雑　誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Okada Y, Shimane K, Kochi Y, Tahira T, Suzuki A, Higasa K, Takahashi A, Horita T, Atsumi T, Ishii T, Okamoto A, Fujio K, Hirakata M, Amano H, Kondo Y, Ito S, Takada K, Mimori A, Saito K, Kamachi M, Kawaguchi Y, Ikari K, Mohammed O W, Matsuda K, Terao C, Ohmura K, Myouzen K, Hosono N, Tsunoda T, Nishimoto N, Mimori T, Matsuda F, Tanaka Y, Sumida T, Yamanaka H, Takasaki Y, Koike T, Horiuchi T, Hayashi K, Kubo M, Kamatani N. Yamada R, Nakamura Y, Yamamoto K.	Meta-analysis identifies nine new loci associated with rheumatoid arthritis in the Japanese population	Nat Genet.z	44	511-6	2012
Kochi Y, Thabet MM, Suzuki A, Okada Y, Daha NA, Toes REM, Huizinga TWJ, Myouzen K, Kubo M, Yamada R, Nakamura Y, Yamamoto K.	PADI4 polymorphism predisposes male smokers to rheumatoid arthritis.	Ann Rheum Dis.	70	515-5	2011

【Ⅴ】研究成果の刊行物・別刷

SUPPLEMENT

Next stage of RA treatment: is TNF inhibitor-free remission a possible treatment goal?

Yoshiya Tanaka

The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan

Correspondence to
Professor Yoshiya Tanaka, The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health Japan, 1-1 Iseigaoka, Kitakyushu 807-8555, Japan; tanaka@med.uoeh-u.ac.jp

Received 12 July 2012
Accepted 11 September 2012
Published Online First 19 December 2012

ABSTRACT

Biological agents targeting tumour necrosis factor (TNF) have revolutionised the treatment of rheumatoid arthritis (RA) and clinical remission has become a realistic treatment goal. Discontinuing anti-TNF therapy after sustained remission has emerged as an important area of investigation in rheumatology from the risk-benefit point of view, including health economic considerations. However, there is little information as to whether 'biologic-free remission' is possible after sustained remission following intensive treatment with TNF inhibitors in RA. European studies such as BeSt and OPTIMA in patients with early RA and Japanese studies such as remission induction by remicade in patients with RA and HONOR in patients with long-standing RA encountered during routine clinical practice have shown that, after a reduction in disease activity to clinical remission or low disease activity by infliximab or adalimumab in combination with methotrexate, patients can successfully remain in clinical remission without TNF inhibitors with no radiological and functional damage progression of articular destruction. Experimental findings in TNF-deficient mouse models suggest that TNF inhibitors may change the disease process of RA and bring about the potential of immunological remission, raising the possibility of a 'treatment holiday' of TNF inhibitors after intensive treatment.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disease that causes significant morbidity and mortality.[1–4] It is recommended that the treatment of RA is initiated with monotherapy or a combination of disease-modifying antirheumatic drugs (DMARDs).[5–8] Patients with active RA, however, are often resistant to DMARD therapy, especially in the context of structural progression. Thus, biological agents targeting proinflammatory cytokines such as tumour necrosis factor (TNF), which plays a pivotal role in the pathological processes of RA leading to joint destruction, have been developed. The combined use of biological agents targeting TNF and methotrexate (MTX) has revolutionised the treatment of RA, producing significant improvements in clinical, structural and functional outcomes that were not previously observed. Accordingly, the concept of treating RA to target by employing a composite measure of disease activity is generally being accepted worldwide.[5] Clinical remission is perceived as an appropriate and realistic primary goal in many patients while, in those with long-standing RA, low disease activity is the aim.

After the induction of clinical remission by combination therapy with TNF inhibitors and MTX, it has to be maintained as described in No. 8 of the 'Treat-to-Target', which leads to structural remission and functional remission.[5] Caution is needed when deciding to reduce or discontinue treatment with synthetic DMARDs because stopping DMARDs in remission was followed by twice as many flare-ups, difficulties in reintroducing remission and a halt in damage, whereas similar studies are not available for the biological agents.[5] However, because of the economic burden associated with expensive biological products and the long-term safety by inhibiting a particular cytokine, the possibility of discontinuation of biological products after the maintenance of remission needs to considered. Thus, treatment strategies with TNF inhibitors targeting induction and/or maintenance of clinical remission can potentially lead to subsequent discontinuation of the TNF inhibitors. However, there is no well-established firm evidence that remission can be sustained even if a biological agent is discontinued (ie, 'biologic-free remission'). In this paper we discuss whether the discontinuation of TNF inhibitors such as adalimumab and infliximab is possible in patients with RA after achieving low disease activity or clinical remission during a certain period with TNF inhibitors.

IS DISCONTINUATION OF ADALIMUMAB POSSIBLE AFTER SUSTAINED REMISSION?

Clinical remission has recently become an achievable goal by the combination therapy of TNF inhibitors and MTX in many patients, and appropriate induction of remission is a prerequisite to halt joint damage and functional disabilities, which revealed improved outcomes with strategic therapeutic approaches.[4–8] If a patient is in persistent remission after tapering of glucocorticoids, one can consider tapering TNF inhibitors, especially if this treatment is combined with DMARDs. However, there is little information about the characteristics of patients with long-standing RA in whom adalimumab can be successfully discontinued.

We have carried out a study (Humira discontinuation without functional and radiographic damage progressiON follOwing sustained Remission, HONOR) to investigate whether adalimumab-free remission is maintained after discontinuation of adalimumab in Japanese patients with established RA in sustained remission obtained with adalimumab plus MTX.[9] In this study, sustained remission was defined as a persistent Disease Activity Score 28 (DAS28)-erythrocyte sedimentation rate (ESR) of < 2.6 for at least 6 months. Informed consent was obtained from patients aged >18 years who had attained sustained remission with adalimumab plus MTX to discontinue adalimumab and those followed up for >6 months were evaluated. The primary endpoint was the proportion of patients who maintained sustained remission for at least another 6 months after discontinuation. DAS28, simplified disease activity index (SDAI), clinical DAI, health assessment questionnaire-disability index (HAQ-DI) and yearly progression of the modified total Sharp score (ΔmTSS) were assessed before and after discontinuation of adalimumab. To predict retaining adalimumab even after withdrawing it, a logistic regression and receiver-operating characteristic analysis were conducted on clinical variables and cut-off values at discontinuation were determined.

Of the 197 patients who started adalimumab treatment between July 2008 and April 2011 in our department, 69 (35.0%) met the criteria for sustained remission and 51 consented to enter the study. The mean age of the 51 patients was 59.5 years and mean disease duration was 7.1 years, indicating that the population included patients with long-established disease. The mean DAS28-ESR score was 5.1, implying that most patients had active disease despite MTX. Furthermore, because the mean ΔmTSS was 11.5, the addition of TNF inhibitors to MTX was needed to control joint destruction as well as disease activity. Fifty-eight percent of the evaluable 50 patients maintained adalimumab-free remission at 6 months. DAS28-ESR at discontinuation was found significantly to predict the retention of remission with a cut-off value of 2.16. Most patients (94.9%) showed no evidence of radiographic progression (ΔmTSS ≤ 0.5) at 1 year. Moreover, HAQ-DI observed at the time of adalimumab discontinuation was almost preserved at 6 months. Therefore, although the sample size is limited, the results of the HONOR study indicated that, after reaching remission with adalimumab plus MTX, most patients could discontinue adalimumab for more than 6 months without disease flare, functional impairment and radiographic damage progression. Also, deep remission at discontinuation was associated with successful biologic-free remission.

Recently, a multinational double-blind randomised controlled study was performed to determine the optimal protocol for treatment initiation with adalimumab plus MTX in patients with early RA (OPTIMA).[10] Outcomes of withdrawal or continuation of adalimumab were assessed in patients who achieved a stable low disease activity target after 26 weeks of initially assigned treatment with adalimumab and MTX. Of the 466 patients with RA treated with adalimumab plus MTX, 207 (44%) achieved stable low disease activity and were re-randomised to placebo or adalimumab plus MTX. At week 78, 86% and 66% of patients treated with adalimumab plus MTX and placebo plus MTX, respectively, achieved DAS28 remission (<2.6). SDAI remission and ΔmTSS remission were comparable for both groups.

Another trial conducted in Germany (HIT HARD) addressed the question of whether early induction therapy with a subsequent step-down strategy leads to a long-term clinical effect in patients with recent onset RA compared with initial and continued MTX monotherapy.[11] During the first 24 weeks, 172 patients were treated with adalimumab or placebo plus MTX and, after week 24, both groups were treated with MTX alone for 24 weeks. During the induction phase 47.9% of patients treated with MTX plus adalimumab achieved DAS28 remission and, at week 48, 42.4% were still in remission with 24 weeks of adalimumab-free treatment.

In the OPTIMA and HIT HARD trials, early induction therapy with adalimumab and MTX followed by withdrawal of adalimumab led to a loss of the response gained with the initial combination treatment in a subgroup of patients, but not in all patients. Unlike the HONOR study, among patients with early RA such as those in both studies, some might be capable of comprehensive disease control with initial and continued MTX monotherapy. However, the results of the HONOR study indicate that a 'treatment holiday' of biological agents by discontinuing adalimumab is now feasible in patients with RA following sustained remission, even in patients with long-standing RA encountered during routine clinical practice (figure 1).

IS DISCONTINUATION OF INFLIXIMAB POSSIBLE AFTER SUSTAINED LOW DISEASE ACTIVITY?

We also conducted a study (Remission induction by Remicade in RA patients, RRR) to examine the possibility of biologic-free remission or low disease activity in patients with RA whose mean disease duration was 5.9 years.[12] This study included a total of 114 patients with RA from 26 centres. The mean DAS28-ESR score was 5.6, implying that most patients had active disease despite MTX therapy. Furthermore, because the mean ΔmTSS was approximately 14, the addition of TNF inhibitors to MTX was needed to control disease activity and joint destruction. The patients enrolled in the study were those who had reached and maintained low disease activity (DAS28<3.2) for more than 24 weeks with infliximab treatment and who then agreed to discontinue the treatment. Among the 102 evaluable patients who completed the study, 56 (55%) maintained low disease activity after 1 year and showed no progression in radiological damage and functional disturbance, and 44 (43%) remained in clinical remission (DAS28<2.6). The mean disease duration of the group who achieved remission or low disease activity in the RRR study was 4.8±5.9 years, which made this study the first to prove that patients with long disease duration may also aim for discontinuation. Furthermore, ΔmTSS≤0.5 was observed in 67% and the HAQ-DI score was only 0.174 in patients who maintained a low disease activity for 1 year after discontinuation. We therefore conclude that more than half of patients who maintain a low disease state for more than 24 weeks on infliximab can discontinue infliximab and maintain low disease activity for a year without radiographic or functional disease progression.

The possibility of biologic-free remission in patients with RA was initially reported by a TNF20 study.[13] The combination of infliximab and MTX in patients with early RA who had symptoms for <12 months provided tight control of the disease activity. Although infliximab was withdrawn at 1 year, low clinical activity and functional abilities were sustained for another year. In the Netherlands, the Behandelstrategieën (BeSt) study was conducted to compare four treatment strategies and to observe clinical outcomes in patients with early RA (disease duration <2 years after onset, mean disease duration 0.8 years).[14–16] In this study, 508 patients with high disease activity were allocated to four groups and evaluated by DAS44 every 3 months. In patients with DAS44>2.4 (intermediate or high disease activity) a change or addition of medications was required, in those with DAS44≤2.4 (remission or low disease activity) the current medication was continued and, in patients with DAS44≤2.4 continued over 6 months, concomitant medications including infliximab were decreased and/or discontinued. In the fourth group who started infliximab, 90 of 120 patients (75%) achieved DAS44≤2.4 and

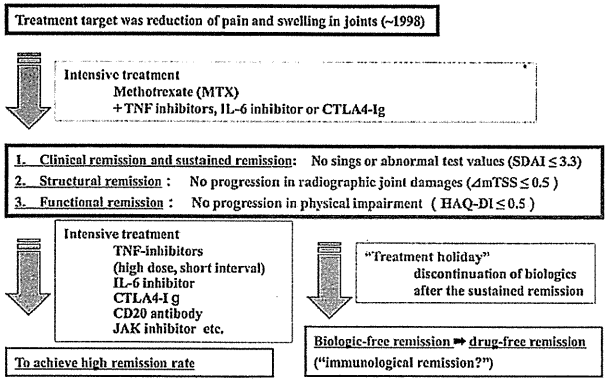

Figure 1 The next stage of the treatment of rheumatoid arthritis: intensive treatment and the possibility of a 'treatment holiday'. IL, interleukin; TNF, tumour necrosis factor.

infliximab was withdrawn in 77 cases because DAS44≤2.4 was maintained for 6 months. In the fourth group started with MTX and infliximab, the total cost of work loss and medical expenses was less than half that of the other groups started with DMARDs.

The biggest difference between the patient populations in the RRR and BeSt studies was disease duration (mean disease duration 0.8 years in the BeSt study vs 5.9 years in the RRR study), implying that biologic-free remission is possible in patients with early onset RA and also in those with long-established disease. It remains unclear whether discontinuation of biological agents targeting TNF is beneficial for comorbidity such as increased cardiovascular and/or cerebrovascular events. Since nearly a decade has passed since the BeSt study was initiated, some answers to this query may be drawn from the study.

IS TNF INVOLVED IN THE DISEASE PROCESSES?

In the BeSt study, 58% of 120 patients discontinued infliximab and 19% of patients have discontinued all DMARDs and remained in clinical remission with minimal joint damage progression 5 years after receiving infliximab and MTX as initial treatment for RA, suggesting the possibility of treatment-free remission.

In our institution, among 577 patients who were treated with infliximab, 88 patients reached biologic-free remission and only five are currently in drug-free remission without MTX. Although both TNF inhibitors and MTX play a role in the treatment, our data suggest that discontinuation of MTX appears to be difficult in patients with long-established RA. The mode of action of MTX is not discussed here, but its continuation is needed as a standard key drug. Discontinuation of biological agents benefits the economic burden of long-term management.

Accumulated studies indicate the involvement of TNF in the disease process in animal arthritis models, especially at the early stages of joint inflammation. Introduction of TNF transgene into the mouse results in typical polyarthritis, with hyperplasia of the synovium, inflammatory infiltrates in the joint space, pannus formation and cartilage and bone destruction. However, the polyarthritis and joint destruction obtained were completely ameliorated by the preventive as well as curative application of TNF inhibitors.[17] Meanwhile, TNF deficiency reduced the incidence of autoimmune arthritis in most models.[18–20] For instance, K/BxN is a model of arthritis which expresses both T cell receptor (TCR) transgene *KRN* and the MHC class II molecule *Ag7*. In the mouse, TCR recognises a self-antigen glucose-6-phosphate isomerase (GPI) and produces anti-GPI antibody, and arthritis is induced by the injection of the serum to naïve mice. Although TNF is highly expressed in K/BxN mice, deficiency of the *TNF* gene markedly reduced both the incidence and severity of the autoimmune arthritis. SKG is also an inflammatory arthritis model with a point mutation of *ZAP-70*, a member of spleen tyrosine kinase (Syk) associated with the TCRζ chain. The knockout mutation of the *TNF* gene in SKG mice showed amelioration of both the incidence and the severity of the arthritis.

If animal data partially reflect the efficacy of TNF inhibitors in patients with RA, it suggests that TNF inhibitors may change the disease course or induce immunological remission in RA. Interestingly, 48% of the 577 patients with RA described became negative for rheumatoid factor (RF) when infliximab was discontinued, although 77% of them were positive for RF at baseline when infliximab was initiated. Although the studies are limited, when the disease course is successfully changed by intensive treatment including the combination of MTX and TNF inhibitors, patients with RA may have the possibility of a 'treatment holiday' of TNF inhibitors.

CONCLUSIONS

Although the studies are limited, after reduction of disease activity to clinical remission by TNF inhibitors such as infliximab and adalimumab in combination with MTX, patients may be able to discontinue TNF inhibitors without clinical flare, radiographic progression of articular destruction and functional impairment. A 'treatment holiday' of biological agents is possible in patients with early RA and also in those with long-established RA. It has to be realised that intensive treatment with a TNF inhibitor is required to bring about the 'treatment holiday' efficiently since deep remission was a major factor affecting the success of discontinuation of TNF inhibitors in two Japanese studies. Discontinuation of biological agents during treatment of RA has become an important area of investigation in rheumatology patients and governments from the risk-benefit viewpoint including health economic considerations. Meanwhile, because treatment with TNF inhibitors can bring about the induction of remission, sustained remission and subsequent biologic-free remission—that is, it may change or modify the course of the disease—a clinical and basic research approach to the 'process-driven disease course' of RA is warranted from wider standpoints, leading to the elucidation of pathological mechanisms and treatment strategies.

Acknowledgements The author thanks all medical staff in all institutions for providing the data.

Funding The series of studies were also supported in part by a Research Grant-In-Aid for Scientific Research by the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan and the University of Occupational and Environmental Health, Japan.

Competing interests YT has received consulting fees, speaking fees and/or honoraria from Mitsubishi-Tanabe Pharma Corporation, Abbott Japan, Eisai, Chugai Pharmaceutical, Janssen Pharmaceutical, Santen Pharmaceutical, Pfizer Japan, Astellas Pharma, Daiichi-Sankyo, GlaxoSmithKline, Astra-Zeneca, Otsuka Pharmaceutical, Actelion Pharmaceuticals Japan, Eli Lilly Japan, Nippon Kayaku, UCB Japan, Quintiles Transnational Japan, Ono Pharmaceutical and Novartis Pharma and has received research grants from Bristol-Myers Squibb, MSD, Chugai Pharmaceutical, Mitsubishi-Tanabe Pharma Corporation, Astellas Pharma, Abbott Japan, Eisai and Janssen Pharmaceutical.

Provenance and peer review Commissioned; externally peer reviewed.

REFERENCES

1. **Scott DL,** Wolfe F, Huizinga TW. Rheumatoid arthritis. *Lancet* 2010;**376**:1094–108.
2. **McInnes IB,** Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med* 2011;**365**:2205–19.
3. **Redlich K,** Smolen JS. Inflammatory bone loss: pathogenesis and therapeutic intervention. *Nat Rev Drug Discovery* 2010;**11**:234–50.
4. **Tanaka Y.** Intensive treatment and treatment holiday of TNF-inhibitors in rheumatoid arthritis. *Curr Opin Rheumatol* 2012;**24**:319–26.
5. **Smolen JS,** Aletaha D, Bijlsma JWJ, *et al.* Treating rheumatoid arthritis to target: recommendations of an international task force. *Ann Rheum Dis* 2010;**69**:631–7.
6. **Felson DT,** Smolen JS, Wells G, *et al.* American College of Rheumatology/European League Against Rheumatism provisional definition of remission in rheumatoid arthritis for clinical trials. *Ann Rheum Dis* 2011;**70**:404–13.
7. **Schoels M,** Knevel R, Aletaha D, *et al.* Evidence for treating rheumatoid arthritis to target: results of a systematic literature search. *Ann Rheum Dis* 2010;**69**:638–43.
8. **Singh JA,** Furst DE, Bharat A, *et al.* 2012 update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. *Arthritis Care Res* 2012;**64**:625–39.
9. **Tanaka Y,** Hirata S, Nawata M, *et al.* Discontinuation of adalimumab without functional and structural progress after attaining remission in patients with rheumatoid arthritis (an interim report of HONOR study) (abstract), ACR2011, #2468.
10. **Kavanaugh A,** Fleischmann RM, Emery P, *et al.* Clinical, functional and radiographic consequences of achieving stable low disease activity and remission with adalimumab plus methotrexate or methotrexate alone in early rheumatoid arthritis: 26-week results from the randomised, controlled OPTIMA study. *Ann Rheum Dis* 2013;**72**:64–71.
11. **Detert J,** Bastian H, Listing J, *et al.* Induction therapy with adalimumab plus methotrexate for 24 weeks followed by methotrexate monotherapy up to week 48 versus methotrexate therapy alone for DMARD-naïve patients with early rheumatoid arthritis: HIT HARD, an investigator-initiated study. *Ann Rheum Dis*. Published Online First 27 June 2012. doi:10.1136/annrheumdis-2012-201612.
12. **Tanaka Y,** Takeuchi T, Mimori T, *et al.* Discontinuation of infliximab after attaining low disease activity in patients with rheumatoid arthritis, RRR (remission induction by remicade in RA) study. *Ann Rheum Dis* 2010;**69**:1265–91.
13. **Quinn MA,** Conaghan PG, O'Connor PJ, *et al.* Very early treatment with infliximab in addition to methotrexate in early, poor-prognosis rheumatoid arthritis reduces magnetic resonance imaging evidence of synovitis and damage, with sustained benefit after infliximab withdrawal: results from a twelve-month randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 2005;**52**:27–35.
14. **Goekoop-Ruiterman YPM,** de Vries-Bouwstra JK, Allaart CF, *et al.* Clinical and radiographic outcomes of four different strategies in patients with early rheumatoid arthritis (the BeSt study). A randomized controlled trial. *Arthritis Rheum* 2005;**52**:3381–90.
15. **Goekoop-Ruiterman YP,** de Vries-Bouwstra JK, Allaart CF, *et al.* Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial. *Arthritis Rheum* 2008;**58**(2 Suppl):S126–35.
16. **van der Bijl AE,** Goekoop-Ruiterman YP, de Vries-Bouwstra JK, *et al.* Infliximab and methotrexate as induction therapy in patients with early rheumatoid arthritis. *Arthritis Rheum* 2007;**56**:2129–34.
17. **Herrak P,** Görtz B, Hayer S, *et al.* Zoledronic acid protects against local and systemic bone loss in tumor necrosis factor-mediated arthritis. *Arthritis Rheum* 2004;**50**:2327–37.
18. **Ji H,** Pettit A, Ohmura K, *et al.* Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis. *J Exp Med* 2002;**196**:77–85.
19. **Hata H,** Sakaguchi N, Yoshitomi H, *et al.* Distinct contribution of IL-6, TNF-alpha, IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J Clin Invest* 2004;**114**:582–8.
20. **Sakaguchi S,** Benham H, Cope AP, *et al.* T-cell receptor signaling and the pathogenesis of autoimmune arthritis: insights from mouse and man. *Immunol Cell Biol* 2012;**90**:277–87.

