conditions favoring Th17 cell differentiation, RORγt expression on CD4+ T cells from T-bet–Tg mice was lower than that on cells from B6 mice. Interestingly, most of the RORγt+ cells also expressed T-bet in T-bet–Tg mice, and the proportion of IL-17–producing RORγt+ T cells in the CD4+ cell subset was lower in T-bet–Tg mice than in B6 mice. These findings support the notion that overexpression of T-bet not only suppresses RORγt expression on CD4+ T cells, but also inhibits the production of IL-17 from RORγt+ T cells.

Previous studies showed that RORγt expression is positively regulated by several transcription factors, such as runt-related transcription factor 1 (RUNX-1), interferon regulatory factor 4, and STAT-3 (26–28). Lazarevic et al (29) recently reported that T-bet prevented RUNX-1–mediated activation of the gene encoding RORγt, followed by the suppression of Th17 cell differentiation. In addition to direct promotion of RORγt expression, RUNX-1 also acts as a coactivator, together with RORγt, and induces the expression of *Il17a* and *Il17f* (26); therefore, T-bet inhibits IL-17 production by RORγt+ cells induced by RUNX-1 (29). Although further studies will be required to identify the effect of T-bet overexpression on the function of RUNX-1, it might be associated with the suppression of Th17 cell differentiation that was observed in the T-bet–Tg mice.

In conclusion, our results demonstrated that overexpression of T-bet in T cells suppressed the development of autoimmune arthritis. The regulatory mechanism of CIA might involve dysfunction of CII-reactive Th17 cell differentiation by overexpression of T-bet via IFNγ-independent pathways. These findings should enhance our understanding of the pathogenesis of autoimmune arthritis and help in the development of new therapies for RA.

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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. Sumida 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.

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Baseline tumour necrosis factor alpha levels predict the necessity for dose escalation of infliximab therapy in patients with rheumatoid arthritis

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ABSTRACT

Objectives To investigate the possible role of baseline plasma tumour necrosis factor alpha levels (baseline-TNF) on the clinical response to infliximab in patients with rheumatoid arthritis (RA).

Methods Patients with RA refractory to methotrexate received 3, 6, or 10 mg/kg of infliximab every 8 weeks, in a randomised, double-blind manner: the RISING study. Clinical response (disease activity score in 28 joints based on C-reactive protein or American College of Rheumatology core set) at week 54 and serum infliximab levels were compared in three patient groups with low, intermediate, or high baseline-TNF (TNF-low, TNF-int, or TNF-high).

Results In TNF-low patients, the clinical response to different doses of infliximab was comparable, whereas TNF-int patients exhibited a dose-dependent trend. In contrast, TNF-high patients (approximately 13% of the total patients) had a clinical response to 10 mg/kg significantly better than the response to 3 and 6 mg/kg of infliximab. In TNF-high patients, the median trough serum levels of infliximab were below the detection limit (<0.1 μg/ml) at 3 and 6 mg/kg but were greater than 2 μg/ml at 10 mg/kg, whereas the levels were approximately 1 μg/ml for each dosage group in TNF-low patients.

Conclusion In patients with RA, baseline-TNF is significantly associated with the clinical response to infliximab in patients with a high baseline-TNF. A higher dose of infliximab may be necessary in these patients, whereas lower doses of infliximab are sufficient for those with a low baseline-TNF. Baseline-TNF may be a useful measure for personalising the treatment of RA using infliximab.

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease that results in joint destruction and disability.[1] Levels of tumour necrosis factor alpha (TNF), an inflammatory cytokine, are elevated in the blood and synovial fluid of patients with RA, and may play a central role in its pathogenesis.[2–7]

Although infliximab, an anti-TNF antibody, exhibits excellent effectiveness in RA,[8–11] insufficient response to the standard treatment of infliximab (3 mg/kg per 8 weeks) has also been observed in some cases in clinical practice. Such patients are usually treated by dose escalation or by shortening the dose interval of the infliximab therapy.[12–15]

The RISING study (NCT00691028) is a randomised, double-blind clinical trial, which has shown that the clinical response to infliximab at a dose of 10 mg/kg is significantly higher than the response to 3 mg/kg infliximab, and that a trough serum level of 1 μg/ml is the threshold for clinical response.[16] However, clinical response to different dose levels of infliximab was significant only for American College of Rheumatology (ACR) improvement criteria and the European League Against Rheumatism (EULAR) responses, and the measurable difference was small.

Several clinical studies have attempted to address whether a higher dose of infliximab provides a better clinical response than standard doses in patients with RA.[8–11 17] However, the results were not consistent among those studies. In addition, a randomised, double-blind study comparing dose escalation and continuation of the standard dose in patients with RA who had an insufficient response to 3 mg/kg of infliximab clearly demonstrated no beneficial response by dose escalation,[18] contrary to our results.

Although the standard dose of infliximab can be efficacious in a large proportion of patients with RA, some patients may require a higher dose of infliximab to achieve clinical response.[19 20] The inconsistent results mentioned above might be explained by the different proportion of patients who might benefit from infliximab dose escalation in each study. Thus the clinical and immunological features of these patients who require higher dose of infliximab are not fully understood.

It is tempting to speculate that the production and resultant plasma levels of TNF, the target molecule of infliximab, exceeds the neutralising capacity of infliximab in insufficient responders who are unable to maintain the threshold serum level of infliximab. Considering that hypothesis, we analysed the RISING study data based on plasma TNF levels. We found that the clinical response of patients with high baseline levels of TNF (baseline-TNF) showed a significant improvement with higher doses of infliximab, whereas patients with low baseline-TNF did not have a better response even with higher doses of infliximab.

METHODS

Patients and study protocol

The study protocol was approved by the local institutional review board and was carried out in accordance with the Helsinki Declaration and good clinical practice. Patient enrollment criteria and study design have previously been described in detail.[16] In the RISING study, 327 patients with active RA, despite receiving previous treatment with methotrexate, were administered 3 mg/kg infliximab at weeks 0, 2 and 6 with methotrexate

(open-label period weeks 0–14). Patients were randomly assigned into three groups using a dynamic allocation procedure based on the clinical response at week 10 and were treated with 3 (n=99), 6 (n=104), or 10 (n=104) mg/kg infliximab, every 8 weeks from weeks 14 to 46, with methotrexate (blinded period weeks 14–54).

Laboratory test values and serum infliximab measurement

Plasma samples for the evaluation of baseline-TNF were collected just before the first infusion of infliximab (week 0). The plasma was separated from EDTA-supplemented blood and was stored at −20 to −80°C. Baseline-TNF levels were measured by ELISA using the QantiGlo ELISA Kit (QTA00B; R&D Systems Inc, Minneapolis, Minnesota, USA) in Mitsubishi Chemical Medience Corporation (Tokyo, Japan). Recombinant human TNF included in the kit was used as reference. Mitsubishi Chemical Medience Corporation has made minor modifications on the assay procedure of the ELISA kit (http://www.rndsystems.com/pdf/qta00b.pdf) and has validated their detection limit. Since the mean relative light units (2 SD) at blank and 0.55 pg/ml were 15.2 (3.4) and 25.2 (5.0) by their modified procedure, respectively, the detection limit was determined as 0.55 pg/ml by adding 2 SD to the relative light units. The coefficient of variation or relative error values of intra-assay or inter-assay TNF were within 3.6% or 6.2%, respectively. They confirmed that freezing and thawing the plasma sample 10 times and storing at −20°C for 22 weeks did not affect the stability of TNF in the plasma.

Sera were collected at weeks 0 and 2 and every 4 weeks thereafter. The serum infliximab level was measured by ELISA using the anti-infliximab monoclonal antibody obtained from Centocor Ortho Biotech Inc. (Horsham, Pennsylvania, USA).[8] Serum infliximab levels below 0.1 μg/ml were undetectable.

Baseline rheumatoid factor (RF) was measured by the latex agglutination test. Antibodies against cyclic citrullinated peptides (CCP) and matrix metalloproteinase-3 (MMP-3) levels were measured by ELISA. The detectable ranges were as follows: RF, 3 IU/ml or greater; anti-CCP antibodies, 0.6 IU/ml or greater to 100 IU/ml or less; and MMP-3, 12.5 ng/ml or greater.

Evaluation of efficacy

The clinical response to infliximab was evaluated using EULAR criteria, and disease activity was assessed using the disease activity score in 28 joints (DAS28) based on C-reactive protein (CRP) and the ACR core set[21] at week 54. The EULAR response was evaluated using the DAS28 based on the erythrocyte sedimentation rate (ESR) assessment method (http://www.reuma-nijmegen.nl/www.das-score.nl/index.html). The cut-off values for DAS28–CRP were as follows, based on a report from Inoue *et al*.[22]: DAS28–CRP remission, less than 2.3; low disease activity (LDA), 2.3 or greater to less than 2.7; moderate disease activity (MDA), 2.7 or greater to 4.1 or less; high disease activity (HDA), greater than 4.1. Joint damage was assessed by the total modified Sharp score (0–390 points).[23]

Analytical method

The clinical responses to infliximab at week 54 were analysed for the full analysis set using the last observation carried forward approach. For the comparison of clinical response among dose groups (3, 6 and 10 mg/kg), logistic regression analysis was performed using the clinical responses at the time of randomisation (week 10) as a covariate.

Because there is no well-established threshold value for an abnormal TNF level in the ELISA kit used, the detection limit, less than 0.55 pg/ml, and a threefold higher level, 1.65 pg/ml, were adopted as cut-off values, and the patients were stratified into the following three patient groups for analysis: low, less than 0.55 pg/ml (TNF-low); intermediate, 0.55 pg/ml or greater to less than 1.65 pg/ml (TNF-int); and high, 1.65 pg/ml or greater (TNF-high).

