autoantibodies [4,5,14,15,21], further replicative investigations may confirm the pathologic role of IFN-α in patients with DM.

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Conflict of interest

None.

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ORIGINAL ARTICLE

Nationwide epidemiological survey of 169 patients with adult Still's disease in Japan

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Abstract

Objectives. A nationwide survey was conducted to assess the number of patients, clinical aspects, treatment, and prognosis of adult Still's disease (ASD) in Japan.
Methods. A primary questionnaire was sent to randomly selected medical institutions in order to estimate the number of patients. We sent a secondary questionnaire to the same institutions to characterize the clinical manifestations and treatment of ASD.
Results. The estimated prevalence of ASD was 3.9 per 100,000. Analysis of 169 patients showed a mean age at onset of 46 years. The main clinical symptoms were fever, arthritis, and typical rash in agreement with previous surveys. Oral glucocorticoids were used to treat 96% of the patients, while methotrexate was used in 41% and biological agents were used in 16%. Lymphadenopathy and macrophage activation syndrome were significantly associated with increased risk of relapse ($P < 0.05$, each). Patients who achieved remission after tocilizumab therapy had significantly longer disease duration (6.2 years) than patients who did not (1.9 years) ($p < 0.05$).
Conclusions. The 2010–2011 nationwide survey of ASD identified important changes in treatment and improvement of prognosis compared with previous surveys.

Keywords

Adult Still's disease, Ferritin, Methotrexate, Multicenter study, Tocilizumab

History

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Introduction

Adult Still's disease (ASD) was first reported by Bywaters in 1971 [1] as an idiopathic systemic inflammatory disease with three main symptoms: quotidian fevers, arthritis, and evanescent rash. ASD is difficult to diagnose due to the lack of specific clinical manifestations and serum biomarkers. The ASD classification proposed by Yamaguchi et al. [2] in 1992 is used worldwide based on its high sensitivity and specificity. Two major epidemiological surveys were conducted in Japan by the research group of the Ministry of Health and Welfare of Japan in 1988 and 1994 [3,4]. Since the last survey, hyperferritinemia has been added to the Yamaguchi's criteria for reference and this has allowed easier and earlier diagnosis of ASD, and probably changed its clinical manifestations and prognosis. In addition, while ASD is generally treated with glucocorticoids, glucocorticoid-resistant ASD has recently been treated with methotrexate (MTX) or biological agents that are effective against rheumatoid arthritis (RA). Such drugs seem to have improved the course and prognosis of ASD over the past two decades. Indeed, some studies have shown the efficacy of immunosuppressive agents like cyclosporine A (CyA) and biologic agents like tumor necrosis factor (TNF) inhibitors or anti-interleukin (IL)-6 receptor antibody in the treatment of small cohort of patients with ASD [5–7]. Therefore, the research group for autoimmune diseases of the Ministry of Health, Labour and Welfare of Japan conducted another nationwide survey of ASD between 2010 and 2011 to estimate the number of ASD patients in Japan and to assess the clinical manifestations, treatment, course, and prognosis of this disease.

Patients and methods

The survey was performed in two parts: the primary survey was designed to estimate the number of ASD patients treated at medical institutions, while the secondary survey assessed the clinical manifestations of ASD. In the primary survey, we randomly selected medical institutions that were stratified according to the number of beds and posted a questionnaire to the Department of Internal Medicine or Rheumatology about the number of ASD patients treated between January 1 and December 31, 2010.

The diagnosis of ASD depended on physician's judgment. We subsequently sent another questionnaire to the same hospitals in 2011 to obtain detailed information about the patients. To comply with the Personal Information Protection Law in Japan, all information that could identify an individual were made anonymous.

Subjects of the survey included ASD patients aged 16 years or older, who met Yamaguchi's criteria, and attended and/or were admitted to the hospital between January 1 and December 31, 2010. In the secondary survey, clinical information was obtained through a structured interview with the patient, physical

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examination, laboratory tests, and review of medical records. Medications were determined by combining the information provided by patients and medical records. Laboratory data on all parameters were obtained at the time when the maximum serum ferritin was detected. Articular X-rays were taken and reviewed in all patients.

Statistical analysis

Demographic characteristics are presented as mean ± SD (median) for continuous variables and as frequencies and percentages for categorical variables. The associations between serum ferritin level and other variables such as leukocyte count, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and albumin were examined in ASD patients using Spearman's rank correlation analysis. Comparison of patients who achieved remission with patients who did not achieve remission after treatment with tocilizumab (TCZ), anti-IL-6 receptor antibody, was performed by Mann–Whitney U test, Fisher's exact test, and Cochran–Armitage test. We defined remission as the absence of articular, systemic, and laboratory evidence of disease activity under the current therapy [8]. Logistic regression analysis was employed to assess the association between clinical features and induction therapy with the risk of relapse in patients with ASD. For this analysis, variables of clinical features were age at onset (per 10 years old), gender, typical rash, lymphadenopathy, splenomegaly, disseminated intravascular coagulation (DIC), macrophage activation syndrome, abnormal liver function, and ferritin level (≧3,000 or not). Variables in relation to medications for induction therapy were administration of oral glucocorticoid only, pulse glucocorticoid therapy, MTX, CyA, non-steroidal anti-inflammatory drugs (NSAIDs), and TCZ. For treating missing data, we used the multiple imputation method. Two hundred imputed datasets were generated using the multiple imputation by chained equations method and their results were synthesized using the ordinary Rubin's rule [9]. We also evaluated potential predictive factors which associate with complication of MAS by logistic regressions. All analyses were performed using SPSS for Windows, version 18.0 (IBM Japan Inc., Tokyo, Japan) and R ver. 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Primary survey

A total of 7,999 Departments of Internal Medicine and 936 Departments of Rheumatology were subsequently stratified into the following seven categories according to the hospital size and number of beds: university hospitals, hospitals with ≥500 beds (excluding university hospitals), 400–499 beds, 300–399 beds, 200–299 beds, 100–199 beds, and ≤99 beds (Table 1). We randomly selected hospitals from among 100% of the university hospitals, the hospitals with 500 beds or over, and the departments of rheumatology, as well as from among 80% of the hospitals with 400–499 beds, 40% of the hospitals with 300–399 beds, 20% of the hospitals with 200–299 beds, 10% of the hospitals with 100–199 beds, and 5% of the hospitals with 99 beds or less. Among the 8,935 departments, we sent the primary questionnaire to 2,586 departments and we received 500 replies (19%). The reported number of patients was 956 (Table 1). The estimated number of patients with ASD across Japan was calculated by the following formula: Sum of [number of reported patients (C)/Returns (B) X Total number of departments (A) in each category]. The total number of ASD patients in 2011 was estimated to be 4,760 in Japan. According to the census conducted in 2010, the population of Japan on December 1, 2011 was 127,799,000; hence, the estimated prevalence rate of ASD was 3.9 patients per 100,000 people.

