Serum CXCL10 levels are associated with better responses to abatacept treatment of rheumatoid arthritis

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3 Short running head: Examination of therapeutic response markers

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ABSTRACT

Objective: This study aimed to identify therapeutic predictors of abatacept (ABT) treatment in rheumatoid arthritis (RA) *in vitro* and in patients.

Methods: T cell cytokine, monokine, and chemokine levels in culture supernatants or serum were determined using flow cytometry bead-based immunoassays. CXCL10 mRNA and protein expressions were also assessed using qPCR and ELISA analyses, respectively. In the patient study, 25 ABT-treated patients were analyzed retrospectively. Patients were divided into low disease activity (LDA) or non-low disease activity (non-LDA) groups at 24 weeks of ABT treatment. Seven T cell cytokines and CXCL10 levels were compared in these two groups.

Results: Peripheral blood mononuclear cells (PBMC) from healthy donors were stimulated by immobilized anti-CD3 with or without ABT for three days, and the levels of 13 T cell cytokines in culture supernatants were determined. ABT significantly inhibited anti-CD3-induced production of IFN-γ. To examine the effect of these T cell cytokines in rheumatoid synovial cells (RSC), RSCs were stimulated with 10 % of culture supernatants from anti-CD3-stimulated PBMCs with or without ABT, and the levels of 23 cytokines were determined. Only CXCL10 was significantly reduced by ABT-treated supernatants. In the patient study, CXCL10 levels at baseline were not

different between the LDA and non-LDA groups, whereas CXCL10 levels at 24 weeks were significantly decreased in the LDA group only.

Conclusion: ABT treatment significantly affected IFN-γ and CXCL10 cytokine levels *in vitro.* In addition, serum CXCL10 levels were associated with better responses in ABT treatment.

Key words: Rheumatoid arthritis, abatacept, C-X-C motif chemokine 10 (CXCL10), cytokine

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovitis, autoantibody production, cartilage and bone destruction, and systemic inflammation, and its development is associated with genetic and environmental factors [1, 2]. The current treatment approach in RA follows a stepwise management, starting from conventional synthetic disease-modifying anti-rheumatic drugs (DMARDs) to biological DMARDs and targeted synthetic DMARDs [3, 4].

Abatacept (ABT), a fully soluble fusion protein, consists of the extracellular domain of human cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) linked to the Fc (hinge, CH2, and CH3 domains) portion of human immunoglobulin G1 that has been modified to avoid complement fixation. Mechanistically, ABT exerts its action by inhibiting the CD80/CD86:CD28 pathway, a key costimulatory pathway required for full T cell activation [5]. When administered alone or in combination with MTX, ABT has been shown to significantly improve the signs and symptoms of RA, joint destruction, and health-related quality of life [6, 7].

However, ABT treatment is costly and fails to produce a response in a substantial

proportion of patients. For example, in more than 20 % of RA patients treated with high doses of biologics, including ABT, treatment was ineffective according to Japanese post-marketing surveillance [8]. As such, a personalized therapeutic approach is desirable to reduce periods of disease activity and patient exposure to the potential side effects of an ineffective treatment [4]. Several studies have reported on the usefulness of clinical and laboratory variables, autoantibodies, cytokines, T cell subsets, and genetic factors as predictors of treatment response to ABT [7, 9-14]. However, a clinically useful biomarker for ABT treatment remains to be identified.

Tweehuyseb et al. [15] reported that a number of cytokines, which are inhibited by biologics *ex vivo*, show some predictive value for drug treatment, suggesting that *ex vivo* or *in vitro* experiments using biologics may be good tools for identification of the corresponding treatment biomarkers. We hypothesized here that cytokines and chemokines, with substantial ABT-induced suppression *in vitro*, would be good predictors of ABT treatment response in RA. In this study, we first identified cytokines and chemokines that were most suppressed by ABT in anti-CD3-stimulated peripheral blood T cells or rheumatoid synovial cells culture *in vitro*. Second, we analyzed RA patients treated with ABT and examined the relationship between serum cytokine levels affected by ABT *in vitro* and disease activity, including achievement of low disease activity (LDA)

and disease activity score (DAS28-CRP) <2.7 at 24 weeks.

MATERIALS AND METHODS

In vitro T cell activation and measurement of T cell cytokines

The *in vitro* study was approved by the Institutional Research and Ethics Committee of Hiroshima University (E-668-1), and written informed consent was obtained from all healthy donors and patients with RA. Mononuclear cells from the peripheral blood of 10 healthy donors were separated by Lympholyte®-H (Cedarlane, NC, USA) density gradient centrifugation. A 96-well microplate was pretreated with 50 μL of 10 μg/mL anti-CD3 (clone OKT3, BioLegend, San Diego, CA, USA) for 2 h. After washing with phosphate-buffered saline (PBS), peripheral blood mononuclear cells (PBMC) were cultured at a cell density of 2×10^5 cells/well in 96-well tissue culture plates with or without ABT (Orencia®, Bristol Myers Squibb Co.) for 3 days. The concentration of ABT used in this study was 500 μg/ml, which is the maximum blood concentration during ABT treatment. T cell cytokines, including TNF-α, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, and IFN-γ levels, in the culture supernatants were then determined by a flow cytometry bead-based immunoassay (LEGENDplex™ Human T Helper cytokine panel, BioLegend) using a CytoFLEX flow cytometer (Beckman Coulter

Co.) according to the manufacturer’s instructions.

Preparation of rheumatoid synovial cells (RSC) and measurement of cytokines and chemokines in RSC culture

Synovia were obtained from three RA patients by surgical treatment after receiving written informed consent. The three patients with RA who provided synovial membranes were all women with a mean age of 63 (51-85) years and IgM-RF-positive and anti-CCP-positive results. These patients were all treated with csDMARDs (two patients with methotrexate, one patients with leflunomide) without biologics. In the disease activity based on the DAS-28 score, one patient had high activity and two patients had low activity. Rheumatoid synovium was obtained by surgical treatment of total knee arthroplasty. Isolated rheumatoid synovia were aseptically dissected free from the surrounding tissues, minced, and enzymatically digested with 1–2 mg/mL clostridium collagenase (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and 5–10 μg/mL deoxyribonuclease 1 (Sigma Chemical Co., St Louis, MO) for 2–3 h at 37 °C. After digestion, the resulting single cell suspension was washed, filtered through sterile gauze and nylon mesh, washed thoroughly again, and finally resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum. Subsequently, the

cells were cultured overnight to adhere to the culture plate. After the plate was washed to remove non-adherent cells, the remaining adherent cells were used as RSCs. The rheumatoid synovial cells used in this study were all CD14-negative, suggesting that they were fibroblast-like cells, not macrophages. To evaluate the effect of anti-CD3-stimulated T cell cytokines, RSC were cultured with 10% of culture supernatants of anti-CD3-stimulated PBMC from healthy donors with or without ABT for 3 days. The cytokine and chemokine levels in the culture supernatants were determined by a flow cytometry bead-based immunoassay (LEGENDplex™ Human macrophage panels and human proinflammatory chemokine panel) using a CytoFLEX flow cytometer according to the manufacturer's instructions. The cytokines and chemokines measured in these experiments were as follows: TNF-α, IL-1β, IL-1RA, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-23, IFN-γ, arginase, C-C motif chemokine (CCL)2, CCL3, CCL4, CCL5, CCL11, CCL17, CCL20, CXCL1, CXCL5, CXCL9, CXCL10, and CXCL11.