The association of baseline-TNF with baseline disease activity and the efficacy of infliximab therapy was analysed for the three groups described above (TNF-low, TNF-int and TNF-high patients) employing the Kendall rank correlation coefficient. Baseline disease activity was analysed for the enrolled patients (n=327). Clinical responses (DAS28–CRP or ACR core set) and efficacy on physical function (improved health assessment questionnaire (HAQ) score) at week 54 were analysed using the last observation carried forward approach (n=307). Radiographic progression was analysed in the patients whose radiographic data were obtained at week 54 (n=273). The association between the serum infliximab level and clinical response or disease activity at week 54 was analysed in the patients for whom both serum levels and DAS28–CRP values were obtained at week 54 (n=271).

Primary non-responders were defined as patients who had not achieved LDA throughout the study period (at weeks 2–54), and secondary non-responders as those who had not achieved LDA at week 54 but had achieved it at least once between weeks 2 and 50. Kruskal–Wallis test analysis was performed to compare the rates of TNF-low, TNF-int and TNF-high patients in primary and secondary non-responders versus responders (those who had achieved LDA at week 54).

RESULTS

Patients' background and association between baseline-TNF and parameters of disease activity at baseline

Table 1 shows the baseline demographics and clinical characteristics of the enrolled patients (n=327). The median (IQR), minimum and maximum baseline-TNF values were 0.92 pg/ml (<0.55–1.29 pg/ml), less than 0.55 pg/ml and 9.68 pg/ml, respectively. The proportion of patients with baseline-TNF values less than 0.55 pg/ml (undetectable), 0.55 pg/ml or greater to less than 1.65 pg/ml, and 1.65 pg/ml or greater (TNF-low, TNF-int and TNF-high patients) was 28.7%, 58.4% and 12.8%, respectively. Details on the distribution of baseline-TNF can be found in supplementary figure 1 (available online only). Baseline-TNF was significantly, but slightly, correlated with most parameters of disease activity and HAQ score, but not with radiographic progression (table 2). The significant correlations of baseline-TNF with RF and anti-CCP antibodies were also observed.

Clinical response to infliximab at week 54

Of the enrolled patients, 307 were treated with 3, 6 or 10 mg/kg infliximab during the blinded period, and clinical response was evaluated at week 54 using DAS28–CRP (figure 1). The clinical responses at week 54 were significantly higher in the 10 mg/kg group than in the 3 mg/kg group, and 45.2% of the patients in the 10 mg/kg group achieved DAS28–CRP remission.

Association between baseline-TNF and the efficacy of infliximab therapy at week 54

The EULAR responses and disease activities at week 54 in TNF-low (n=87), TNF-int (n=180), and TNF-high (n=40) patients are shown in figure 2A,B. In TNF-low patients, the clinical response was similar among the 3, 6 and 10 mg/kg groups, showing no dose dependency. In TNF-int patients, the EULAR

Table 1 Baseline demographics and clinical characteristics of patients enrolled in the RISING study (n=327)

	Baseline value
Age, year	49.9 (12.0)
Gender, female	267 (81.7)*
Disease duration, years	8.2 (8.0)
Steinbrocker stage	
I	32 (9.8)*
II	114 (34.9)*
III	105 (32.1)*
IV	76 (23.2)*
Steinbrocker functional class	
1	58 (17.7)*
2	239 (73.1)*
3	30 (9.2)*
4	0 (0.0)*
Duration of methotrexate use, years	2.7 (2.8)
Methotrexate dose, mg/week	7.8 (1.7)
Glucocorticoid use	223 (68.2)*
DAS28–CRP	5.5 (4.9, 6.2)†
DAS28–ESR	6.2 (5.6, 6.8)†
TJC, 68 joints	15.0 (10.0, 23.0)†
SJC, 66 joints	12.0 (9.0, 17.0)†
CRP, mg/dl	2.4 (1.2, 4.1)†
ESR, mm/h	52 (35, 72)†
Patient pain VAS, mm	54 (39, 72)†
Patient global VAS, mm	54 (36, 72)†
Physician global VAS, mm	64 (50, 78)†
TSS‡	35.5 (11.5, 73.4)†
Estimated yearly progression of TSS, point/year‡	6.1 (3.1, 11.4)†
HAQ	1.1 (0.8, 1.6)†
RF value, IU/ml	90 (36, 223)†
Anti-CCP antibodies value, IU/ml	≥100 (28, ≥100)†
MMP-3 value, ng/ml	219 (105, 419)†

Data in baseline values are mean (SD), *number of patients (%), or †median (IQR).
Health assessment questionnaire (HAQ) score: scores can range from 0 (no difficulty) to 3 (unable to perform this activity).
Total modified Sharp score (TSS): scores can range from 0 to 390 (erosion score 0–230, joint space narrowing score 0–160), with high scores indicating more joint damage. Estimated yearly rate of progression of the TSS was based on the duration of disease and baseline TSS for every patient.
‡n=325.
CCP, cyclic citrullinated peptide; CRP, C-reactive protein; DAS 28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; MMP-3, matrix metalloproteinase 3; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count; VAS, visual analogue scale.

response rate increased and disease activity decreased as the dose increased, but without significant difference. For TNF-high patients, the clinical response and disease activity were significantly better at 10 mg/kg than at 3 mg/kg and 6 mg/kg. The good response rates were 14%, 31% and 60% in the 3, 6 and 10 mg/kg groups, respectively, and the DAS28–CRP remission rates were 7%, 31% and 50%, respectively. A similar influence by baseline-TNF was also noted on the ACR core set (figure 2C). Significant dose dependency was observed only in TNF-high patients.

Baseline-TNF at clinical response is shown in supplementary table 1 (available online only). Clinical response was significantly influenced by baseline-TNF in the 3 mg/kg group, but not in the 6 and 10 mg/kg groups. Baseline-TNF also influenced the improving physical function, in which the improvement in the HAQ score values and the rates of patients with meaningful improvement (>0.22) in all dosage groups were significantly different in TNF-high patients (supplementary table 2, available online only). Meanwhile, none of the groups showed significant association with radiographic progression.

The proportions of TNF-low, TNF-int and TNF-high patients in responders (n=148) were 34.5%, 56.8% and 8.8%, respectively. On the other hand, those of primary non-responders (n=88) were 21.6%, 62.5% and 15.9%, and those of secondary non-responders (n=71) were 23.9%, 57.7% and 18.3%, respectively. Significant differences were observed between the responders and both of the non-responders (p=0.016 and p=0.032, respectively).

Association between baseline-TNF and serum infliximab level

Clinical response to infliximab has been shown to be influenced by trough serum infliximab levels, and the threshold level for clinical response is approximately 1 μg/ml.[20 24] In this study, the median (IQR) serum infliximab level at week 54 in patients with EULAR good response, moderate response and no response was 3.1 (1.2–7.1), 1.2 (<0.1–3.6) and less than 0.1 (<0.1–0.4) μg/ml. The median (IQR) level in patients in DAS28–CRP remission, with LDA, with MDA and with HDA at week 54 was 3.4 (1.5–7.5), 2.1 (0.4–4.7), 1.4 (<0.1–4.0) and less than 0.1 (<0.1–0.7) μg/ml, respectively. A significant association was observed between clinical response or disease activity and the serum infliximab level (both p<0.001). In patients with high trough serum infliximab levels, better clinical responses were attained. Meanwhile, in patients with poor clinical response (EULAR no response, high disease activity), the median trough serum level was undetectable (<0.1 μg/ml).

Baseline-TNF greatly affected the serum infliximab levels (figure 3A–C). In TNF-low patients, the median trough serum infliximab levels were equivalent to or greater than the threshold level (1 μg/ml) in all groups (3, 6 and 10 mg/kg). For TNF-int patients, the 3 mg/kg dose did not achieve the threshold level of infliximab but did provide a detectable level (≥0.1 μg/ml). However, in TNF-high patients, the median trough serum infliximab levels were undetectable at both 3 and 6 mg/kg doses, in contrast to the 10 mg/kg dose, at which a high level (>2 μg/ml) was maintained.

The positive rates for anti-infliximab antibodies (human anti-chimeric antibodies; HACA), which is reported to influence the serum infliximab level[25] were 27.3%, 23.1% and 12.5% in the 3, 6 and 10 mg/kg dose levels, respectively. In patients who were negative for HACA, the serum infliximab level was also lower in TNF-high patients than in TNF-low patients, and the median serum infliximab levels at the 3 mg/kg dose at week 54 in TNF-low patients (n=23), TNF-int patients (n=37) and TNF-high patients (n=8) were 1.0, 0.6 and 0.2 μg/ml, respectively.

Association between safety and baseline-TNF or serum infliximab level

The incidences of adverse events (total, serious events, infection, serious infection, or infusion reaction) were almost comparable among patients receiving 3, 6 and 10 mg/kg doses in TNF-low, TNF-int and TNF-high patients. We could find no clear association between the incidence of adverse events and the trough serum level of infliximab either (supplementary table 3, available online only).

