited the clinical information of the enrolled patients. HKM wrote the manuscript and did the statistical analysis. All authors read and approved the final manuscript.

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Tables

Table 1.

Selected over-expressed 34 genes in ACPA-positive, MTX-naïve eRA by 6-fold change

Gene Accession	Gene Symbol	ACPA-positive, MTX-naïve eRA/OA. Fold change	*P* value	Gene Description
NM_001565	*CXCL10*	166.062	0.022	chemokine (C-X-C motif) ligand 10
NM_001031683	*IFIT3*	64.937	0.005	interferon-induced protein with tetratricopeptide repeats 3
NM_001547	*IFIT2*	36.972	0.006	interferon-induced protein with tetratricopeptide repeats 2
NM_001291469	*CCL4L2*	26.572	0.001	chemokine (C-C motif) ligand 4-like 2
NM_002984	*CCL4*	24.244	0.002	chemokine (C-C motif) ligand 4
AK092813	*IFIT1*	23.994	0.009	interferon-induced protein with tetratricopeptide repeats 1
NM_000575	*IL1A*	23.857	0.003	interleukin 1, alpha
NM_080657	*RSAD2*	18.130	0.014	radical S-adenosyl methionine domain containing 2
AB208912	*GBP1*	14.195	0.008	guanylate binding protein 1, interferon-inducible
NM_001001437	*CCL3L3*	13.427	0.001	chemokine (C-C motif) ligand 3-like 3
NM_000600	*IL6*	12.948	0.027	interleukin 6
ENST00000325042	*LOC541472*	11.680	0.019	uncharacterized LOC541472
NM_007115	*TNFAIP6*	11.397	0.019	tumor necrosis factor, alpha-induced protein 6
NM_001130046	*CCL20*	11.061	0.003	chemokine (C-C motif) ligand 20
NM_006417	*IFI44*	10.553	0.005	interferon-induced protein 44
NM_001282588	*SLAMF7*	9.976	0.034	SLAM family member 7
NM_020980	*AQP9*	9.652	0.007	aquaporin 9
NM_022168	*IFIH1*	9.363	0.003	interferon induced with helicase C domain 1
NM_000963	*PTGS2*	8.455	0.010	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
NM_006820	*IFI44L*	8.321	0.032	interferon-induced protein 44-like

NM_001261825	*OASL*	8.057	0.004	2-5-oligoadenylate synthetase-like
NM_016323	*HERC5*	7.906	0.006	HECT and RLD domain containing E3 ubiquitin protein ligase 5
NM_018370	*DRAM1*	7.287	0.019	DNA-damage regulated autophagy modulator 1
NM_014314	*DDX58*	7.063	0.006	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
AB044545	*OAS3*	7.027	0.012	2-5-oligoadenylate synthetase 3, 100kDa
NM_002852	*PTX3*	7.022	0.023	pentraxin 3, long
NM_001143818	*SERPINB2*	6.843	0.012	serpin peptidase inhibitor, clade B (ovalbumin), member 2
NM_152703	*SAMD9L*	6.520	0.014	sterile alpha motif domain containing 9-like
NM_207315	*CMPK2*	6.423	0.007	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial
NM_001032409	*OAS1*	6.315	0.007	2-5-oligoadenylate synthetase 1, 40/46kDa
NM_014398	*LAMP3*	6.240	0.049	lysosomal-associated membrane protein 3
NR_001458	*MIR155HG*	6.213	0.013	MIR155 host gene (non-protein coding)
NR_037867	*LOC731424*	6.143	0.045	uncharacterized LOC731424
NM_012420	*IFIT5*	6.083	0.002	interferon-induced protein with tetratricopeptide repeats 5

Table 2.

List of the 19 IFN-γ related genes of 6-fold ACPA-positive, MTX-naïve eRA specific genes