Quantitative real-time PCR and ELISA analyses for CXCL10 expression

qPCR analysis of CXCL10. RSCs were cultured with IL-1β, TNFα, IL-6, IL-6 receptor α (Rα), IL-17A (all from BioLegend), or IFN-γ (Peprotech Inc, Rocky Hill, NJ, USA) for 24 h. After culture, treated RSCs were lysed, and total RNA was isolated using a

NucleoSpin® RNA kit (Takara-Bio, Japan). Subsequently, total RNA (200 ng) was reverse transcribed to cDNA using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara-Bio, Japan). Real-time RT-PCR was then performed using TB Green™ *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara-Bio, Japan) with the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc). PCR amplification was performed with 40 cycles at 95 °C for 5 s and 60 °C for 10 s. The following primes were used: CXCL10 (sense, 5'-AAAGCAGTTAGCAAGGAAAG-3' and antisense, 5'-TCATTGGTCACCTTTT-3'); β-actin (sense, 5'-GACGACATGGAGAAAATCTG-3' and antisense, 5'-ATGATCTGGGTCATCTTCTC-3'). CXCL10 sense mRNA expression was normalized to β-actin for each sample.

ELISA assay for CXCL10. RSCs were incubated with the indicated cytokines for 48 h. After culture, CXCL10 levels in the culture supernatant were determined using an ELISA kit (BioLegend) according to the manufacturer's instructions.

Patient study

This study retrospectively analyzed data from 25 patients diagnosed with RA between September 2010 and April 2017 who received intravenous ABT treatment. All patients

fulfilled the 2010 European League against Rheumatism (EULAR) and American College of Rheumatology (ACR) classification criteria for RA [16, 17]. This study followed the guidelines of Helsinki Declaration and ethical guidelines for epidemiologic research in Japan. The study protocol was approved by the Institutional Ethical Committee of Higashi-Hiroshima Memorial Hospital (permission; HMH-18-01) and Hiroshima University (permission; E-1383). ABT was introduced through intravenous infusion according to baseline weight (< 60 kg received 500 mg, 60–100 kg received 750 mg, and > 100 kg received 1000 mg) on days 1, 15, and 29, followed by monthly treatments. The same doctor at Higashi Hiroshima Memorial Hospital determined the clinical outcome of all patients at week 24 to potentially eliminate bias. Evaluation of clinical disease activity in patients was conducted by assessing the following parameters: C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), matrix metalloproteinase 3 (MMP-3), rheumatoid factor (RF), anti-CCP antibody, tender joints count, swollen joints count, and DAS28-CRP. Serum samples collected from patients were immediately stored at −20 °C. Serum levels of IL-2, IL-17A, TNF-α, and CXCL10 were assessed by a flow cytometry bead-based immunoassay according to the manufacturer's instructions (LEGENDplex, BioLegend). IL-4, IL-6, IL-10, and IFN-γ levels were assessed using ELISA kits (Biolegend).

Statistical Analyses

Values in Figure 1 and 2 are displayed as mean ± SE. Values in Figure 3 are displayed as mean ± SD. Values in tables are expressed as median (interquartile range), number, or percentage. The differences between pairs of groups were compared using unpaired t test, Mann-Whitney U test, or Wilcoxon's rank test. Categorical variables were analyzed using the Fisher' exact test or the $\chi 2$ test, $p < 0.05$ was considered to be statistically significant. All analyses were performed using the statistical software package EZR (Easy R) (Jichi Medical University Saitama Medical Center, Saitama, Japan), version 1.30, which is based on R and R commander.

RESULTS

Effects of ABT on T cell cytokine production by anti-CD3-stimulated PBMC

To determine which T cell cytokines are most reduced by ABT *in vitro*, we evaluated the effect of ABT on anti-CD3-induced production of T cell cytokines in PBMC culture. As shown in Figure 1, ABT significantly inhibited IL-9, IL-10, IL-13, IL-17A, IL-17F, and IFN-γ production. Among which, IFN-γ was most significantly inhibited by ABT (Figure 1).

Effect of ABT-treated PBMC culture supernatant on monokine and chemokine production by RSCs

Several T cell cytokines has been reported to modulate production of pro-inflammatory cytokines by RSCs. Therefore, we first cultured PBMCs in anti-CD3 pre-coated microplates with or without ABT for 3 days. Subsequently, their supernatants were added to the RSC culture at a final concentration of 10%. After culture for 48 h, the levels of inflammatory cytokines and chemokines in the supernatant were determined. Among 23 cytokines tested, only CXCL10 was significantly inhibited by ABT (Figure 2A, 2B).

Effect of inflammatory cytokines on CXCL10 expression in RSC culture

IFN-γ is known to be a potent inducer of CXCL10 [18] and may mediate ABT-induced inhibition of CXCL10. However, its effect does not exclude the possible involvement of other inflammatory cytokines. Therefore, we examined the effect of inflammatory cytokines on CXCL10 mRNA and protein expression levels in RSC culture. As shown in Figure 3A, and 3B, IFN-γ potently induced CXCL10. In contrast, there were minimal effects by other inflammatory cytokines, suggesting that ABT may primarily affect the IFN-γ/CXCL10 pathway in T cells and RSC culture *in vitro*.

Characteristics of RA patients

Of the 25 study participants, 72% of patients were females, and their median age was 70 (65–73) years, of which 84% and 96% of patients were IgM-RF-positive and anti-CCP-positive, respectively. Among patients with anti-CCP, median levels were 111 U/mL (32.4–264). The median tender joint count, swollen joint count, patient's visual analog scale, and DAS28-CRP were 6 (3–7), 6 (4–9), 50 (40–70), and 4.9 (4.4–5.3), respectively.