DISCUSSION

Although dose escalation or shortening of the administration interval between administrations has been used for patients with RA who show an insufficient response to the standard dose of infliximab (3 mg/kg for 8 weeks after initial induction), the effectiveness of these countermeasures is still controversial.[18–20 26–32] In this report, we hypothesised that the production and resultant plasma level of TNF exceeds the neutralising capacity of infliximab in insufficient responders; and that infliximab is consumed

Table 2 Baseline disease activities in TNF-low, TNF-int and TNF-high patients

	Baseline-TNF			
	TNF-low (n=94)	TNF-int (n=191)	TNF-high (n=42)	p Value (overall)
DAS28-CRP	5.3 (4.9, 5.9)	5.5 (5.0, 6.2)	5.9 (5.2, 6.7)	0.002
DAS28-ESR	5.9 (5.5, 6.4)	6.2 (5.7, 6.8)	6.8 (6.2, 7.3)	<0.001
TJC, 68 joints	14.0 (10.0, 21.0)	15.0 (11.0, 23.0)	16.5 (12.0, 23.0)	0.153
SJC, 66 joints	11.0 (8.0, 16.0)	13.0 (9.0, 17.0)	12.5 (10.0, 18.0)	0.041
Patient pain VAS, mm	49.0 (37.0, 65.0)	58.0 (40.0, 72.0)	68.5 (46.0, 88.0)	<0.001
Patient global VAS, mm	51.0 (36.0, 64.0)	56.0 (36.0, 72.0)	65.0 (43.0, 86.0)	0.016
Physician global VAS, mm	62.0 (50.0, 75.0)	65.0 (49.0, 77.0)	72.0 (51.0, 82.0)	0.081
CRP (mg/dl)	2.0 (1.0, 3.9)	2.4 (1.2, 3.9)	2.8 (1.4, 5.8)	0.028
ESR (mm/h)	46.0 (33.0, 65.0)	53.0 (35.0, 72.0)	67.5 (40.0, 89.0)	0.001
HAQ	1.0 (0.5, 1.4)	1.1 (0.9, 1.6)	1.4 (1.0, 2.1)	0.002
TSS	37.0 (10.5, 76.0)*	36.0 (12.5, 72.3)†	22.5 (13.0, 56.0)	0.960
Estimated yearly progression of TSS, point/year	5.8 (2.8, 12.0)*	6.3 (3.4, 11.3)†	5.5 (2.5, 10.0)	0.833
RF value, IU/ml	85 (27, 215)	81 (37, 167)	238 (126, 554)	0.007
Anti-CCP antibodies value, IU/ml	91 (17, ≥100)	≥100 (30, ≥100)	≥100 (≥100, ≥100)	0.012
MMP-3 value, ng/ml	205 (105, 377)	216 (102, 391)	268 (114, 640)	0.214

Data in baseline value are median (IQR).
Baseline disease activities were evaluated in the patients enrolled in the RISING study (n=327). The associations of baseline-tumour necrosis factor alpha (TNF) with baseline disease activities were analysed in the three groups (TNF-low, TNF-int and TNF-high patients) employing the Kendall rank correlation coefficient.
*n=93.
†n=190.
CCP, cyclic citrullinated peptide; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; ESR, erythrocyte sedimentation rate; HAQ, health assessment questionnaire; MMP-3, matrix metalloproteinase 3; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count; TSS, total modified Sharp score; VAS, visual analogue scale.

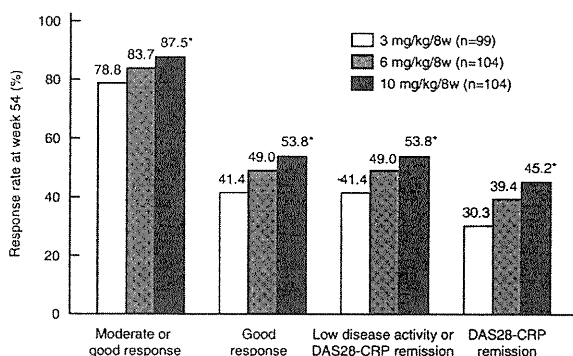

Figure 1 Clinical responses to infliximab therapy in patients with rheumatoid arthritis at week 54. The European League Against Rheumatism response criteria and disease activity were measured by the disease activity score in 28 joints (DAS28)–C-reactive protein (CRP) using the last observation carried forward method. Disease activity was defined as high disease activity, DAS28–CRP greater than 4.1; moderate disease activity, 2.7 or greater to 4.1 or less; low disease activity, 2.3 or greater to less than 2.7; and DAS28–CRP remission, less than 2.3. *p<0.05 versus the 3 mg/kg groups.

to neutralise the TNF, decreasing the serum infliximab level in those patients.

In our study, clinical responses at doses of 3, 6 and 10 mg/kg of infliximab were comparable in TNF-low patients, and the minimum serum infliximab level required for clinical response (approximately 1 μg/ml) was maintained, even in the 3 mg/kg group. Accordingly, the maximal clinical responses can be achieved even with 3 mg/kg therapy in TNF-low patients. In other words, dose escalation does not provide any benefit in this population. In contrast, in TNF-high patients, clinical responses at 3 and 6 mg/kg were significantly decreased in comparison with the response to a 10 mg/kg dose. The trough serum infliximab levels were 'undetectable' at 3 and 6 mg/kg but were greater than 2 μg/ml with a 10 mg/kg dose. As the association between baseline-TNF and the serum infliximab level was also observed in HACA-negative patients, this association was not reflected by the presence of HACA status.

It has been suggested that the threshold serum level for infliximab efficacy on radiographic progression might be lower than that for clinical response.[33] In this study, approximately 90% of the patients whose serum level could be maintained over the threshold for radiographic progression, including even TNF-high patients, showed no radiographic progression. It may explain why baseline-TNF status had no influence on radiographic progression as observed in this study.

The present report has demonstrated that higher doses of infliximab exhibited significantly better clinical and functional response in patients with high baseline-TNF, whereas clinical and functional response was comparable among patients administered 3, 6 and 10 mg/kg of infliximab with low baseline-TNF.

We had reported previously that there was no difference in the safety profile among groups receiving 3, 6 or 10 mg/kg.[16] The rates of adverse events were almost comparable among all dosage groups in TNF-low, TNF-int and TNF-high patients. However, it has been reported that high-dose infliximab therapy might increase the risk of adverse events.[11 34 35] So, the balance between risk and benefit should be considered at the time of infliximab dose escalation, even in TNF-high patients.

There have been several reports on the association between TNF level and the efficacy of infliximab.[36–39] Although the reason for the inconsistent results is unclear, the serum TNF level was measured by bioassay in the report by Marotte *et al*,[37] which is different from this study, in which ELISA was employed. In the studies by Wijbrandts *et al*,[38] clinical response was evaluated at week 16, 2 weeks after infliximab administration when a high serum infliximab level may have been maintained even in patients with a high baseline-TNF. These differences may have led to the different conclusions.

In the RISING study, baseline-TNF was measured in one institution (Mitsubishi Chemical Medience Corporation) concurrently, where minor modifications were made in the original assay procedure and validated for the commercial use of clinical samples.