Downloaded from ard.bmj.com on March 25, 2013 - Published by group.bmj.com

In vitro and in vivo analysis of a JAK inhibitor in rheumatoid arthritis

Y Tanaka, Y Maeshima, K Yamaoka

The First Department of Internal Medicine, University of Occupational and Environmental Health, Japan, Kitakyushu, Japan

Correspondence to
Yoshiya Tanaka, The First Department of Internal Medicine, School of Medicine, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Kitakyushu, 807-8555, Japan; tanaka@med.uoeh-u.ac.jp

Received 9 August 2011
Accepted 15 November 2011

ABSTRACT

Multiple cytokines play a pivotal role in the pathogenesis of rheumatoid arthritis (RA). The appropriate intracellular signalling pathways must be activated via cytokine receptors on the cell surface, and the tyrosine kinases transduce the first 'outside to in' signals to be phosphorylated after receptor binding to its ligand. Among them, members of the Janus kinase (JAK) family are essential for the signalling pathways of various cytokines and are implicated in the pathogenesis of RA. The in vitro, ex vivo and in vivo effects of a JAK inhibitor CP-690,550 (tofacitinib) for the treatment of RA are reported. In vitro experiments indicated that the effects of tofacitinib were mediated through suppression of interleukin 17 (IL-17) and interferon γ production and proliferation of CD4 T cells, presumably Th1 and Th17. A treatment study was conducted in the severe combined immunodeficiency (SCID)-HuRAg mice, an RA animal model using SCID mice implanted with synovium and cartilage from patients. Tofacitinib reduced serum levels of human IL-6 and IL-8 in the mice and also reduced synovial inflammation and invasion into the implanted cartilage. A phase 2 double-blind study using tofacitinib was carried out in Japanese patients with active RA and inadequate response to methotrexate (MTX). A total of 140 patients were randomised to tofacitinib 1, 3, 5, 10 mg or placebo twice daily and the American College of Rheumatology 20% improvement criteria (ACR20) response rate at week 12, a primary end point, was significant for all tofacitinib treatment groups. Thus, an orally available tofacitinib in combination with MTX was efficacious and had a manageable safety profile. Tofacitinib at 5 and 10 mg twice a day appears suitable for further evaluation to optimise the treatment of RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a representative autoimmune disease characterised by chronic and destructive inflammatory synovitis that causes severe disability and mortality. A new concept of 'treat-to-target' is emerging in treatments of RA, whereby patients are treated according to prespecified goals, such as remission. Conventional disease-modifying antirheumatic drugs (DMARDs), most commonly methotrexate (MTX), remain the cornerstone of RA treatment. Patients for whom MTX produces an inadequate response are treated with biological agents targeting tumour necrosis factor (TNF) and interleukin 6 (IL-6). The combined use of a TNF inhibitor and MTX has produced previously unseen significant improvements in clinical, structural and functional outcomes and has revolutionised the treatment goal of RA to clinical remission. However, since only about 30% of patients treated in this way attained clinical remission, next-generation treatments are a prerequisite for patients with refractory RA.[1–3]

The importance of inflammatory cytokines in the pathogenesis of RA has become apparent from the clinical efficacy of biological agents. For such cytokines to exert their biological activities, the appropriate signalling pathways must be activated via their specific receptors on the cell surface. Tyrosine kinases are the first intracellular signalling molecules to be activated after receptor binding in a cytokine response and play a part in inflammation. Among them, a Janus kinase (JAK) family has received particular attention since JAKs are essential to the signalling pathways of various cytokines and are implicated in the pathogenesis of RA. JAK3 expression is mainly limited to lymphocytes and dendritic cells and constitutively binds to the common γ (γc) chain, which is a common receptor subunit for many cytokines involved in RA (figure 1).

In view of this background, an orally available JAK inhibitor CP-690,550, which is now designated tofacitinib, was developed with the expectation that it would be a new immunosuppressant agent with few side effects.[4 5] Tofacitinib improved end points of both murine collagen-induced arthritis and rat adjuvant-induced arthritis. Tofacitinib at low concentration was also reported to greatly suppress JAK3 with few side effects in a graft-versus-host disease experiment.[4–6] Tofacitinib is currently being used in clinical trials for RA, with satisfactory effects and acceptable safety. However, the mode of action of tofacitinib in patients with RA remains unclear. We here document the in vitro, ex vivo and in vivo effects of a JAK inhibitor for the treatment of RA.

JAK-STAT PATHWAY IN INFLAMMATION

RA is characterised by systemic, chronic and destructive inflammatory synovitis. Various intercellular signalling pathways have a pivotal role during its pathological process, some of which are mediated by soluble ligands, such as cytokines and growth factors, and others are affected by cognate interaction through costimulatory molecules and adhesion molecules. For such intercellular signals to exert their biological activities, the appropriate intracellular signalling pathways must be activated by engagement of their specific receptors on the cell surface, that is 'outside to in' signalling. The intracellular signals are represented by the following pathway: (1) phosphorylation of a protein kinase such as serine/threonine kinase, tyrosine kinase and mitogen-activated protein kinase; (2) guanosine triphosphate-binding proteins, including small G-protein such as Rho and Ras and heterotrimeric G-protein consisting of Gα, Gβ, Gγ; (3) second messengers such as cyclic adenosine cyclic monophosphate and guanosine cyclic monophosphate; (4) protease-activating, apoptotic-related proteins such as caspase; (5) ubiquitination. The transduction of these intracellular signals leads to various cellular functions through directly, sequentially activating or regulating one another.

More than 99% of kinase proteins are serine/threonine kinases under physiological conditions. In contrast, although tyrosine kinase accounts for <1% of them under ordinary conditions, it is the first intracellular signalling molecule to be phosphorylated after receptor binding in a cytokine response and is involved in fundamental functions, such as cell proliferation, differentiation and adhesion in various pathological processes, including inflammation and cancer. Therefore, many investigators have examined tyrosine kinases as a target for the treatment of various diseases. More than 90 genes encoding tyrosine kinases have been identified from human genome-wide studies and 14 tyrosine kinases are known to be involved in RA.[7]

Of the tyrosine kinases, a JAK family, consisting of JAK1, JAK2, JAK3 and Tyk2, has received particular attention since JAKs are essential for the signalling pathways of various cytokines. JAKs are phosphorylated just after cytokines bind to their receptors and consecutively activate transcription factor signal transducers and activators of transcription (STAT) (figure 1).[8–13] After the engagement of homodimeric or heterodimeric receptors for cytokines and growth factors, which are constitutively bound to JAKs, JAKs are activated by a conformational change in the receptor that allows trans- and/or autophosphorylation of the two bound JAKs. These in turn phosphorylate the cytokine receptors. STAT proteins bind the phosphorylated receptor chains, which allows the JAKs to phosphorylate the STATs. Phosphorylated STATs form dimers and translocate into the nucleus, where they regulate gene transcription and expression.

Thus, the JAK-STAT pathway regulates multiple immune functions. For instance, different STATs are involved in differential cytokine production from CD4 T cell subsets: STAT1 and STAT4 mainly induce interferon γ (IFNγ) from Th1; STAT6 induces IL-4 from Th2; STAT5 induces transforming growth factor β from regulatory T cells and STAT3 induces IL-17 from Th17. JAK3 expression is essentially limited to lymphocytes and dendritic cells and constitutively binds to the γc chain, which is a common receptor subunit for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. The deficiency or dysfunction of JAK3 leads to severe combined immunodeficiency (SCID) in both humans and mice. Thus, a number of tyrosine kinase inhibitors have recently been evaluated in clinical trials. Selective inhibition of JAK3 was considered as a potential target in the treatment of RA without affecting other organ systems.

IN VITRO EFFECTS OF A JAK INHIBITOR IN RA

JAKs are essential for the signalling pathways of various cytokines and are implicated in the pathogenesis of RA. An orally available JAK inhibitor CP-690,550 (tofacitinib) is undergoing clinical trials for RA, with satisfactory effects and acceptable safety. However, the mode of action of tofacitinib in patients with RA remains unclear. Walker *et al* reported that JAK3, STAT1, STAT4 and STAT6 were highly expressed in the synovium of patients with RA, whereas their expression was rare in the synovium of normal volunteers and patients with osteoarthritis and spondyloarthritis.[14]

In view of the important role of JAK3 in lymphocyte development, differentiation and proliferation, we assessed the effects of tofacitinib on CD4 T cells at local inflammatory sites in patients with RA. We have previously assessed the effects of tofacitinib on immune cells prepared from the peripheral blood and synovium tissue of patients with RA.[15] The proliferation of CD4 synovial T cells in patients with RA stimulated with anti-CD3 and anti-CD28 antibodies was inhibited by tofacitinib in a dose-dependent manner. Treatment of synovial CD4 T cells with tofacitinib inhibited production of IL-17 and IFNγ, but had no effect on IL-6 and IL-8 production. However, CD14 monocytes and synovial fibroblasts isolated from the synovium in patients with RA were not affected by tofacitinib. Our in vitro results suggested that the effects of tofacitinib in RA are mediated through the suppression of IL-17 and IFNγ production and proliferation of CD4 T cells without affecting synovial fibroblasts and monocytes. Since IFNγ and IL-17 are produced by Th1 and Th17 cells, respectively, and are important drivers of destructive arthritis in mice and humans, JAK3 in CD4 T cells, presumably Th1 and Th17 cells, plays a crucial role in rheumatoid synovitis.

EX VIVO EFFECTS OF A JAK INHIBITOR IN RA

We next conducted a treatment study in SCID-HuRAg mice, an RA animal model using SCID mice implanted with synovium and cartilage from patients with RA, in which tofacitinib was administered via an implanted osmotic mini-pump.[15] Male SCID mice (C.B-17/Icr), 6–8 weeks old, were housed in specific pathogen-free conditions at our university animal centre. Synovial tissue and articular cartilage and bone obtained from two patients

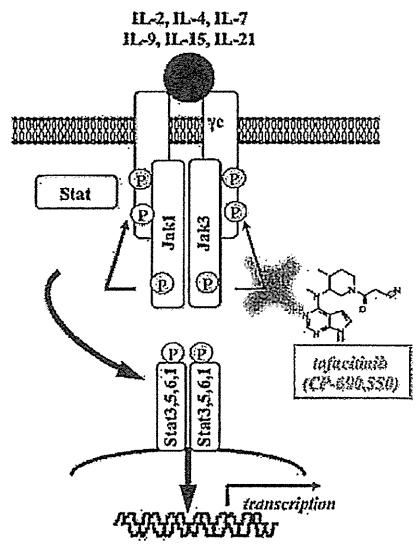

Figure 1 The JAK-STAT signalling pathway. JAK3 expression is essentially limited to haematopoietic cells and constitutively binds to the γc chain which is a common receptor subunit for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 and IL-21. An orally available JAK3 inhibitor CP-690,550 (tofacitinib) is currently in clinical trials for rheumatoid arthritis.

Downloaded from ard.bmj.com on January 23, 2013 - Published by group.bmj.com

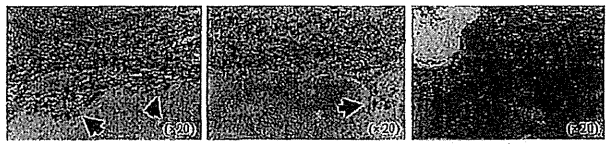

Figure 2 A JAK3-inhibitor tofacitinib used for (SCID)-HuRAg mice with severe combined immunodeficiency. Rheumatoid arthritis (RA) synovium with articular cartilage was co-implanted onto the back of SCID mice. Treatment with vehicle or tofacitinib (1.5 and 15 mg/kg/day) was started on day 7, and thereafter serum was collected weekly. The co-implants were removed on day 35 and then stained for histological evaluation. The histological section was stained with haematoxylin and eosin. Light microscopic features of cartilage erosion in the engrafted specimens are shown. Arrows show the invasive front of the synovial tissue (original magnification ×400). These experiments were repeated with two patients with RA and the results were similar.

with RA at the time of joint replacement surgery were used. The synovium was cut into sections, 5–10 mm in diameter, cartilage was cut into 2 mm^3 pieces, and then synovium and cartilage were transplanted onto the back of nine SCID mice. A week after the implantation, the nine mice were randomly divided into three groups, and tofacitinib dissolved in polyethylene glycol 300 was administered continuously at 0 (n=3), 1.5 (n=3) or 15 (n=3) mg/kg/day via Alzet osmotic mini-pumps implanted subcutaneously on the back. Blood samples were collected and the serum samples were stored at −80°C until measurement of IL-6 and IL-8. Treatment of SCID-HuRAg mice with tofacitinib reduced their serum levels of human IL-6 and IL-8. However, we have previously shown that tofacitinib did not affect IL-6 and IL-8 production from CD4 T cells, synovial fibroblasts and CD14 monocytes in vitro. On the other hand, IL-17 and IFNγ production is known to induce cytokine production from monocytes and fibroblast, and IL-6 has been reported to be mainly derived from macrophages and fibroblasts of the synovium.[13 14] These findings led us to speculate that tofacitinib specifically inhibited IL-17 and IFNγ production by CD4 T cells (presumably Th1 and Th17 cells), which in turn regulated synovitis by indirectly suppressing IL-6 and IL-8 from synovial fibroblasts and CD14 monocytes.

Next, implanted tissues were removed from the SCID-HuRAg mice 5 weeks after implantation, paraffin embedded and stained with haematoxylin and eosin. Histological evaluation was carried out and showed that in mice treated with vehicle alone, prominent invasion of the synovial tissue into the implanted cartilage had occurred. However, treatment with tofacitinib markedly inhibited this invasion, indicating that tofacitinib has the potential to inhibit the progression of structural damage of joints in patients with RA (figure 2).

IN VIVO EFFECTS OF A JAK INHIBITOR IN RA

In view of these results, an orally available JAK inhibitor, tofacitinib, is currently undergoing clinical trials for RA. Kremer *et al* reported a phase II dose-ranging trial which was carried out to investigate the efficacy, safety and tolerability of oral tofacitinib in 264 patients with active RA in whom MTX, etanercept, infliximab or adalimumab caused an inadequate or toxic response.[16 17] Patients were randomised to placebo, 5, 15 or 30 mg tofacitinib twice daily for 6 weeks, and were followed up for an additional 6 weeks after treatment. The American College of Rheumatology 20% improvement criteria (ACR20) response rate was 26.9%, 70.5%, 81.2% and 76.8% in the placebo, 5, 15 and 30 mg twice daily groups, respectively, at 6 weeks. Thus, patients treated with tofacitinib in all treatment groups achieved the primary efficacy end point—ACR20 response rate at 6 weeks. Rapid improvements in disease activity were seen in patients treated with tofacitinib, and ACR50 and ACR70 response rates significantly improved in all treatment groups by week 4. The most common adverse events (AEs) reported were headache and nausea. The infection rate in the 15 mg twice daily group and the 30 mg twice daily group was 30.4% (26.2% in placebo) and opportunistic infections or deaths were not seen.

A phase II, double-blind study was also carried out to investigate the efficacy and safety of orally available tofacitinib in Japanese patients with active RA in whom MTX had produced an inadequate response.[18] A total of 140 patients were randomised to tofacitinib 1, 3, 5, 10 mg, or placebo twice daily in this 12-week trial and continued to receive background MTX. The ACR20 response rates at week 12, a primary end point, were significant for all tofacitinib treatment groups—14.3%, 64.3%, 77.8%, 96.3%, 80.8% in the placebo, 1, 3, 5 and 10 mg twice daily groups, respectively, at 12 weeks (figure 3). Significant improvements in ACR50, ACR70 and the Health Assessment Questionnaire-Disability Index were also obtained by the use of 5 or 10 mg tofacitinib. Furthermore, in patients with high disease activity at baseline (28-joint Disease Activity Score (DAS28) >5.1), the greatest percentage of patients achieving DAS remission at week 12 was seen in the group receiving tofacitinib 10 mg twice daily (45.5%). In patients with low to moderate disease activity at baseline (DAS28 ≤5.1), the group receiving tofacitinib 5 mg twice daily contained the greatest percentage of patients achieving DAS remission at week 12 (80.0%).

The most commonly reported AEs were nasopharyngitis (n=13), and increased alanine aminotransferase (n=12) and aspartate aminotransferase (n=9). These AEs were mild or moderate in severity. Serious AEs were reported by five patients, but no deaths occurred. It is noteworthy that dose-dependent decreases in mean neutrophil counts were seen but did not result in any patients discontinuing treatment. At week 12, the mean decrease in neutrophil counts from baseline was significantly different from placebo for all tofacitinib treatment groups. Also, small changes in mean haemoglobin levels were observed across all of the tofacitinib treatment groups, although no potentially life-threatening anaemia was seen for any treatment group. Furthermore, dose-dependent increases in low-density lipoprotein, high-density lipoprotein and total cholesterol were seen, and appeared to plateau between weeks 4 and 12, although no patients stopped treatment owing to increases in serum lipids.

Neutrophil changes and anaemia may result from treatment with tofacitinib, which can inhibit signalling by haematopoietic cytokines, such as erythropoietin and granulocyte–macrophage colony-stimulating factor, through JAK2. It is interesting that some of the AEs seen here for tofacitinib, including decreases in neutrophils and increases in cholesterol and liver transaminases, were similar to those previously reported for tocilizumab, a humanised anti–IL-6 receptor antibody that blocks IL-6 signalling.[19] Results from animal models of arthritis, showing decreased IL-6 levels in tofacitinib-treated animals, and the dose-dependent increase in blood lipids levels seen in clinical trials, suggest that tofacitinib may have an inhibitory effect on IL-6. Therefore, although tofacitinib has been reported to be highly specific to JAK3, it is now known that it functions as a pan-JAK inhibitor because the inhibition of JAK1 and JAK2 should also be taken into account.

In Japanese patients with active RA and in whom MTX had produced an inadequate response, an orally available JAK inhibitor tofacitinib, in combination with MTX over 12 weeks was efficacious and had a manageable safety profile. Accordingly, longer dose-ranging studies of this new JAK inhibitor tofacitinib in the treatment of patients with RA who are MTX-naïve, or who have an inadequate response to MTX/DMARDs or TNF inhibitors are continuing. Multiple global clinical examinations and efficacy studies examining the regulation of progress in structural damage and functional disability are continuing.

CONCLUSION

Biological DMARDs such as TNF inhibitors have changed the treatment strategy of RA. Clinical remission is, however, obtained in only one-third of patients treated with biological agents, and antibodies to these agents are found. Accordingly, orally available low molecular weight products such as tofacitinib, targeting intracellular signalling molecules, would provide enormous power and flexibility in the treatment of RA. It has become clear that JAK inhibition with tofacitinib in patients with RA results in a rapid and remarkable clinical effect equivalent to that of TNF inhibitors. However, the mechanism of action of tofacitinib had not been determined. Our in vitro and in vivo studies have shown that tofacitinib mainly acts on CD4 T cells, subsequently suppressing cell proliferation and production of inflammatory cytokines such as IL-17 and IFNγ and reducing synovial inflammation (figure 4). Since it is possible to design low molecular weight products recognising a particular conformation of target molecules in the signalling cascade, the success of tofacitinib will accelerate new development of multiple products for RA and for many inflammatory diseases.