Correlation between candidate predictors and therapeutic response

All patients were intravenously treated with ABT, and treatment response was defined according to the EULAR response criteria of DAS28-CRP score at week 24 after treatment: remission (n = 8), low disease activity (n = 5), moderate disease activity (n = 8), and high disease activity (n = 4) (Figure 4). We divided the study population into two groups: achieved LDA (LDA) group (remission and LDA, n=13) and non-achieved LDA (non-LDA) group (Moderate and high disease activity, n = 12). Further, we evaluated clinical usefulness of candidate predictors in these two groups. As shown in Tables 1 and 2, no significant difference between LDA and non-LDA groups was observed in baseline data of clinical features, inflammatory parameters, autoantibodies, and serum levels of T

cell cytokines and CXCL10. We next analyzed differences in T cell cytokines and CXCL10 levels at baseline and 24 weeks in these two groups. As shown in Table 3, significant changes at baseline and 24 weeks were not observed in serum levels of IL-2, IL-4, IL-17A, TNF-α, and IFN-γ of both groups. IL-6 and IL-10 were significantly reduced after ABT treatment; however, this reduction was observed in both LDA and non-LDA groups. Interestingly, CXCL10 was significantly reduced only in the LDA group, but not in the non-LDA group, suggesting that serum CXCL10 levels may be associated with the therapeutic response to ABT.

DISCUSSION

ABT is an effective drug in RA treatment, although there are no clinically useful biomarkers for ABT treatment. In this study, we found that the IFN-γ and CXCL10 pathway was most affected by ABT *in vitro* in T cell and RSC cultures. In addition, we showed that serum CXCL10 levels are associated with better responses in ABT treatment of RA, although the CXCL10 value at baseline was not different between response and non-response groups. Among 13 cytokines tested, IFN-γ was the most reduced by ABT in the CD3-stimulated PBMC culture. However, IFN-γ was not a suitable predictor and marker of disease activity in ABT-treated patients. In contrast,

previous study has reported decreased serum IFN-γ levels in RA patients treated with ABT [19]. This discrepancy may be explained by differences in sensitivity of the assay systems used. Scarsi et al. [20] demonstrated a reduction in IFN-γ-producing peripheral blood T cells after *in vitro* stimulation in ABT-treated patients, suggesting that the IFN-γ-producing T cell subset would be a good predictor for ABT treatment.

Activation of the inflamed rheumatoid synovium is induced by direct interaction with activated T cells [21]. In addition, synovial T cells show similar characteristics as activated human peripheral blood lymphocytes [22]. These findings demonstrate the importance of the interplay between RSCs and activated T cells in RA pathogenesis [21]. In this study, anti-CD3-stimulated T cells secreted cytokines that induced RSCs to produce large amounts of cytokines and chemokines. ABT-treated T cell cytokines reduced the enhanced expression of CXCL10. This inhibition is likely mediated by IFN-γ. Most importantly, CXCL10 is consistently measurable in the serum of RA patients, thus associated with the therapeutic response of ABT.

CXCL10 is known to play important roles in the perpetuation of inflammation and tissue destruction at inflamed rheumatoid joints. For example, CXCL10 is actively

produced by inflammatory stimulation of interferon, IL-1β, and TNF in rheumatoid synovial cells [18, 23, 24], and its level is highly elevated in the rheumatoid synovial fluid of patients, as compared to serum levels [25, 26]. Additionally, CXCL10 is involved in homing inflammatory cells, such as activated T cells, monocytes, and NK cells, to inflamed tissue [27-29]. Furthermore, CXCL10 can induce RANKL expression in RA synoviocytes and CD4+ T cells [30], suggesting possible involvement in bone resorption at the rheumatoid joint. Previous studies have reported higher levels of CXCL10 in serum or plasma in patients with established RA [31, 32], early RA [33], and untreated early RA [34], as compared to healthy controls. However, the association of CXCL10 with the therapeutic response to ABT has yet to be reported.

There are some limitations in our study. First, the patient population was relatively small and heterogeneous. Thus, the clinical usefulness of CXCL10 should be evaluated in a large-scale study. Second, serum CXCL10 levels at 24 weeks were reduced in the response group, but the levels at baseline were not associated with therapeutic response to ABT. Third, we were not able to assess joint destruction using total sharp score in the two groups. Furthermore, CXCL10 was found to be related to treatment response of ABT treatment. Yet, it is important to clarify whether this association is also related to

other biologics.

Based on *in vitro* study, it is likely that IFN-γ and CXCL10 are target molecules of ABT in inflamed rheumatoid joints. In addition, we showed that serum CXCL10 levels may be a possible indicator of therapeutic response to ABT treatment. However, further studies are needed to evaluate the clinical utility of CXCL10 as a disease marker to predict the therapeutic efficacy of biological DMARDs.

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The contribution of authors are as follows. E.S. and K.Y. designed the study; K.Y., E.S., T.K., K.O., T.T., H.K, and Y.Y. performed the experiments; E.S., K.Y. and S.M. drafted the manuscript; and J.Y., M.I., S.Y., and S.H. for critical revision of the manuscript for important intellectual content.

Conflict of Interest:

E.S and S.H. have received speakers fee from Bristol-Myers Squibb. Co.

References

1. CROIA C, BURSI R, SUTERA D, PETRELLI F, ALUNNO A, PUXEDDU I: One year in review 2019: pathogenesis of rheumatoid arthritis. Clin Exp Rheumatol 2019;37(3):347-57.
2. MCINNES IB, SCHETT G: The pathogenesis of rheumatoid arthritis. N Engl J Med 2011;365(23):2205-19.
3. SILVAGNI E, DI BATTISTA M, BONIFACIO AF, ZUCCHI D, GOVERNATO G, SCIRE CA: One year in review 2019: novelties in the treatment of rheumatoid arthritis. Clin Exp Rheumatol 2019;37(4):519-34.
4. SMOLEN JS, LANDEWE R, BIJLSMA J*, et al.*: EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2016 update. Ann Rheum Dis 2017;76(6):960-77.
5. EMERY P: The therapeutic potential of costimulatory blockade with CTLA4Ig in rheumatoid arthritis. Expert Opin Investig Drugs 2003;12(4):673-81.
6. SCHIFF M, WEINBLATT ME, VALENTE R*, et al.*: Head-to-head comparison of subcutaneous abatacept versus adalimumab for rheumatoid arthritis: two-year efficacy and safety findings from AMPLE trial. Ann Rheum Dis 2014;73(1):86-94.

7. SOKOLOVE J, SCHIFF M, FLEISCHMANN R, *et al.*: Impact of baseline anti-cyclic citrullinated peptide-2 antibody concentration on efficacy outcomes following treatment with subcutaneous abatacept or adalimumab: 2-year results from the AMPLE trial. Ann Rheum Dis 2016;75(4):709-14.
8. TAKEUCHI T, KAMEDA H: The Japanese experience with biologic therapies for rheumatoid arthritis. Nat Rev Rheumatol 2010;6(11):644-52.
9. D'AGOSTINO MA, WAKEFIELD RJ, BERNER-HAMMER H, *et al.*: Value of ultrasonography as a marker of early response to abatacept in patients with rheumatoid arthritis and an inadequate response to methotrexate: results from the APPRAISE study. Ann Rheum Dis 2016;75(10):1763-9.
10. GOTTENBERG JE, RAVAUD P, CANTAGREL A, *et al.*: Positivity for anti-cyclic citrullinated peptide is associated with a better response to abatacept: data from the 'Orencia and Rheumatoid Arthritis' registry. Ann Rheum Dis 2012;71(11):1815-9.
11. LEE JS, AHMAD H, SHIM SC, BAE SC, SONG YW, LEE EY: High proportion of subjective component to the disease activity score is associated with favorable response to abatacept in rheumatoid arthritis. Patient 2018;12(3):319-26.
12. PIANTONI S, REGOLA F, SCARSI M, TINCANI A, AIRO P: Circulating follicular helper T cells (CD4+CXCR5+ICOS+) decrease in patients with rheumatoid arthritis

treated with abatacept. Clin Exp Rheumatol 2018;36(4):685.