Gene Accession	Gene symbol	ACPA-positive, MTX-naïve eRA/OA. Fold change	*P* value	Gene Description
NM_001565	*CXCL10*	166.062	0.022	chemokine (C-X-C motif) ligand 10
NM_001031683	*IFIT3*	64.937	0.005	interferon-induced protein with tetratricopeptide repeats 3
NM_001547	*IFIT2*	36.972	0.006	interferon-induced protein with tetratricopeptide repeats 2
NM_001291469	*CCL4L2*	26.572	0.001	chemokine (C-C motif) ligand 4-like 2
NM_002984	*CCL4*	24.244	0.002	chemokine (C-C motif) ligand 4
AK092813	*IFIT1*	23.994	0.009	interferon-induced protein with tetratricopeptide repeats 1
NM_080657	*RSAD2*	18.130	0.014	radical S-adenosyl methionine domain containing 2
AB208912	*GBP1*	14.195	0.008	guanylate binding protein 1, interferon-inducible
NM_007115	*TNFAIP6*	11.397	0.019	tumor necrosis factor, alpha-induced protein 6
NM_001130046	*CCL20*	11.061	0.003	chemokine (C-C motif) ligand 20
NM_006417	*IFI44*	10.553	0.005	interferon-induced protein 44
NM_001282588	*SLAMF7*	9.976	0.034	SLAM family member 7
NM_022168	*IFIH1*	9.363	0.003	interferon induced with helicase C domain 1
NM_006820	*IFI44L*	8.321	0.032	interferon-induced protein 44-like
NM_001261825	*OASL*	8.057	0.004	2-5-oligoadenylate synthetase-like
NM_016323	*HERC5*	7.906	0.006	HECT and RLD domain containing E3 ubiquitin protein ligase 5
AB044545	*OAS3*	7.027	0.012	2-5-oligoadenylate synthetase 3, 100kDa
NM_001032409	*OAS1*	6.315	0.007	2-5-oligoadenylate synthetase 1, 40/46kDa
NM_012420	*IFIT5*	6.083	0.002	interferon-induced protein with tetratricopeptide repeats 5

Table 3.

List of the 8 IL-17A related genes of 6-fold ACPA-positive, MTX-naïve eRA specific genes

Gene Accession	Gene symbol	ACPA-positive, MTX-naïve eRA/OA. Fold change	*P* value	Gene Description
NM_001031683	*IFIT3*	64.937	0.005	interferon-induced protein with tetratricopeptide repeats 3
NM_080657	*RSAD2*	18.130	0.014	radical S-adenosyl methionine domain containing 2
AB208912	*GBP1*	14.195	0.008	guanylate binding protein 1, interferon-inducible
NM_001130046	*CCL20*	11.061	0.003	chemokine (C-C motif) ligand 20
NM_001261825	*OASL*	8.057	0.004	2-5-oligoadenylate synthetase-like
NM_152703	*SAMD9L*	6.520	0.014	sterile alpha motif domain containing 9-like
NM_207315	*CMPK2*	6.423	0.007	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial
NM_014398	*LAMP3*	6.240	0.049	lysosomal-associated membrane protein 3