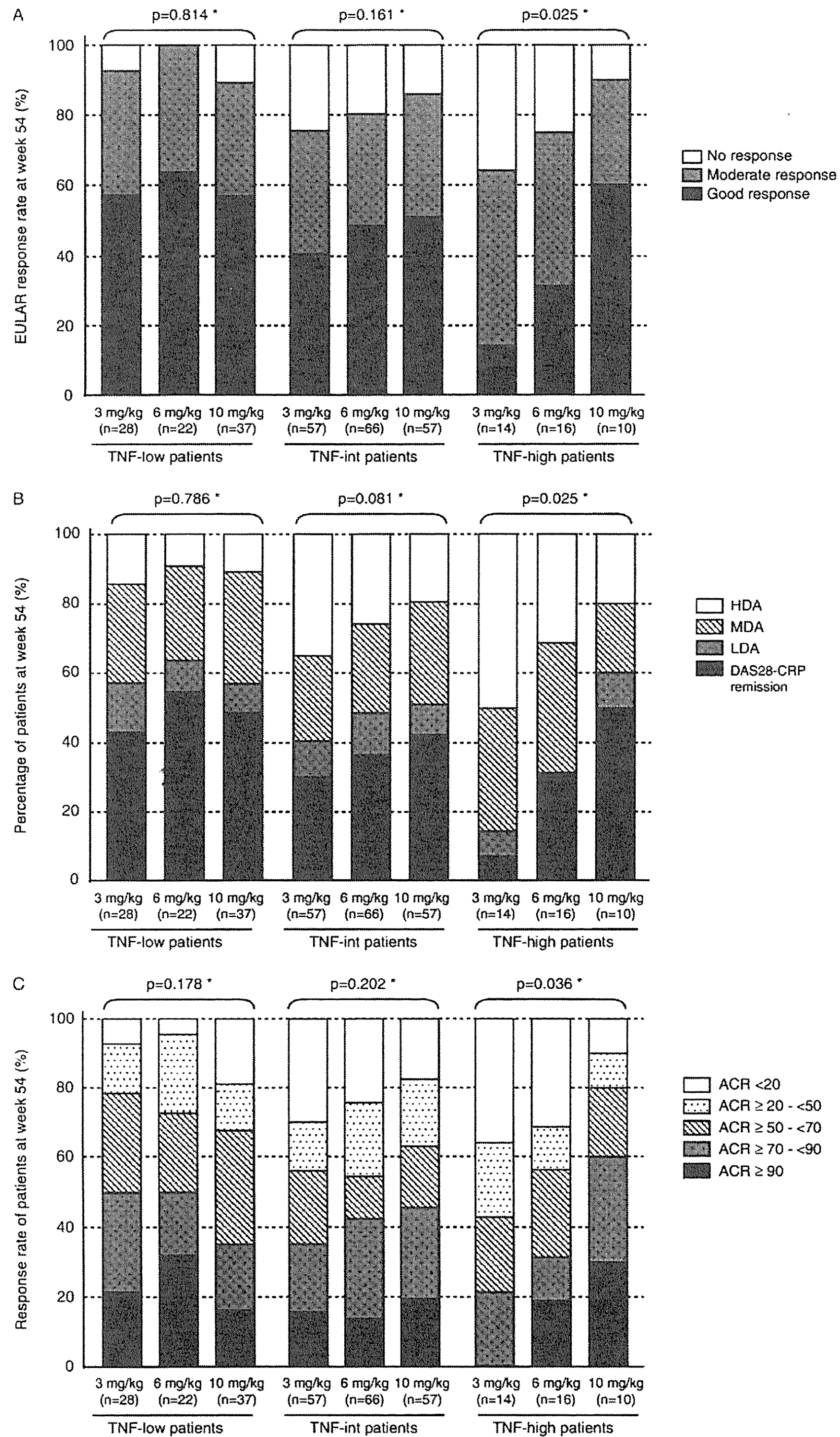

Figure 2 Correlation of plasma tumour necrosis factor alpha (TNF) levels before infliximab therapy (baseline-TNF) with (A) the European League Against Rheumatism (EULAR) response, (B) the disease activity score in 28 joints (DAS28) and (C) American College of Rheumatology (ACR) core set at week 54. Patients were divided into three patients groups by the baseline-TNF level as follows: TNF-low patients, less than 0.55 pg/ml; TNF-int patients, 0.55 pg/ml or greater to less than 1.65 pg/ml; and TNF-high patients, 1.65 pg/ml or greater. EULAR response criteria for disease activity were measured by DAS28–C-reactive protein (CRP). Disease activity was defined as high disease activity (HDA), DAS28–CRP greater than 4.1; moderate disease activity (MDA), 2.7 or greater to 4.1 or less; low disease activity (LDA), 2.3 or greater to less than 2.7; and DAS28–CRP remission, less than 2.3. All clinical responses were evaluated by using the last observation carried forward method. *Kendall rank correlation coefficient.

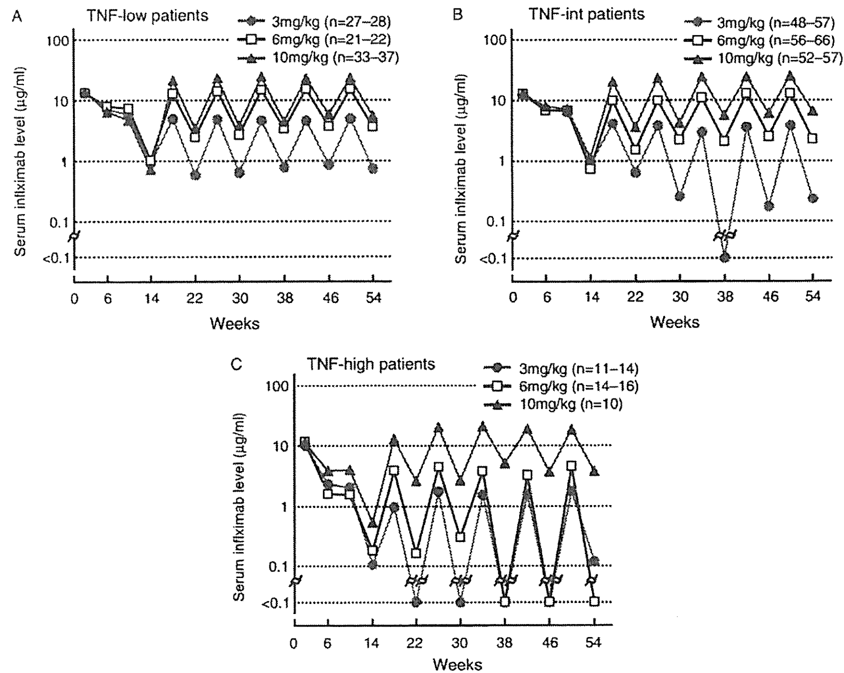

Figure 3 Serum infliximab levels in (A) tumour necrosis factor alpha (TNF)-low, (B) TNF-int and (C) TNF-high patients. Patients were divided into three groups by the plasma TNF level before infliximab therapy (baseline-TNF) as follows: TNF-low patients, less than 0.55 pg/ml; TNF-int patients, 0.55 pg/ml or greater to less than 1.65 pg/ml; and TNF-high patients, 1.65 pg/ml or greater. Serum infliximab levels were measured by ELISA.

It was reported that RF in the samples may cause false-positive results in immunoassays by cross-linking the capture and detection antibodies.[40] In our study, a significant correlation was observed between TNF and RF at baseline (τ value 0.129, $p<0.001$). However, the maximum baseline RF level was 1560 IU/ml in patients with undetectable baseline-TNF (supplementary figure 2, available online only). Therefore, we considered that RF might interfere with the assay of TNF in patients with greater than 1560 IU/ml, but not in patients with less than 1560 IU/ml. Furthermore, we freshly collected the plasma samples from 39 patients with RA (in which backgrounds were shown in supplementary table 4, available online only), and carried out several tests to evaluate the effects of RF on the ELISA for TNF: 'RF-blocking test', using 40% mouse serum, 20% goat serum and 20% rabbit serum;[41 42] 'IgM-RF adding test', using purified polyclonal IgM–RF (Interference Check RF Plus; Sysmex Corporation, Kobe, Japan); 'mismatch simplex sandwich test',[42] using anti-interleukin 6 antibodies conjugated to horseradish peroxidase from the QantiGlo ELISA Kit for interleukin 6 (Q6000B; R&D Systems Inc) as the detection antibodies. As we did not find a clear signal indicating that RF interacted with our ELISA for TNF, we considered that the TNF levels in our study were not influenced by RF (supplementary tables 5–7, available online only).

The circulating TNF level in patients with RA varied from several pg/ml to greater than 100 pg/ml in different reports.[43–46] Although the patient background should be considered in determining the cause of the variation, the measurement systems used for detecting circulating TNF levels in individual studies may largely account for the variation. In that regard, the cut-off values for baseline-TNF used in this report (0.55 and 1.65 pg/ml) were not absolute, and the titre should be standardised in the future.

So far, some of the patient backgrounds have been investigated for predictors of their clinical response to infliximab. Baseline CRP was reported to be associated with the serum infliximab level,[47] but no consensus has been obtained about the correlation between baseline CRP and clinical response.[36 48] We investigated the influence of baseline CRP on the clinical response as an index of EULAR response as done by baseline-TNF. However, we could find no clear relationship between them (supplementary figure 3A,B, available online only). Given that TNF is one of the key cytokines that induce CRP in various inflammatory diseases such as RA, it is reasonable that baseline-TNF, rather than CRP, would be a good predictor of clinical response to the TNF inhibitor, infliximab.

Many contradictory reports have been published regarding the effectiveness of dose escalation with infliximab.[18–20 26–32] The reason why no consensus has been reached is that dose escalation is significant only in TNF-high patients, so the results of reports have been influenced by the number of patients with high baseline-TNF included in those studies. In the RISING study, TNF-high patients accounted for approximately 13% of the total.

This study may provide insight into our understanding of the relationship between the concentration of circulating cytokines and the dose of antibodies against the cytokines in the treatment of RA. As mentioned by van Vollenhoven,[26] an attempt to apply results obtained under highly controlled circumstances at the group level to the unique individual is necessary. These results may be useful for establishing treatment strategies, such as the appropriate dose of infliximab in accordance with baseline-TNF in patients with RA, not only in daily clinical practice but also in relation to the economics of health care.

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Competing interests TT and NM have received research support and consulting or lecture fees from Mitsubishi Tanabe Pharma Corporation. YT, TY and TY are employees of Mitsubishi Tanabe Pharma Corporation. TA and TK have received consulting and lecture fees from Mitsubishi Tanabe Pharma Corporation.

Patient consent Obtained.

Ethics approval The study protocol was approved by the local institutional review board and was carried out in accordance with the Helsinki Declaration and good clinical practice.

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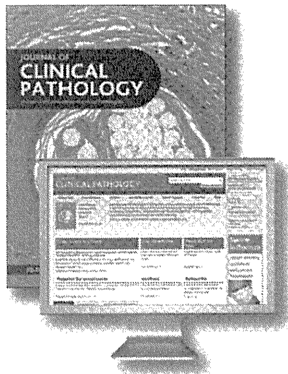

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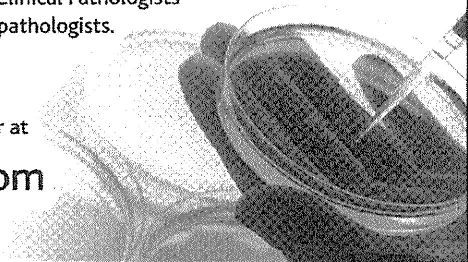

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RESEARCH ARTICLE Open Access

Correlation of antinuclear antibody and anti-double-stranded DNA antibody with clinical response to infliximab in patients with rheumatoid arthritis: a retrospective clinical study

Naoichiro Yukawa*, Takao Fujii, Seiko Kondo-Ishikawa, Hajime Yoshifuji, Daisuke Kawabata, Takaki Nojima, Koichiro Ohmura, Takashi Usui and Tsuneyo Mimori

Abstract

Introduction: The induction of antinuclear antibodies (ANAs) or anti-double-stranded (ds) -DNA antibodies (Abs) after infliximab (IFX) therapy in rheumatoid arthritis (RA) is a well-known phenomenon, but the correlation of such Abs with the clinical response to IFX has not yet been determined. The aims of this retrospective observational study were to examine the prevalence of positive ANA and anti-ds-DNA Abs before and after IFX therapy in patients with RA and to investigate whether an increased titer of such Abs is associated with the clinical efficacy of IFX.