Secondary survey

Clinical characteristics

We received 40 replies to the secondary questionnaire survey and obtained clinical information on 169 patients, including 121 females, with a male:female ratio of 1:2.57 (Table 2). The information about 94 (55.6%) out of 169 patients with ASD was obtained from university hospitals in secondary survey. Estimated number of ASD patients in university hospitals was 1,516 (31.8%) out of 4,760 (estimated number of ASD patients in Japan). Thus, prevalence of the patients in university hospitals was higher in the secondary survey than that in the primary survey.

All patients satisfied Yamaguchi's criteria. Eight of the one sixty-nine patients developed ASD when they were at the age of younger than sixteen, while the other one fifty-eight patients had adult-onset ASD. The mean age at onset was 46 ± 19 years (Table 2). Disease duration at presentation of the patients was 0.4 ± 1.6 years (mean ± SD; median: 0.1 year). Observation period of this study was 4.9 ± 4.6 years (mean ± SD; median: 3.3 years). Two (1.3%) out of one fifty-eight patients had a family history of juvenile Still's disease, while none had family history of ASD. Seven (4.5%) out of one fifty-seven patients had family

Table 1. Reported patients in primary survey.

Category by specialty and number of beds	Total number of departments (A)	Surveys for target departments	Returns (B)	Response rate (%)	Number of reported patients with ASD (C)	Estimated number of patients with ASD (D)
Internal medicine						
University hospital	147	147	34	23	122	527
500 beds and over	384	384	60	16	118	755
400–499 beds	321	256	39	15	13	107
300–399 beds	662	265	39	15	7	119
200–299 beds	1023	204	39	19	10	262
100–199 beds	2367	236	55	23	7	301
Under 100 beds	3095	158	35	22	0	0
Subtotal	7999	1650	301	18	277	2072
Rheumatology						
University hospital	48	48	15	31	309	989
500 beds and over	67	67	16	24	79	331
400–499 beds	48	48	10	21	6	29
300–399 beds	67	67	12	18	12	67
200–299 beds	130	130	28	22	257	1193
100–199 beds	270	270	52	19	10	52
Under 100 beds	306	306	66	22	6	28
Subtotal	936	936	199	21	679	2688
Total	8935	2586	500	19	956	4760

ASD adult Still's disease

Table 2. Clinical characteristics of patients with ASD .

	Present study ($n = 169$) Values (Frequency)	Previous survey in 1988 ($n = 90$) Values (Frequency)
General characteristics		
Infant onset:Adult onset	8 (4.8%):158 (95.2%)	NA
Age at onset, years	46 ± 19 (median 46)	NA
Female (%)	121/168 (72.0%)	60/90 (66.7%)
Family history		
Juvenile idiopathic arthritis	2/158 (1.3%)	NA
ASD	0/159 (0.0%)	NA
RA	7/157 (4.5%)	NA
Other autoimmune disease	3/157 (1.9%)	NA
Clinical characteristics		
Fever > 39.0°C, ≧ 1 week	152/166 (91.6%)	71/90 (78.9%)
Arthralgia > 2 weeks	138/166 (83.1%)	90/90 (100.0%)
Arthritis	77/152 (50.7%)	62/86 (72.1%)
Typical rash	102/164 (62.2%)	72/83 (86.7%)
Sore throat	96/162 (59.3%)	58/83 (69.9%)
Lymphadenopathy	72/161 (44.7%)	59/86 (68.6%)
Splenomegaly	52/161 (32.3%)	56/86 (65.1%)
Pericarditis	5/161 (3.1%)	9/87 (10.3%)
Pleuritis	6/161 (3.7%)	11/89 (12.4%)
Interstitial pneumonia	4/161 (2.5%)	NA
Myalgia	42/162 (25.9%)	50/89 (56.2%)
Drug allergy	29/165 (17.6%)	44/82 (53.7%)
Complications		
Amyloidosis	0/127 (0.0%)	NA
DIC	8/127 (6.3%)	NA
Macrophage activation syndrome	19/127 (15.0%)	NA

There were some missing data in the database of this study. Total number of enrolled patients in this study was 169. Information about disease onset and gender was not obtained from 3 patients and 1 patient, each.

DIC disseminated intravascular coagulation, *NA* not applicable

Infant onset was defined as ASD developed at the age of less than sixteen. Adult onset was defined as ASD developed at sixteen years old or older.

history of RA and 3 patients had family history of autoimmune diseases (Graves' disease, $n = 1$; Sjögren's syndrome, $n = 2$) (Table 2). Clinical manifestations detected in the 169 ASD patients were mainly fever (> 39°C for at least 1 week, 91.6%), arthralgia (persisting for at least 2 weeks, 83.1%), and typical rash (62.2%). The features corresponded to the results of previous survey in Japan [3].

Arthritis was found in 44.4% of patients with arthralgia ($n = 138$) (Table 3). The number of ASD patients with monoarthritis, oligoarthritis, and polyarthritis was 3, 33, and 41, respectively. Polyarthritis was the most common in patients with ASD. The involved joints were the wrists (27.0%), knees (27.0%), and shoulders (15.8%) in order of descending prevalence. Articular X-rays of suffered joints were carried out in each patient by the attending physician's decision. Because we assumed that joint destruction did not exist when no articular X-ray was taken in the patients, we count such patients as having no joint destruction. Fifteen patients (11.7%) showed joint destruction, such as bone erosion (11 patients, 8.6%), joint space narrowing (10 patients, 7.8%), and/or ankylosis (2 patients, 1.6%). Three patients showed other radiographic abnormalities (osteoporosis, spur formation at the distal interphalangeal joints, and unspecified changes in 1 case each), while 110 other patients (85.9%) showed no radiographic abnormalities. Of 128 ASD patients with available X-ray findings,

Table 3. Articular manifestations on each joints during 6 months after disease onset in patients with ASD.