Figures

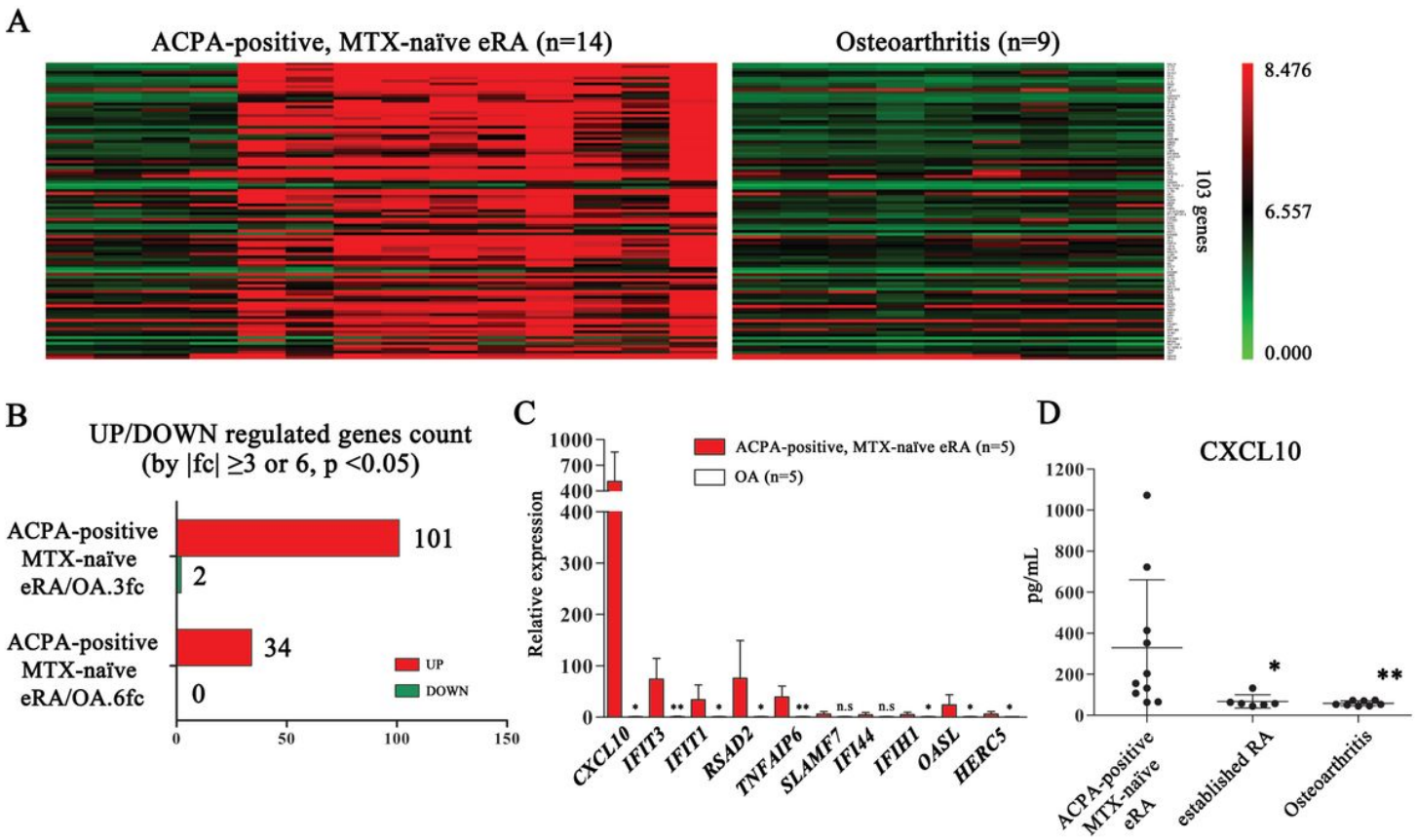

Figure 1

Microarray analysis and RT-qPCR of ACPA-positive, MTX-naïve early RA (eRA). (A) Total microarray analysis data of CD4+ T cells from patients with ACPA-positive, MTX-naïve eRA or OA. (B) Three-fold and six-fold upregulated genes in ACPA-positive, MTX-naïve eRA. (C) Validation of 10 upregulated genes in ACPA-positive, MTX-naïve eRA by RT-qPCR. (D) Plasma levels of CXCL-10 in ACPA-positive, MTX-naïve eRA, established RA, and OA.