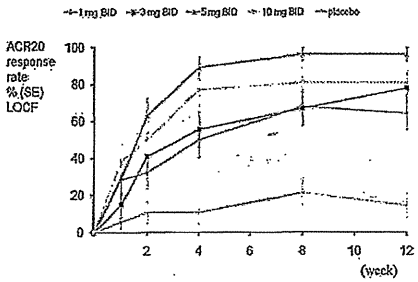

Figure 3 Efficacy of tofacitinib for patients with active rheumatoid arthritis (RA). A phase II double-blind study was carried out to investigate the efficacy and safety of orally available tofacitinib in 140 Japanese patients with active RA and inadequate response to methotrexate. Patients were randomised to tofacitinib 1, 3, 5, 10 mg or placebo twice daily (bid) for 12 weeks. The ACR20 response rates for weeks 0–12 (non-responder imputation) are shown. Modified from Tanaka et al.[18]. LOCF, last observation carried forward.

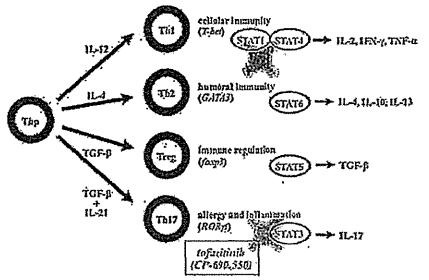

Figure 4 Schematic diagram of the JAK-STAT pathway and tofacitinib in T cell subsets. Effects of tofacitinib in rheumatoid arthritis were mediated through suppression of interleukin (IL)-17 and interferon γ (IFNγ) production and proliferation of CD4 T cells, presumably Th1 and Th17 cells. TGFβ, transforming growth factor β; TNF, tumour necrosis factor.

Acknowledgements The authors thank all medical staff in all institutions for providing the data.

Funding The clinical examination regarding tofacitinib (CP-690,550) was sponsored by Pfizer Inc and the compound CP-690,550 for in vitro studies was provided by Pfizer Inc. The series of studies were also supported in part by a research grant-in-aid for scientific research by the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan and the University of Occupational and Environmental Health, Japan.

Competing interests YT has received consulting fees, speaking fees, and/or honoraria from Mitsubishi-Tanabe Pharma, Chugai Pharma, Eisai Pharma, Pfizer, Abbott Immunology Pharma, Daiichi-Sankyo, Janssen Pharma, Astra-Zeneca, Takeda Industrial Pharma, Astellas Pharma, Asahi-kasei Pharma and GlaxoSmithKline and has received research grant support from Mitsubishi-Tanabe Pharma, Bristol-Myers Squibb, Takeda Industrial Pharma, MSD, Astellas Pharma, Eisai Pharma, Chugai Pharma, Pfizer and Daiichi-Sankyo.

Provenance and peer review Commissioned; externally peer reviewed.

REFERENCES

1. Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. *Lancet* 2010;376:1094–108.
2. Smolen JS, Aletaha D, Bijlsma JW, *et al*. Treating rheumatoid arthritis to target: recommendations of an international task force. *Ann Rheum Dis* 2010;69:631–7.
3. Nam JL, Winthrop KL, van Vollenhoven RF, *et al*. Current evidence for the management of rheumatoid arthritis with biological disease-modifying antirheumatic drugs: a systematic literature review informing the EULAR recommendations for the management of RA. *Ann Rheum Dis* 2010;69:976–86.
4. Changelian PS, Flanagan ME, Ball DJ, *et al*. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. *Science* 2003;302:875–8.
5. Flanagan ME, Blumenkopf TA, Brissette WH, *et al*. Discovery of CP-690,550: a potent and selective Janus kinase (JAK) inhibitor for the treatment of autoimmune diseases and organ transplant rejection. *J Med Chem* 2010;53:8468–84.
6. Milici AJ, Kudlacz EM, Audoly L, *et al*. Cartilage preservation by inhibition of Janus kinase 3 in two rodent models of rheumatoid arthritis. *Arthritis Res Ther* 2008;10:R14.
7. D'Aura Swanson C, Pantaqua RT, Lindstrom TM, *et al*. Tyrosine kinases as targets for the treatment of rheumatoid arthritis. *Nat Rev Rheumatol* 2009;5:317–24.
8. Johnston JA, Kawamura M, Kirken RA, *et al*. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. *Nature* 1994;370:151–3.
9. Yamaoka K, Saharinen P, Pesu M, *et al*. The Janus kinases (Jaks). *Genome Biol* 2004;5:253.
10. Yamaoka K, Min B, Zhou YJ, *et al*. Jak3 negatively regulates dendritic-cell cytokine production and survival. *Blood* 2005;106:3227–33.
11. Yamaoka K, Maeshima K, Nakano K, *et al*. Involvement of Stat6 in inflammation by negatively regulating IL-10 production. *Ann Rheum Dis* 2010;69:333.

Downloaded from ard.bmj.com on January 23, 2013 - Published by group.bmj.com

12. Ghoreschi K, Laurence A, O'Shea JJ. Janus kinases in immune cell signaling. *Immunol Rev* 2009;228:273–87.
13. Pesu M, Laurence A, Kishore N, *et al.* Therapeutic targeting of Janus kinases. *Immunol Rev* 2008;223:132–42.
14. Walker JG, Ahern MJ, Coleman M, *et al.* Expression of Jak3, STAT1, STAT4, and STAT6 in inflammatory arthritis: unique Jak3 and STAT4 expression in dendritic cells in seropositive rheumatoid arthritis. *Ann Rheum Dis* 2006;65:149–56.
15. Maeshima K, Yamaoka K, Kubo S, *et al.* A JAK inhibitor tofacitinib regulates synovitis through inhibition of IFN-γ and IL-17 production by human CD4+ T cells. *Arthritis Rheum* 2011. doi:10.1002/art.34329.
16. Kremer JM, Bloom BJ, Breedveld FC, *et al.* The safety and efficacy of a JAK inhibitor in patients with active rheumatoid arthritis: Results of a double-blind, placebo-controlled phase IIa trial of three dosage levels of CP-690,550 versus placebo. *Arthritis Rheum* 2009;60:1895–905.
17. Coombs JH, Bloom BJ, Breedveld FC, *et al.* Improved pain, physical functioning and health status in patients with rheumatoid arthritis treated with CP-690,550, an orally active Janus kinase (JAK) inhibitor: results from a randomised, double-blind, placebo-controlled trial. *Ann Rheum Dis* 2010;69:413–16.
18. Tanaka Y, Suzuki M, Nakamura H, *et al.* Phase II study of tofacitinib (CP-690,550) combined with methotrexate in patients with rheumatoid arthritis and an inadequate response to methotrexate. *Arthritis Care Res (Hoboken)* 2011;63:1150–8.
19. Nishimoto N, Miyasaka N, Yamamoto K, *et al.* Study of active controlled tocilizumab monotherapy for rheumatoid arthritis patients with an inadequate response to methotrexate (SATORI): significant reduction in disease activity and serum vascular endothelial growth factor by IL-6 receptor inhibition therapy. *Mod Rheumatol* 2009;19:12–19.

Amplification of Toll-like receptor–mediated signaling through spleen tyrosine kinase in human B-cell activation

Shigeru Iwata, MD, PhD,[a] Kunihiro Yamaoka, MD, PhD,[a] Hiroaki Niiro, MD, PhD,[b] Kazuhisa Nakano, MD, PhD,[a] Sheau-Pey Wang, MS,[a] Koichi Akashi, MD, PhD,[b] and Yoshiya Tanaka, MD, PhD[a] *Kitakyushu and Fukuoka, Japan*

Background: B cells are activated by combined signals through the B-cell receptor (BCR) and CD40. However, the underlying mechanisms by which BCR signals synergize with Toll-like receptor (TLR) signaling in human B cells remain unclear.
Objective: We sought to elucidate a role of spleen tyrosine kinase (Syk), a key molecule of BCR signaling, in TLR-mediated activation of human B cells.
Methods: Human naive and memory B cells were stimulated with combinations of anti-BCR, soluble CD40 ligand, and CpG. Effects of the Syk inhibitors on several B-cell functions and expression of TLR9, TNF receptor–associated factors (TRAFs), and phospho–nuclear factor κB in B cells were assessed.
Results: Activation of BCR synergized with CD40- and TLR9-mediated signals in driving robust proliferation, cell-cycle progression, expression of costimulatory molecules, cytokine production, and immunoglobulin production of human B-cell subsets, especially memory B cells. However, the Syk inhibitors remarkably abrogated these B-cell functions. Notably, after stimulation through all 3 receptors, B-cell subsets induced marked expression of *TLR9*, TRAF6, and phospho–nuclear factor κB, which was again significantly abrogated by the Syk inhibitors.
Conclusion: Syk-mediated BCR signaling is a prerequisite for optimal induction of TLR9 and TRAF6, allowing efficient propagation of TLR9-mediated signaling in memory B cells. These results also underscore the role of Syk in aberrant B-cell activation in patients with autoimmune diseases. (J Allergy Clin Immunol 2012;129:1594-601.)

Key words: Syk, Toll-like receptor 9, TNF receptor–associated factor 6, B cells

Abbreviations used
AICDA: Activation-induced cytidine deaminase
BCR: B-cell receptor
FITC: Fluorescein isothiocyanate
NF-κB: Nuclear factor κB
PI: Propidium iodide
SLE: Systemic lupus erythematosus
Syk: Spleen tyrosine kinase
TLR: Toll-like receptor
TRAF: TNF receptor–associated factor
XBP-1: X-box binding protein 1

B cells play a pivotal role in initiation and perpetuation of autoimmune diseases, including systemic lupus erythematosus (SLE). Activated self-reactive B cells not only are a source of pathogenic autoantibodies but also exert effector functions, including antigen presentation, cytokine production, and modulation of the T-cell repertoire. We recently reported that B-cell depletion therapy with rituximab for refractory patients with SLE not only rapidly depleted both naive and memory B cells in peripheral blood but also rapidly downregulated the expression levels of CD69, CD40 ligand, and inducible costimulator on $CD4^+$ T cells.[1] Thus B cells can facilitate autoimmune processes in both antibody-dependent and antibody-independent manners.

B cells are effectively activated by combined signals through B-cell receptor (BCR) and CD40; however, they require additional signals for efficient proliferation and differentiation. Accordingly, when combined with BCR and CD40 stimulation, Toll-like receptor (TLR) signaling by nucleic acids[2] induces the most robust B-cell activation.[3] In patients with SLE, RNA- or DNA-containing self-antigens coligate BCRs and TLR7 or TLR9, causing activation, proliferation, and differentiation of self-reactive B cells. However, the underlying mechanisms by which BCR signals potentiate TLR signaling in human B cells remain unclear.

On BCR ligation by antigens, protein kinases, including Lyn, an Src family kinase Lyn, and spleen tyrosine kinase (Syk), are initially activated.[4] Activation of Syk is a key event for further propagation of downstream signaling molecules in B cells.[5] In addition to BCR, Syk is activated through T-cell receptor and Fc receptor.[6,7] Notably, Syk inhibitors exert potent therapeutic efficacy against rheumatoid arthritis, as well as bronchial asthma and idiopathic thrombocytopenic purpura.[8-10] Moreover, Syk blockade prevents the development of skin and kidney lesions in mice with lupus.[11,12] Our current understanding of BCR-mediated Syk activation, however, extrapolates mainly from rodent studies.

In this study we demonstrate that Syk-mediated BCR signaling is a prerequisite for optimal induction of TLR9, TNF receptor–associated factor (TRAF) 6, and nuclear factor κB (NF-κB),

From [a]the First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, and [b]the Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka.
Supported in part by a Research Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan; the Ministry of Education, Culture, Sports, Science and Technology of Japan; and the University of Occupational and Environmental Health, Japan.
Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.
Received for publication September 25, 2011; revised February 3, 2012; accepted for publication March 7, 2012.
Available online April 25, 2012.
Corresponding author: Yoshiya Tanaka, MD, PhD, First Department of Internal Medicine, School of Medicine, University of Occupational & Environmental Health, Japan, 1-1 Iseigaoka, Yahata-nishi, Kitakyushu 807-8555, Japan. E-mail: tanaka@med.uoeh-u.ac.jp.
0091-6749/$36.00

doi:10.1016/j.jaci.2012.03.014

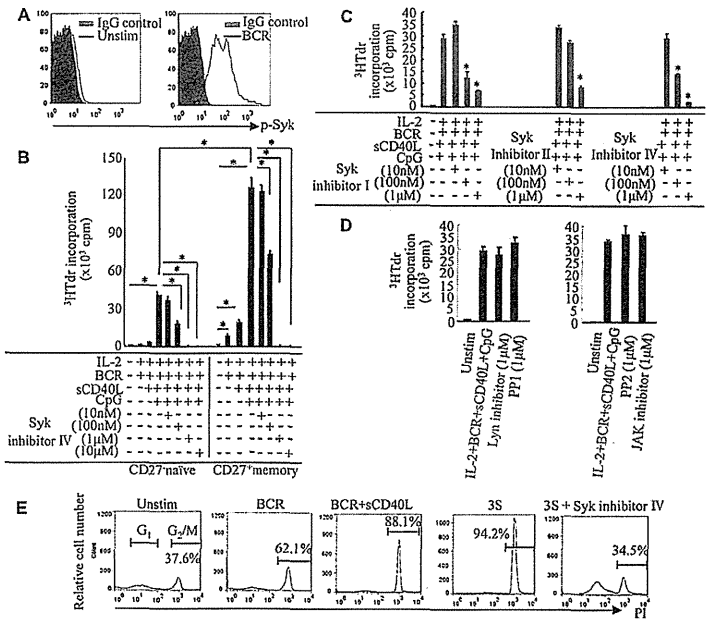

FIG 1. Syk regulates proliferation and cell-cycle progression in B-cell subsets on BCR, CD40, and TLR9 stimulation. A, BCR-induced phosphorylation of Syk (15 minutes). B-D, Tritiated thymidine (*³HTdr*) incorporation of human B cells was measured during the last 18 hours of the 72-hour culture. The data are shown as means ± SDs. **P* < .05. *sCD40L*, Soluble CD40 ligand. E, FACS histograms of nuclear DNA content in memory B cells 24 hours later. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR9 stimulation. Results are representative of 3 independent experiments.

thereby driving efficient TLR9 signaling that is critical for the proliferation and differentiation of human memory B cells.

METHODS

Reagents

Syk inhibitor I, Syk inhibitor II, Syk inhibitor IV, BAY61-3606, PP1, and PP2 were purchased from Merck (Darmstadt, Germany). Lyn peptide inhibitor was purchased from Tocris Bioscience (Ellisville, Mo). PF-956980 (JAK3 kinase inhibitor) was provided from Pfizer, Inc (New York, NY). Anti-BCR mAbs (anti-Igλ and anti-Igκ), recombinant human IL-2, recombinant human CD40 ligand, and phosphorothioate-protected CpG-oligonucleotide 2006 (CpG-ODN 2006; 5′-TCGTCGTTTTGTCGTTTTGTCG TT-3′) were from BD PharMingen (San Diego, Calif), R&D Systems (Minneapolis, Minn), PeproTec (Rocky Hill, NJ), and Greiner Bio-One (Tokyo, Japan), respectively.

Isolation, culture, and stimulation of B-cell subsets

This study protocol has been approved by the ethics committee of our university. PBMCs from 3 healthy donors were isolated with lymphocyte separation medium (ICN/Cappel Pharmaceuticals, Aurora, Ohio). B cells were obtained by means of negative selection from PBMCs by using the memory B-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD27⁺ memory B cells were then isolated by means of positive selection from B cells with CD27 microbeads. The negative fraction of this isolation was assigned to CD27⁻ naive B cells. Purity of naive and memory B cells was greater than 90% (see Fig E1 in this article's Online Repository at www.jacionline.org). B cells were cultured in RPMI 1640 (Wako Pure Clinical Industries, Osaka, Japan) supplemented with 10% FCS (Tissue Culture Biologicals, Tulare, Calif), 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen, Carlsbad, Calif). According to a previous study,[13] we used the combination of anti-Igκ and anti-Igλ mAbs for BCR stimulation and initially ensured strong induction of Syk phosphorylation by these antibodies (Fig 1, *A*). CD40 stimulation with recombinant human CD40 ligand is hereafter referred to simply as CD40 stimulation. CpG-ODN 2006 is a type B CpG-ODN specific for human TLR9 and mainly activates B cells but only weakly stimulates IFN-α secretion in plasmacytoid dendritic cells.[14]

Proliferation assay

Purified B cells were stimulated in 96-well plates (1×10^5 per well) with anti-BCR mAbs (anti-Igλ and anti-Igκ, 1 μg/mL each), soluble CD40 ligand (2 μg/mL), and CpG-ODN (2.5 μg/mL) with or without IL-2 (10 ng/mL). Cells were cultured for 72 hours and pulsed with 0.5 μCi (18.5 kBq) per well of tritiated thymidine during the last 18 hours of culture and then harvested with a semiautomatic cell harvester (Abe Kagaku, Chiba, Japan), and

their uptake of tritiated thymidine was determined with a scintillation counter (Aloka LSC-3500ETM, Tokyo, Japan).

Flow cytometric analysis

After washing, B-cell subsets were incubated in blocking buffer (0.25% human globulin, 0.5% human albumin [Yoshitomi, Osaka, Japan], and 0.1% NaN_3 in PBS) in a 96-well plate at 4°C for 15 minutes. Cells were then suspended in 100 μL of FACS solution (0.5% human albumin and 0.1% NaN_3 in PBS) and treated with fluorescein isothiocyanate (FITC)–labeled murine IgG1κ, anti-human CD80 (BD PharMingen, San Diego, Calif), or anti-human CD86 (Dako Japan, Kyoto, Japan) for 30 minutes at 4°C. Cells were washed 3 times with FACS solution and analyzed with a FACSCalibur (Becton–Dickinson, San Jose, Calif) and FlowJo software (Tomy Digital Biology, Tokyo, Japan). For intracellular staining of phosho-Syk, Blimp-1, TRAF2, TRAF3, TRAF5, TRAF6, and phospho–NF-κB, cells were fixed with PBS containing 1% formaldehyde and permeabilized with saponin-PBS (PBS containing 0.1% saponin, 0.1% BSA, 0.1% NaN3, and 0.01 mol/L HEPES). After washing, cells were resuspended in saponin-PBS and stained with mouse anti-human phospho-Syk (pY348) (BD PharMingen), goat anti-human Blimp-1 (N-20; Santa Cruz Biotechnology, Santa Cruz, Calif), rat anti-human TRAF2 (MBL), rabbit anti-human TRAF3 (Santa Cruz Biotechnology), rabbit anti-human TRAF5 (Santa Cruz Biotechnology), mouse anti-human TRAF6 (Santa Cruz Biotechnology), or rabbit anti-human phospho–NF-κB p65 (Ser 536, 93H1; Cell Signaling Technology, Tokyo, Japan), followed by washing with saponin-PBS. FITC-labeled donkey anti-goat IgG (Santa Cruz Biotechnology), phycoerythrin-labeled goat anti-rat (BD PharMingen), phycoerythrin-labeled goat anti-rabbit (CALTAG), FITC-labeled rat anti-mouse (BD PharMingen), and FITC-labeled goat anti-rabbit IgG (BD PharMingen) were used as secondary antibodies. Isotype-matched goat IgG, rat IgG, rabbit IgG, or mouse IgG controls (all from Sigma-Aldrich, St Louis, Mo) were used to evaluate the background.

Apoptosis assay

Purified B cells were stimulated for 72 hours in 96-well plates (2×10^5 per well) with anti-BCR mAbs (anti-Igλ and anti-Igκ, 1 μg/mL each), soluble CD40 ligand (2 μg/mL), and CpG-ODN (2.5 μg/mL) with or without Syk inhibitor IV. After culture, cells were double-stained with FITC–Annexin V and propidium iodide (PI) in Apoptosis Detection kit I (BD PharMingen). The percentage of apoptotic cells was measured by using flow cytometry.

Cell-cycle analysis

For cell-cycle analysis, cells were suspended in PI staining buffer (50 μg/mL PI, 5 mmol/L EDTA, 1 μg/mL DNase-free RNase, and 0.1% saponin in PBS). The samples were then incubated for 30 minutes at 37°C, and DNA content was analyzed by using flow cytometry.

Cytokine production

Levels of IL-6, IL-10, IL-12 p70, and TNF-α in culture were determined by using the BD Cytometric Bead Array human Flex set, according to the manufacturer's instructions (BD PharMingen).

IgG ELISA

For quantification of *in vitro* IgG secretion, B-cell subsets were cultured with anti-BCR mAbs, CD40 ligand, and CpG-ODN 2006 in 96-well plates (1×10^5 per well) for 5 days. IgG levels in culture were determined by using a human IgG ELISA Quantitation Kit (Bethyl Laboratories, Inc, Montgomery, Ala).