	Patients who were assessed by X-ray ($N = 128$)							
Joint	No of Pts with erosion in each joint	Erosion (%)	No of Pts with JSN in each joint	JSN (%)	No of Pts with ankylosis in each joint	Ankylosis (%)	No of Pts who had each joint destruction (erosion or JSN or ankylosis)	each joint destruction (%)
TMJ	0	0.0	0	0.0	0	0.0	0	0.0
Shoulder	0	0.0	1	0.8	0	0.0	1	0.8
SCJ	0	0.0	0	0.0	0	0.0	0	0.0
Elbow	2	1.6	1	0.8	0	0.0	2	1.6
Wrist	7	5.5	7	5.5	1	0.8	10	7.8
Hip	3	2.3	1	0.8	0	0.0	3	2.3
Knee	3	2.3	3	2.3	1	0.8	6	4.7
Ankle	2	1.6	1	0.8	0	0.0	3	2.3
MCP	1	0.8	4	3.1	0	0.0	4	3.1
PIP	3	2.3	6	4.7	0	0.0	8	6.3
DIP	1	0.8	3	2.3	0	0.0	3	2.3
ITJ	0	0.0	1	0.8	0	0.0	1	0.8
MTP	0	0.0	1	0.8	0	0.0	1	0.8

Pts patients, *JSN* joint space narrowing, *No* number, *TMJ* Temporomandibular joint, *SCJ* Sternoclavicular joint, *MCP* Metacarpophalangeal, *PIP* Proximal interphalangeal, *DIP* Distal interphalangeal, *ITJ* Intertarsal joints, *MTP* Metatarsophalangeal

Several joints were affected at the same time in some of the patients.

the radiographic abnormalities of erosion, joint space narrowing, and ankylosis were commonly seen in the wrists, knees, and proximal interphalangeal joints (Table 3). Several joints were affected at the same time in some of the patients. Erosions and joint space narrowing were detected in several joints in 8 patients and in 6 patients, each. Other findings included sore throat (57.3%), lymphadenopathy (44.7%), splenomegaly (32.3%), and drug allergy (17.6%) (Table 1). With respect to complications, 15.8% of the patients had macrophage activation syndrome (MAS) and 6.3% developed DIC, while none of the patients had secondary amyloidosis (Table 2).

Laboratory findings

The results of various laboratory tests are summarized in Table 4. In general, the laboratory tests demonstrated an inflammatory response, with high leukocyte count ($\geq$ 10,000/mm^3, 79.4%), polymorphonuclear cells ($\geq$ 80%, 71.5%), erythrocyte sedimentation rate (ESR; $\geq$ 40 mm/hr, 68.9%), and C-reactive protein (CRP; positive CRP, 91.5%), together with liver dysfunction (abnormal liver function tests, 73.9%) and hyperferritinemia (presence of hyperferritinemia, 88.5%). Severe hyperferritinemia (ferritin, $\geq$ 3,000 ng/mL) was noted in 60% of the patients. Serum rheumatoid factor (RF) and antinuclear antibody were negative in 79.9% and 74.2% of the patients, respectively. Serum IL-6 levels were high in all of the 15 patients tested. Plasma IL-18 levels were also elevated in 11 out of the 12 patients tested (91.7%).

Various factors, such as leukocyte count ($r = 0.289$, $P < 0.001$), AST ($r = 0.561$, $P < 0.001$), LDH ($r = 0.677$, $P < 0.001$), and hypoalbuminemia ($r = -0.445$, $P < 0.001$), correlated significantly with serum ferritin level at time of peak hyperferritinemia.

Treatment

The drugs used to treat 166 ASD patients are listed in Table 5. The most common was oral glucocorticoid, which was used in 160 patients (96.4%), followed by NSAIDs in 73 patients (44.0%). With respect to immunosuppressants, MTX was used in 68 patients (41.0%), followed by CyA in 45 patients (27.1%). Fifty-two patients (31.3%) were treated with glucocorticoid pulse therapy (Table 5, left column). Biological drugs were used on 33 occasions in 27 patients (16.3%) (Table 5, left column, and Table 6). Among them, four patients received two biologic agents (Patients 1, 5, 15, and 24) and one patient received three biologics (Patient 14, Table 6). As induction therapy for ASD ($n = 161$), oral glucocorticoid alone was the most common choice and used in 82 patients (50.9%), among whom 47 (29.2%) were treated with glucocorticoid pulse therapy. MTX was combined with glucocorticoid in 37 patients (23.0%) and CyA was administered with steroids in 30 patients (18.6%) (Table 5, middle column).

With regard to treatment of relapses ($n = 67$), oral glucocorticoid monotherapy was the most common, being used in 28 patients (41.8%). Twelve patients (17.9%) were treated with steroid pulse therapy. MTX was combined with glucocorticoid in 17 patients (25.4%), and CyA was administered with glucocorticoid in 8 patients (11.9%) (Table 5, right column).

Table 6 lists the demographic profiles of 27 patients treated with biologic agents, comprising TNF inhibitors in 12 patients (infliximab in 7 patients, etanercept in 4 patient, and adalimumab in 1 patient) and TCZ in 21 patients. We obtained clinical information on 19 patients out of 21 patients who received TCZ. Nine patients were treated with TCZ for induction therapy; however, only 2 achieved remission and 5 patients stopped TCZ because of adverse events (allergic reaction, hypotension, MAS, rash, and infection). On the other hand, 10 patients were treated with TCZ for maintenance therapy (2 patients) or for relapse (8 patients), among whom 7 patients achieved remission and 1 developed fungal infection. Comparison of clinical features of patients who achieved remission ($n = 9$) with patients who did not achieve remission ($n = 10$) by TCZ indicated significantly longer disease duration (6.2 ± 5.6 years) in the former compared with the latter (1.9 ± 2.8 years, $P = 0.03$). Age, gender, treatment period, and prevalence of patients with oral glucocorticoid or with other immunosuppressant were not related to TCZ-treated patients who did or did not achieve remission.

Clinical outcome

Of the 146 patients with available data on the clinical course, 58 (39.7%) and 50 patients (34.2%) showed monocyclic and polycyclic systemic patterns, respectively, while 15 (10.3%) and 23 patients (15.8%) showed monocyclic and polycyclic systemic

Table 4. Laboratory findings in patients with ASD.