Methods: One hundred eleven RA patients who had received IFX were studied. ANA (indirect immunofluorescence with HEp-2 cells) and anti-ds-DNA Abs (Farr assay) results were examined before and after IFX therapy.

Results: The overall clinical response assessed by EULAR response criteria was as follows: good response in 55%, including remission in 38%; moderate response in 18%; and no response (NOR) in 27%. The positivity of ANA (≥ 1:160) and anti-ds-DNA Abs significantly increased from 25% to 40% ($P = 0.03$) and from 3% to 26% ($P < 0.001$) after IFX, respectively. EULAR response differed significantly according to the ANA titer before IFX ($P = 0.001$), and the efficacy of IFX became worse as the ANA titer before starting IFX increased. Furthermore, the differences in the clinical response of the ANA titer before IFX ≤ 1:80 and ≥ 1:160 were significant (good, moderate, and no response were 66%, 9%, and 25% in ≤ 1:80 group versus 26%, 33%, 41% in ≥ 1:160 group, respectively; $P < 0.001$). In 13 patients whose ANA had increased after IFX, 10 showed NOR, only one showed a good response, and none reached remission. These clinical responses were significantly different from ANA no-change patients. In 21 patients with positive anti-ds-DNA Abs after IFX, 16 showed NOR, only two showed a good response, and none reached remission.

Conclusions: The present study suggests that the ANA titer before starting IFX predicts the clinical response to IFX. The increased titers of ANA or anti-ds-DNA Abs after IFX may be useful markers of NOR.

Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory disease with the potential to cause substantial joint damage and disability. Tumor necrosis factor (TNF)-α plays a central role in the pathogenesis of RA, as demonstrated by the clinical benefit of anti-TNF-α therapy [1-6]. Infliximab (IFX), a chimeric anti-human TNF-α monoclonal antibody, has enabled great advances in the treatment strategy for RA, resulting in a paradigm shift of RA treatment. Although IFX therapy concomitant with methotrexate (MTX) is effective in the majority of RA patients, some patients have persistent active disease, and others lose efficacy after prolonged treatment [5-7]. However, no useful clinical marker has been established to predict such nonresponse (NOR) to IFX.

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The induction of antinuclear antibodies (ANAs) and anti-double stranded (ds)-DNA antibodies (Abs) during IFX therapy is a well-known phenomenon that has already been observed in earlier clinical trials [1-3]. It has been reported that the induction of ANAs is independent of the IFX dose [2,8] and is not modified by concomitant treatment with MTX [9,10], leflunomide, and corticosteroid [8]. Furthermore, the production of ANA is not associated with the clinical response to IFX [11], and even when the development of anti-ds-DNA Abs is observed, onset of lupus-like symptoms is extremely rare [12]. Thus, the significance of the development of such antibodies, including correlations of ANAs and anti-ds-DNA Abs with NOR in RA, has not yet been determined.

Recently, it was reported that the development of ANAs and anti-ds-DNA Abs with anti-TNF therapies may act as a marker of forthcoming treatment failure in patients with psoriasis [13]. Conversely, as in RA patients, it has been reported that ANAs are a predictive factor of infusion reactions during IFX as well as without MTX [14]. On the basis of these findings, the aims of this retrospective observational study were to examine the prevalence of positive ANAs and anti-ds-DNA Abs before and after IFX therapy in patients with RA, and to investigate whether the induction or increased titer of such Abs is associated with the clinical efficacy of IFX.

Materials and methods

Patients and administration of infliximab

One hundred eleven Japanese patients with RA, who had started using IFX as the first biologic agent from November 2003 to June 2009 in our hospital, were studied. All the patients had met the 1987 revised criteria of the American College of Rheumatology (ACR) for the classification of RA [15]. IFX concomitant with MTX was given at 0 (initial dose of 3 mg/kg), 2, and 6 weeks, and then every 8 weeks. If the efficacy of IFX was insufficient, we were permitted to increase the dosage up to the full-bottled dose (for example, 150 mg to 200 mg in a patient weighing 50 kg) or to shorten the administration interval up to every 6 weeks from 8 weeks, according to the judgment of the attending physician. Disease activity was assessed by the disease-activity score in 28 joints (DAS28 ESR) [16], and clinical responses to IFX were evaluated with the European League against Rheumatism (EULAR) response criteria [17]. In contrast to primary NOR patients who had never achieved moderate or good response, loss of response (LOR) was defined as DAS28 score returned to NOR according to the EULAR criteria, after having maintained moderate or good response during at least 3 times of administration of IFX. The present study was conducted in compliance with the Declaration of Helsinki and was approved by the Kyoto University Ethics Committee Review Board, and written informed consent was obtained from all patients.

Determination of study point

Determination of the study point after the IFX therapy is different in each group. In moderate- or good-response patients, the data were collected at a stable point after at least three consecutive administrations of IFX after an achievement of a moderate or good response. In LOR patients, the data were collected within 3 months after LOR was observed. In the IFX withdrawal group (including primary NOR patients), the data were collected just before IFX was discontinued. The intervals between before and after IFX were 6 to 286 weeks (mean, 87 ± 57 weeks), and the total number of IFX administrations was 3 to 38 times (mean, 13 ± 7.3 times), respectively.

Determination of antinuclear antibody and anti-ds-DNA antibody

ANAs and anti-ds-DNA Abs were examined before and after IFX therapy. ANAs were determined by indirect immunofluorescence with HEp-2 cells, and anti-ds-DNA Abs by the Farr assay (normal, < 6 U/ml).

Statistical analysis

Statistical analysis was performed with PAWS version 18 software, by using the Fisher Exact test for changes of ANAs and anti-ds-DNA Abs before and after therapy, the Jonckheere-Terpstra trend test for correlations between ANA titers and clinical response to IFX, and the χ^2 test for comparison between two groups (including ANA ≤ 1:80 group versus ≥ 1:160 group, and ANA titers in the no-change group versus increased group after therapy, respectively), respectively. A value of $p < 0.05$ was considered significant.

Results

Characteristics of the patients and clinical efficacy of IFX

The characteristics of 111 RA patients are shown in Table 1: 82% were female patients; mean age was 51 years; and mean disease duration was 6.6 years at the baseline. Mean DAS28 before IFX was 5.37, and MTX was used in all patients at a mean dosage of 8.1 mg/week. Corticosteroids were used in 61%, and the mean dosage of prednisolone (PSL) was 6.2 mg/day. At the study point, the total number of IFX administrations was 3 to 38 times (mean, 13 times). DAS28 had fallen to 3.55 from 5.37, and EULAR responses were as follows: good response in 55%, including remission in 38%; moderate response in 18%; and NOR in 27%, including LOR in 21%, respectively.

Table 1 Characteristics of 111 RA patients

Baseline (before starting IFX)	
Female, age (mean ± SD, range)	91/111 (82%), 51.6 ± 13.3 years (21 ~ 80)
Disease duration (mean ± SD, range)	6.6 ± 6.4 years (4 months ~ 32 years)
DAS28 (ESR) (mean ± SD, range)	5.37 ± 1.33 (1.71 ~ 8.41)
MTX (mean ± SD, range)	(used in all the patients), 8.1 ± 1.6 mg/week (4 ~ 14 mg)
Corticosteroids users	68/111 patients (61%)
PSL dosage (mean ± SD, range)	6.2 ± 3.2 mg/day (2 ~ 16 mg)
At the study point	
Total number of IFX (mean ± SD, range)	13.0 ± 7.3 times (3 ~ 38)
DAS28(ESR) (mean ± SD, range)	3.55 ± 1.64 (0.54 ~ 7.84)
EULAR response	Good response 55% (including remission 38%)
	Moderate response 18%
	NOR 27% (including LOR 21%)
Discontinuation	45/111 (41%)
Reasons for discontinuation	Remission, eight; NOR, 21; adverse events, 14;
	financial reasons, two

IFX, infliximab; LOR, loss of response; MTX, methotrexate; NOR, no response; PSL, prednisolone.

Positivity of ANA and anti-ds-DNA Abs before and after therapy

The prevalence of positive ANA (≥ 1:40) did not change before and after IFX (78% to 82%), but with ANA ≥ 1:160, the prevalence significantly increased from 25% to 40% (Table 2; $P = 0.03$, Fisher Exact test). Furthermore, the positivity of anti-ds-DNA Abs significantly increased from 3% to 26% ($P < 0.001$; Fisher Exact test). The changes of ANA titer between before and after IFX are shown in Figure 1.

Correlation between ANA titer and clinical response to IFX

Next, we studied the correlation between the ANA status before starting therapy with IFX and the clinical response to IFX. EULAR response (Figure 2(a), upper) and DAS28 after IFX (Figure 2(a), lower) were significantly different by ANA titer before starting IFX ($P = 0.001$ and 0.002, respectively, by the Jonckheere-Terpstra trend test), and the efficacy of IFX became worse if the ANA titer before IFX increased. No correlation was found between ANA titer before IFX and DAS28 before IFX (Figure 2(a), middle). In addition, clinical responses

Table 2 Positivity of ANAs and anti-DNA Abs before and after IFX

		Before IFX	After IFX	*P* value[a]
ANA	≥ 1:40	83/106	77/94	NS
		78%	82%	
	≥ 1:160	27/106	38/94	0.03
		25%	40%	
anti-DNA	≥ 6 U/ml	3/93	21/80	< 0.001
		3%	26%	

ANA, antinuclear antibodies; anti-DNAs, anti-ds-DNA antibodies; IFX, infliximab; NS, not significant. [a]Analyzed with the Fisher Exact test.