Quantitative real-time PCR

Total RNA was prepared by using the RNeasy Mini Kit (Qiagen, Chatsworth, Calif). First-strand cDNA was synthesized, and quantitative real-time PCR was performed in the Step One Plus instrument (Applied Biosystems, Foster City, Calif) in triplicate wells in 96-well plates. TaqMan target mixes for X-box binding protein 1 *(XBP-1)* (Hs00152973-m1), AICDA (Hs00757808-m1), and *TLR9* (Hs00964360-m1) were purchased from Applied Biosystems. *XBP-1*, activation-induced cytidine deaminase *(AICDA)* and *TLR9* mRNA expression levels were normalized to the levels of 18S ribosomal RNA (Hs99999901-m1, Applied Biosystems) as an endogenous control, and the relative quantity compared with the PBMC sample as a reference was calculated by using the quantification-comparative cycle threshold ($\Delta\Delta C_T$) formula. Relative quantity was calculated by using the $\Delta\Delta C_T$ formula–referenced sample of PBMCs.

Western blot analysis

Raji cells were lysed in an NP-40 buffer containing NaCl, Tris-HCL (pH 8.0), distilled water, and protease inhibitor. Lysates were then mixed with an equal volume of sample buffer solution (2-mercaptoethanol; Wako Pure Chemical Industries) and boiled for 5 minutes. Proteins were separated by means of SDS-PAGE, transferred onto nitrocellulose membranes (Whatman, Tokyo, Japan), blocked with 5% skim milk, and immunoblotted with anti-human Syk, anti-human phospho-Syk (pY348), anti-human TRAF6, anti-human phospho–NF-κB p65 (Ser 536, 93H1), and horseradish peroxidase–labeled anti-secondary (#NA931V and #NA934V; GE Healthcare, Osaka, Japan) by using immunoreaction enhancer solution (Can Get Signal; Toyobo, Osaka, Japan). Blots were developed with ECL Western Blotting Detection Reagents (GE Healthcare) and visualized with a light-capture instrument (ATTO, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed with JMP version 8.0.2 statistical software (SAS Institute Inc, Cary, NC). Statistical significance of differences between the pretreatment and posttreatment values was tested by using the Wilcoxon test. *P* values of less than .05 were considered statistically significant.

RESULTS

Syk is critical for proliferation and cell-cycle progression in memory B cells

We investigated the effect of BCR, CD40, and TLR9 stimulation on the proliferation of B-cell subsets. BCR stimulation alone remarkably induced Syk phosphorylation; however, it had only marginal effects on DNA synthesis in B cells (Fig 1, *A* and *B*). Combined stimulation of BCR, CD40, and TLR9 strongly induced DNA synthesis in both naive and memory B cells, although significantly more so in the latter. This robust proliferation was inhibited by Syk inhibitor IV (BAY61-3606) in a dose-dependent manner (Fig 1, *B*). Similar data were obtained with another Syk inhibitor (Syk inhibitors I and II; Fig 1, *C*). In contrast to these Syk inhibitors, non-Syk inhibitors (PP1, PP2, and JAK inhibitor) were not effective, even at high concentrations (Fig 1, *D*). Syk inhibitor IV was hereinafter used for further experiments. We next tested cell-cycle progression in memory B cells after BCR, CD40, and TLR9 stimulation (Fig 1, *E*). The percentage of cells in the G_2/M phase without stimulation was 37.6%. This value increased further up to 94.2% with combined stimulation of BCR, CD40, and TLR9. Consistent with our results (Fig 1, *B* and *C*), Syk inhibitor IV significantly inhibited G_2/M phase progression in memory B cells. Together, these results suggest a critical role for Syk in BCR-, CD40-, and TLR-induced proliferation and cell-cycle progression in human memory B cells.

Syk regulates expression of costimulatory molecules and cytokine production in B-cell subsets

We tested expression of the costimulatory molecules CD80 and CD86 in B cells (Fig 2). Both were only marginally expressed in

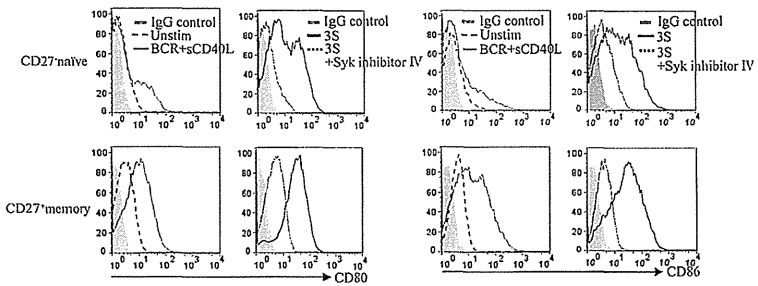

FIG 2. Syk regulates expression of CD80 and CD86 in B-cell subsets on stimulation. *Overlay histograms* depict relative fluorescence intensities of human naive and memory B cells cultured for 72 hours. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR9 stimulation. Results are representative of 3 independent experiments.

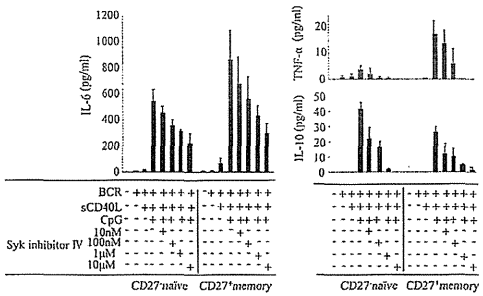

FIG 3. Syk regulates cytokine production in B-cell subsets on stimulation. Human peripheral blood naive and memory B cells were cultured for 72 hours, and supernatants were harvested and assayed by using the cytometric bead array for IL-6, TNF-α, and IL-10 content. Data are shown as means ± SDs and are representative of 3 independent experiments. *sCD40L*, Soluble CD40 ligand.

memory but not naive B cells without stimulation. Combined stimulation of BCR, CD40, and TLR9 induced significant expression of CD80/CD86 in memory B cells compared with that seen in naive cells. Syk inhibitor IV almost completely canceled CD80/CD86 expression in both subsets, suggesting a role of Syk in expression of costimulatory molecules in B cells.

We next analyzed cytokine production (IL-6, IL-10, and TNF-α) by B-cell subsets (Fig 3). Combined stimulation with BCR, CD40, and TLR9 induced production of the proinflammatory cytokines IL-6 and TNF-α in naive and memory cells, although more markedly in the latter. Syk inhibitor IV clearly inhibited production of these cytokines in both subsets in a dose-dependent fashion. In contrast to proinflammatory cytokines, anti-inflammatory IL-10 production was more pronounced in naive than memory B cells, which is consistent with a recent study that IL-10–producing B cells are enriched in human $CD27^{-}CD38^{hi}$ B cells.[15] Again, dose-dependent suppression of IL-10 production by Syk inhibitor IV was observed in both subsets. We failed to detect IL-12 p70, IL-2, IFN-α, and IFN-γ under any conditions (data not shown). These results suggest the critical role of Syk in BCR-, CD40-, and TLR-induced cytokine production in B-cell subsets and also underscore the therapeutic efficacy of Syk inhibitors in decreasing the inflammatory consequences of autoimmune diseases by modulating proinflammatory cytokines, such as TNF-α and IL-6.

Syk regulates B-cell differentiation on BCR, CD40, and TLR9 stimulation

On strong stimulation, B cells differentiate to plasma cells and undergo class-switching along with expression of critical molecules, such as *AICDA*, *XBP-1*, and Blimp-1. Both naive and memory B cells strongly induced expression of *AICDA*, *XBP-1*, and Blimp-1 after BCR, CD40, and TLR9 stimulation, which was inhibited by Syk inhibitor IV (Fig 4, *A* and *B*). In addition, IgG production induced by BCR, CD40, and TLR9 stimulation, which was particularly high in memory B cells, was again greatly reduced by Syk inhibitor IV in a dose-dependent manner (Fig 4, *C*).

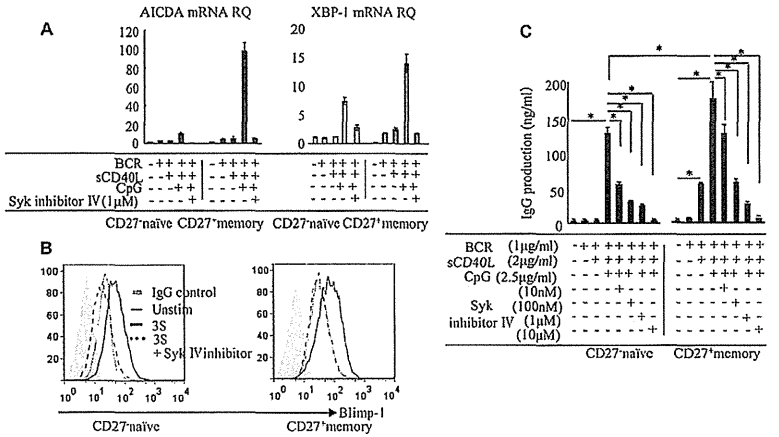

FIG 4. Syk regulates B-cell differentiation on BCR, CD40, and TLR9 stimulation. Naive and memory B cells were cultured for 48 hours (*AICDA* and *XBP-1* mRNA and Blimp-1) or for 5 days (IgG production). *A*, The level of *AICDA* and *XBP-1* mRNA was measured by using real-time PCR. *RQ*, Relative quantity. *B*, Blimp-1 expression was measured by means of flow cytometry. *Unstim*, Before stimulation; *3S*, BCR, CD40, and TLR stimulation. *C*, IgG in the supernatant was quantified by using ELISA. Data are shown as means ± SDs and are representative of 3 independent experiments. $^{*}P < .05$. *sCD40L*, Soluble CD40 ligand.

These results suggest that Syk also regulates B-cell differentiation induced by BCR, CD40, and TLR9 stimulation.

TRAF6 is a key Syk-regulated molecule in B-cell subsets on stimulation

Syk is a key downstream signaling molecule of BCR, but not CD40 or TLR9, in B cells.[16,17] Considering that Syk blockade significantly abrogates proliferation, cytokine production, and differentiation after BCR, CD40, and TLR9 stimulation (Figs 1-4), we particularly sought to elucidate the mechanisms by which Syk regulates TLR9 signaling in human B-cell subsets. Given that TLR9 expression is significantly induced in BCR-stimulated B cells and that TRAFs are the critical downstream molecules in CD40 and TLR9 signaling in B cells,[18,19] we reasoned that TLR9 and TRAFs were possible candidates. Memory B cells constitutively expressed more *TLR9* mRNA than naive B cells (Fig 5, *A*). On BCR, CD40, and TLR9 stimulation, *TLR9* mRNA expression was more drastically induced in memory than naive B cells. Syk inhibitor IV inhibited expression of *TLR9* mRNA in memory B cells to the level seen in unstimulated naive B cells (Fig 5, *A*). Among TRAFs, expression of TRAF2, TRAF3, and TRAF5 was constitutively detected; however, their expression was not affected by BCR stimulation (Fig 5, *B*). In contrast, TRAF6 expression was only slightly detected in memory B cells without stimulation. BCR stimulation alone, however, potently increased TRAF6 expression in both subsets (Fig 5, *B*). TRAF6 expression was further pronounced by additional CD40 and TLR9 stimulation, and strong NF-κB phosphorylation was correlatively observed. Expression of these molecules was blocked by Syk inhibitor IV (Fig 5, *B* and *C*).

Without stimuli, Raji cells exhibit higher basal (tonic) signaling that supports proliferation and survival.[20] In these cells TLR9 mRNA was expressed at a much higher level than in unstimulated naive B cells, which was markedly reduced by Syk inhibitor IV (Fig 6, *A*). In addition, these cells constitutively exhibited pronounced expression and phosphorylation of Syk. Syk inhibitor IV clearly inhibited Syk phosphorylation without affecting its protein levels. Of note, TRAF6 expression and NF-κB phosphorylation were strongly reduced as well by Syk inhibitor IV (Fig 6, *B*). These suggest that Syk blockade exerts an inhibitory action on expression of TLR9, TRAF6, and NF-κB phosphorylation, even in B cells with high basal BCR signaling.

DISCUSSION

In this study we demonstrate that engagement of BCR in conjunction with ligation of CD40 and TLR9 induces remarkable proliferation, expression of costimulatory molecules, cytokine production, and immunoglobulin production in human B cells, especially the memory subset. Moreover, the Syk inhibitor suppresses all of these functions to background levels, at least in part through inhibition of expression of TLR9 and TRAF6, resulting in decreased phosphorylation of NF-κB.

We show that combined stimulation with BCR and CD40 was sufficient to activate memory B cells, whereas it had less effect on naive B cells. However, Additional CpG stimulation caused potent activation of both subsets, although always more strongly in the memory subset, suggesting that memory B cells exhibit a lower threshold for activation compared with naive B cells. Memory B cells can survive without antigenic stimulation, and they can be fully activated only by cognate T-cell help and

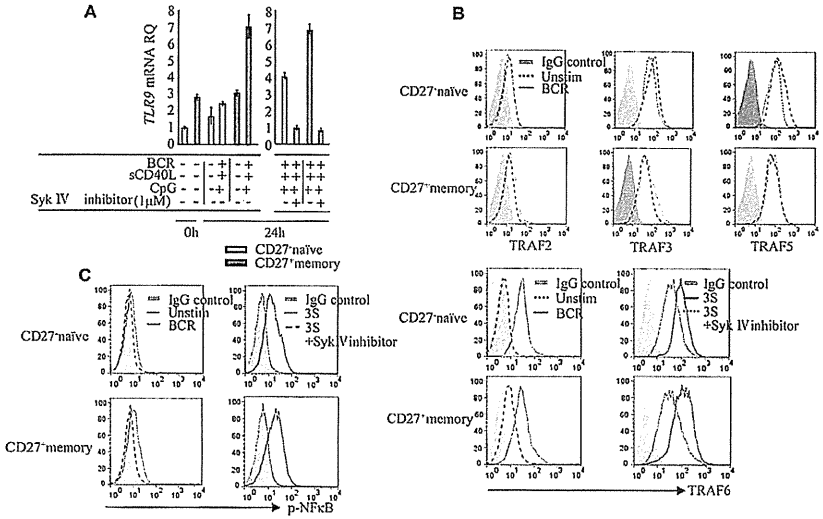

FIG 5. TLR9 and TRAF6 are key Syk-regulated molecules in B-cell subsets on stimulation. A, *TLR9* mRNA was quantified by using real-time PCR (TaqMan PCR kit) 24 hours later. *RQ*, Relative quantity; *sCD40L*, soluble CD40 ligand. B and C, TRAF2, TRAF3, TRAF5, and TRAF6 levels (48 hours later) and NF-κB phosphorylation (p65; 12 hours later) were measured by means of flow cytometry (intracellular staining). *Unstim*, Before stimulation; *3S*, combination of BCR, CD40, and TLR9 stimulation. Data are representative of 3 independent experiments.

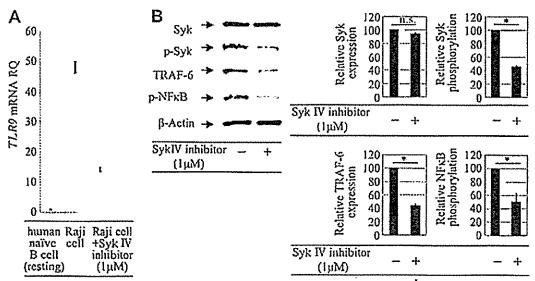

FIG 6. Syk inhibitor exerts marked inhibitory action, even at an activated state of B cells. Raji cells were cultured with RPMI containing 2% FCS for 48 hours. A, *TLR9* mRNA was quantified by means of real-time PCR. *RQ*, Relative quantity. B, Expression of Syk, phospho-Syk (Y348), TRAF6, and phospho-NF-κB (p65) was assessed by means of Western blotting. The intensity of bands was quantified and normalized with respect to those of corresponding β-actin. The resulting values were expressed as the percentage in reference to that of cells without Syk inhibitor IV. Data are shown as means ± SDs and are representative of 3 independent experiments. *$P < .05$. *n.s.*, Not significant.

cytokines.[21-23] In addition, the costimulatory molecules CD80 and CD86, as well as TLR9 and TRAF6, are weakly expressed in memory B cells in the nonstimulated (steady) state (Figs 2 and 5). These findings suggest that a basal BCR tonic signal in memory B cells is higher than in naive B cells, which might account for the maintenance of serologic memory.[24,25]

What signaling molecules are responsible for a basal BCR tonic signal in memory B cells? We recently showed that without

BCR stimulation, weak activation of Syk is constitutively observed in memory B cells.[26] Given that Syk activation is a key event for further propagation of the BCR signaling pathway,[4] these findings support our rationale that blockade of Syk activation regulates the functions of memory B cells. Surprisingly, the effects of the Syk inhibitor on B-cell functions were more dramatic than we had initially expected: it almost completely abrogated B-cell proliferation, activation, cytokine production, and differentiation induced by a combinatorial stimulation of BCR, CD40, and TLR9 (Figs 1-4). We also evaluated B-cell survival by determining the percentage of apoptotic cells with FITC–Annexin V and PI. Consistent with our previous study,[26] without stimuli, a considerable fraction of B cells spontaneously underwent apoptotic cell death *in vitro*, and such cell death was not affected by the Syk inhibitor, excluding nonspecific cytotoxic effects of this inhibitor on B-cell survival (see Fig E2 in this article's Online Repository at www.jacionline.org). On stimulation with BCR, CD40, and TLR9, apoptotic cell death (Annexin V^+PI^- and AnnexinV^+PI^+) was considerably protected. This protection was indeed abrogated by the Syk inhibitor in a dose-dependent manner, suggesting that Syk provides survival signals as well for B cells after stimulation through all 3 receptors (see Fig E2).

It remains somewhat unclear whether Syk is directly activated in CD40 and TLR9 signaling pathways in B cells.[16,17] Ying et al[27] showed that Syk is synergistically activated in B cells on BCR/CD40 costimulation, suggesting a role for Syk in CD40 signaling. Sanjuan et al[28] showed, using human monocytic cell lines, that tyrosine phosphorylation of TLR9 by the Src family kinases leads to the recruitment and activation of Syk, suggesting a role for Syk in TLR9 signaling. In contrast to these findings, we found that robust proliferation in memory B cells after CD40, TLR9, or both stimulation is not influenced by the Syk inhibitor (data not shown). Thus other regulatory mechanisms of B-cell activation by the Syk inhibitor are more likely to exist.

We show here that Syk is a regulator of expression of TLR9 and TRAF6, both of which are critical for TLR9-induced NF-κB activation. Consistent with our results, a previous study showed that *TLR9* mRNA is expressed at high levels in memory B cells and its expression is enhanced by BCR cross-linking,[18] although involvement of Syk in this process was not investigated. NF-κB activation regulates *TLR9* mRNA expression induced by BCR, CD40, and TLR9 stimulation,[29] suggesting that NF-κB–induced TLR9 expression forms a novel feed-forward loop in NF-κB activation in B cells. Blockade of Syk-mediated BCR signaling could thus shut off this loop, thereby inhibiting NF-κB activation and TLR9 expression. Indeed, we found that Syk inhibition reduces expression of TLR9 mRNA in memory B cells to the levels seen in unstimulated, steady-state naive B cells (Fig 5, *A*).

TRAF6 plays a pivotal role in TLR9-induced c-Jun N-terminal kinase activation, CD80 expression,[30] and IL-6 production.[31] B cell–specific disruption of TRAF6 results in a lower number of mature B cells, as well as inhibition of antibody class-switching and impaired differentiation to plasma cells.[32] We found that BCR stimulation alone strongly induces TRAF6 expression, which is further enhanced by additional CD40 and TLR9 stimulation (Fig 5, *B*). TRAF6 expression, as well as NF-κB phosphorylation, on B-cell activation is markedly inhibited by Syk blockade. These findings clearly suggest that Syk-mediated BCR signaling is a prerequisite for optimal induction of TRAF6, allowing efficient propagation of TLR9 signaling.

Our current findings provide a novel insight into B-cell aberrations in patients with SLE. The prevailing hypothesis of B cell–mediated autoimmunity is that both autoantigen-triggered BCR signals and costimulatory signals are required for activation of autoreactive (pathogenic) B cells, which are particularly enriched in the memory subset. However, recent studies showed that TLR7 and TLR9 can recognize self-derived RNA and DNA, respectively, and that TLR signaling is necessary for autoantibody production in mice with lupus.[33,34] BCR-induced calcium mobilization and protein tyrosine phosphorylation were both pronounced in B cells from mice with SLE,[35] indicating that alterations in B-cell signaling already occur at the proximity of the BCR. We here demonstrate that Syk-mediated BCR signaling is a prerequisite for optimal induction of TLR9 and TRAF6, thereby allowing efficient propagation of CD40 and TLR9 signaling, which are critical for the proliferation and differentiation of human memory B cells. Our current findings also underscore the potential role of Syk in B cell–mediated pathologic processes in patients with autoimmune diseases, namely Syk-mediated BCR signaling, could be already activated probably by autoantigens and that Syk inhibitors have potential as new drugs in the treatment of autoimmune diseases, including SLE and RA.