	Present survey in 2011 ($n = 169$) Values (Frequency)	Previous survey in 1988 ($n = 90$) Values (Frequency)
Leukocytosis (Leucocytes $\geqq$ 10,000/μL)	131/165 (79.4%)	80/90 (88.9%)
Glanulocytosis (Neutrophils $\geqq$ 80%)	118/165 (71.5%)	74/89 (83.1%)
Anemia (Hemoglobin $\leqq$ 10 g/dL)	68/169 (40.2%)	53/90 (58.9%)
Thrombocytopenia (Platelets $< 15 \times 10^4$)	23/169 (13.6%)	NA
Elevated ESR (ESR $\geqq$ 40mm/hr)	113/164 (68.9%)	85/89 (95.5%)
Hypoproteinemia#	32/169 (18.9%)	NA
Hypoalbuminemia#	107/139 (77.0%)	44%
Abnormal liver function†	122/165 (73.9%)	74/87 (85.1%)
Positive CRP	151/165 (91.5%)	NA
Hyperferritinemia*	146/165 (88.5%)	28/34 (82.4%)
Serum ferritin levels above 3,000 ng/mL	99/165 (60.0%)	NA
Positive RF	33/164 (20.1%)	5/89 (5.6%)
Positive ANA	42/163 (25.8%)	6/88 (6.8%)
Elevation of serum IL-6 (pg/mL)	15/15 (100.0%)	NA
Elevation of plasma IL-18 (pg/mL)	11/12 (91.7%)	NA

ESR erythrocyte sedimentation rate, *CRP* C-reactive protein, *RF* rheumatoid factor, *ANA* anti-nuclear antibodies, *IL-6* interleukin-6, *IL-18* interleukin-18, *NA* not applicable

†Abnormal liver function was defined as any elevated liver enzymes (aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase) above the normal range in each medical facility.

#Hypoproteinuria and hypoalbuminemia were defined as total protein and serum albumin levels lower than a normal reference value in each medical facility.

*Hyperferritinemia was defined as serum ferritin levels higher than a normal reference value in each medical facility.

Table 5. Treatment of patients with ASD.

Medication for ASD ($N = 166$)		Induction therapy for ASD ($N = 161$)		Therapy for relapse of ASD ($N = 67$)	
Drugs	Numbers of patients (%)	Therapy	Numbers of patients (%)	Therapy	Numbers of patients (%)
Medication	163 (98.2%)	Pulse GC therapy	47 (29.2%)	Pulse GC therapy	12 (17.9%)
Pulse GC therapy	52 (31.3%)	GC only	82 (50.9%)	GC only	28 (41.8%)
GC	160 (96.4%)	GC + MTX	21 (13.0%)	GC + MTX	10 (14.9%)
NSAIDs	73 (44.0%)	GC + CyA	20 (12.4%)	GC + CyA	8 (11.9%)
MTX	68 (41.0%)	GC + NSAIDs	9 (5.6%)	GC + TCZ	3 (4.5%)
CyA	45 (27.1%)	GC + MTX + CyA	6 (3.7%)	MTX + TCZ	2 (3.0%)
TAC	12 (7.2%)	GC + MTX + TAC	3 (1.9%)	GC + MTX + NSAID	2 (3.0%)
Salazosulfapyridine	4 (2.4%)	TCZ	3 (1.9%)	GC + MTX + TAC	2 (3.0%)
Mizoribine	3 (1.8%)	GC + MTX + NSAID	2 (1.2%)	Other therapy	10 (14.9%)
Azathioprine	3 (1.8%)	Other therapy	15 (9.3%)		
Cyclophosphamide	2 (1.2%)				
Leflunomide	1 (0.6%)				
Auranofin	2 (1.2%)				
Gold	1 (0.6%)				
Etoposide	1 (0.6%)				
Infliximab	7 (4.2%)				
Etanercept	4 (2.4%)				
Adalimumab	1 (0.6%)				
Tocilizumab	21 (12.7%)				

ASD adult Still's disease, *GC* glucocorticoid, *MTX* methotrexate, *CyA* cyclosporine, *NSAIDs* non-steroidal anti-inflammatory drugs, *TAC* tacrolimus, *TCZ* tocilizumab.

Other therapy means the therapies carried out in only one patient, respectively.

As induction therapy, the group of other therapy includes 5 patients treated with MTX + GC+ some other drugs and 2 patients treated with CyA + GC+ some other drugs. As therapy for relapse, the group of other therapy includes 3 patients treated with MTX + GC+ some other drugs.

patterns with chronic articular involvement, respectively. Furthermore, a self-limited pattern was seen in 11 patients. During the observation period, 66 out of 169 patients (39.1%) experienced relapse. However, there were no deaths during the observation period. At the last medical examination, 145 out of 164 patients (88.4%) had achieved remission.

Relationship between clinical features/induction therapy and risk of relapse

Association of clinical features at baseline and induction therapy with risk of relapse was investigated by logistic regression analysis. Relapse was observed in 66 out of 169 patients with ASD. According to the previous report [10], serum ferritin levels of above 3000 ng/mL in a patient with compatible symptoms should lead to suspicion of ASD in the absence of a bacterial or viral infection. Therefore, we defined cutoff levels of ferritin as more than 3000 ng/ml. Univariate analysis showed that lymphadenopathy (odds ratio [OR] = 1.99, 95% confidence interval [CI]: 1.04–3.78; $p = 0.037$), and complication of MAS (OR = 2.88, 95%CI: 1.04–8.00; $p = 0.043$) were associated with risk of relapse in patients with ASD; however, marked hyperferritinemia (≥3,000 ng/mL), complication of DIC, and medications of induction therapy were not. Multivariate analysis identified lymphadenopathy as the only significant factor for risk of relapse after adjustment for age, sex, other clinical features, and medications for induction therapy (OR = 2.40, 95%CI: 1.08–5.33; $p = 0.032$).

Potential predictive factors for complication of MAS in patients with ASD

We conducted logistic regression analyses to explore potential predictive factors for complication of MAS in ASD. Here, the number of MAS events was small (only 19), so we evaluated univariate associations. As a result, AST (OR = 1.84, 95%CI: 1.24–2.74; $p = 0.003$), LDH (OR = 5.07, 95%CI: 1.98–12.97; $p = 0.001$), and hyperferritinemia (OR = 4.36, 95%CI: 1.30–14.68; $p = 0.017$) were significantly associated with complication of MAS. These factors have been known to be included in laboratory findings of MAS and the strong associations were also observed in the clinical data.