13. SCARSI M, ZIGLIOLI T, AIRO P: Baseline numbers of circulating CD28-negative T cells may predict clinical response to abatacept in patients with rheumatoid arthritis. J Rheumatol 2011;38(10):2105-11.

14. WEISMAN MH, DUREZ P, HALLEGUA D*, et al.*: Reduction of inflammatory biomarker response by abatacept in treatment of rheumatoid arthritis. J Rheumatol 2006;33(11):2162-6.

15. TWEEHUYSEN L, DEN BROEDER AA, SCHRAA K, NETEA MG, VAN DEN HOOGEN FHJ, JOOSTEN LAB: Predictive value of ex-vivo drug-inhibited cytokine production for clinical response to biologic DMARD therapy in rheumatoid arthritis. Clin Exp Rheumatol 2019;37(3):367-72.

16. ALETAHA D, NEOGI T, SILMAN AJ*, et al.*: 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis 2010;69(9):1580-8.

17. ARNETT FC, EDWORTHY SM, BLOCH DA*, et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31(3):315-24.

18. LUSTER AD, RAVETCH JV: Biochemical characterization of a gamma interferon-

inducible cytokine (IP-10). J Exp Med 1987;166(4):1084-97.

19. MARTI L, GOLMIA R, GOLMIA AP*, et al.*: Alterations in cytokine profile and dendritic cells subsets in peripheral blood of rheumatoid arthritis patients before and after biologic therapy. Ann N Y Acad Sci 2009;1173:334-42.

20. SCARSI M, ZANOTTI C, CHIARINI M*, et al.*: Reduction of peripheral blood T cells producing IFN-gamma and IL-17 after therapy with abatacept for rheumatoid arthritis. Clin Exp Rheumatol 2014;32(2):204-10.

21. ROBERTS CA, DICKINSON AK, TAAMS LS: The interplay between monocytes/macrophages and CD4(+) T cell subsets in rheumatoid arthritis. Front Immunol 2015;6:571.

22. BRENNAN FM, SMITH NM, OWEN S*, et al.*: Resting CD4+ effector memory T cells are precursors of bystander-activated effectors: a surrogate model of rheumatoid arthritis synovial T-cell function. Arthritis Res Ther 2008;10(2):R36.

23. GROOM JR, LUSTER AD: CXCR3 ligands: redundant, collaborative and antagonistic functions. Immunol Cell Biol 2011;89(2):207-15.

24. UENO A, YAMAMURA M, IWAHASHI M*, et al.*: The production of CXCR3-agonistic chemokines by synovial fibroblasts from patients with rheumatoid arthritis. Rheumatol Int 2005;25(5):361-7.

25. HANAOKA R, KASAMA T, MURAMATSU M*, et al.*: A novel mechanism for the regulation of IFN-gamma inducible protein-10 expression in rheumatoid arthritis. Arthritis Res Ther 2003;5(2):R74-81.
26. PATEL DD, ZACHARIAH JP, WHICHARD LP: CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. Clin Immunol 2001;98(1):39-45.
27. LEE EY, LEE ZH, SONG YW: CXCL10 and autoimmune diseases. Autoimmun Rev 2009;8(5):379-83.
28. LEE EY, LEE ZH, SONG YW: The interaction between CXCL10 and cytokines in chronic inflammatory arthritis. Autoimmun Rev 2013;12(5):554-7.
29. TAUB DD, LLOYD AR, CONLON K*, et al.*: Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. J Exp Med 1993;177(6):1809-14.
30. LEE EY, SEO M, JUHNN YS*, et al.*: Potential role and mechanism of IFN-gamma inducible protein-10 on receptor activator of nuclear factor kappa-B ligand (RANKL) expression in rheumatoid arthritis. Arthritis Res Ther 2011;13(3):R104.
31. ERIKSSON C, RANTAPAA-DAHLQVIST S, SUNDQVIST KG: Changes in chemokines and their receptors in blood during treatment with the TNF inhibitor infliximab in patients with rheumatoid arthritis. Scand J Rheumatol 2013;42(4):260-

5.

32. HUEBER W, TOMOOKA BH, ZHAO X, *et al.*: Proteomic analysis of secreted proteins in early rheumatoid arthritis: anti-citrulline autoreactivity is associated with up regulation of proinflammatory cytokines. Ann Rheum Dis 2007;66(6):712-9.
33. KOKKONEN H, SODERSTROM I, ROCKLOV J, HALLMANS G, LEJON K, RANTAPAA DAHLQVIST S: Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. Arthritis Rheum 2010;62(2):383-91.
34. PANDYA JM, LUNDELL AC, ANDERSSON K, NORDSTROM I, THEANDER E, RUDIN A: Blood chemokine profile in untreated early rheumatoid arthritis: CXCL10 as a disease activity marker. Arthritis Res Ther 2017;19(1):20.

Figure legends

Figure 1. *Effects of abatacept (ABT) on T cell cytokine production by anti-CD3 stimulated peripheral blood mononuclear cells (PBMC).*

PBMCs were cultured on immobilized monoclonal anti-CD3 pre-coated microplates with or without ABT (500 μg/mL) for 3 days, and T cell cytokine levels in cultured supernatants were analyzed using flow cytometry bead-based immunoassays. Data below the lower limit of detection were regarded as 50% of the lower limit in some samples. Results are shown as the mean ± SE (n = 10). * $P < 0.05$, ** $P < 0.01$.

Figure 2. *Effect of abatacept (ABT)-treated PBMC culture supernatant on monokine and chemokine production by rheumatoid synovial cells (RSC).*

PBMCs were cultured on immobilized monoclonal anti-CD3 pre-coated microplates with or without ABT (500 μg/mL) for 3 days. The culture supernatants were added to the RSC culture at a final concentration of 10% for 48 h. The levels of 23 monokines and chemokines in the culture supernatants were determined using flow cytometry bead-based immunoassays. The net concentrations of each cytokine were obtained by subtracting the values of the cell-free supernatant from those of the RSC culture supernatant. Results are shown as the mean ± SE (n = 10). * $P < 0.05$.