We thank Ms T. Adachi, Ms N. Sakaguchi, and Ms K. Noda for their excellent technical assistance.

Clinical implications: Syk inhibitors might be promising for controlling the aberrant TLR9 signaling that is related to the proliferation and differentiation of pathogenic memory B cells in patients with autoimmune diseases, including SLE and RA.

REFERENCES

1. Iwata S, Saito K, Tokunaga M, Yamaoka K, Nawata M, Yukawa S, et al. Phenotypic changes of lymphocytes in patients with systemic lupus erythematosus who are in longterm remission after B cell depletion therapy with rituximab. J Rheumatol 2011;38:633-41.
2. Krug A. Nucleic acid recognition receptors in autoimmunity. Handb Exp Pharmacol 2008;183:129-51.
3. Ruprecht CR, Lanzavecchia A. Toll-like receptor stimulation as a third signal required for activation of human naïve B cells. Eur J Immunol 2006;36:810-6.
4. Taniguchi T, Kobayashi T, Kondo J, Takahashi K, Nakamura H, Suzuki J, et al. Molecular cloning of a porcine gene syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis. J Biol Chem 1991;266:15790-6.
5. Kulathu Y, Grothe G, Reth M. Autoinhibition and adapter function of Syk. Immunol Rev 2009;232:286-99.
6. Wong WS, Leong KP. Tyrosine kinase inhibitors: a new approach for asthma. Biochim Biophys Acta 2004;1697:53-69.
7. Beaven MA, Baumgartner RA. Downstream signals initiated in mast cells by Fc epsilon RI and other receptors. Curr Opin Immunol 1996;8:766-72.
8. Meltzer EO, Berkowitz RB, Grossbard EB. An intranasal Syk-kinase inhibitor (R112) improves the symptoms of seasonal allergic rhinitis in a park environment. J Allergy Clin Immunol 2005;115:791-6.
9. Podolanczuk A, Lazarus AH, Crow AR, Grossbard E, Bussel JB. Of mice and men: an open-label pilot study for treatment of immune thrombocytopenic purpura by an inhibitor of Syk. Blood 2009;113:3154-60.
10. Weinblatt ME, Kavanaugh A, Genovese MC, Musser TK, Grossbard EB, Magilvay DB. An oral spleen tyrosine kinase (Syk) inhibitor for rheumatoid arthritis. N Engl J Med 2010;363:1303-12.
11. Bahjat FR, Pine PR, Reitsma A, Cassafer G, Baluom M, Grillo S. An orally bioavailable spleen tyrosine kinase inhibitor delays disease progression and prolongs survival in murine lupus. Arthritis Rheum 2008;58:1433-44.
12. Deng GM, Liu L, Bahjat FR, Pine PR, Tsokos GC. Suppression of skin and kidney disease by inhibition of spleen tyrosine kinase in lupus-prone mice. Arthritis Rheum 2010;62:2086-92.
13. Sakurai D, Kanno Y, Hase H, Kojima H, Okumura K, Kobata T. TACI attenuates antibody production costimulated by BAFF-R and CD40. Eur J Immunol 2007;37:110-8.

14. Krug A, Rothenfusser S, Hornung V, Jahrsdörfer B, Blackwell S, Ballas ZK, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol 2001;31:2154-63.
15. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. Immunity 2010;32:129-40.
16. Graham JP, Arcipowski KM, Bishop GA. Differential B-lymphocyte regulation by CD40 and its viral mimic, latent membrane protein 1. Immunol Rev 2010;237:226-48.
17. Krieg AM. A role for Toll in autoimmunity. Nat Immunol 2002;3:423-4.
18. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003;101:4500-4.
19. Xie P, Kraus ZJ, Stunz LL, Bishop GA. Roles of TRAF molecules in B lymphocyte function. Cytokine Growth Factor Rev 2008;19:199-207.
20. Guo Q, Qian L, Guo L, Shi M, Chen C, Lv X, et al. Transactivators Zta and Rta of Epstein-Barr virus promote G0/G1 to S transition in Raji cells: a novel relationship between lytic virus and cell cycle. Mol Immunol 2010;47:1783-92.
21. Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. Nature 2000;407:636-42.
22. Tangye SG, Avery DT, Deenick EK, Hodgkin PD. Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. J Immunol 2003;170:686-94.
23. Kindler V, Zubler RH. Memory, but not naïve, peripheral blood B lymphocytes differentiate into Ig-secreting cells after CD40 ligation and costimulation with IL-4 and the differentiation factors IL-2, IL-10, and IL-3. J Immunol 1997;159:2085-90.
24. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002;298:2199-202.
25. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell 1997;90:1073-83.
26. Tabrizi SJ, Niiro H, Masui M, Yoshimoto G, Iino T, Kikushige Y, et al. T cell leukemia/lymphoma 1 and galectin-1 regulate survival/cell death pathways in human naive and IgM+ memory B cells through altering balances in Bcl-2 family proteins. J Immunol 2009;182:1490-9.
27. Lin YC, Huang DY, Chu CL, Lin WW. Anti-inflammatory actions of Syk inhibitors in macrophages involve non-specific inhibition of toll-like receptors-mediated JNK signaling pathway. Mol Immunol 2010;47:1569-78.
28. Sanjuan MA, Rao N, Lai KT, Gu Y, Sun S, Fuchs A, et al. CpG-induced tyrosine phosphorylation occurs via a TLR9-independent mechanism and is required for cytokine secretion. J Cell Biol 2006;172:1057-68.
29. An H, Yu Y, Zhang M, Xu H, Qi R, Yan X, et al. Involvement of ERK, p38 and NF-kappaB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. Immunology 2002;106:38-45.
30. Rowland SL, Tremblay MM, Ellison JM, Stunz LL, Bishop GA, Hostager BS. A novel mechanism for TNFR-associated factor 6-dependent CD40 signaling. J Immunol 2007;179:4645-53.
31. Jalukar SV, Hostager BS, Bishop GA. Characterization of the roles of TNF receptor-associated factor 6 in CD40-mediated B lymphocyte effector functions. J Immunol 2000;164:623-30.
32. Kobayashi T, Kim TS, Jacob A, Walsh MC, Kadono Y, Fuentes-Panana E, et al. TRAF6 is required for generation of the B-1a B cell compartment as well as T cell-dependent and -independent humoral immune responses. PLoS One 2009;4:e4736.
33. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchick MJ. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity 2006;25:417-28.
34. Viglianti GA, Lau CM, Hanley TM, Miko BA, Shlomchik MJ, Marshak-Rothstein A. Activation of autoreactive B cells by CpG dsDNA. Immunity 2003;19:837-47.
35. Liossis SN, Kovacs B, Dennis G, Kammer GM, Tsokos GC. B cells from patients with systemic lupus erythematosus display abnormal antigen receptor-mediated early signal transduction events. J Clin Invest 1996;98:2549-57.

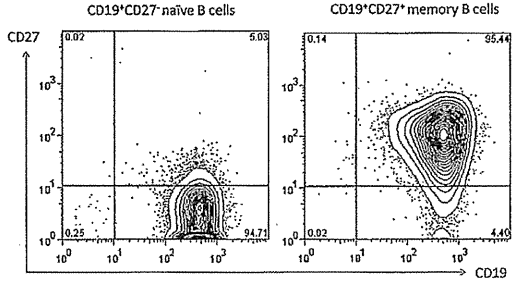

FIG E1. Phenotypic analysis of B-cell subsets in human peripheral blood. B cells were obtained by means of negative selection from PBMCs. $CD27^+$ memory B cells were then isolated by using positive selection from B cells with CD27 microbeads. The negative fraction of this isolation was assigned to $CD27^-$ naive B cells. The purity of naive and memory B cells was greater than 90% (*x-axis*, CD19; *y-axis*, CD27).

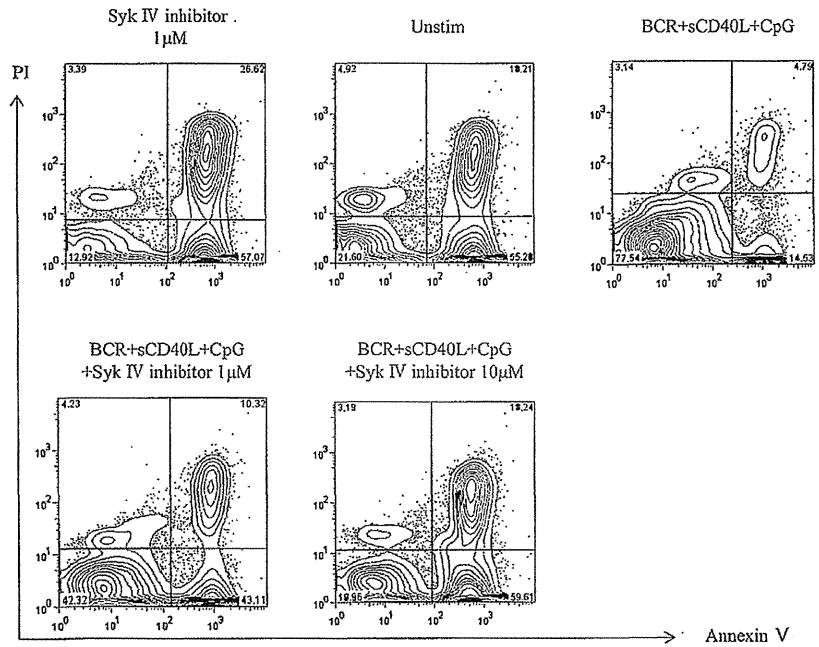

FIG E2. Syk provides survival signals for B cells after stimulation through all 3 receptors. B cells (2×10^5 per well) were cultured in triplicate in 96-well plates with anti-Igλ and anti-Igk antibodies (1 μg/mL), soluble CD40 ligand (*sCD40L*; 2 μg/mL), and CpG-ODN 2006 (2.5 μg/mL) with or without Syk inhibitor IV for 72 hours. The percentage of apoptotic B cells was assessed by means of double-staining with FITC–Annexin V and PI (*x-axis*, PI; *y-axis*, Annexin V).

ARTHRITIS & RHEUMATISM
Vol. 64, No. 6, June 2012, pp 1790–1798
DOI 10.1002/art.34329

The JAK Inhibitor Tofacitinib Regulates Synovitis Through Inhibition of Interferon-γ and Interleukin-17 Production by Human CD4+ T Cells

Keisuke Maeshima,[1] Kunihiro Yamaoka,[2] Satoshi Kubo,[2] Kazuhisa Nakano,[2] Shigeru Iwata,[2] Kazuyoshi Saito,[2] Masanobu Ohishi,[3] Hisaaki Miyahara,[3] Shinya Tanaka,[2] Koji Ishii,[4] Hironobu Yoshimatsu,[4] and Yoshiya Tanaka[2]

Objective. **Tofacitinib (CP-690,550) is a novel JAK inhibitor that is currently in clinical trials for the treatment of rheumatoid arthritis (RA). The aim of this study was to examine the effects of tofacitinib in vitro and in vivo in RA, in order to elucidate the role of JAK in the disease process.**

Methods. **CD4+ T cells, CD14+ monocytes, and synovial fibroblasts (SFs) were purified from the synovium and peripheral blood of patients with RA and were evaluated for the effect of tofacitinib on cytokine production and cell proliferation. For in vivo analysis, synovium and cartilage samples obtained from patients with RA were implanted in immunodeficient mice (SCID-HuRAg mice), and tofacitinib was administered via an osmotic minipump.**

Results. **Tofacitinib treatment of CD4+ T cells originating from synovium and peripheral blood inhibited the production of interleukin-17 (IL-17) and interferon-γ (IFNγ) in a dose-dependent manner, affecting both proliferation and transcription, but had no effect on IL-6 and IL-8 production. Tofacitinib did not affect IL-6 and IL-8 production by RASFs and CD14+ monocytes. However, conditioned medium from CD4+ T cells cultured with tofacitinib inhibited IL-6 production by RASFs and IL-8 production by CD14+ monocytes. Treatment of SCID-HuRAg mice with tofacitinib decreased serum levels of human IL-6 and IL-8 and markedly suppressed invasion of synovial tissue into cartilage.**

Conclusion. **Tofacitinib directly suppressed the production of IL-17 and IFNγ and the proliferation of CD4+ T cells, resulting in inhibition of IL-6 production by RASFs and IL-8 production by CD14+ cells and decreased cartilage destruction. In CD4+ T cells, presumably Th1 and Th17 cells, JAK plays a crucial role in RA synovitis.**

The importance of inflammatory cytokines in the pathogenesis of rheumatoid arthritis (RA) has become apparent based on the clinical efficacy of biologic agents targeting tumor necrosis factor α (TNFα), interleukin-1 (IL-1) receptor, and IL-6. For such cytokines to exert their biologic activities, the appropriate intracellular signaling pathways must be activated via their specific receptors on the cell surface. Tyrosine kinases are the first intracellular signaling molecules to be activated following receptor binding in a cytokine response. Therefore, various tyrosine kinases are involved at the sites of inflammation (1,2). Several recent studies have focused on tyrosine kinases as potential targets for the treatment of RA. Among these, the JAK family, consisting of JAK-1, JAK-2, JAK-3, and tyrosine kinase 2

Supported in part by the Ministry of Health, Labor, and Welfare of Japan (Research Grant-In-Aid for Scientific Research), by the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the University of Occupational and Environmental Health, Japan.

[1]Keisuke Maeshima, MD: University of Occupational and Environmental Health, Japan, Kitakyushu, Japan, and Oita University, Oita, Japan; [2]Kunihiro Yamaoka, MD, PhD, Satoshi Kubo, MD, Kazuhisa Nakano, MD, PhD, Shigeru Iwata, MD, PhD, Kazuyoshi Saito, MD, PhD, Shinya Tanaka, MD, PhD, Yoshiya Tanaka, MD, PhD: University of Occupational and Environmental Health, Japan, Kitakyushu, Japan; [3]Masanobu Ohishi, MD, PhD, Hisaaki Miyahara, MD, PhD: National Hospital Organization Kyushu Medical Center, Fukuoka, Japan; [4]Koji Ishii, MD, PhD, Hironobu Yoshimatsu, MD, PhD: Oita University, Oita, Japan.

Dr. Y. Tanaka has received consulting fees, speaking fees, and/or honoraria from Eisai, Pfizer, Abbott, Janssen Pharma, Takeda, AstraZeneca, Astellas, Asahi Kasei Pharma, and GlaxoSmithKline (less than $10,000 each) and from Chugai and Mitsubishi Tanabe Pharma (more than $10,000 each).

Address correspondence to Yoshiya Tanaka, MD, PhD, First Department of Internal Medicine, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu, Fukuoka 807-8555, Japan. E-mail: tanaka@med.uoeh-u.ac.jp.

Submitted for publication April 28, 2011; accepted in revised form December 1, 2011.

(Tyk-2), has gathered particular attention, because JAKs are essential for the signaling pathways of various cytokines and growth factors that have been implicated in the pathogenesis of RA (e.g., IL-2, IL-6, IL-7, IL-12, IL-15, IL-17, IL-23, granulocyte–macrophage colony-stimulating factor, and interferon-γ [IFNγ]).

The importance of JAKs in development of the immune system has been demonstrated by gene deletion or mutation. According to the abundant expression of JAK-1 and JAK-2, deletion or mutation of either gene in mice has been shown to be lethal, whereas mutation of Tyk-2 or JAK-3 results in immunodeficiency in both humans and mice (3,4). In contrast to other members of the JAK family that are widely expressed, JAK-3 expression is essentially limited to hematopoietic cells, and JAK-3 constitutively binds to the common γ-chain (γ_c-chain). The γ_c-chain is a common receptor subunit for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, some of which are known to be involved in RA. In fact, tofacitinib, an orally available selective inhibitor of JAK, "dose-dependently decreased endpoints of disease" in both murine collagen-induced arthritis and rat adjuvant-induced arthritis (5). Furthermore, tofacitinib is currently in clinical trials for the treatment of RA, and satisfactory effects, acceptable safety, and, surprisingly, efficacy comparable with that of known biologic agents have been observed (6–8).

Tofacitinib is a selective inhibitor of JAKs with nanomolar potency resulting in the inhibition of transmigration of STAT molecules to the nucleus. Initially, the high specificity of tofacitinib for JAK-3 attracted attention; however, recent efforts to investigate the mechanism of action have shown that tofacitinib interacts with multiple JAKs and presumably other kinases (9–11). Tofacitinib preferentially inhibits JAK-1, JAK-3, and STAT-1 activation, resulting in potent inhibition of γ_c-chain cytokines, IL-6, and IFNγ in naive CD4+ T cells (12).

Although the biologic roles of JAK in lymphocytes are well known, its function in monocyte-lineage cells remains elusive. Previously, we reported that dendritic cells (DCs) from $Jak3^{-/-}$ mice produce increased IL-10, but not IL-6 or TNFα, compared with wild-type DCs, in response to Toll-like receptor ligands (13). Ghoreschi et al also showed increased IL-10 production in mouse plasma after the mice received an intraperitoneal injection of lipopolysaccharide (LPS) following tofacitinib pretreatment (12). Accordingly, tofacitinib may affect not only lymphoid cells but also myeloid cells and other cells that do not express JAK-3, such as mesenchymal cells involved in synovitis, as an off-target effect.

The remarkable effects of tofacitinib observed in clinical studies thus far indicate that this agent will be widely used for the treatment of RA. Although the precise action of tofacitinib on the JAK/STAT pathway in mice has been investigated, the exact mechanism of action under inflammatory conditions in humans remains unclear. Improved knowledge of the underlying mechanisms of tofacitinib would contribute to a better understanding of the pathogenesis of RA and to further application of the drug in other diseases. In this study, we used synovium from patients with RA for in vitro and in vivo experiments to evaluate the effect of tofacitinib and elucidate the role of JAKs at the sites of inflammation in RA.

PATIENTS AND METHODS

Tofacitinib (CP-690,550). Tofacitinib (kindly provided by Pfizer) was dissolved in DMSO (Wako) and kept as a 20-μ*M* stock solution at −80°C.

Cell isolation. Human synovial tissue specimens were obtained from patients undergoing joint replacement surgery or synovectomy at our university and at the National Hospital Organization Kyushu Medical Center. All patients fulfilled the 1987 American College of Rheumatology criteria for the classification of RA (14) and provided written informed consent. All patients had active RA but had never received treatment with biologic agents. The tissue was digested with collagenase (1 mg/ml; Wako) and Dispase (1,000 proteolytic units/ml; Godo Shusei) for at least 2 hours at 37°C. After being filtrated, the cells were cultured in 10-cm culture dishes with RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Following overnight culture, CD14+ cells were isolated from adherent cells by positive selection, and CD4+ T cells were isolated from nonadherent cells by negative selection using a magnetic cell separation system (Miltenyi Biotec). Purities were >90%, as determined by flow cytometry (FACSCalibur; BD PharMingen). Adherent cells were subcultured in Dulbecco's modified Eagle's medium to purify RA synovial fibroblasts (RASFs). Cells between passages 3 and 6 were used for the experiments, as a homogeneous population of RASFs. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation over Lymphocyte Separation Medium (ICN Pharmaceuticals), and CD4+ T cells and CD14+ monocytes were obtained as described above.

Cell proliferation, apoptosis, and cytokine production. Synovial and peripheral blood CD4+ T cells were plated at 2×10^5 cells/200 μl with the indicated concentrations of tofacitinib and stimulated with plate-bound anti-CD3 antibodies (100 ng/well; R&D Systems) and soluble anti-CD28 antibodies (1 μg/ml; R&D Systems) for 72 hours; supernatants were harvested to measure cytokine levels and to use as culture medium for RASFs and CD14+ cells. Peripheral blood CD4+ T cells were prestimulated with anti-CD3 and anti-CD28 antibodies for 72 hours, collected and washed, and re-plated at 2×10^5 cells/200 μl with the indicated concentrations of tofacitinib; the cells were stimulated with recombinant IL-2 (100 ng/ml; R&D Systems) for 72 hours, and supernatants were harvested. A BD Cytometric Bead Array (BD Biosciences) and a DuoSet enzyme-linked immunosorbent assay (R&D Systems) were used to measure cytokine concentrations. To analyze cell proliferation, cells were pulsed with ^{3}H-thymidine for the last 16 hours of culture. Cell apoptosis was evaluated by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide (PI) (BD PharMingen). RASFs were plated at a density of 5×10^3 cells/200 μl and stimulated with LPS from *Escherichia coli* 055:B5 (10 ng/ml; Sigma-Aldrich), IL-1β (10 pg/ml; Relia Tech), or IL-17 (10 ng/ml; R&D Systems) for 48 hours. CD14+ cells were plated at 1×10^5 cells/200 μl and cultured for 24 hours.