Discussion

According to the 1994 nationwide survey conducted by the research group of the Ministry of Health and Welfare of Japan [4], the prevalence rate of ASD was approximately 2 patients per 100,000 and the male:female ratio was 1:2. The current survey conducted in 2010 showed a prevalence rate of 3.7 per 100,000 people in Japan. The tendency for ASD to show female predominance was still noted. A French retrospective study published in 1995 showed an estimated prevalence rate of ASD of 0.16 cases per 100,000 people and no difference in prevalence between males and females [11]. These results suggest a higher prevalence rate in Japan than that in France. Yamaguchi's classification criteria were used in the 1994 survey, but serum ferritin was not included at that time [4]. The research group of the Ministry of Health and Welfare of Japan assessed the diagnostic value of serum ferritin and concluded that the inclusion of ferritin did not improve the diagnostic accuracy. Hence, serum ferritin is only used for reference in Yamaguchi's criteria. In the present survey, 89% of ASD patients showed hyperferritinemia and serum ferritin level was more than 3,000 ng/mL in 60% of patients. In the 1988 epidemiological survey of ASD, the major clinical manifestations consisted of fever (100%), high fever (81%), arthralgia (100%), typical rash (87%), sore throat (70%), lymphadenopathy (69%), splenomegaly (65%), pleuritis (12%), and pericarditis (10%) (Table 2) [3]. Laboratory findings included leukocytosis (89%), liver dysfunction (85%), negative RF (94%), and negative antinuclear antibody (93%) (Table 4). In the present survey, fewer patients were positive for each of these items (Table 4). Since earlier diagnosis would have been possible due to the wide-spreading knowledge of this disease, the prevalence of clinical and laboratory findings decreased in ASD patients of this study in 2011 compared with the 1988 epidemiological survey of ASD. Also, drug allergy was only found in 18% of the patients in this survey compared with 54% in the 1988 survey (Table 2). Initiation of steroids at an early stage of the disease might have resulted in a decrease in the number of ASD patients with drug allergy.

Recent advances have demonstrated the major role of proinflammatory cytokines, such as IL-6 and IL-18, in the pathogenesis of ASD [12,13]. IL-6 levels are associated with disease activity, and IL-18 levels are thought to be a marker of disease severity and

Table 6. Demographic profile of 27 patients treated with biologic agents.

Patient	Age (years)	Gender	Disease duration (years)	Biologic agent	Therapy	Treatment period (months)	Adverse events	Treatment progress	With GC	With other immunosuppressant
1–1	21	M	0.3	TCZ	Induction therapy	5	Allergy	Withdrawal (adverse event)	Yes	None
1–2	21	M	0.3	ADA	Induction therapy	NA		Ongoing (active)	NA	NA
2	20	M	0.1	TCZ	Induction therapy	10	Hypotension	Withdrawal (adverse event)	No	None
3	47	M	0.1	TCZ	Induction therapy	6		Withdrawal (remission)	Yes	CyA
4	26	F	0.1	TCZ	Induction therapy	1	MAS	Withdrawal (adverse event)	Yes	None
5–1	48	M	0.1	TCZ	Induction therapy	7		Withdrawal (unavailable)	No	None
5–2	48	M	1.2	IFX	Therapy at relapse	9		Ongoing (remission)	Yes	MTX
6	36	F	1.7	TCZ	Induction therapy	38		Ongoing (remission)	No	None
7	48	F	0.2	TCZ	Induction therapy	2	Generalized rash	Withdrawal (adverse event)	No	CyA
8	71	F	5.7	TCZ	Therapy at relapse	12		Ongoing (remission)	Yes	MTX
9	57	F	9.4	TCZ	Therapy at relapse	5		Ongoing (remission)	Yes	None
10	23	M	8.5	TCZ	Therapy at relapse	12		Ongoing (active)	Yes	None
11	78	F	1.8	TCZ	Therapy at relapse	18	Fungal infection	Ongoing (remission)	Yes	None
12	65	F	NA	IFX	Maintenance therapy	NA		NA	Yes	MTX
13	46	M	4.6	TCZ	Maintenance therapy	10		Ongoing (remission)	Yes	MTX
14–1	35	F	1.4	IFX	Induction therapy	17		Withdrawal (unavailable)	Yes	MTX
14–2	35	F	2.8	TCZ	Induction therapy	20	Infection	Withdrawal (adverse event)	Yes	MTX
14–3	35	F	4.8	ETN	Maintenance therapy	3		Withdrawal (unavailable)	Yes	MTX
15–1	34	F	NA	IFX	NA	NA		NA	NA	NA
15-2	34	F	15.1	TCZ	Therapy at relapse	22		Ongoing (remission)	Yes	MTX
16	39	F	NA	TCZ	Therapy at relapse	10		Ongoing (remission)	Yes	TAC
17	72	F	4.3	TCZ	Therapy at relapse	96		Ongoing (active)	None	MTX
18	42	M	1.7	IFX	Maintenance therapy	41		Withdrawal (remission)	Yes	MTX
19	50	F	0.2	TCZ	Induction therapy	8		Withdrawal (economic reason)	Yes	MTX
20	76	M	NA	TCZ	NA	NA		NA	NA	NA
21	32	F	NA	ETN	NA	NA		NA	NA	NA
22	52	M	NA	ETN	Maintenance therapy	NA		Withdrawal (remission)	Yes	CyA
23	60	F	NA	TCZ	NA	NA		NA	NA	NA
24–1	49	F	3.9	TCZ	Therapy at relapse	11		Withdrawal (unavailable)	Yes	MTX
24–2	49	F	4.8	IFX	Therapy at relapse	10		Ongoing (remission)	Yes	MTX
25	50	F	NA	IFX	Maintenance therapy	NA		Ongoing (remission)	Yes	MTX
26	37	F	2.5	ETN	Therapy at relapse	NA		Ongoing (active)	Yes	TAC
27	24	F	7.3	TCZ	Maintenance therapy	40		Ongoing (remission)	Yes	None

M male, *F* female, *GC* glucocorticoid, *MTX* methotrexate, *CyA* cyclosporine A, *TAC* tacrolimus, *ETN* etanercept, *IFX* infliximab, *ADA* adalimumab, *TCZ* tocilizumab, *MAS* macrophage activation syndrome, *NA* not applicable.