Figure 3. *Effect of inflammatory cytokines on CXCL10 expression in RSC culture.*

(A) Rheumatoid synovial cells (RSC) were cultured with inflammatory cytokines, including IL-1β (50 ng/mL), TNF-α (50 ng/mL), IL-6 (50 ng/mL), IL-6 receptor α (50 ng/mL), and IFN-γ (50 ng/mL), or in combination for 24 h. Total cellular RNA was then extracted and analyzed using real-time PCR with the CXCL10 mRNA primer set. The data were normalized to β-actin (n = 2).

(B) RSCs were cultured with inflammatory cytokines, including IL-1β (50 ng/mL), TNF-α (50 ng/mL), IL-6 (50 ng/mL) with IL-6 receptor α (50 ng/mL), IL-17A (50 ng/mL), and IFN-γ (50 ng/mL), or in combination for 48 h, and CXCL10 levels in the cultured supernatants were analyzed using ELISA assays. Results are shown as the mean ± SD (n = 3). ** $P < 0.01$.

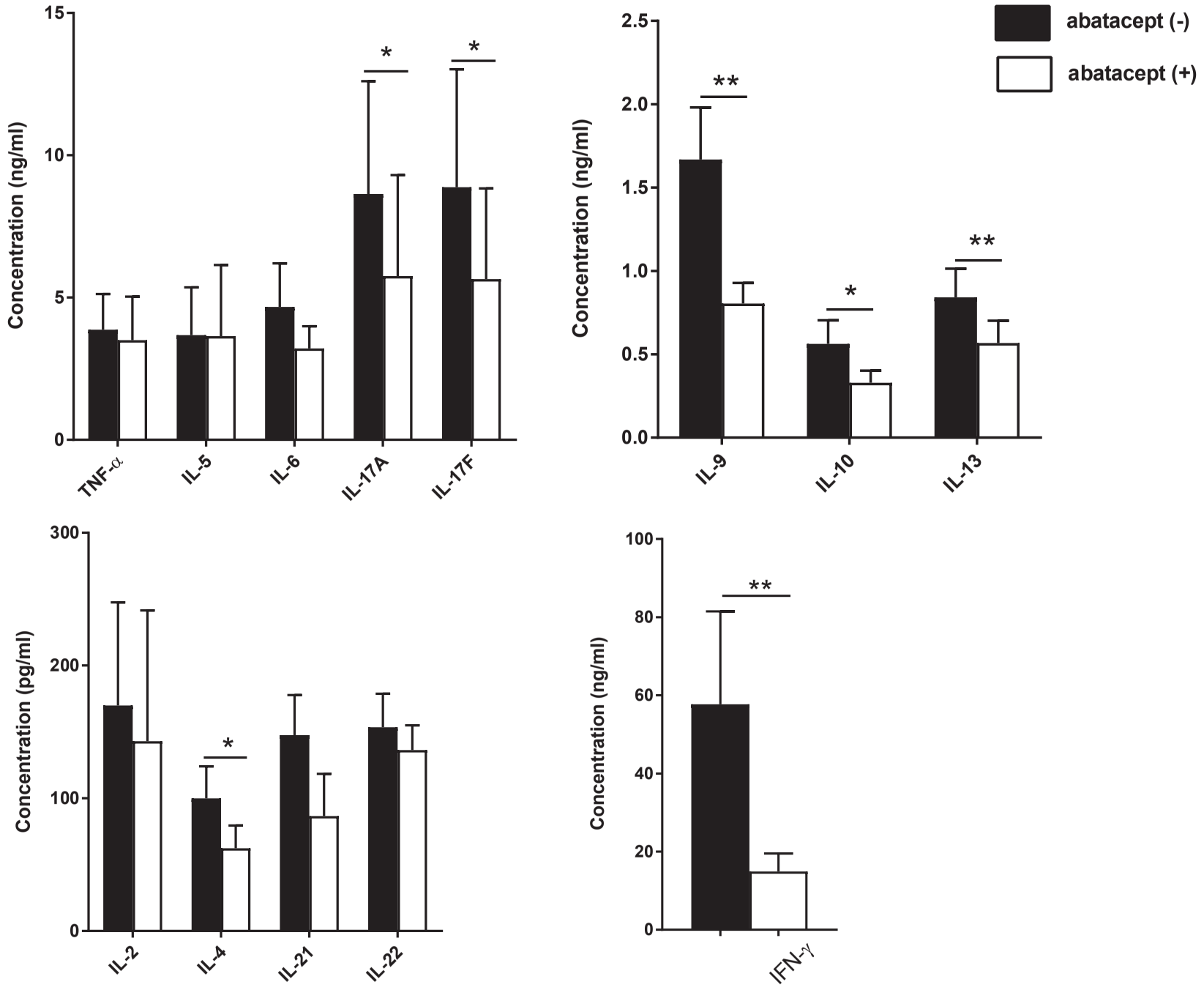
Figure 4. *Study design.*

(A) Changes in DAS28-CRP values during the 24-week study of patients treated with abatacept (ABT). The ratios of patients who demonstrated high disease activity (defined as DAS28-CRP > 4.1), moderate activity (2.7–4.1), low activity (< 2.7), and remission (< 2.3) at each observation point at day 0 and week 24 are shown.

(B) Patients with ABT treatment were divided into two groups: achieved LDA (LDA) group (remission and LDA, n = 13) and non-achieved LDA (non-LDA) group (moderate and high disease activity, n = 12), and clinical indices, inflammatory parameters, and autoantibody and serum cytokine levels were compared in the two groups.

Figure 5. *Change in serum levels of IL-6 (A), IL-10 (B), and CXCL10 (C) at day 0 and week 24 in achieved LDA (LDA) and non-achieved LDA (non-LDA) groups.* The differences between the two groups were compared using Wilcoxon's rank test. $P<0.05$ was considered to be statistically significant.

Figure 1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IL-17F
Concentration (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
IL-9
IL-10
IL-13
Concentration (pg/ml)
300
200
100
0
*
IL-2
IL-4
IL-21
IL-22
Concentration (ng/ml)
100
80
60
40
20
0
**
IFN-γ