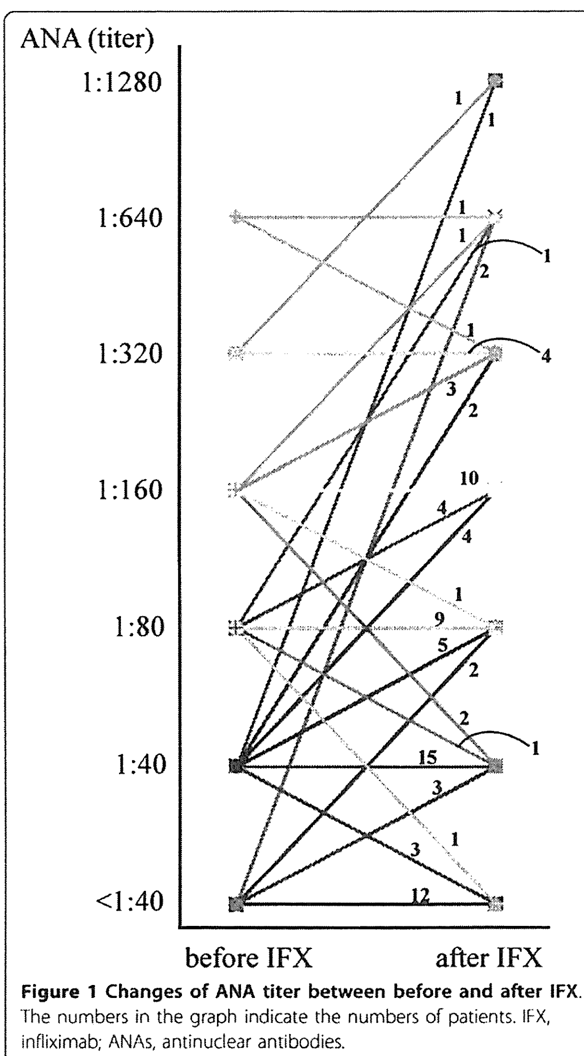

Figure 1 Changes of ANA titer between before and after IFX. The numbers in the graph indicate the numbers of patients. IFX, infliximab; ANAs, antinuclear antibodies.

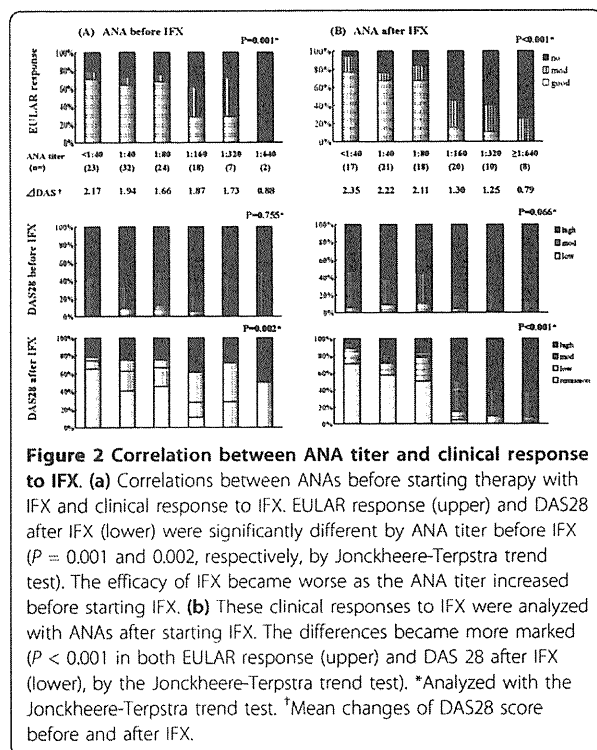

Figure 2 Correlation between ANA titer and clinical response to IFX. **(a)** Correlations between ANAs before starting therapy with IFX and clinical response to IFX. EULAR response (upper) and DAS28 after IFX (lower) were significantly different by ANA titer before IFX (P = 0.001 and 0.002, respectively, by Jonckheere-Terpstra trend test). The efficacy of IFX became worse as the ANA titer increased before starting IFX. **(b)** These clinical responses to IFX were analyzed with ANAs after starting IFX. The differences became more marked (P < 0.001 in both EULAR response (upper) and DAS 28 after IFX (lower), by the Jonckheere-Terpstra trend test). *Analyzed with the Jonckheere-Terpstra trend test. †Mean changes of DAS28 score before and after IFX.

seemed to be divided into two groups between the ANA titer before IFX with ≤ 1:80 and ≥ 1:160 in this figure. As shown in Table 3, the differences in the EULAR response between these two groups were significant (P < 0.001, χ^2 test). Furthermore, when these clinical responses to IFX were analyzed by the ANA titers after starting IFX, such tendencies became more marked, as shown in Figure 1(b) (P < 0.001 in both EULAR response (upper) and DAS 28 after IFX (lower), by the Jonckheere-Terpstra trend test). In the comparison of the ANA titer before IFX and the EULAR response, a significant difference in the ANA titer after IFX ≤ 1:80 and ≥ 1:160 was observed.

Table 3 Comparison of clinical response between ANA titer ≤ 1:80 and ≥ 1:160

EULAR response	ANA titer: Before IFX ≤ 1:80 (n = 79)	Before IFX ≥ 1:160 (n = 27)	Before IFX P value[a]	After IFX ≤ 1:80 (n = 56)	After IFX ≥ 1:160 (n = 38)	After IFX P value[a]
Good	66%	26%	< 0.001	77%	11%	< 0.001
(remission)	(16%)	(7%)	< 0.001	(59%)	(3%)	< 0.001
Moderate	9%	33%	< 0.001	7%	29%	< 0.001
No	25%	41%	< 0.001	16%	60%	< 0.001

ANA, antinuclear antibodies; IFX, infliximab; [a]Analyzed with a χ^2 test.

Clinical response of patients with increased ANA titer after IFX

Based on these results, we next examined patients whose ANAs had increased by two or more dilution levels (for example, ANA titer from < 1:40 to 1:80, from 1:80 to 1:320, and so on) after treatment with IFX (Table 4). Among these 13 patients, 10 showed NOR, only one showed a good response, and none reached remission. These clinical responses were significantly different from those of the ANA no-change patients (Table 5).

Clinical response of patients with positive anti-ds-DNA antibodies after IFX

The characteristics of 21 patients with positive anti-ds-DNA antibodies after IFX are shown in Table 6. Sixteen of 21 patients showed NOR, only two showed a good response, and none reached remission. Interestingly, the second biologic agents, including other TNF antagonists switched from IFX, were all effective in these patients. Furthermore, three patients' clinical responses to IFX were restored by 20-mg PSL before each IFX infusion.

Discussion

In this study, we found that the high titer of ANAs (≥ 1:160) significantly increased from 25% to 40% in RA patients after using IFX, and the positivity of anti-ds-DNA Abs was also significantly increased from 3% to 26%. These results are similar to those of previous reports. Whereas the induction of ANAs and anti-ds-DNA Abs during IFX therapy is a well-known phenomenon, the mechanisms of autoantibody production are poorly understood, and their clinical significance is

Table 4 Clinical response of the 13 patients with increased ANA titer after IFX

	ANA titer Before	ANA titer After	EULAR response
1	< 40	80	Good
2	< 40	80	No
3	< 40	640	No
4	< 40	640	No
5	40	160	No
6	40	160	No
7	40	160	Moderate
8	40	320	No
9	40	320	No
10	40	1,280	No
11	80	640	Moderate
12	160	640	No
13	320	1,280	No

ANAs, antinuclear antibodies; IFX, infliximab.

Table 5 Comparison of clinical response between ANA titer increased and no-change patients

EULAR response	ANA after IFX		P value[a]
	Increased (n = 13)	No change (n = 76)	
Good	8%	54%	0.001
(Remission)	(0)	(42%)	0.001
Moderate	15%	20%	0.001
No	77%	26%	0.001

ANAs, antinuclear antibodies; IFX, infliximab.
[a]Analyzed with χ^2 test.

unknown. Several reasons for the production of ANAs and anti-ds-DNA Abs have been speculated on.

First, the direct effect of a decreased serum TNF level caused by a TNF blocker is considered. It has been reported that low levels of TNF-α may promote SLE in predisposed mice and that the treatment of (NZB/NZW) F_1 mice with TNF-α ameliorates nephritis [18], and low TNF-α expression may be implicated also in human SLE patients [19]. Thus the blockade of TNF-α itself may favor a lupus-like autoimmunity phenomenon.

Second, anti-TNF treatment also reduces CRP levels, and CRP is known to help clear nuclear material after apoptosis. Low CRP levels would result in the prolonged exposure of nuclear material and hence further increase the chance of antibody formation [20,21]; however, serum CRP levels can be reduced not only by anti-TNF treatment but also by other antirheumatic drugs, including MTX and corticosteroids, and actually do not differ between patients with and without TNF blockade induced by autoantibodies [22]. Thus, these phenomena alone are not sufficient to explain the production of ANAs or anti-ds-DNA Abs. Moreover, because the reduction of serum TNF levels is caused by etanercept (ETN) and adalimumab (ADA), as well as IFX, it is difficult to explain the differences in the positivity of ANAs after treatment between IFX and ETN and the successful treatment of switching from IFX to ETN in patients with positive anti-ds-DNA Abs.