Patient 1 was treated with TCZ and ADA. Patients 5, 15, and 24 were treated with TCZ and IFX. Patient 14 was treated with IFX, TCZ, and ETN.

MAS in patients with ASD [12,13]. Serum IL-6 and plasma IL-18 were elevated in patients with ASD in this study, despite the small number of patients tested for these parameters.

Pouchot et al. [8] reported that 41% (16 of 39 patients) of ASD patients had abnormal X-ray findings of joint space narrowing at the carpometacarpal or intercarpal joints of the wrist, which progressed to ankylosis in 25% of the patients. In the present study, 12% of patients exhibited radiographic abnormalities (erosion, 9%; joint space narrowing, 8%), and only 2% showed ankylosis. With respect to the radiographic joint damage, the prognosis of joints in ASD seems to be relatively better than that before 1990s. A study of 90 ASD patients in 1990 reported that the polycyclic systemic pattern was the most common, being seen in 41% of patients, while more than half of the patients experienced relapse (55%) and 4 patients died (4%) [3]. At that time, glucocorticoid and NSAIDs were used for treating 92% and 79% of the patients, respectively, while only 10% were treated with immunosuppressants (cyclophosphamide and azathioprine) and neither MTX nor CyA was used. In the present survey, the monocyclic systemic pattern was the most common (in 40% of the patients), while 39% of patients experienced relapse and none of the patients died. The majority of the large observational studies of ASD were performed before MTX became widely used and before the marketing of biologic agents. The long-term benefits of MTX in limited joint destruction have been demonstrated in RA but not in ASD. One possible reason for the improved prognosis is that ASD was better controlled by steroid therapy combined with immunosuppressants (MTX or CyA) or biological agents, all of which have become available after the previous survey. ASD patients occasionally develop severe complications such as MAS or reactive hemophagocytotic syndrome (RHS). According to a retrospective study of 50 patients with ASD, 6 patients (12%) experienced RHS [14]. Another retrospective observational study showed that RHS was complicated in 8 out of 57 patients (14.0%) in ASD patients [15]. In our survey, 16% of patients developed MAS or RHS, which was similar to that in the previous reports. We evaluated potential predictive factors which were associated with complication of MAS in ASD. In our study patients with ASD, AST, LDH, and hyperferritinemia were associated with complication of MAS. Although the above factors have been already known to be included in laboratory findings of MAS and might not be novel findings, the information obtained in this epidemiological study would be meaningful. Secondary amyloidosis was also identified as a complication of ASD in previous surveys, but was not recognized in any of the patients in the present survey [2,15,16]. This could be due to early treatment, which resulted in inhibition of production and deposition of amyloid proteins associated with chronic inflammation.

Glucocorticoids were the most commonly used medications on the treatment of ASD. Furthermore, glucocorticoid pulse therapy, DMARDs, or biologic agents were added to control the disease based on the degree of disease activity and severity. The prognosis of ASD is known to be relatively good; however, 39% of the patients with ASD experienced relapse while on therapy or after discontinuation of treatment during the observation period (the mean observation period between the first and last examinations was 4.9 years in this survey). We investigated whether clinical features and induction therapy were associated with risk of relapse. Previous studies reported significant correlation between hyperferritinemia and disease activity, and recommended the use of hyperferritinemia as a marker to monitor the response to treatment in ASD [17,18]. Analysis of our survey data showed no relation between serum ferritin levels above 3,000 ng/mL and risk of relapse. Furthermore, pulse glucocorticoid therapy, and MTX, CyA, NSAIDs, and TCZ for the induction therapy did not reduce the risk of relapse. Univariate analysis showed that lymphadenopathy and MAS were associated with increased risk of relapse in patients with ASD, suggesting that these two complications could be considered as risk factors for relapse in patients with ASD.

Twenty-one ASD patients in the present survey were treated with TCZ, and clinical information was available for 19 of these 21 patients. Patients who achieved remission after treatment with TCZ had longer duration of disease compared with those who did not. A few studies have reported the efficacy of TCZ in refractory ASD [6,7]. Furthermore, 94% of 35 patients with ASD reported in the literature were resistant to other immunosuppressive agents, such as MTX, TNF blockers, and anakinra. TCZ induced remission and allowed reduction of the dose or discontinuation of corticosteroids [19]. Based on the above results and those of the present survey, we recommend the use of TCZ for treatment in patients with refractory and long-standing ASD.

There have been some papers about successful treatment experience with TCZ in patients with ASD [6,7,20,21]. MAS is a life-threatening syndrome with excess immune activation. MAS occurs either in ASD or in systemic juvenile idiopathic arthritis (sJIA) [14,22]. Major findings are fever, hepatosplenomegaly, rash, lymphadenopathy, neurologic symptoms, cytopenias, hyperferritinemia, and abnormal liver function. The propriety of TCZ therapy in MAS complicated with ASD patients has been controversial. In some cases of ASD and sJIA, MAS aggravated during TCZ therapy [6,19,21,23]. Although the contribution of TCZ to occurrence of MAS has not been determined, careful observation should be required during TCZ therapy in patients with active ASD.

Conclusion

We conducted a nationwide survey of ASD, which showed changes in treatment and improvement of prognosis compared with previous surveys. Our findings suggest that lymphadenopathy and MAS are potential risk factors for relapse in patients with ASD. The use of immunosuppressants like MTX or CyA and biologics for ASD has increased in recent years. We also recommend the use of TCZ for treatment of relapse in patients with long-standing ASD.

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Conflict of interest

None.