TaqMan polymerase chain reaction analysis. Total RNA was isolated using an RNeasy Mini Kit (Qiagen), and complementary DNA was synthesized. TaqMan Gene Expression Assays for human IL-17A (Hs99999082_m1), IFNγ (Hs99999041_m1), and GAPDH (Hs99999905_m1) (Applied Biosystems) were used to evaluate gene expression. The relative quantities were obtained using the comparative threshold (C_t) method and were normalized to GAPDH. Stimulation-dependent fold induction was calculated relative to the C_t value obtained in the unstimulated cells. All experiments were performed in triplicate.

SCID-HuRAg mice. Male SCID mice (CB17/Icr; CLEA Japan), 6–8 weeks of age, were housed in specific pathogen–free conditions at our university animal center. Synovial tissue, articular cartilage, and bone obtained as a mass from 2 patients with RA at the time of joint replacement surgery were used. Synovium was cut into pieces 5–10 mm in diameter, and cartilage was cut into 2-mm^3 pieces. Mice were anesthetized according to the guidelines established by our animal ethics committee, and synovium and cartilage were transplanted onto the backs of 9 SCID mice (day 0). One week after implantation, the 9 mice were randomly divided into 3 groups, and tofacitinib dissolved in polyethylene glycol 300 (Sigma-Aldrich) was administered continuously at dosages of 0 mg/kg/day (n = 3), 1.5 mg/kg/day (n = 3), or 15 mg/kg/day (n = 3) via Alzet osmotic minipumps (DURECT Corporation) (5,15) implanted subcutaneously on the backs. Blood samples were collected, and the sera were stored at −80°C until measurement of IL-6 and IL-8.

Histologic evaluation of SCID-HuRAg mice. Implanted tissues were removed from the SCID-HuRAg mice 5 weeks after implantation, paraffin embedded, and stained with hematoxylin and eosin. Immunostaining was performed with anti–IL-6 antibodies (R&D Systems) and anti–IL-8 antibodies (R&D Systems). Invasion of synovial tissue into the cartilage was quantified according to a semiquantitative score ranging from 0 to 4, based on the number of invading cell layers and the number of affected cartilage sites. Erosion was classified as previously described (16), as follows: 0 = no or minimal invasion, 0.5 = invasion of 1–2 cell layers, 1 = invasion of 3–5 cell layers, 1.5 = invasion of 3–5 cell layers at 3 independent sites of the cartilage, 2 = invasion of 6–10 cell layers, 2.5 = invasion of 6–10 cell layers at 3 independent sites, 3 = invasion of >10 cell layers, 3.5 = invasion of >10 cell layers at 2 independent sites, and 4 = invasion of >10 cell layers at ≥3 independent sites. The invasion scores were determined by counting cells at 400× magnification in 7 high-power fields in each specimen. Histologic assessments were made under double-blind conditions. Three animal researchers recorded the data on separate case record forms without exchanging any information.

Statistical analysis. All data were evaluated by one-way analysis of variance with Dunnett's post hoc test. *P* values less than 0.05 were considered significant.

RESULTS

Tofacitinib-induced inhibition of proliferation of CD4+ T cells from synovium and peripheral blood. We first analyzed the effect of tofacitinib on the proliferation of CD4+ T cells isolated from the synovium and peripheral blood of patients with active RA. When CD4+ T cells were stimulated with anti-CD3 and anti-CD28 antibodies, marked proliferation was induced. However, the addition of tofacitinib to the culture inhibited the proliferation in a dose-dependent manner, with a statistically significant difference starting at 10 n*M*) (Figures 1A and B). Similar inhibitory effects were observed in CD4+ T cells from healthy subjects (data

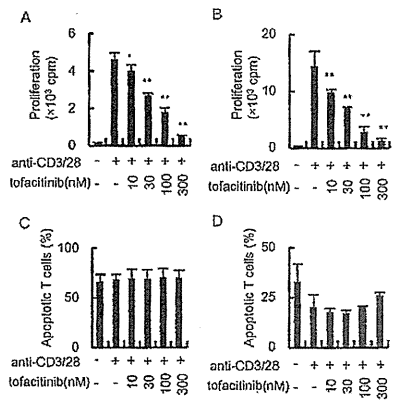

Figure 1. Tofacitinib inhibits proliferation of CD4+ T cells derived from the synovium and peripheral blood of patients with rheumatoid arthritis (RA), without cell toxicity. Synovial (A and C) and peripheral blood (B and D) CD4+ T cells were stimulated with anti-CD3/anti-CD28 antibodies in the presence of increasing doses of tofacitinib. A and B, To analyze cell proliferation, cells were pulsed with ^{3}H-thymidine for the last 16 hours of culture. Values are the mean ± SD of triplicate cultures. The experiments were repeated in 3 RA patients, and the results were similar; representative data are shown. * = $P < 0.05$; ** = $P < 0.01$ versus stimulated untreated cells. C and D, Cell apoptosis was evaluated by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide. Values are the mean ± SD results of all experiments.

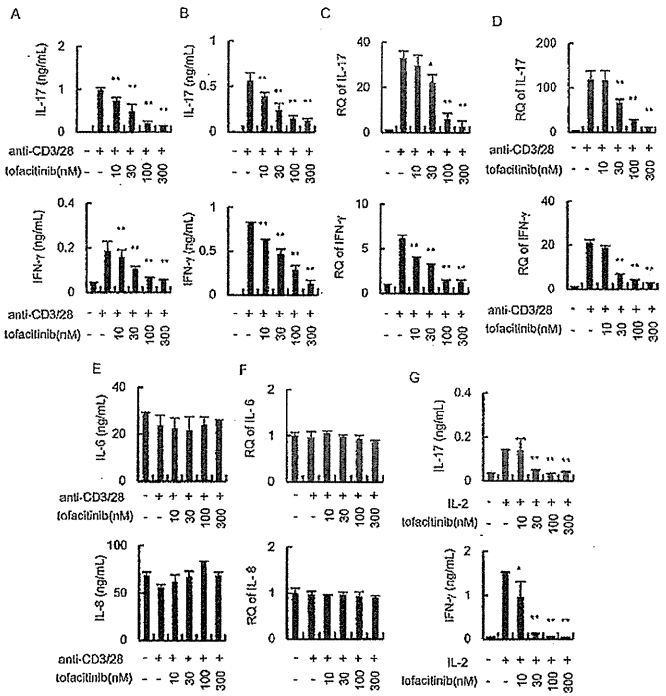

Figure 2. Inhibitory effect of tofacitinib on interleukin-17 (IL-17) and interferon-γ (IFNγ) production, but not IL-6 and IL-8 production, by CD4+ T cells derived from the synovium and peripheral blood of patients with rheumatoid arthritis (RA). Synovial (A, C, E, and F) and peripheral blood (B and D) CD4+ T cells were stimulated with anti-CD3/anti-CD28 antibodies for 72 hours in the presence of increasing concentrations of tofacitinib. A, B, and E, Culture supernatants were collected for analysis of cytokine production. C, D, and F, Messenger RNA expression (fold induction versus unstimulated cells) was determined by TaqMan polymerase chain reaction. G, After prestimulation with anti-CD3/anti-CD28 antibodies for 72 hours, peripheral blood CD4+ T cells were restimulated with IL-2 (100 ng/ml) for 72 hours, and supernatants were harvested. Values are the mean ± SD of triplicate cultures. The experiments were repeated in 3 RA patients, and the results were similar; representative data are shown. * = $P < 0.05$; ** = $P < 0.01$ versus stimulated untreated cells. RQ = relative quantity.

not shown). Next, in order to evaluate whether these inhibitory effects were mediated by the cytotoxicity of tofacitinib, synovial and peripheral blood CD4+ T cells were stained with annexin V and PI. The addition of tofacitinib did not significantly affect the percentage of apoptotic cells (annexin V–positive/PI-negative and annexin V–positive/PI-positive) even at the highest concentration of tofacitinib (300 n*M*) (Figures 1C and D), indicating that the effects of tofacitinib were not mediated by apoptosis.

Tofacitinib-induced inhibition of IL-17 and IFNγ production by CD4+ T cells. We next assessed the effects of tofacitinib on cytokine production by CD4+ T cells obtained from patients with RA. Although stimulation of CD4+ T cells derived from both the synovium and peripheral blood with anti-CD3/anti-CD28 antibodies strongly induced the production of IL-17 and IFNγ, the addition of tofacitinib to the culture inhibited production of these cytokines in a dose-dependent manner, starting at the minimum concentration of 10 n*M* (Figures 2A and B). Furthermore, tofacitinib decreased messenger RNA (mRNA) levels of IL-17 and IFNγ in a dose-dependent manner, indicating inhibitory effects on gene transcription (Figures 2C and D). In contrast to its effect on IL-17 and IFNγ, tofacitinib did not affect IL-6 and IL-8 production by synovial and peripheral blood CD4+ T cells, at both the protein and mRNA levels (Figures 2E and F, and data not shown). Furthermore, when peripheral blood CD4+ T cells were restimulated with IL-2 (100 ng/ml) for 72 hours in the presence or absence of tofacitinib after prestimulation with anti-CD3/anti-CD28 antibodies, production of IL-17 and IFNγ by peripheral blood CD4+ T cells was inhibited by tofacitinib in a dose-dependent manner (Figure 2G). These results suggest that inhibition of IL-17 and IFNγ production could be associated with inhibition of the IL-2–mediated JAK-1/3/STAT-5 pathway by tofacitinib.

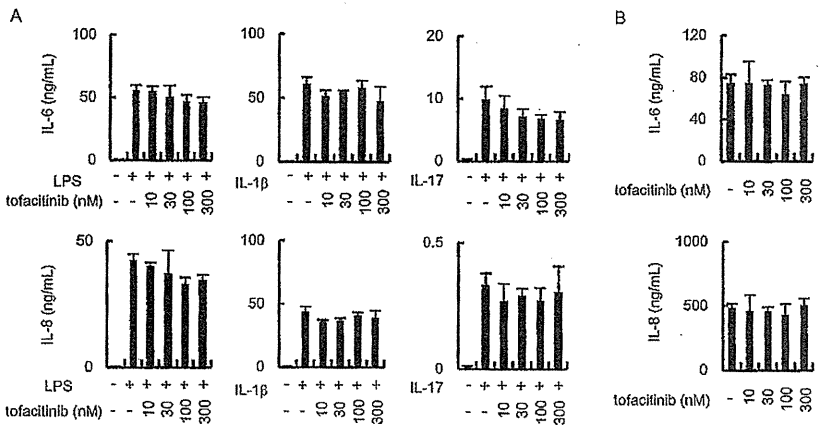

Figure 3. Tofacitinib does not affect interleukin-6 (IL-6) and IL-8 production by rheumatoid arthritis synovial fibroblasts (RASFs) and CD14+ monocytes originating from RA synovium. A, RASFs were stimulated with 10 ng/ml of lipopolysaccharide (LPS), 10 pg/ml of IL-1β, or 10 ng/ml of IL-17 for 48 hours, alone or with tofacitinib in increasing concentrations. B, CD14+ monocytes were cultured for 24 hours with increasing concentrations of tofacitinib, and supernatant was collected for analysis of IL-6 and IL-8 production. Values are the mean ± SD of triplicate cultures. The experiments were repeated in 3 RA patients, and the results were similar; representative data are shown.

Lack of effect of tofacitinib on IL-6 and IL-8 production by RASFs and CD14+ monocytes. We next investigated the effect of tofacitinib on RASFs and CD14+ monocytes, which together are the major source of cytokines in RA synovium. Because RASFs expressed JAK-1 and JAK-2 abundantly but did not express JAK-3 (data not shown), we expected off-target effects of tofacitinib on RASFs. Both LPS and IL-1β, which are not implicated in JAK/STAT signaling, strongly induced the production of IL-6 and IL-8 by RASFs. The production of these cytokines was not, however, affected by the addition to the culture of tofacitinib at any concentration (Figure 3A). Furthermore, although CD14+ monocytes isolated from RA synovium produced large amounts of IL-6 and IL-8 without any stimulation, the addition of tofacitinib did not affect production of these cytokines (Figure 3B). Based on our previous studies of JAK-3–deficient DCs, we expected tofacitinib to increase IL-10 production by CD14+ monocytes. However, we did not observe the parallel phenotype in vitro (data not shown). These results indicate that the mode of action of tofacitinib appeared to be restricted to

proliferation and particular cytokine production by CD4+ T cells rather than CD14+ monocytes and RASFs in patients with RA.

Indirect effect of tofacitinib on IL-6 production by RASFs and IL-8 production by CD14+ monocytes. Because direct inhibitory effects of tofacitinib on IL-6 and IL-8 production by RASFs and CD14+ monocytes were not observed, we next investigated the possibility of an indirect effect of tofacitinib through CD4+ T cells. We collected the culture supernatants from purified CD4+ T cells that were stimulated with anti-CD3/anti-CD28 antibodies in the presence of tofacitinib, added the obtained conditioned medium to RASFs or CD14+ monocytes from RA synovium, and assessed IL-6 and IL-8 levels in the supernatants of RASFs and CD14+ monocytes after further incubation. When RASFs were cultured with supernatant from CD4+ T cells treated with tofacitinib, IL-6 production was reduced significantly at doses of 30 n*M* and higher, while IL-8 production was not affected (Figure 4A). When CD14+ monocytes were cultured with the supernatant, IL-8 production decreased significantly at doses of 30 n*M* and higher, whereas IL-6 production was not affected (Figure 4B). Thus, tofacitinib-induced inhibition of cytokine production by CD4+ T cells appeared to result in reduced production of IL-6 and IL-8 from RASFs and CD14+ monocytes in a cell-trophic manner.

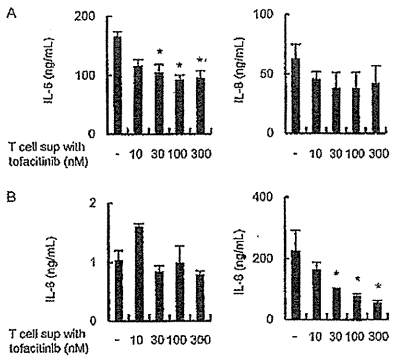

Figure 4. Tofacitinib indirectly suppresses IL-6 production by RASFs and IL-8 production by CD14+ monocytes. Peripheral blood CD4+ T cells were stimulated with anti-CD3/anti-CD28 antibodies for 72 hours in the presence of increasing concentrations of tofacitinib. Culture supernatants (sup) were harvested and cultured with RASFs or peripheral blood CD14+ cells. RASFs (**A**) and CD14+ monocytes (**B**) were cultured for 48 hours and 24 hours, respectively, and the supernatants were collected to measure cytokine concentration. Values are the mean ± SD of 3 individual experiments. The experiments were repeated in 3 RA patients, and the results were similar. * = $P < 0.05$; ** = $P < 0.01$ versus stimulated untreated cells. See Figure 3 for other definitions.

Effect of tofacitinib on production of human IL-6 and IL-8 and invasion of synovial cells into cartilage in SCID-HuRAg mice. In order to clarify the mode and mechanism of action of tofacitinib, we assessed the in vivo effects of tofacitinib in SCID-HuRAg mice. Tofacitinib was continuously administered to these mice by osmotic minipump. We observed increased production of human IL-6 and IL-8 in serum from the SCID-HuRAg mice, which peaked 7–14 days after implantation and then gradually decreased and became undetectable within 21 to 28 days (data not shown). Human TNF and IL-10 were not detected in the serum. Because of the variable levels of cytokine production depending on the condition of synovium samples, IL-6 and IL-8 levels on day 14 were compared with those on day 7 to evaluate the effect of tofacitinib in vivo (Figure 5A). In the groups receiving tofacitinib at dosages of 1.5 mg/kg/day and 15 mg/kg/day, the serum level of human IL-8 was significantly lower compared with that in the control group. Human IL-6 was also inhibited by tofacitinib, although there was no significant difference compared with control.

To further investigate the effect of tofacitinib on cytokine expression and cartilage destruction, we removed the implanted specimens on day 35 and performed histologic evaluation. Immunohistochemical analysis demonstrated that IL-6 was highly expressed in the RA synovium grafts in mice treated with vehicle, but that the number of IL-6–positive cells was markedly reduced in the tofacitinib-treated group (Figure 5C). Tofacitinib also decreased the expression of IL-8 (Figure 5C) and IL-17 (data not shown) in the implanted RA synovium graft. Furthermore, the mice treated with vehicle alone showed prominent invasion of synovial tissue into the implanted cartilage. However, treatment with tofacitinib markedly inhibited this invasion (Figure 5D). Histologic evaluation according to the erosion score also showed a dose response, with significant differences between mice treated with high-dose tofacitinib (15 mg/kg/day) and placebo-treated controls (Figure 5B).

DISCUSSION

The JAK/STAT pathway is a common signaling pathway activated by inflammatory cytokines, which has recently received attention as a new potential molecular target for the treatment of RA. In this study, we used

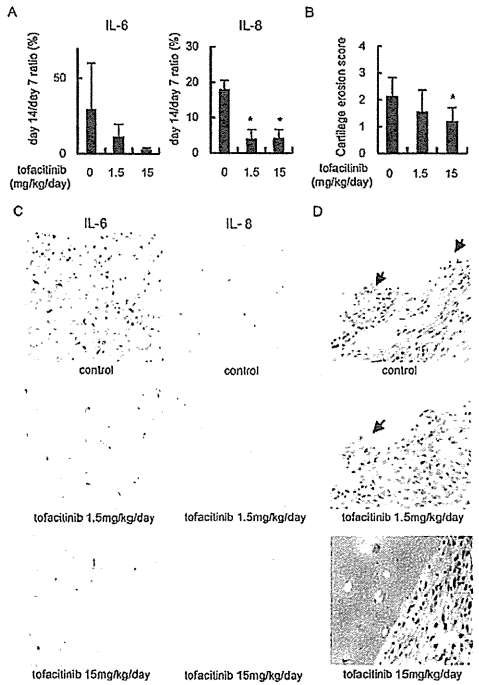

Figure 5. Tofacitinib suppresses human interleukin-6 (IL-6) and IL-8 production and cartilage destruction in SCID-HuRAg mice. Rheumatoid arthritis (RA) synovium and articular cartilage were co-implanted onto the backs of SCID mice. Treatment with vehicle or tofacitinib (1.5 or 15 mg/kg/day) was initiated on day 7, and thereafter serum was collected weekly. The co-implants were removed on day 35 and stained for histologic evaluation. **A**, Markedly decreased production of human IL-6 and IL-8 in the tofacitinib-treated groups compared with the vehicle-treated group. **B**, Cartilage erosion score in each treatment group. The experiments were repeated in 2 RA patients, and the results were similar. Bars show the mean ± SEM of 3 individual experiments. * = $P < 0.05$ versus control. **C** and **D**, Immunohistochemical evaluation of human IL-6– and IL-8–positive cells (**C**), and light microscopic features of cartilage erosion in the engrafted specimens (**D**). Arrows show the invasive front of the synovial tissue. Original magnification × 400.

CD4+ T cells, RASFs, and CD14+ monocytes purified from the synovium and peripheral blood of patients with RA to clarify the mechanism of the JAK inhibitor tofacitinib, which has shown clinical benefit in trials involving patients with active RA (6–8). Although the high specificity of tofacitinib for JAK-3 was shown in earlier studies (9), recent in vitro evidence (10,12) and the emergence of anemia and neutropenia in clinical trials (6,8) have indicated that tofacitinib also exerts an inhibitory effect on other JAKs. The present study showed that the therapeutic potency of tofacitinib in patients with RA could occur via the inhibition of CD4+ T cells, especially proliferation and cytokine production for which JAK plays a critical role in physiologic processes.

IL-6 plays a pivotal role in the pathologic pro-

cesses in RA, and anti–IL-6 receptor antibody is therapeutically useful in RA (17,18). Although previous studies have shown that tofacitinib decreased the serum IL-6 level in a rodent model of arthritis (5,11), this study is the first to show that tofacitinib inhibited both human IL-6 and IL-8 derived from RA synovium implanted in SCID mice. However, we observed that tofacitinib did not directly affect IL-6 and IL-8 production by RASFs, CD14+ monocytes, and CD4+ T cells in vitro, whereas IL-17 and IFNγ production by CD4+ T cells was markedly decreased by tofacitinib in vitro. In contrast, we also observed that production of IL-6 and IL-8 by RASFs and CD14+ monocytes was significantly reduced in a concentration-dependent manner when these cells were cultured with supernatant from CD4+ T cells treated with tofacitinib. This suggests that tofacitinib inhibited IL-6 production by RASFs and IL-8 production by CD14+ monocytes in an indirect manner through the inhibition of CD4+ T cells. Moreover, the numbers of IL-6– and IL-8–positive cells were significantly reduced, with decreased cartilage destruction in SCID-HuRAg mice treated with tofacitinib. Thus, it appears that tofacitinib-induced specific inhibition of IL-17 and IFNγ production by CD4+ T cells (presumably Th1 and Th17 cells) resulted in the suppression of IL-6 and IL-8 production by RASFs and CD14+ monocytes, with decreased cartilage destruction in SCID-HuRAg mice.