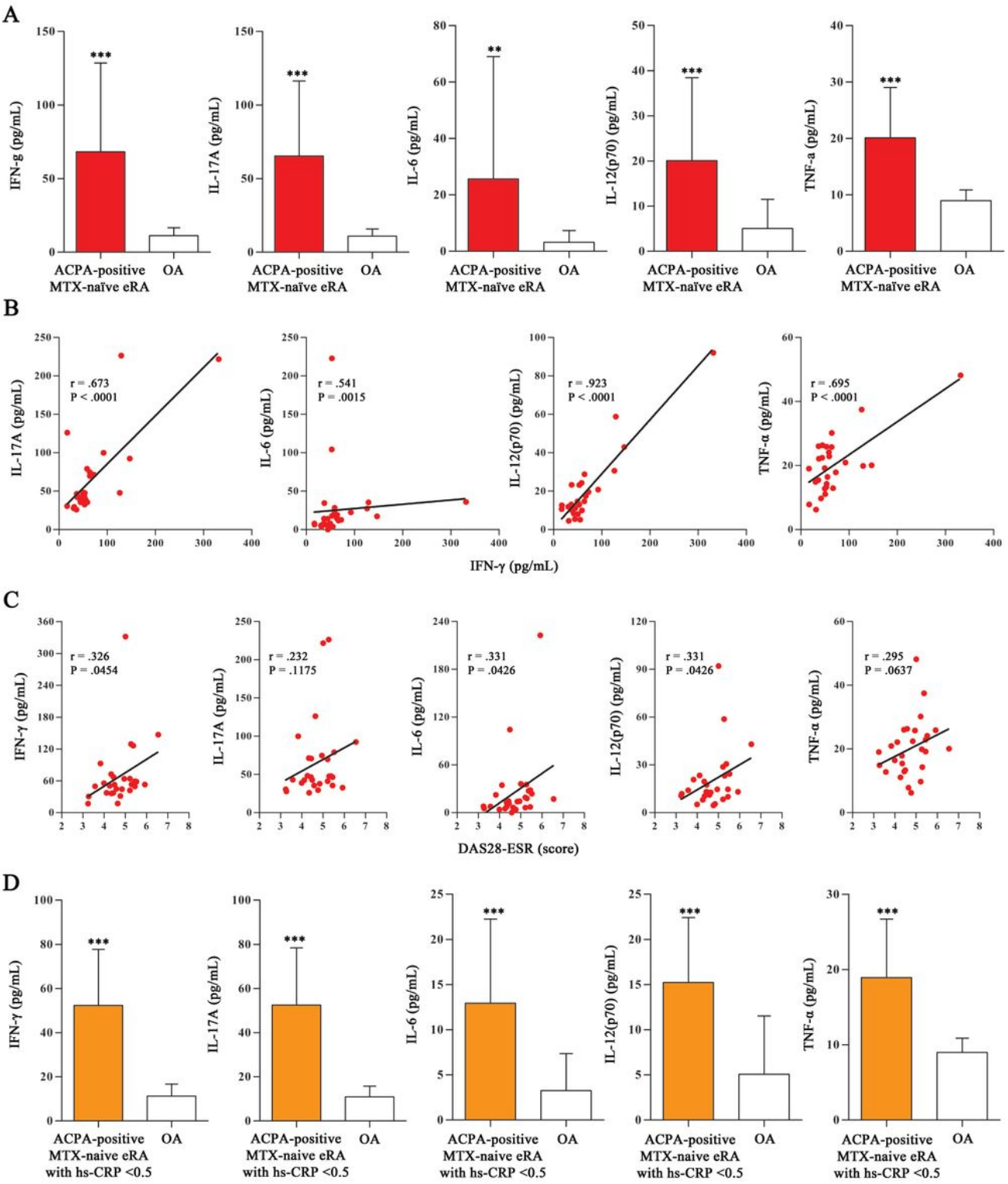

Figure 2

Multiplex cytokine assay of ACPA-positive, MTX-naïve eRA. (A) Plasma cytokine levels of five cytokines (IFN-γ, IL-17A, IL-6, IL-12, TNF-α) in patients with ACPA-positive, MTX-naïve eRA. (B) Correlation between plasma IFN-γ and IL-17A/IL-6/IL-12/TNF-α. (C) Correlation between DAS28-ESR and plasma cytokine levels. (D) Plasma cytokine levels in patients with ACPA-positive, MTX-naïve eRA with hs-CRP under 0.5 mg/dL.

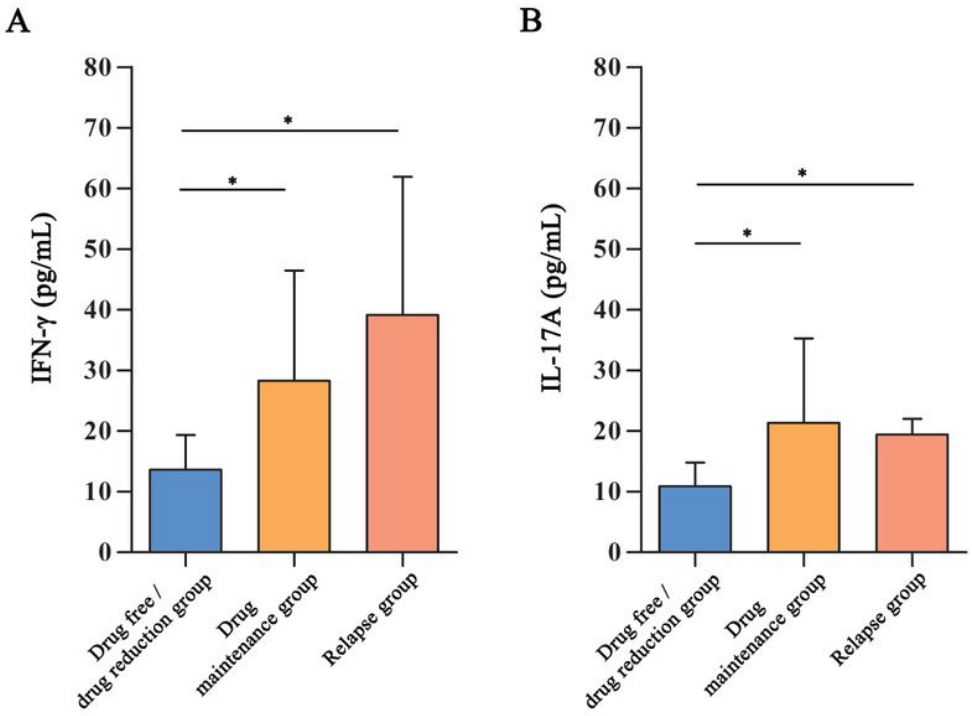

Figure 3

IFN-γ and IL-17A levels in established RA with low disease activity (DAS28-ESR ≤ 3.2). (A) Comparison of baseline plasma levels of IFN-γ in the established RA with drug-free or drug reduction, drug maintenance, and relapse after dose reduction groups. (B) Comparison of baseline plasma levels of IL-17A in the established RA with drug-free or drug reduction, drug maintenance, and relapse after dose reduction groups.

Supplementary Files

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