As already is known, IFX inhibits not only soluble TNF-α but also transmembrane TNF (tmTNF)-α; the binding of IFX to tmTNF-α may provoke apoptotic cell death with the expression of autoantigens that could trigger the development of anti-ds-DNA antibodies [9]. In contrast, ETN binds mainly soluble TNF-α; the ability of IFX to bind tmTNF-α may explain why IFX but not ETN induces the apoptosis of monocytes and T lymphocytes in Crohn disease [23]. A recent article demonstrated that all three anti-TNF drugs were able to bind tmTNF-α exposed by Jurkat cells, a human

Table 6 Twenty-one patients with positive anti-ds-DNA Abs after IFX

	age/sex	anti-DNA before	anti-DNA after	ANA before	ANA after	EULAR response	symptoms	IFX	following biologics
1	51/F	< 2	300	40	320	No		Discontinue	ADA- > TOC*4
2	41/F	6	300	320	320	No		Discontinue	ETN
3	45/F	51	300	160	320	No	SLE like*1	Discontinue	ETN
4	32/F	NA	52	320	1280	No			
5	31/F	5	42	80	160	No	SLE like*2	Discontinue	ETN
6	36/F	5	38	40	1280	No- > good		PSL 20 mg iv*3	
7	43/F	4	26	160	640	No		Discontinue	TOC
8	56/F	3	26	80	160	No- > good		PSL 20 mg iv*3	
9	62/M	21	25	80	80	Good	PCP	Discontinue	TOC
10	65/F	2	23	160	160	No		Discontinue	TOC- > ETN*5
11	52/F	NA	22	NA	160	No			
12	56/M	< 2	20	320	320	No	allergy	Discontinue	ETN
13	33/F	5	19	40	80	No			
14	51/F	< 2	13	40	80	Mod			
15	56/M	< 2	11	160	160	No- > mod		PSL 20 mg iv*3	
16	75/F	< 2	11	< 40	320	No		Discontinue	ETN
17	58/F	11	11	80	640	Mod			
18	30/M	< 2	10	80	80	No		Discontinue	ETN
19	35/F	< 2	10	80	80	Good			
20	40/F	3	8	80	160	No		Discontinue	ETN
21	59/F	NA	7	NA	640	Mod			

anti-DNA, anti-ds-DNA antibodies; ANA, antinuclear antibodies; IFX, infliximab; SLE, systemic lupus erythematosus; PCP; pneumocystis jirovecii pneumonia; PSL, prednisolone; iv, intravenous injection; ADA, adalimumab; ETN, etanercept; TOC, tocilizumab; *1, leukocytopenia and alopecia; *2, erythema; *3, premedication of each IFX administration; *4, ADA was also getting no response and successful swiched to TOC; *5, TOC ineffective, and ETN effective

lymphoblastoid cell line, and to induce similar antibody-dependent cell-mediated cytotoxicity; in contrast, complement-dependent cytotoxicity was more pronounced with anti-TNF-α monoclonal antibodies in comparison with ETN [24]. These data suggest that ETN may be less effective than both IFX and ADA in the elimination of tmTNF-α-expressing cells. The partially different mechanism of TNF-α inhibition between ETN and anti-TNF-α monoclonal antibodies may explain the lesser generation of autoantibodies in patients treated with ETN in comparison with IFX, as well as the clinical efficacy of IFX and ADA but not of ETN in the treatment of granulomatous diseases such as Crohn disease and Wegener granulomatosis.

Thus, some reasonable explanations exist for the production of ANAs; however, it is unclear why their formation should be associated with the clinical response to IFX. Recently, it was shown that ANAs are a predictive factor of infusion reactions during IFX as well as without MTX in RA patients [14]. Furthermore, it was reported that the development of ANA and anti-ds-DNA Abs in anti-TNF therapies may act as a marker of forthcoming treatment failure in patients with psoriasis [13]. To our knowledge, the present study is the first report to clarify the correlation of ANAs and anti-ds-DNA Abs with the efficacy of IFX in RA patients. Moreover, surprisingly, the clinical response to IFX differed by the ANA titer before IFX, and the predictive value of the baseline ANA titer of ≤ 1:80 and ≥ 1:160 for the efficacy of IFX was observed. This result suggests that preexisting autoimmune abnormalities indicated by ANAs may influence the effect of IFX. Conversely, NOR of IFX was more markedly associated with the increase of ANAs or the induction of anti-ds-DNA Abs. Furthermore, in NOR patients, switching to second TNF antagonists and premedication of PSL without discontinuation of IFX were both effective, suggesting that IFX-induced autoimmune responses may affect the efficacy of IFX.

The mechanisms underlying treatment failure in RA patients treated with IFX have not been entirely clarified; however, one important factor may be the development of anti-drug antibody, or human anti-chimeric antibody (HACA) [1,2]. Because these antibodies cannot be routinely measured in the clinical setting, currently no clinically accessible markers of forthcoming treatment failure are available. Although HACA could not be determined in all patients in this study, we could measure serum HACA in three patients who were successfully restored from NOR by premedication with PSL without discontinuation of IFX, and HACA was negative, at least in these three patients.

Recently, Takeuchi *et al.* [7] reported that the clinical efficacy of IFX was correlated with the trough serum IFX level in a prospective randomized control trial (RISING study). The authors speculated that anti-IFX antibody may be an important factor influencing the efficacy of IFX by increasing the serum clearance of IFX; however, anti-IFX antibody and autoantibodies including ANAs were not measured in the RISING study. Although the IFX trough level was also not measured in our study, ANAs and anti-ds-DNA Abs observed in the NOR patients in our study seemed to be indicators of the immunologic response to the decreased clinical efficacy of IFX. Recently, Hoffmann *et al.* [25] reported that ANAs and anti-ds-DNA Abs in psoriasis patients are predictors for LOR and anti-IFX antibody induction [25], and these data strongly supported our speculation. To confirm this speculation, a large-scale clinical study in RA patients is necessary to examine the correlations between the clinical efficacy of IFX and various factors, including the trough serum IFX level, anti-IFX antibody, and autoantibodies (including ANAs and anti-ds-DNA Abs).

One important limitation of this study was that the approved dosage of IFX and MTX in Japan during the study period was only 3 mg

kg every 8 weeks (IFX) and 8 mg/week (MTX), respectively. This approved dosage of IFX and MTX in Japan is much lower than that in Western countries, and it may result in insufficient clinical efficacy of IFX or suppression of immune responses to IFX. We therefore consider that it is necessary to research the production of ANAs and anti-ds-DNA Abs and their correlation with the clinical efficacy of IFX with a sufficient dosage of IFX and MTX.

Recently, several prognostic markers for the efficacy of IFX, including plasma platelet factor 4 [26] and the gene or mRNA profile in peripheral blood cells [27,28], have been reported; however, the measurement of these markers is complicated and commercially unavailable, and the prognostic value remains insufficient. Regarding these points, ANA and anti-ds DNA Abs are routine laboratory tests and can be measured easily and simply in daily clinical practice. In addition, IFX is the only drug showing clinical evidence of the possibility of biologics-free remission and even drug-free remission [29,30] among the several TNF antagonists, so it is necessary to establish prognostic markers of IFX efficacy, especially clinical remission.

Conclusions

The present study suggests that the ANA titer before starting IFX predicts the clinical response to IFX. Moreover, increased titers of ANAs or the development of anti-ds-DNA Abs after IFX may be useful markers of NOR. Large-scale prospective studies are required to assess the importance of this observation.

Abbreviations
Abs: antibodies; ACR: American College of Rheumatology; ADA: adalimumab; ANAs: antinuclear antibodies; ds: double stranded; ETN: etanercept; EULAR: European League against Rheumatism; HACA: human anti-chimeric antibody; IFX: infliximab; LOR: loss of response; MTX: methotrexate; NOR: no response; PSL: prednisolone; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; TNF: tumor necrosis factor; tmTNF: transmembrane TNF.

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Authors' contributions
NY drafted the manuscript and performed the statistical analysis. NY and TF designed the study. NY, TF, SKI, HY, DK, TN, KO, TU, and TM collected the clinical data. NY, TF, HY, DK, TN, KO, TU, and TM enrolled patients for the study. TM supervised the study design and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Arthritic Joint-Targeting Small Interfering RNA-Encapsulated Liposome: Implication for Treatment Strategy for Rheumatoid Arthritis

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ABSTRACT

RNA interference, mediated by small interfering RNA (siRNA), is effective in silencing genes with a high degree of specificity. To explore the therapeutic potential of systemically administered siRNA for rheumatoid arthritis (RA), we tested the complex of siRNA and the recently developed wrapsome (siRNA/WS) containing siRNA-encapsulated liposome in mice with collagen-induced arthritis (CIA). Mice with CIA received an intravenous injection of Cy5-labeled siRNA/WS. Fluorescence stereoscopic microscopy and flow cytometry were used to assess the siRNA/WS tissue distribution. The efficacy of siRNA-targeting tumor necrosis factor (TNF)-α/WS in CIA was evaluated by arthritis score. TNF-α mRNA levels in the joints were measured by real-time reverse transcriptase-polymerase chain reaction. The intensity of Cy5 fluorescence was higher in arthritic joints than in nonarthritic sites in Cy5-siRNA/WS-treated mice and remained higher up to 48 h after injection, compared with that in naked Cy5-siRNA-treated mice. Cy5 fluorescence intensity was higher in synovial cells than in splenocytes, bone marrow cells, and peripheral blood leukocytes. The majority of Cy5-positive synovial cells were CD11b$^+$ with only a few CD3$^+$ cells. Treatment with TNF-α siRNA/WS resulted in significant decreases in severity of arthritis and TNF-α mRNA level in the joints compared with control siRNA/WS. In conclusion, the use of our WS allowed efficient and targeted delivery of siRNAs to arthritic joints. The siRNA/WS was mainly incorporated into CD11b$^+$ cells, including macrophages and neutrophils, in the inflamed synovium, suggesting its potential therapeutic effects in RA by silencing the expression of inflammatory molecules produced by these cells.