Figure 2

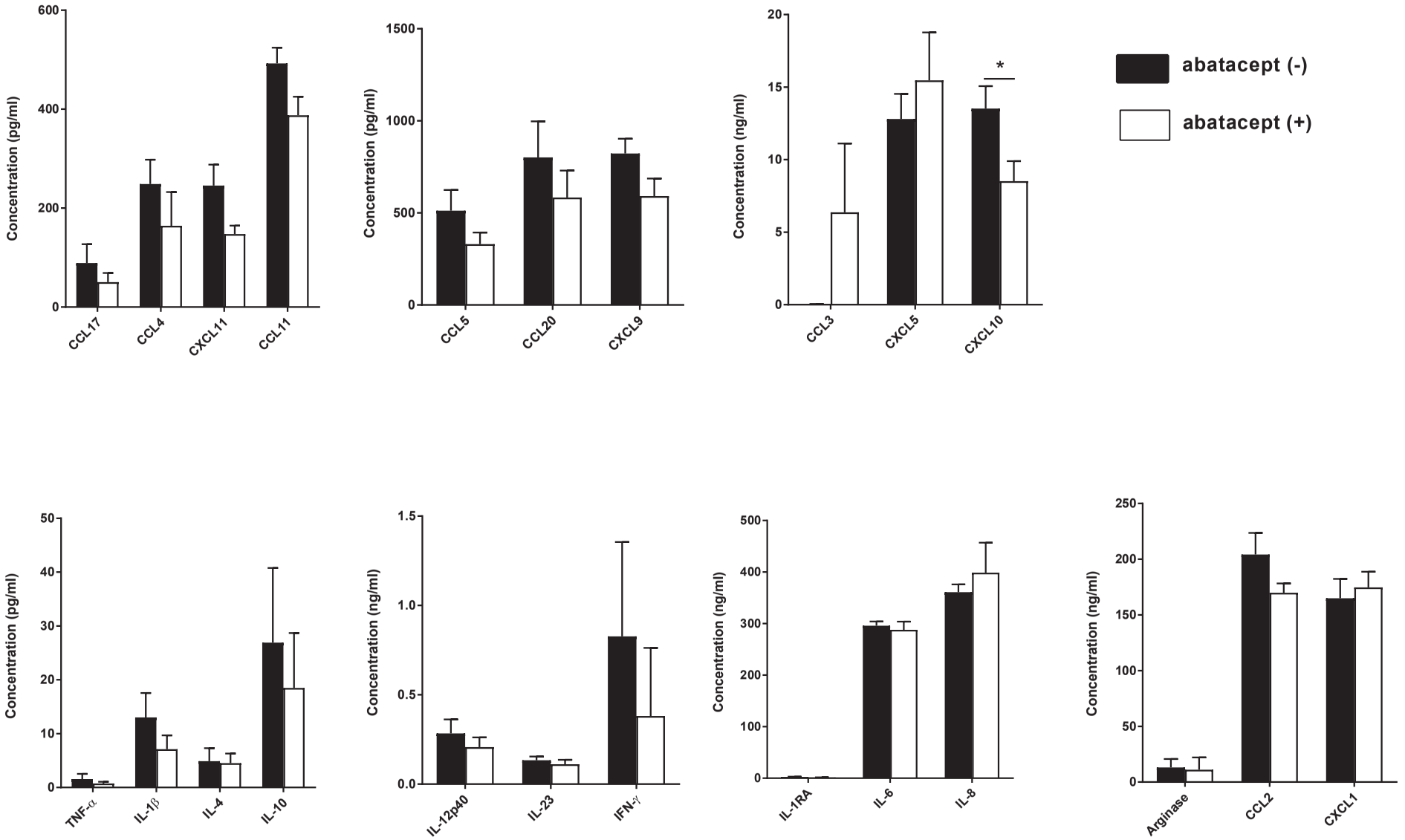

Concentration (pg/ml)
600
400
200
0
CCL17
CCL4
CXCL11
CCL11
Concentration (pg/ml)
1500
1000
500
0
CCL5
CCL20
CXCL9
Concentration (ng/ml)
20
15
10
5
0
*
CCL3
CXCL5
CXCL10
abatacept (-)
abatacept (+)
Concentration (pg/ml)
50
40
30
20
10
0
TNF-α
IL-1β
IL-4
IL-10
Concentration (ng/ml)
1.5
1.0
0.5
0.0
IL-12p40
IL-23
IFN-γ
Concentration (ng/ml)
500
400
300
200
100
0
IL-1RA
IL-6
IL-8
Concentration (ng/ml)
250
200
150
100
50
0
Arginase
CCL2
CXCL1