The mechanism of tofacitinib-induced inhibition of IL-17 and IFNγ production by CD4+ T cells remains unknown. We observed that the concentration of IL-2 in the culture supernatant of CD4+ T cells stimulated with anti-CD3/anti-CD28 antibodies was apparently increased when tofacitinib was added to the culture (data not shown), as reported by other investigators (19). This suggests that tofacitinib might inhibit the consumption of IL-2, which is produced by CD4+ T cells stimulated with anti-CD3/anti-CD28 antibodies. Alternatively, IL-2 production by CD4+ T cells is also thought to be enhanced by tofacitinib, because tofacitinib might cancel the IL-2–mediated negative feedback loop through activating STAT-5 (20). Furthermore, it has been reported that tofacitinib inhibited IL-2–enhanced IFNγ production by T cells in the peripheral blood of the cynomolgus monkey (21). Our study also showed that tofacitinib significantly decreased IL-2–induced production of IL-17 and IFNγ by peripheral blood CD4+ T cells (Figure 2G). Taken together, our observations suggest that IL-2–dependent activation of CD4+ T cells may be important in the pathologic processes of RA, and that tofacitinib could inhibit the IL-2–mediated JAK/STAT signaling pathway.

Although the present study confirmed the specificity of the action of tofacitinib on CD4+ T cells, it remains possible that other immune cells expressing JAKs could be targeted. Dose-related decreases in neutrophil counts (6,8) have been observed that can be related to attenuation of inflammation (11); however, it is highly likely that neutropenia can occur through inhibition of JAK-1. Because active RA responds to rituximab, a monoclonal antibody selectively targeting CD20+ B cells (22–24), and because B cells also express JAKs, it is possible that tofacitinib directly affects B cell function. Treatment with tofacitinib was previously reported to decrease the absolute number of CD3+ CD16+CD56+ natural killer cells (25), suggesting an antiinflammatory effect through natural killer cells. Additionally, a growing body of evidence indicates the involvement of mast cells in the pathogenesis of RA (26,27), and these immune cells also express JAKs. Indeed, the majority of IL-17A–expressing cells in synovium have been reported to be mast cells (28), suggesting another possible underlying mechanism of the anti-inflammatory effect.

Here, we demonstrated that tofacitinib functions through a mechanism different from that of biologic agents that target IL-6 or TNF. Our results indicate that tofacitinib is potentially useful in various autoimmune diseases involving autoreactive T cells. This JAK inhibitor could therefore be indicated widely for immunologic abnormalities and inflammatory conditions in the future. In addition to the effect of tofacitinib in RA, the clinical benefit of orally available tofacitinib in other immune diseases such as inflammatory bowel disease and psoriasis has been observed in ongoing clinical trials. We await with interest the results of these and future trials, to establish the usefulness of tofacitinib in clinical practice.

Finally, we conclude that JAKs in CD4+ T cells play an important role in RA synovitis. However, considering the dramatic effect of tofacitinib in RA, further basic and clinical research is needed to fully determine the mechanism of tofacitinib and the importance of immune cells expressing JAKs and mesenchymal cells expressing only JAK-1 and JAK-2 in RA pathology.

ACKNOWLEDGMENTS

We thank Ms N. Sakaguchi, Ms K. Noda, and Ms T. Adachi for the excellent technical assistance. We also thank Dr. John O'Shea (National Institutes of Health, Bethesda, MD) for his helpful review of the manuscript.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Y. Tanaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Maeshima, Yamaoka, Kubo, Nakano, Iwata, Saito, Ishii, Yoshimatsu, Y. Tanaka.

Acquisition of data. Maeshima, Yamaoka, Kubo, Nakano, Iwata, Ohishi, Miyahara, S. Tanaka, Y. Tanaka.

Analysis and interpretation of data. Maeshima, Yamaoka, Kubo, Nakano, Iwata, Saito, Ishii, Yoshimatsu, Y. Tanaka.

REFERENCES

1. Tristano AG. Tyrosine kinases as targets in rheumatoid arthritis. Int Immunopharmacol 2009;9:1–9.
2. D'Aura Swanson C, Paniagua RT, Lindstrom TM, Robinson WH. Tyrosine kinases as targets for the treatment of rheumatoid arthritis. Nat Rev Rheumatol 2009;5:317–24.
3. Russell SM, Tayebi N, Nakajima H, Riedy MC, Roberts JL, Aman MJ, et al. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. Science 1995;270:797–800.
4. Macchi P, Villa A, Giliani S, Sacco MG, Frattini A, Porta F, et al. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). Nature 1995;377:65–8.
5. Milici AJ, Kudlacz EM, Audoly L, Zwillich S, Changelian P. Cartilage preservation by inhibition of Janus kinase 3 in two rodent models of rheumatoid arthritis. Arthritis Res Ther 2008; 10:R14.
6. Kremer JM, Bloom BJ, Breedveld FC, Coombs JH, Fletcher MP, Gruben D, et al. The safety and efficacy of a JAK inhibitor in patients with active rheumatoid arthritis: results of a double-blind, placebo-controlled phase IIa trial of three dosage levels of CP-690,550 versus placebo. Arthritis Rheum 2009;60:1895–905.
7. Coombs JH, Bloom BJ, Breedveld FC, Fletcher MP, Gruben D, Kremer JM, et al. Improved pain, physical functioning and health status in patients with rheumatoid arthritis treated with CP-690,550, an orally active Janus kinase (JAK) inhibitor: results from a randomised, double-blind, placebo-controlled trial. Ann Rheum Dis 2010;69:413–6.
8. Tanaka Y, Suzuki M, Nakamura H, Toyoizumi S, Zwillich SH, and the Tofacitinib Study Investigators. Phase II study of tofacitinib (CP-690,550) combined with methotrexate in patients with rheumatoid arthritis and an inadequate response to methotrexate. Arthritis Care Res (Hoboken) 2011;63:1150–8.
9. Changelian PS, Flanagan ME, Ball DJ, Kent CR, Magnuson KS, Martin WH, et al. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. Science 2003;302:875–8.
10. Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, et al. A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol 2008;26:127–32.
11. Meyer DM, Jesson MI, Li X, Elrick MM, Funckes-Shippy CL, Warner JD, et al. Anti-inflammatory activity and neutrophil reductions mediated by the JAK1/JAK3 inhibitor, CP-690,550, in rat adjuvant-induced arthritis. J Inflamm (Lond) 2010;7:41.
12. Ghoreschi K, Jesson MI, Li X, Lee JL, Ghosh S, Alsup JW, et al. Modulation of innate and adaptive immune responses by tofacitinib (CP-690,550). J Immunol 2011;186:4234–43.
13. Yamaoka K, Min B, Zhou YJ, Paul WE, O'Shea JJ. Jak3 negatively regulates dendritic-cell cytokine production and survival. Blood 2005;106:3227–33.
14. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
15. Lefevre S, Knedla A, Tennie C, Kampmann A, Wunrau C, Dinser R, et al. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. Nat Med 2009;15:1414–20.
16. Jia J, Wang C, Shi Z, Zhao J, Jia Y, Zhao-Hui Z, et al. Inhibitory effect of CD147/HAb18 monoclonal antibody on cartilage erosion and synovitis in the SCID mouse model for rheumatoid arthritis. Rheumatology (Oxford) 2009;48:721–6.
17. Choy EH, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H, et al. Therapeutic benefit of blocking interleukin-6 activity with an anti–interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum 2002;46:3143–50.
18. Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, et al. Treatment of rheumatoid arthritis with humanized anti–interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum 2004;50:1761–9.
19. Park HB, Oh K, Garmaa N, Seo MW, Byoun OJ, Lee HY, et al. CP-690550, a Janus kinase inhibitor, suppresses CD4+ T-cell-mediated acute graft-versus-host disease by inhibiting the interferon-γ pathway. Transplantation 2010;90:825–35.
20. Villarino AV, Tato CM, Stumhofer JS, Yao Z, Cui YK, Hennighausen L, et al. Helper T cell IL-2 production is limited by negative feedback and STAT-dependent cytokine signals. J Exp Med 2007;204:65–71.
21. Paniagua R, Si MS, Flores MG, Rousvoal G, Zhang S, Aalami O, et al. Effects of JAK3 inhibition with CP-690,550 on immune cell populations and their functions in nonhuman primate recipients of kidney allografts. Transplantation 2005;80:1283–92.
22. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med 2004;350:2572–81.
23. Emery P, Fleischmann R, Filipowicz-Sosnowska A, Schechtman J, Szczepanski L, Kavanaugh A, et al, for the DANCER Study Group. The efficacy and safety of rituximab in patients with active rheumatoid arthritis despite methotrexate treatment: results of a phase IIb randomized, double-blind, placebo-controlled, dose-ranging trial. Arthritis Rheum 2006;54:1390–400.
24. Cohen SB, Emery P, Greenwald MW, Dougados M, Furie RA, Genovese MC, et al, for the REFLEX Trial Group. Rituximab for rheumatoid arthritis refractory to anti–tumor necrosis factor therapy: results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. Arthritis Rheum 2006;54:2793–806.
25. Van Gurp EA, Schoordijk-Verschoor W, Klepper M, Korevaar SS, Chan G, Weimar W, et al. The effect of the JAK inhibitor CP-690,550 on peripheral immune parameters in stable kidney allograft patients. Transplantation 2009;87:79–86.
26. Nigrovic PA, Lee DM. Synovial mast cells: role in acute and chronic arthritis. Immunol Rev 2007;217:19–37.
27. Eklund KK. Mast cells in the pathogenesis of rheumatic diseases and as potential targets for anti-rheumatic therapy. Immunol Rev 2007;217:38–52.
28. Hueber AJ, Asquith DL, Miller AM, Reilly J, Kerr S, Leipe J, et al. Mast cells express IL-17a in rheumatoid arthritis synovium. J Immunol 2010;184:3336–40.

Mod Rheumatol (2013) 23:582–586
DOI 10.1007/s10165-012-0680-3

CASE REPORT

Usefulness of ultrasonography-proven tenosynovitis to monitor disease activity of a patient with very early rheumatoid arthritis treated by abatacept

Shin-ya Kawashiri · Keita Fujikawa · Ayako Nishino · Takahisa Suzuki · Akitomo Okada · Hideki Nakamura · Atsushi Kawakami

Received: 7 February 2012 / Accepted: 16 May 2012 / Published online: 7 July 2012

Abstract We introduced abatacept (ABT) in a very early rheumatoid arthritis (RA) patient with active tenosynovitis of hands defined by musculoskeletal ultrasonography (MSKUS). MSKUS-proven tenosynovitis remarkably improved at 2 months in spite of clinical exacerbation, followed by clinical remission at 5 months. MSKUS abnormalities also disappeared. Although ABT was discontinued due to an adverse event after the sixth infusion, she remained in clinical remission as well as imaging remission by MSKUS at 13 months.

Keywords Rheumatoid arthritis · Musculoskeletal ultrasonography · Abatacept

List of Abbreviations

ACPA	Anti-cyclic citrullinated peptide antibodies
ACR	American College of Rheumatology
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
DAS28	Disease Activity Score 28
DMARDs	Disease-modifying antirheumatic drugs
ESR	Erythrocyte sedimentation rate
eVAS	Evaluator global visual analog scale
MRI	Magnetic resonance imaging
MSKUS	Musculoskeletal ultrasonography
MTX	Methotrexate
PD	Power Doppler
pVAS	Patient global visual analog scale
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SDAI	Simplified Disease Activity Index
SJC	Swollen joint counts
TJC	Tender joint counts
UA	Undifferentiated arthritis

S. Kawashiri (✉) · A. Nishino · T. Suzuki · A. Okada · H. Nakamura · A. Kawakami
Department of Immunology and Rheumatology, Unit of Translational Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: shin-ya@hotmail.co.jp

K. Fujikawa
Department of Internal Medicine, Isahaya Health Insurance General Hospital, Isahaya, Japan

Introduction

Musculoskeletal ultrasonography (MSKUS) in rheumatic disease has become widely used in Western countries as well as in Japan. MSKUS is more sensitive and may be more reliable than clinical examination for detecting joint injury in patients with rheumatoid arthritis (RA) [1–3]. Synovitis, tenosynovitis, and bone erosion are major joint injuries that are frequently found in patients with RA examined by MSKUS [1–4]. The grayscale mode detects morphological abnormalities such as hypertrophy of synovial tissues, while the power Doppler mode identifies their vascularity [1–5], such that MSKUS is very useful in diagnosis and evaluation of the disease activity of RA.

Most previous reports have focused on the severity of articular synovitis, considering the therapeutic efficacy of disease-modifying antirheumatic drugs (DMARDs) [6, 7], while other components such as tenosynovitis and bone erosion might also reflect the disease activity of RA [8, 9]. We describe herein a case of very early RA in which the findings of MSKUS-proven tenosynovitis were very useful in understanding the patient's clinical condition and deciding upon a therapeutic strategy. The patient achieved biologic-free clinical remission and imaging remission by MSKUS in a short time by the introduction of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4)-IgG, or abatacept (ABT), in the early phase of RA.

Case report

The patient was a 63-year-old Japanese woman. She had noticed arthralgia of right wrist in July 2010. She visited our hospital in November 2010. On physical examination, she had 3 tender joints and 8 swollen joints of the right wrist and fingers (Fig. 1a). Her laboratory tests showed C-reactive protein (CRP) of 0.14 mg/dl (normal range <0.17 mg/dl), erythrocyte sedimentation rate (ESR) of 26 mm/h (normal range <15 mm/h), rheumatoid factor (RF) of 25.1 IU/ml (normal range <15 IU/ml), and anti-cyclic citrullinated peptide antibodies (ACPA) of >100 U/ml (normal range <4.5 U/ml). Plain radiography of bilateral hands and feet was normal. She was diagnosed with RA according to the 2010 RA classification criteria [10]: at least 1 joint with definite clinical synovitis (swelling) not better explained by another disease, and a total disease score of 8 [joint involvement, 8 small joints (+3); serology, high-positive ACPA (+3); acute-phase reactants, abnormal ESR (+1); and duration of symptoms, longer than 6 weeks (+1)]. MSKUS showed mild articular synovitis of grayscale 1 without power Doppler (PD) signal of 2nd to 4th proximal interphalangeal (PIP) joints, but rather remarkable tenosynovitis of the extensor tendon of the right wrist and the flexor tendon of the right index finger (Fig. 1b, c). Clinical disease activity was moderate with Disease Activity Score 28 (DAS28) of 4.74 and Simplified Disease Activity Index (SDAI) of 19.1.

Her clinical course is shown in Fig. 2. Treatment with 6 mg/week methotrexate (MTX) was initiated in December 2010. The dose of MTX could not be increased because of leukocytopenia. Moderate disease activity continued, and the MSKUS-proven tenosynovitis was not improved at 3 months after initiation of MTX therapy. Therefore, she additionally received 500 mg abatacept (ABT)

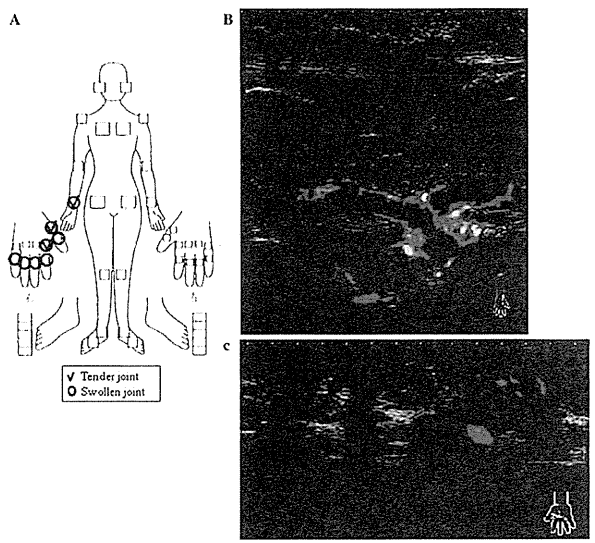

Fig. 1 The findings of physical examination and musculoskeletal ultrasonography (MSKUS) at initial visit. a She had 3 tender joints and 8 swollen joints of right wrist and fingers on physical examination. b First compartment (abductor pollicis longus and extensor pollicis brevis tendons) of the extensor tendons on the dorsal aspect of the wrist. Images obtained in longitudinal scan using grayscale (*upper*) and power Doppler mode (*lower*). Images show typical findings of tenosynovitis, which is defined by irregular hypertrophy of the synovium of the tendon sheath with positive-sign Doppler signals. c The flexor tendon on the volar aspect of the finger. Images obtained in transverse scan using grayscale (*left*) and power Doppler mode (*right*). Images show typical findings of tenosynovitis, which is defined by hypertrophy of the synovium of the tendon sheath with positive-sign Doppler signals

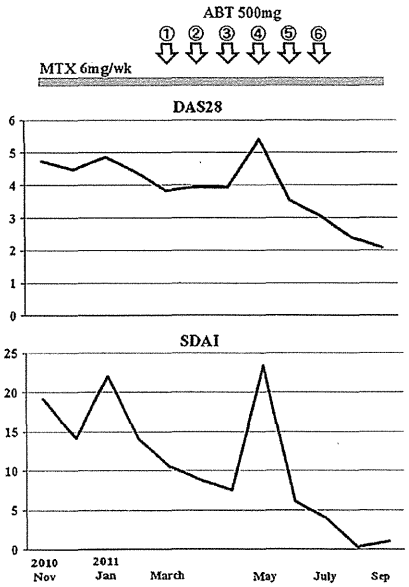

Fig. 2 Clinical course of the present case. *MTX* methotrexate, *DAS28* Disease Activity Score 28, *SDAI* Simplified Disease Activity Index

intravenously every 4 weeks from March 2011 (DAS28, 3.83; SDAI, 10.5; tender joint count (TJC), 4; swollen joint count (SJC), 4; patient global visual analog scale (pVAS), 15; evaluator global visual analog scale (eVAS), 15; ESR, 16 mm/h; CRP, 0.03 mg/dl). Treatment with ABT seemed not to exert an effect, and her clinical disease activity was exacerbated at 2 months after initiation especially due to left knee joint arthritis (DAS28, 5.41; SDAI, 23.4; TJC, 4; SJC, 5; pVAS, 70; eVAS, 70; ESR, 46 mm/h; CRP, 0.40 mg/dl). However, because the tenosynovitis had remarkably improved and articular synovitis of wrist and finger joints was not detected on MSKUS by this time (Fig. 3a), ABT was continued. Her symptoms gradually improved from 3 months, and she achieved low disease activity scores (DAS28, 3.07; SDAI, 3.92) at 4 months and clinical remission at 5 months. The MSKUS findings disappeared altogether at 4 months (Fig. 3b).

In August 2011, she was admitted because of a subcutaneous abscess of the left gluteal region. An examination revealed that she was implanted the placenta, which was a supplement, in the site of lesion before the initiation of ABT therapy. ABT therapy was discontinued after the sixth infusion, but MTX administration (6 mg/week) continued for the following 6 months. At 13 months after the initiation of MTX (6 months after discontinuation of ABT), the patient remained in clinical remission (DAS28, 2.08; SDAI, 1.1) with imaging remission (no abnormalities) as determined by MSKUS.

Discussion

In this case, we found that MSKUS was very important in monitoring the efficacy of ABT in a patient with very early RA.

First, we were able to accurately understand the patient's clinical condition by MSKUS. In fact, MSKUS predominantly showed active tenosynovitis rather than articular synovitis. Tender and swollen joints on physical examination were suggested to be due to remarkable tenosynovitis adding to articular synovitis. Tenosynovitis is widely accepted to be common in RA and postulated to be the first manifestation in some cases [11]. Previous studies have described MSKUS as the gold-standard imaging method for assessing tendon involvement in RA [12], even in the early phase [11]. Tenosynovitis is defined by abnormal hypoechoic or anechoic material with or without fluid inside the tendon sheath and with positive-sign Doppler signals in two perpendicular planes [11, 13, 14]. We have recently revealed the utility of MSKUS for clinical diagnosis toward patients with early RA [15]. In this issue, we have also found a high frequency of tenosynovitis in these patients [15]. It may be caused by invasion of the pannus into the tendon or by compression of the pannus, either of which would cause edema, ischemia, and necrosis [11]. Hypervascularity determined by an intense PD signal was found in this case, confirming that this patient was suffering from active tenosynovitis by RA.