Introduction

RNA interference, mediated by small interfering RNA (siRNA), is a powerful way to silence genes with a high degree of specificity and is a potential therapeutic approach in various diseases including viral infections and proliferative vascular retinopathies (de Fougerolles et al., 2007; Whitehead et al., 2009). To apply this technology to the treatment of rheumatoid arthritis (RA), it is important to develop means to deliver siRNA via systemic injection to multiple affected joints. The delivery tool should be stable and protect siRNA from nuclease attack in vivo. Our group used the wrapsome (WS), which was recently designed with a core composed of a cationic lipid bilayer and siRNA complex enveloped in a neutral lipid bilayer with polyethylene glycol on the surface, as a vehicle for siRNA delivery (Yagi et al., 2009). In contrast to the widely used cationic liposomes, the WS is unique in that it is uncharged. The cationic liposomes are prone to bind in vivo to plasma proteins because of their cationic nature. Because the WS carries a neutral surface charge, the loss of WS function by the plasma protein is less likely. For the same reason, attachment of the WS to the endothelial cell membrane and its entrapment by the reticuloendothelial systems are avoidable, which can otherwise lead to adverse effects, including embolism, a complication reported with the use of cationic liposomes (Litzinger et al., 1996). In addition, the surface pegylation of the WS confers the long half-life of the WS in the systemic circulation ($t_{1/2}$ of 17.6 h). Investigators in our group have recently demonstrated that the use of the WS results in accumulation of

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ABBREVIATIONS: siRNA, small interfering RNA; RA, rheumatoid arthritis; WS, wrapsome; TNF-α, tumor necrosis factor-α; CIA, collagen-induced arthritis; mAb, monoclonal antibody; RT, reverse transcriptase; PCR, polymerase chain reaction; MFI, mean fluorescence intensity; IL, interleukin.

siRNA in tumors in vivo without any signs of significant toxicity (Yagi et al., 2009).

In the present study, we evaluated the accumulation of siRNA/WS complex into the inflamed joints of mice with experimentally induced arthritis. In addition, the therapeutic potential of the siRNA/WS complex targeting tumor necrosis factor (TNF)-α, which is produced primarily by macrophages in the synovia of the affected joints, was assessed in the arthritic mice.

Materials and Methods

Preparation of siRNA-Encapsulated Liposome. All siRNAs including fluorescence-labeled randomized siRNA were synthesized and purified by Hokkaido System Science (Hokkaido, Japan). The siRNA sequences were as follows: control randomized siRNA sense 5′-GGU ACA CAU GUG CAC ACA CdTdT-3′ and antisense 5′-GUG UGU GCA CAU GUG UAC CdTdT-3′; and TNF-α-siRNA sense 5′-GUG CCU AUG UCU CAG CCU CdTdT-3′ and antisense 5′-GAG GCU GAG ACA UAG GCA CdTdT-3′ (Schiffelers et al., 2005). Preparation of the complex of siRNAs and WS, which was developed as delivery system for siRNAs, was described previously (Yagi et al., 2009). The siRNA/WS complex consists of siRNA and cationic lipids enveloped by a neutral lipid bilayer containing egg phosphatidyl choline and is ~100 nm in diameter.

Imaging of siRNA/WS Accumulation in Arthritic Joints of Mice with Collagen-Induced Arthritis. Murine collagen-induced arthritis (CIA) was induced in 8-week-old male DBA/1J mice (Charles River Japan, Tokyo, Japan) by immunization twice with bovine type II collagen (Collagen Research Center, Tokyo, Japan) and complete Freund's adjuvant (Difco, Detroit, MI) (Nanki et al., 2005). From the day of the second immunization (day 0), mice were examined for signs of joint inflammation. The severity of arthritis was evaluated using the following clinical scoring method (Nanki et al., 2005): 0, normal; 1, erythema and mild swelling confined to the midfoot or ankle joint; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joint; and 4, erythema and severe swelling of the ankle, foot, and digits. The clinical score was defined as the sum of the scores of all four paws of each mouse. At day 7, CIA mice showing joint inflammation scored more than 2 with the above definition at least in a paw, were randomly injected either with a single injection of Cy5-labeled siRNA/WS, naked Cy5-labeled siRNA, or saline via the tail vein. Considering the limited sensitivity for detection of fluorescence in the in vivo setting, the doses of the Cy5-labeled siRNA were selected to be 50 and 150 μg for whole-body imaging (Yagi et al., 2009). Cy5 fluorescence was monitored for up to 96 h after administration using a fluorescence stereoscopic microscope (Macro Imaging System, Nippon Roper, Tokyo). The level of fluorescence labeled-siRNA in the affected joints was quantified by analyzing regions of interest using image analysis software (Nippon Roper). Approval for the experimental protocol was obtained from the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Analysis of siRNA/WS Tissue Distribution in the CIA Mice. At day 7, CIA mice showing joint inflammation as described above were randomly injected either with 50 μg of Cy5-labeled siRNA/WS, naked Cy5-labeled siRNA, or saline via the tail vein. On the basis of the results of the whole-body imaging, the dosage of Cy5-labeled siRNA/WS for fluorescence-activated cell sorting analysis was selected. Twenty-four hours later, tissues (synovium, spleen, and bone marrow) were harvested, and single cell suspensions were prepared using standard procedures. Synovial tissues were harvested from arthritic joints (scored more than 2). Peripheral blood leukocytes were isolated by depletion of red blood cells with NH_4Cl hemolytic solution. The cells were analyzed for Cy5 fluorescence using a FACSCalibur system (BD Biosciences, San Jose, CA). The following mAbs were used for staining: CD11b-phycoerythrin (eBioscience, San Diego, CA), F4/80-fluorescein isothiocyanate (eBioscience), CD3-fluorescein isothiocyanate (BD Pharmingen, San Diego, CA), and CD19-phycoerythrin (eBioscience).

Analysis of the Effect of TNF-α siRNA/WS in CIA Mice. At the day of the second immunization (day 0), mice were grouped on the basis of equal average clinical scores and were given intravenous injections of TNF-α siRNA/WS, naked TNF-α siRNA, control randomized-siRNA/WS (1 or 10 μg/day), or buffered saline three times per week until day 10. The clinical arthritis score and the incidence of arthritis were evaluated. In addition, the thickness of each paw was measured using calipers.

Real-Time RT-PCR. CIA mice were sacrificed on day 12, and paws were pulverized using a cryopress (Microtec, Chiba, Japan). Total RNA was prepared from 50 mg of the sample using an RNeasy RNA isolation kit (QIAGEN, Valencia, CA). The levels of TNF-α mRNA were measured by a real-time RT-PCR assay using TaqMan probes [TNF-α (Mm00443258) and glyceraldehyde-3-phosphate dehydrogenase (Mm99999915) (Applied Biosystems, Foster City, CA)]. Amplification was performed in a thermal cycler (ABI Prism 7000; Applied Biosystems).

Statistical Analysis. Data are expressed as mean ± S.E.M. For comparing clinical scores between groups, the nonpaired Student's *t* test was used. For comparing incidence of arthritis between groups, Fisher's exact test was used. Analyses were performed using StatView (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered significant.

Results

Accumulation of Systemically Administered siRNA/WS in Arthritic Joints of Murine CIA. The potential of the WS as a delivery tool of siRNA was investigated in CIA. At day 7, when the CIA mice developed arthritis (scored more than 2 at least in a paw), they were given injections with either fluorescence (Cy5)-labeled siRNA/WS or naked Cy5-labeled siRNA (50 or 150 μg). After 12 h, observation under the stereoscopic microscope showed accumulation of fluorescence in the arthritic joints but not in nonarthritic sites in mice treated with Cy5-siRNA/WS (150 μg), and the levels of the accumulation appear higher in severely swollen joints compared with that in less swollen or normal joints (Figs. 1, A and B). Although the intensity was weaker, fluorescence was detected in arthritic joints in mice treated with 50 μg of Cy5-siRNA/WS (data not shown). In mice treated with naked Cy5-siRNA (150 μg), although a low level of fluorescence was observed in arthritic joints immediately after the injection, the fluorescence disappeared within 12 h (Fig. 1C). The intensity of the fluorescence level remained high up to 48 h after injection in the arthritic joints of the mice treated with Cy5-siRNA/WS (Fig. 1D). Although the intensity was weaker, fluorescence was detected in arthritic joints in mice treated with 50 μg of Cy5-siRNA/WS (data not shown). In contrast, the intensity of the fluorescence level in the arthritic joints of the mice treated with naked Cy5-siRNA (150 μg) was much lower than that in the mice treated with Cy5-siRNA/WS (Fig. 1D).

Next, the tissue distribution of systemically administered Cy5-siRNA/WS was examined in the CIA mice. At 24 h after injection of 50 μg of Cy5-siRNA/WS, naked Cy5-siRNA, or saline, the Cy5 fluorescence levels in cells from the synovium, spleen, bone marrow, and peripheral blood leukocytes were examined by flow cytometry. Compared with that in saline-injected mice, the fluorescence intensity of Cy5 was higher in cells harvested from the