Figure 3

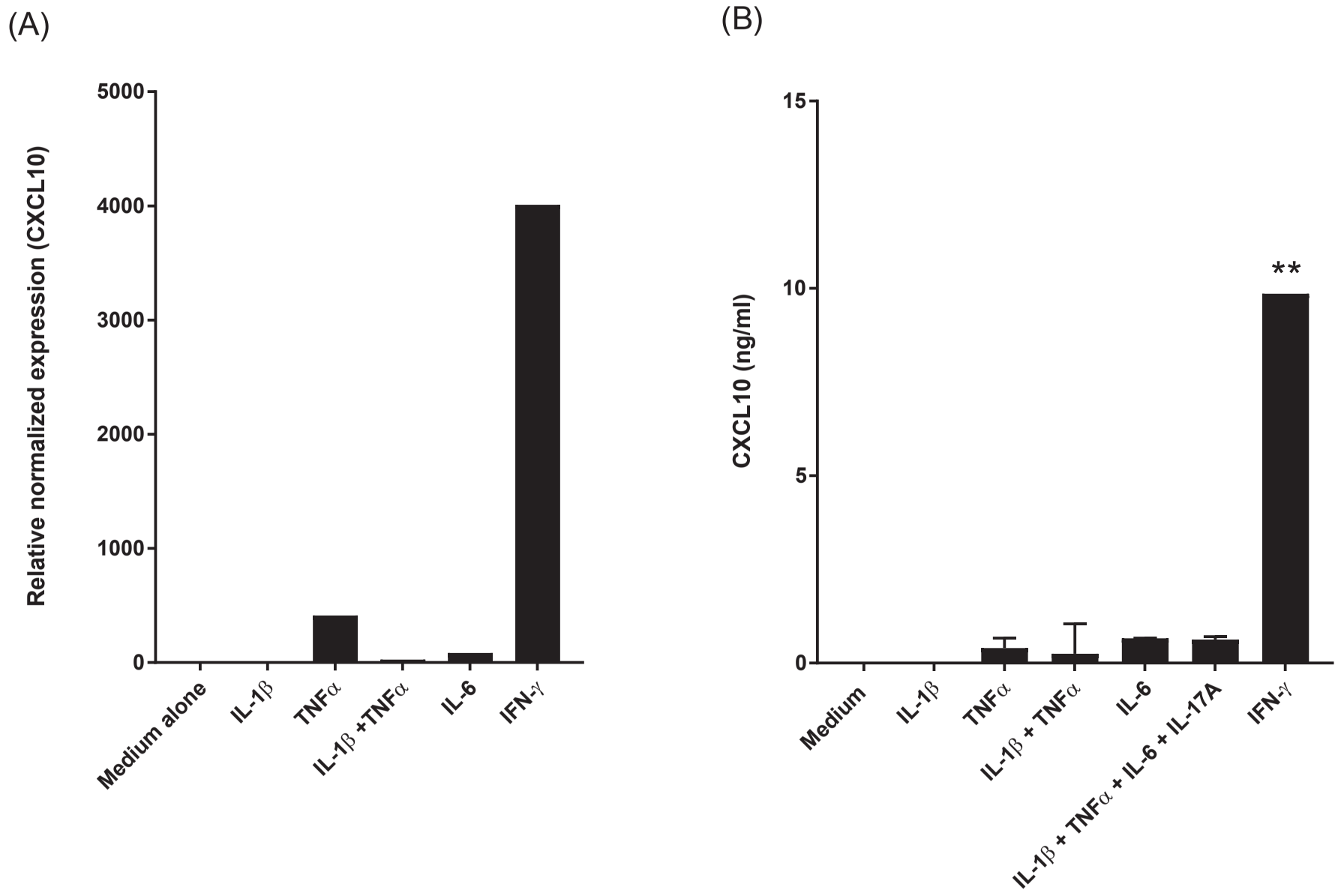
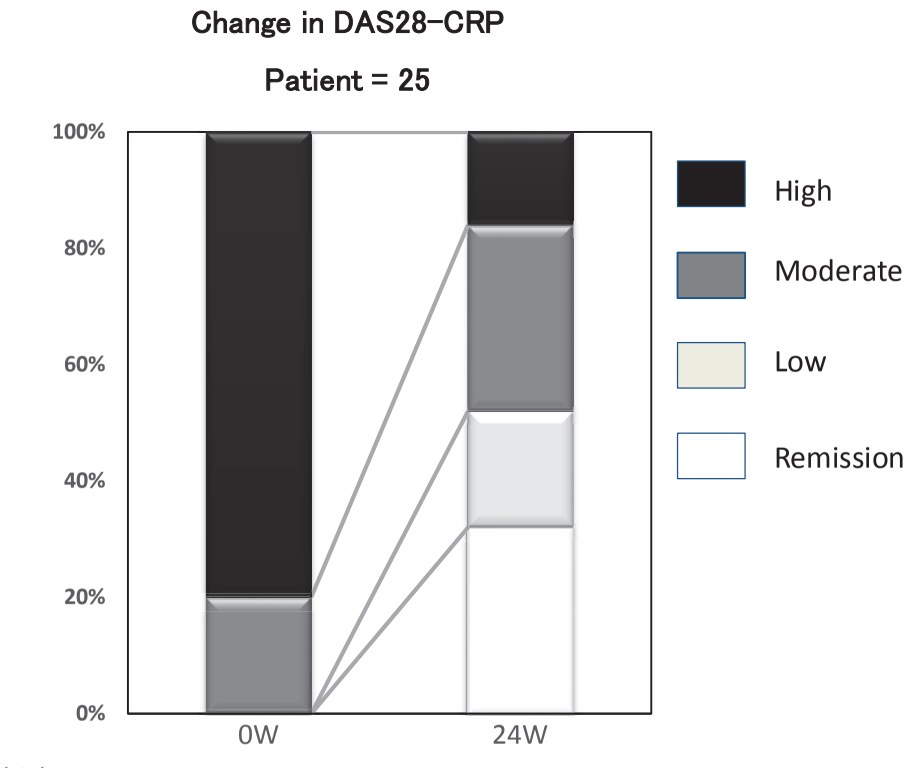
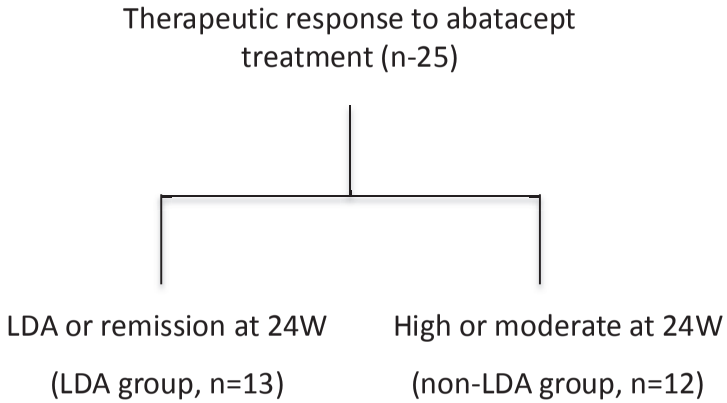

(A)
Relative normalized expression (CXCL10)
5000
4000
3000
2000
1000
0
Medium alone
IL-1β
TNFα
IL-1β +TNFα
IL-6
IFN-γ
(B)
CXCL10 (ng/ml)
15
10
5
0
**
Medium
IL-1β
TNFα
IL-1β + TNFα
IL-6
IL-1β + TNFα + IL-6 + IL-17A
IFN-γ