Secondly, the findings of MSKUS were very helpful in considering therapeutic strategies in this case. ABT therapy was initiated because the abnormal findings of MSKUS were persistent at 3 months after initiation of MTX therapy. ABT therapy was continued for 2 months despite exacerbation of clinical disease activity estimated by DAS28 and SDAI, because the abnormal findings of MSKUS of wrist and finger joints had obviously improved. A previous study of RA patients treated with anti-tumor necrosis factor (TNF) therapy found that the PD score of articular synovitis correlated well with DAS28 [16]. Although knowledge is limited regarding the response of tenosynovitis to anti-inflammatory treatment, a recent report revealed that the PD tenosynovitis score of RA patients was also improved by adalimumab [8]. Another report found a positive association between tenosynovitis of the ankle joints and RF [17], suggesting that we should

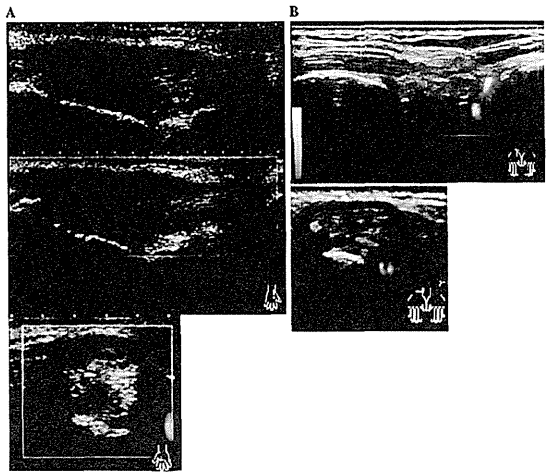

Fig. 3 The findings of musculoskeletal ultrasonography (MSKUS) after treatment with abatacept. a The findings of MSKUS at 2 months, when clinical disease activity worsened. First compartment (abductor pollicis longus and extensor pollicis brevis tendons) of the extensor tendons on the dorsal aspect of the wrist (*upper* longitudinal scan, grayscale; *middle* longitudinal scan, power Doppler mode) and the flexor tendon on the volar aspect of the finger (*lower* transverse scan, power Doppler mode). Although hypertrophy of tendon sheath persisted, power Doppler signals disappeared. b The findings of MSKUS at 4 months, when clinical disease activity improved. First compartment of the extensor tendons on the dorsal aspect of the wrist (*upper* longitudinal scan, power Doppler mode; *lower* transverse scan, power Doppler mode). The hypertrophy of the tendon sheath and power Doppler signals disappeared

be aware of the importance of tenosynovitis in pathogenesis as well as in response to DMARD therapy. There are no case reports regarding the change of MSKUS findings including tenosynovitis in patients with RA treated by ABT. The present case has firstly shown that ABT therapy induces clinical as well as imaging remission in early RA patients.

The efficacy of ABT in early RA is prominent compared with its effect in established RA [18]. The abatacept study to determine its effectiveness in preventing development of rheumatoid arthritis in patients with undifferentiated inflammatory arthritis and to evaluate safety and tolerability (the ADJUST trial) focused on patients with undifferentiated arthritis (UA) or very early RA [19]. In the ADJUST trial, improvement of magnetic resonance imaging score of synovitis was achieved by ABT at 6 months; furthermore, its effect was maintained after cessation of ABT [19]. Although the follow-up period in the present patient was short, this is the first observation that tenosynovitis of a very early RA patient could disappear by ABT even after incidental cessation of ABT.

According to previous reports, the presence of abnormalities detected by MSKUS (especially PDUS) and MRI (especially MRI osteitis) predict further radiographic progression even in RA patients with clinical remission [20]. Therefore, the ideal imaging remission is considered as no abnormality found in MSKUS and MRI. Although the present case did not receive MRI study, abnormal MSKUS findings including articular synovitis and tenosynovitis had disappeared after 4 months of introduction of ABT. Therefore, we concluded that the patient achieved imaging remission in addition to clinical remission.

In this case, biologic-free remission might also be induced in a short time by the introduction of ABT in the early phase of RA. Although the clear definition of biologic-free remission is still obscure at present, biologic-free duration longer than 1 year may be a criterion, since Tanaka et al. have recently examined the characteristics of RA patients who were able to discontinue infliximab longer than 1 year [21]. The findings of MSKUS were very helpful in observing the clinical status of this patient; however, more longitudinal observation is needed to determine whether the present case achieved biologic-free remission.

Conflict of interest None.

References

1. Naredo E, Bonilla G, Gamero F, Uson J, Carmona L, Laffon A. Assessment of inflammatory activity in rheumatoid arthritis: a comparative study of clinical evaluation with grey scale and power Doppler ultrasonography. Ann Rheum Dis. 2005;64: 375–81.
2. Salaffi F, Filippucci E, Carotti M, Naredo E, Meenagh G, Ciapetti A, et al. Inter-observer agreement of standard joint counts in early rheumatoid arthritis: a comparison with grey scale ultrasonography—a preliminary study. Rheumatology (Oxford). 2008;47:54–8.
3. Saleem B, Brown AK, Keen H, Nizam S, Freeston J, Karim Z, et al. Disease remission state in patients treated with the combination of tumor necrosis factor blockade and methotrexate or with disease-modifying antirheumatic drugs: a clinical and imaging comparative study. Arthritis Rheum. 2009;60:1915–22.
4. Backhaus M, Burmester GR, Gerber T, Grassi W, Machold KP, Swen WA, et al. Working Group for Musculoskeletal Ultrasound in the EULAR Standing Committee on International Clinical Studies including Therapeutic Trials. Guidelines for musculoskeletal ultrasound in rheumatology. Ann Rheum Dis. 2001; 60:641–9.
5. Kawashiri SY, Kawakami A, Iwamoto N, Fujikawa K, Satoh K, Tamai M, et al. The power Doppler ultrasonography score from 24 synovial sites or 6 simplified synovial sites, including the metacarpophalangeal joints, reflects the clinical disease activity and level of serum biomarkers in patients with rheumatoid arthritis. Rheumatology (Oxford). 2011;50:962–5.
6. Hartung W, Kellner H, Strunk J, Sattler H, Schmidt WA, Ehrenstein B, et al. Development and evaluation of a novel ultrasound score for large joints in rheumatoid arthritis: one year experience in daily clinical practice. Arthritis Care Res (Hoboken);2011 [Epub ahead of print]
7. Hammer HB, Kvien TK. Comparisons of 7- to 78-joint ultrasonography scores: all different joint combinations show equal response to adalimumab treatment in patients with rheumatoid arthritis. Arthritis Res Ther. 2011;13:R78.
8. Hammer HB, Kvien TK. Ultrasonography shows significant improvement in wrist and ankle tenosynovitis in rheumatoid arthritis patients treated with adalimumab. Scand J Rheumatol. 2011;40:178–82.
9. Baillet A, Gaujoux-Viala C, Mouterde G, Pham T, Tebib J, Saraux A, et al. Comparison of the efficacy of sonography, magnetic resonance imaging and conventional radiography for the detection of bone erosions in rheumatoid arthritis patients: a systematic review and meta-analysis. Rheumatology (Oxford). 2011;50:1137–47.
10. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO 3rd, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62: 2569–81.
11. Hmamouchi I, Bahiri R, Srifi N, Aktaou S, Abouqal R, Hajjaj-Hassouni N. A comparison of ultrasound and clinical examination in the detection of flexor tenosynovitis in early arthritis. BMC Musculoskelet Disord. 2011;12:91.
12. Grassi W, Filippucci E, Farina A, Cervini C. Sonographic imaging of tendons. Arthritis Rheum. 2000;43:969–76.
13. Backhaus M. Ultrasound and structural changes in inflammatory arthritis: synovitis and tenosynovitis. Ann N Y Acad Sci. 2009; 1154:139–51.
14. Filippucci E, Gabba A, Di Geso L, Girolimetti R, Salaffi F, Grassi W. Hand Tendon Involvement in Rheumatoid Arthritis: An Ultrasound Study. Semin Arthritis Rheum. 2011 [Epub ahead of print]
15. Kawashiri SY, Suzuki T, Okada A, Yamasaki S, Tamai M, Nakamura H, et al. Musculoskeletal ultrasonography assists the diagnostic performance of the 2010 classification criteria for rheumatoid arthritis. Mod Rheumatol. 2012 [Epub ahead of print]
16. Naredo E, Möller I, Cruz A, Carmona L, Garrido J. Power Doppler ultrasonographic monitoring of response to anti-tumor necrosis factor therapy in patients with rheumatoid arthritis. Arthritis Rheum. 2008;58:2248–56.
17. Suzuki T, Tohda E, Ishihara K. Power Doppler ultrasonography of symptomatic rheumatoid arthritis ankles revealed a positive association between tenosynovitis and rheumatoid factor. Mod Rheumatol. 2009;19:235–44.
18. Yazici Y, Moniz Reed D, Klem C, Rosenblatt L, Wu G, Kremer JM. Greater remission rates in patients with early versus long-standing disease in biologic-naive rheumatoid arthritis patients treated with abatacept: a post hoc analysis of randomized clinical trial data. Clin Exp Rheumatol. 2011;29:494–9.
19. Emery P, Durez P, Dougados M, Legerton CW, Becker JC, Vratsanos G, et al. Impact of T-cell costimulation modulation in patients with undifferentiated inflammatory arthritis or very early rheumatoid arthritis: a clinical and imaging study of abatacept (the ADJUST trial). Ann Rheum Dis. 2010;69:510–6.
20. Brown AK, Conaghan PG, Karim Z, Quinn MA, Ikeda K, Peterfy CG, et al. An explanation for the apparent dissociation between clinical remission and continued structural deterioration in rheumatoid arthritis. Arthritis Rheum. 2008;58:2958–67.
21. Tanaka Y, Takeuchi T, Mimori T, Saito K, Nawata M, Kameda H, et al. Discontinuation of infliximab after attaining low disease activity in patients with rheumatoid arthritis: RRR (remission induction by Remicade in RA) study. Ann Rheum Dis. 2010; 69:1286–91.

Mod Rheumatol (2012) 22:654–658
DOI 10.1007/s10165-011-0575-8

ORIGINAL ARTICLE

Magnetic resonance imaging (MRI) detection of synovitis and bone lesions of the wrists and finger joints in early-stage rheumatoid arthritis: comparison of the accuracy of plain MRI-based findings and gadolinium-diethylenetriamine pentaacetic acid-enhanced MRI-based findings

Mami Tamai · Atsushi Kawakami · Masataka Uetani · Aya Fukushima · Kazuhiko Arima · Keita Fujikawa · Naoki Iwamoto · Toshiyuki Aramaki · Makoto Kamachi · Hideki Nakamura · Hiroaki Ida · Tomoki Origuchi · Kiyoshi Aoyagi · Katsumi Eguchi

Received: 25 July 2011 / Accepted: 25 November 2011 / Published online: 28 December 2011

Abstract

Objective To explore whether synovitis and bone lesions in the wrists and finger joints visualized by plain magnetic resonance imaging (MRI)-based findings correspond exactly or not to those judged by gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA)-enhanced MRI-based findings.

Methods Magnetic resonance imaging of the wrists and finger joints of both hands were examined in 51 early-stage rheumatoid arthritis (RA) patients whose median disease duration from the onset of articular manifestations to entry was 5 months, by both plain (T1 and short-time inversion recovery images) and Gd-DTPA-enhanced MRI (post-contrast fat-suppressed T1-weighted images) simultaneously. We focused on 15 sites per hand, to examine the presence of synovitis and bone lesions (bone edema and bone erosion). Gd-DTPA-enhanced MRI-based findings

M. Tamai and A. Kawakami contributed equally to this work.

M. Tamai (✉) · A. Kawakami · N. Iwamoto · M. Kamachi · H. Nakamura
Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: tamaim@nagasaki-u.ac.jp

M. Tamai
Center for Health and Community Medicine, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

M. Uetani · A. Fukushima
Department of Radiology and Radiation Research, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

K. Arima
Department of Medical Gene Technology, Atomic Bomb Disease Institute, Nagasaki University School of Health Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

K. Fujikawa
Department of Rheumatology, Isahaya Healthy Insurance General Hospital, 24-1 Eisyohigashi-machi, Isahaya 854-8501, Japan

T. Aramaki
Department of Rheumatology, Japanese Red Cross Nagasaki Genbaku Hospital, 3-15 Mori-machi, Nagasaki 852-8511, Japan

H. Ida
Division of Respirology, Neurology and Rheumatology, Department of Medicine, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan

T. Origuchi
Department of Rehabilitation Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

K. Aoyagi
Department of Public Health, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

K. Eguchi
Sasebo City General Hospital, 9-3 Hirase-machi, Sasebo 857-8511, Japan

were considered "true" lesions, and we evaluated the accuracy of plain MRI-based findings in comparison to Gd-DTPA-enhanced MRI-based findings.

Results Synovitis, judged by plain MRI-based findings, appeared as false-positive at pretty frequency; thus, the specificity, positive predictive value and accuracy of the findings were low. The rate of enhancement (E-rate) in false-positive synovitis sites was significantly low compared with true-positive synovitis sites where Gd-DTPA enhancement appears. In contrast to synovitis, the false-positivity of bone lesions, judged by plain MRI-based findings, was very low compared with Gd-DTPA-enhanced MRI-based findings.

Conclusion Synovitis judged by plain MRI-based findings is sometimes considered false-positive especially in sites where synovitis is mild. However, plain MRI is effective in identifying bone lesions in the wrist and finger joints in early-stage RA.

Keywords Early-stage RA · Plain MRI · Gd-DTPA-enhanced MRI · Synovitis · Bone lesions

Abbreviations

ACR	American College of Rheumatology
CRP	C-reactive protein
E-rate	Rate of enhancement
Gd-DTPA	Gadolinium–diethylenetriamine pentaacetic acid
HLA-DRB1*SE	HLA-DRB1*shared epitope
RA	Rheumatoid arthritis
UA	Undifferentiated arthritis

Introduction

Magnetic resonance imaging (MRI) reveals joint inflammation and damage in early-stage rheumatoid arthritis (RA) [1–4] that take the form of synovitis and bone lesions, including bone edema and bone erosion [1–4]. As active synovial lesions in patients with RA are rich in vascularity, gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA)-enhanced MRI-based findings have become the gold standard to evaluate joint inflammation and damage in RA [1]. Accordingly, by assessing Gd-DTPA-enhanced MRI-based findings of the wrists and finger joints of both hands, we have determined that symmetrical synovitis and bone lesions are important predictors of the development of RA in patients with undifferentiated arthritis (UA) [5–8]. In these earlier studies, we did not specifically compare Gd-DTPA-enhanced MRI-based findings with plain MRI-based findings. However, Gd-DTPA-enhanced MRI is an expensive diagnostic tool compared to plain MRI, and Gd-DTPA can induce serious adverse events [9]. Thus, if plain MRI is sufficiently sensitive for the purpose, it should be possible to reduce both the cost and the adverse events associated with Gd-DTPA by using plain MRI.

The aim of the study reported here was to determine whether plain MRI-based findings are effective in evaluating joint inflammation and damage in early-stage RA in comparison to Gd-DTPA-enhanced MRI-based findings. Our results suggest that plain MRI is a sufficiently sensitive diagnostic tool to evaluate bone lesions, but that synovitis determined by plain MRI-based findings may on occasion appear as a false-positive, especially at sites where synovitis is mild.

Patients and methods

Patients

The Early Arthritis Clinic opened in 2001 as part of the Unit of Translational Medicine of the Department of Immunology and Rheumatology of the Graduate School of Biomedical Sciences of Nagasaki University. It is a regional center for the treatment of arthritis, with patients from the whole western part of Japan, Nagasaki Prefecture (approx. 450,000 inhabitants) being referred there for treatment. For our study, we recruited 51 early-stage RA patients from this clinic. The disease status of these patients was formally confirmed by a rheumatologist in our department, and a diagnosis of RA was based on the 1987 criteria for RA of the American College of Rheumatology (ACR) [10]. Baseline clinical manifestations and variables included sex, age, localization of arthritis, morning stiffness, number of tender joints, number of swollen joints, C-reactive protein level (CRP; measured by latex turbidimetric immunosorbent assay; Daiichi Pure Chemicals, Fukuoka, Japan), immunoglobulin M-rheumatoid factor (IgM-RF) positivity (measured by latex-enhanced immunonephelometric assay; cut-off value 14 IU/ml; Dade Behring, Marburg, Germany), positive status for anti-cyclic citrullinated peptide (CCP) antibodies (measured by enzyme-linked immunosorbent assay; cut-off value 4.5 U/ml; DIASTAT Anti-CCP; Axis-Shield, Dundee, UK), HLA-DRB1 genotyping, and MRI findings for both the wrists and finger joints, as previously described [5–8, 11]. All variables were examined on the same day, as previously reported [5–8, 11]. Each patient provided a signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University.

MRI of wrists and finger joints

Magnetic resonance scan images of both the wrists and finger joints were acquired using a 1.5 T system (Signa; GE Medical Systems, Milwaukee, WI) with an extremity coil. T1-weighted spin-echo (TR 450 ms, TE 13 ms) images, short-time inversion recovery (STIR; TR 3000 ms, TE 12 ms, T1 160 ms) images, and Gd-DTPA-enhanced images were simultaneously acquired. The images were evaluated for bone edema, bone erosion, and synovitis in 15 sites in each finger and wrist: the distal radioulnar joint, the radiocarpal joint, the midcarpal joint, the first carpometacarpal joint, the second-fifth carpometacarpal joints (together), the first–fifth metacarpophalangeal joints, and the first–fifth proximal interphalangeal joints (PIP joints) separately (a total of 30 sites in both hands), as recently reported [5–8, 11]. The presence of synovitis, bone edema, and bone erosion was evaluated according to the methods described by Lassere et al. [12] and Conaghan et al. [13], by two experienced radiologists (M.U. and A.F.), and decisions were reached by consensus, as previously described [5–8, 11]. Since the focus of our study was to compare MRI-based findings and Gd-DTPA-enhanced MRI-based findings in terms of their accuracy in determining synovitis and bone change, we included bone edema and bone erosion as bone lesions in our study. Gd-DTPA-enhanced images were obtained by intravenous injection of 0.1 mmol/kg of Gd-DTPA (Magnevist; Bayer Schering Pharma, Berlin, Germany). A dynamic study was performed to evaluate the vascularity of the affected joints as a rate of enhancement (E-rate), which was determined by examining coronal sections taken at 4-s intervals over a 150-s time period with fast spoiled gradient recalled acquisition in the steady state (SPGR) sequences, as previously described [5–8, 11].

Comparison of plain MRI-based findings and Gd-DTPA-enhanced MRI-based findings

Gd-DTPA-enhanced MRI-based findings are the gold standard for evaluating joint inflammation and damage by MRI in RA [1]. Thus, we assumed that Gd-DTPA-enhanced MRI-based findings represented "true" lesions and subsequently calculated the accuracy of plain MRI-based findings, comparing sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy.

Statistical analysis

Differences between the groups shown in Table 4 were examined for statistical significance using the Mann–Whitney *U* test. A *P* value of <0.05 was taken to indicate a statistically significant difference.

Results

Patient characteristics

Table 1 shows the baseline characteristics of the 51 patients with RA enrolled in our study. Since the median disease duration from the onset of articular manifestations to entry was 5 months, this study population was considered to have early-stage RA. The median Genant-modified Sharp score of the 51 patients at baseline was 0.49, which also identifies them as early-stage RA patients. The rates of seropositivity of IgM-RF and anti-CCP antibodies were 62.7 and 74.5%, respectively, and the rates of carriership of the HLA-DRB1*0405 allele and HLA-DRB1*shared epitope (SE) allele were 44.0 and 56.0%. These characteristics of autoantibodies and HLA-DR typing indicate that our study population manifested typical RA characteristics.

Synovitis and bone lesions of the wrists and finger joints of both hands according to plain MRI-based findings and Gd-DTPA-enhanced MRI-based findings

Among the 1530 sites of interest, we were able to evaluate synovitis in 1416 sites on both plain MR and Gd-DTPA-enhanced MR scan images. Synovitis was considered positive in 65.6% of sites (929/1416) according to plain MRI-based findings, but was not found in 316 of these 929 sites by Gd-DTPA-enhanced MRI-based findings

Table 1 Demographic features of 51 early-stage rheumatoid arthritis patients

Demographic feature	Value
Gender (M:F, % F)	8:43 (84.3%)
Age (years)	52 (19–80)
Duration (months)	5 (1–28)
Distribution of arthritis	
Symmetric (%)	82.4
Only upper extremities (%)	27.5
Both upper and lower extremities (%)	72.5
Genant-modified Sharp score	0.49 (0–8.58)
Positivity of IgM-RF (%)	62.7
IgM-RF (IU/ml)	18.0 (4.5–395)
Positivity of anti-CCP antibodies (%)	74.5
Anti-CCP antibodies (IU/ml)	24.3 (0.6–2115.3)
Positivity of CRP (%)	70.0
CRP (mg/dl)	1.14 (0.03–11.13)
Carriership of HLA-DRB1*0405 (%)	44.0 (diploid: 8.0%)
Carriership of HLA-DRB1*shared epitope (%)	56.0 (diploid: 8.0%)

Values are given as the median with the range in parenthesis, unless otherwise stated

M Male, *F* female, *IgM* immunoglobulin M, *RF* rheumatoid factor, *CCP* cyclic citrullinated peptide, *CPR* C-reactive protein