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Aberrant histone acetylation contributes to elevated interleukin-6 production in rheumatoid arthritis synovial fibroblasts

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ABSTRACT

Accumulating evidence indicates that epigenetic aberrations have a role in the pathogenesis of rheumatoid arthritis (RA). However, reports on histone modifications are as yet quite limited in RA. Interleukin (IL)-6 is an inflammatory cytokine which is known to be involved in the pathogenesis of RA. Here we report the role of histone modifications in elevated IL-6 production in RA synovial fibroblasts (SFs). The level of histone H3 acetylation (H3ac) in the IL-6 promoter was significantly higher in RASFs than osteoarthritis (OA) SFs. This suggests that chromatin structure is in an open or loose state in the IL-6 promoter in RASFs. Furthermore, curcumin, a histone acetyltransferase (HAT) inhibitor, significantly reduced the level of H3ac in the IL-6 promoter, as well as IL-6 mRNA expression and IL-6 protein secretion by RASFs. Taken together, it is suggested that hyperacetylation of histone H3 in the IL-6 promoter induces the increase in IL-6 production by RASFs and thereby participates in the pathogenesis of RA.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease which is characterized by persistent synovitis and progressive joint destruction [1]. RA synovial fibroblasts (SFs) produce the inflammatory cytokine interleukin (IL)-6 that is important in the pathogenesis of RA [2]. IL-6 is a 21 kDa glycoprotein which consists of 184 amino acids and functions as a pleiotropic cytokine which is involved in the acute phase response and immune regulation [3]. IL-6 induces the activation and differentiation of T cells, immunoglobulin production in B cells, platelet maturation and the production of acute phase proteins, such as C-reactive protein, in hepatocytes. IL-6 deficient mice are resistant to arthritis induction [4]. The remarkable role of IL-6 in the pathogenesis of RA is supported by the striking therapeutic success of Tocilizumab, an IL-6 receptor inhibitor [5].

Multiple lines of evidence suggest that genetic and environmental factors participate in the pathogenesis of RA. Twin studies have shown that the concordance of monozygotic twins (12–15%) is higher than dizygotic twins (3%) in RA [6,7]. The heritability of RA was estimated to be 65% from these studies in twins [8]. A family study reported that the relative risk of RA was 3.0 in offspring, 4.6 in siblings and 6.4 in twins [9]. Serotyping studies demonstrated a significant association between RA and the HLA allele HLA-DRB1. HLA-DRB1 molecules contain a conserved amino acid sequence QKRAA/QRRAA, or "shared epitope", which may contribute to RA susceptibility [10]. Candidate gene studies and genome-wide association studies (GWAS) have identified approximately 60 susceptibility loci for RA [11]. On the other hand, environmental factors such as cigarette smoking, viral infection, *Porphyromonas gingivalis* and silica exposure are suggested to trigger RA [12–15] . However, in spite of these data, the etiological mechanisms of RA are not well understood.

Epigenetic mechanisms including histone modifications have been shown to determine chromatin assembly and influence gene transcription [16–18]. Histone amino-terminal tails are subject to covalent post-translational modifications such as acetylation,

Abbreviations: RA, rheumatoid arthritis; OA, osteoarthritis; SFs, synovial fibroblasts; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; H3ac, acetylation of histone H3; H3K4me3, tri-methylation of histone H3 lysine 4; HAT, histone acetyltransferase.

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methylation, phosphorylation and ubiquitination [19]. The acetylation of histone H3 (H3ac) and tri-methylation of histone H3 lysine 4 (H3K4me3) are associated with the activation of gene transcription [20]. Recently, there have been increasing reports on epigenetic alterations in RA, although most of them have focused on DNA methylation or microRNAs (miRNA) [21]. We have hypothesized that another epigenetic gene-regulator, histone modification, is associated with the pathogenesis of RA. In the present study, we sought to clarify the role of histone modifications in the elevated IL-6 production that takes place in RASFs. The level of H3ac in the IL-6 promoter was significantly higher in RASFs than in osteoarthritis (OA) SFs. Additionally, curcumin, a histone acetyltransferase (HAT) inhibitor, decreased the level of H3ac in the IL-6 promoter, IL-6 mRNA and IL-6 protein secretion in RASFs.

2. Materials and methods

2.1. Materials

Anti-H3ac and an isotype control IgG were purchased from Upstate. Anti-H3K4me3 was obtained from Abcam. Human tumor necrosis factor (TNF)-α was purchased from Miltenyi Biotech. Sodium butyrate, phenyl methylsulfonyl fluoride (PMSF), micrococcal nuclease (MNase), and curcumin were obtained from Sigma.

2.2. Preparation and culture of SFs

Synovial tissues were obtained from RA and OA patients during total knee joint replacement at the Saitama Medical University Hospital. All of the RA patients fulfilled the American College of Rheumatology 1987 revised criteria for RA. This study was approved by the ethics committee of Saitama Medical University and a written informed consent was obtained from every patient in this study. Synovial tissues were minced into small pieces and incubated with 1.5 mg/ml collagenase and 0.04% hyaluronidase for 2 h at 37 °C as previously described [22]. After overnight culture, nonadherent cells were removed and SFs from passages 4 through 8 were used in this study.

2.3. Real-time quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from SFs using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. 2 μg of total RNA were used to synthesize cDNA using SuperScript III reverse transcriptase (Invitrogen). PCR was conducted in 20 μl of total volume with 0.2 μM primers using Power SYBR Green PCR Master Mix (Applied Biosystems) for 40 cycles in a StepOne Plus Real-Time PCR System (Applied Biosystems). For standardization, 18s ribosomal RNA (18s rRNA) was amplified simultaneously. The sequences of the primers used are available upon request.

2.4. Enzyme-linked immunosorbent assay (ELISA)

SFs (1×10^5 cells) were cultured in DMEM containing 0.5% FBS in 6-well plates for 24 h. The supernatants were collected at 0, 24, 48, and 72 h after stimulation with 10 ng/ml TNF-α. The concentrations of the IL-6 protein were measured using an ELISA kit (PeproTech) according to the manufacturer's instructions and corrected by the cell number.

2.5. Chromatin immunoprecipitation (ChIP) assay

SFs (5×10^5 cells) were incubated with digestion buffer (50 mM Tris–HCl pH 7.6, 1 mM $CaCl_2$, 0.2% Triton X-100, 5 mM sodium butyrate and 0.5 mM PMSF) containing protease inhibitor cocktail (Roche) along with 0.2 U MNase for 10 min at 37 °C. The chromosomal DNA was sonicated using SONIFIER W-150 (Branson), dialyzed with RIPA buffer for 2 h at 4 °C, and incubated with Dynabeads Protein G (Invitrogen) and antibody (anti-H3ac or anti-H3K4me3) overnight at 4 °C. After treatment with 0.2 mg/ml RNase A and 1 mg/ml proteinase K, the immunoprecipitated DNA was used for analysis by real-time PCR using Taqman universal PCR master mix (Applied Biosystems). The sequences of the primers and the probe used are available upon request.

2.6. Treatment of RASFs with curcumin

RASFs were pretreated with 20 μM curcumin for 2 h and followed by stimulation with 10 ng/ml TNF-α. The cells were harvested at 8 h for the analyses using RT-qPCR and ChIP assay. The supernatants were replaced with DMEM containing 0.5% FBS at 8 h and collected at 24 h for ELISA.