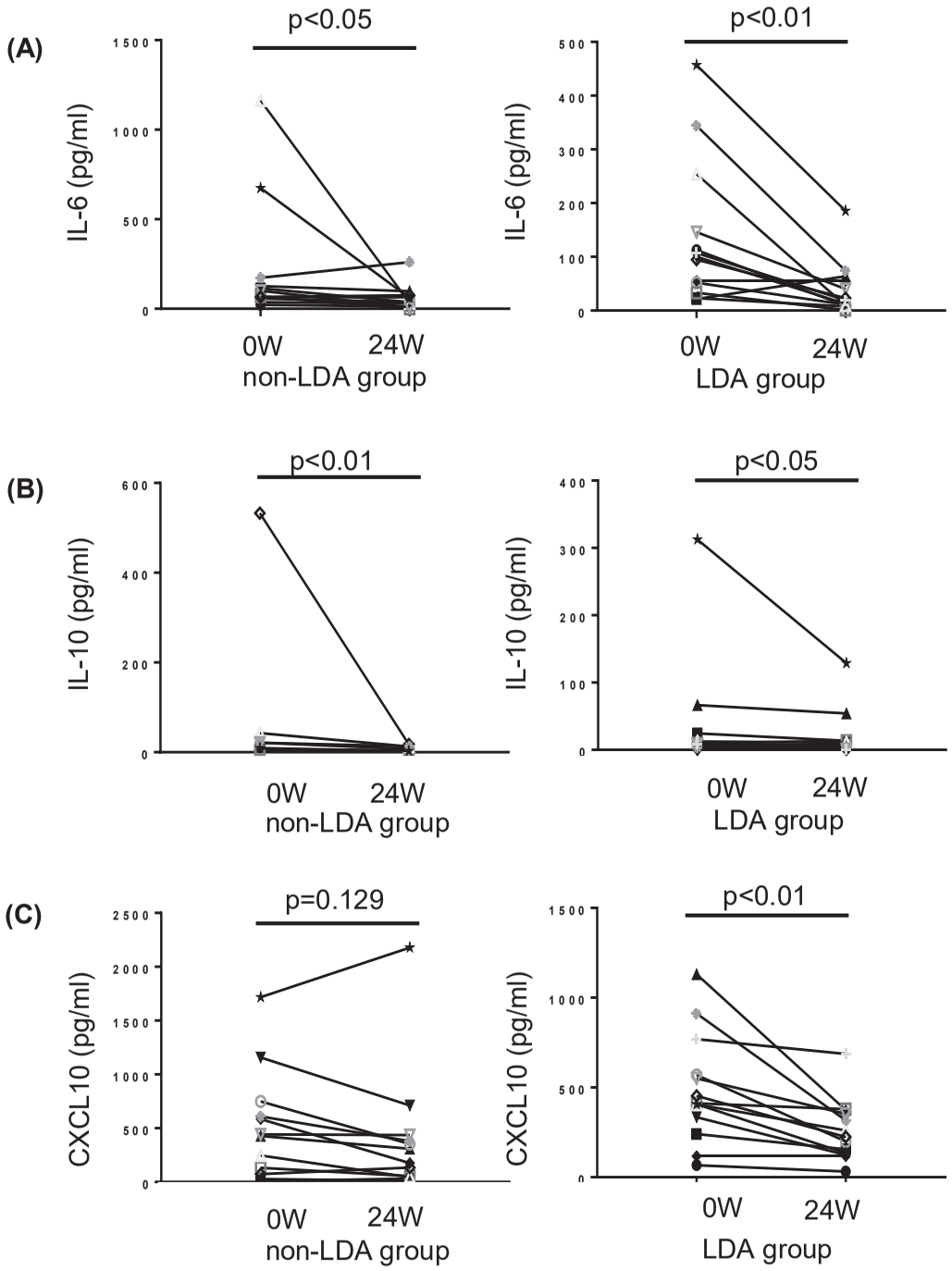
Figure 4





LDA: Low Disease Activity

Figure 5



Wilcoxon's rank test

Table 1 Baseline characteristics of patients in LDA and non-LDA group

Characteristic	Whole data (n=25)	LDA group (n=13)	non-LDA group (n=12)	p value
Female, %	72.0	53.8	91.7	0.073[a]
Age, y	70 (65-73)	70 (65-82)	72 (66-89)	0.382[c]
Disease duration, y	6 (2-12)	6 (1.2-14)	5 (2.4-10)	0.913[c]
Stage (Ⅰ/Ⅱ/Ⅲ/Ⅳ)	5/8/7/5	3/4/3/3	2/4/4/2	0.918[b]
Class (Ⅰ/Ⅱ/Ⅲ/Ⅳ)	0/19/5/1	0/11/2/0	0/8/3/1	0.441[b]
Tender joints count, n	6 (3-7)	6 (2-6)	5 (4-9)	0.527[c]
Swollen joints count, n	6 (4-9)	6 (4-9)	7 (4-9)	0.764[c]
DAS28-CRP	4.9 (4.4-5.3)	4.9 (4.4-5.2)	5.0 (4.3-5.3)	0.785[c]
Dose of MTX, mg/wk	8 (0-9)	8 (0-9)	6 (0-8)	0.520[c]
Use of MTX, %	64.0	61.5	66.7	1.000[a]
Dose of PSL, mg/d	5 (2.0-5.0)	5 (2.0-5.0)	5 (2.4-5.3)	0.654[c]
Use of PSL, %	96.0	92.3	100	1.000[a]
RF titer, IU/ml	72 (29-227)	58 (21-292)	94 (55.5-224)	0.728[c]
Positive rate of RF, %	84.0	76.9	91.7	0.593[a]
Anti-CCP titer, U/ml	127.1 (35.3-358.3)	112.7 (30.6-399.9)	149.2 (72.5-294.3)	0.936[c]
Positive rate of CCP, %	96.0	100	91.7	0.480[a]
MMP-3, ng/ml	273 (175.5-455.0)	238.0 (216.0-435.0)	276.5 (110.3-427.5)	0.651[c]

LDA: low disease activity, DAS: disease activity score, MTX: methotrexate, PSL: prednisolone,
RF: rheumatoid factor, CCP: cyclic citrullinated peptide, MMP: matrix metalloproteinase

a: Fisher's exact test b: $\chi 2$ test c: Mann-Whitney U test

Table 2 Baseline characteristics of patients in LDA and non-LDA groups

Cytokines	LDA group (n=13)	non-LDA group (n=12)	p value
IL-2 (pg/ml)	8.4 (2.0-8.4)	8.4 (8.1-16.4)	0.596
IL-4 (pg/ml)	0.1 (0.1-1.8)	0.1 (0.1-0.2)	0.192
IL-6 (pg/ml)	100.2 (51.9-145.9)	84.6 (63.4-138.2)	0.765
IL-10 (pg/ml)	8.6 (6.3-12.4)	6.9 (3.7-21.3)	0.568
IL-17A (pg/ml)	23.0 (9.8-37.6)	9.8 (9.8-34.9)	0.759
TNF-α (ml)	9.8 (2.0-11.3)	9.8 (9.5-9.8)	0.731
IFN-γ (pg/ml)	65.8 (14.7-308.9)	120.0 (25.0-387.2)	0.586
CXCL10 (pg/ml)	411.7 (334.4-569.1)	435.5 (117.7-644.1)	0.936

LDA: low disease activity, TNF: tumor necrosis factor, IFN: interferon, CXCL: C-X-C motif chemokine ligand

Mann-Whitney U test

Table 3 Change in cytokine parameters after treatment of abatacept in LDA and non-LDA groups

	LDA group (n=13)			non-LDA group (n=12)		
Cytokines	0W	24W	p value	0W	24W	p-value
IL-2 (pg/ml)	8.4 (2.0-8.4)	8.4 (2.0-13.2)	0.201	8.4 (8.1-16.4)	8.4 (7.5-55.1)	0.59
IL-4 (pg/ml)	0.1 (0.1-1.8)	0.4 (0.1-5.3)	0.108	0.1 (0.1-0.2)	0.4 (0.1-6.8)	0.107
IL-6 (pg/ml)	100.2 (51.9-145.9)	19.6 (9.4-55.3)	<0.01	84.6 (63.4-138.2)	37.5 (25.5-66.8)	<0.05
IL-10 (pg/ml)	8.6 (6.3-12.4)	7.1 (3.1-13.5)	<0.05	6.9 (3.7-21.3)	3.6 (2.5-8.8)	<0.01
IL-17A (pg/ml)	23.0 (9.8-37.6)	25.9 (9.8-29.9)	0.477	9.8 (9.8-34.9)	9.8 (9.8-14.5)	0.787
TNF-α (pg/ml)	9.8 (2.0-11.3)	9.8 (2.0-9.8)	0.106	9.8 (9.5-9.8)	9.8 (9.8-13.8)	1.000
IFN-γ (pg/ml)	65.8 (14.7-308.9)	28.8 (7.6-258.4)	0.311	120.0 (25.0-387.2)	29.1 (19.4-87.9)	0.129
CXCL10 (pg/ml)	411.7 (334.4-569.1)	224.4 (134.6-353.0)	<0.01	435.5 (117.7-644.1)	242.7 (51.9-394.0)	0.129

LDA: low disease activity, TNF: tumor necrosis factor, IFN: interferon, CXCL: C-X-C motif chemokine ligand

Wilcoxon’s rank test