2.7. Statistical analysis

The differences between the groups were determined by a Mann–Whitney *U* test or a Wilcoxson's signed rank test. All the results are expressed as the means ± SEM. A p value of <0.05 was defined as statistically significant.

3. Results

3.1. IL-6 mRNA expression was significantly higher in RASFs than in OASFs

Previous reports have shown that the inflammatory cytokine IL-6 is involved in the pathogenesis of RA [2]. We examined whether IL-6 production was increased in RASFs. We isolated SFs enzymatically from synovial tissues of RA and OA patients and compared IL-6 gene expression in RASFs and OASFs. IL-6 mRNA was significantly higher by 3.1-fold in RASFs than OASFs (Fig. 1A). GAPDH mRNA was not significantly different between RASFs and OASFs (Fig. 1A). ELISA was used to measure the IL-6 protein in supernatants secreted by RASFs and OASFs. Although not statistically significant, IL-6 protein production was higher in RASFs than in OASFs (Fig. 1B). These results indicate that IL-6 production is substantially enhanced in RASFs and may thus contribute to synovial inflammation in RA.

3.2. H3ac and H3K4me3 in the IL-6 promoter were significantly higher in RASFs than in OASFs

It has been demonstrated that epigenetic mechanisms, including histone modifications, alter chromatin structure and have an affect on trasnscriptional activity. To determine whether histone modifications in the IL-6 promoter were associated with elevated IL-6 gene expression in RASFs, we examined H3ac, which is correlated with active gene transcription, in the IL-6 promoter with a ChIP assay and quantitative PCR in both RASFs and OASFs. The level of H3ac was significantly higher in the proximal IL-6 promoter (from −101 to −16) in RASFs than OASFs, whereas that of H3ac was comparable in the distal IL-6 promoter (from −586 to −528) (Fig. 2A). The level of H3ac was not significantly different in the GAPDH promoter between RASFs and OASFs. (Fig. 2C). We wondered whether other active histone marker profiles were similar to H3ac in the IL-6 promoter in RASFs. We subsequently found that the level of H3K4me3 was significantly higher in the proximal and distal IL-6 promoters in RASFs than OASFs (Fig. 2B). The levels of H3ac and H3K4me3 were higher in the proximal IL-6 promoter than in the distal IL-6 promoter in RASFs. The level of H3K4me3

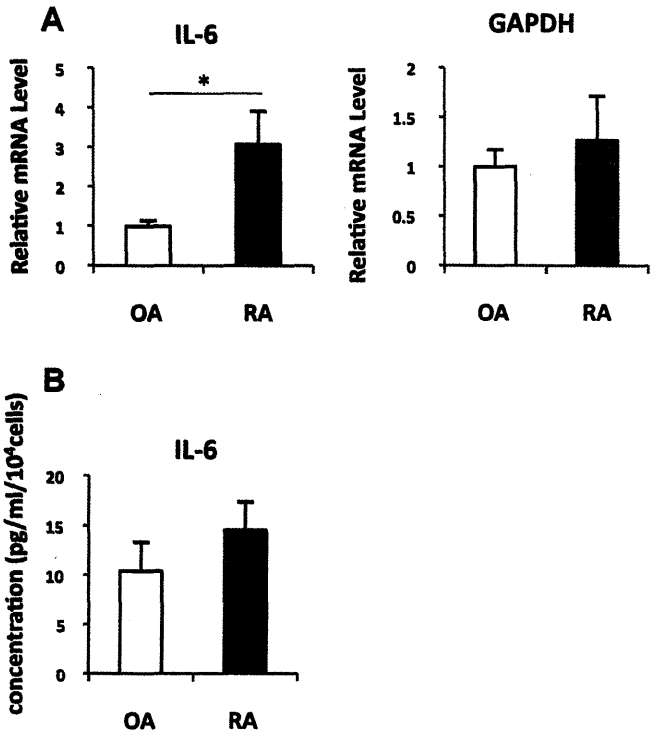

Fig. 1. IL-6 production increases in RASFs. (A) IL-6 and GAPDH mRNAs were examined by quantitative RT-PCR in RASFs ($n = 19$) and OASFs ($n = 21$). Values were expressed as the fold increase versus the value in OASFs. (B) The IL-6 protein in the supernatant of RASFs ($n = 10$) and OASFs ($n = 11$) was measured by ELISA. Values were expressed as the concentration (pg/ml) per 10^4 cells. The data are the means ± SEM. *$p < 0.05$ (Mann–Whitney U test).

was not significantly different in the GAPDH promoter (Fig. 2D). These results suggest that the chromatin structure is open in the IL-6 promoter, resulting in IL-6 mRNA elevation in RASFs.

3.3. IL-6 mRNA expression in RASFs significantly increased after stimulation with TNF-α

We considered that the high levels of H3ac and H3K4me3 in the IL-6 promoter affected the IL-6 gene expression after activation in RASFs. Therefore, we investigated whether IL-6 transcription was activated in response to TNF-α in RASFs. IL-6 mRNA significantly increased at 4, 24 and 72 h after incubation with 10 ng/ml TNF-α in RASFs compared with OASFs (Fig. 3A), while GAPDH mRNA did not change after stimulation with TNF-α (Fig. 3A). IL-6 protein secretion also significantly increased at 24 and 72 h after stimulation with TNF-α in RASFs compared with OASFs (Fig. 3B). These data imply that the open chromatin in the IL-6 promoter results in robust IL-6 production by RASFs in response to TNF-α.

3.4. Curcumin significantly reduced H3ac in the IL-6 promoter and IL-6 production in RASFs

It is reasonable to expect that the high levels of H3ac in the IL-6 promoter would be associated with the elevated IL-6 production in RASFs. Histone acetylation is increased by the catalytic activity of HATs, including the CREB binding protein (CBP)/p300. Curcumin, a yellow pigment found in turmeric, specifically inhibits CBP/p300 [23]. It has been reported that curcumin reduces histone acetylation with a consequent decrease of gene expression [24]. We thus examined whether curcumin decreased the level of H3ac in the proximal IL-6 promoter after TNF-α stimulation in RASFs. In curcumin-pretreated RASFs, the level of H3ac in the IL-6 proximal promoter was significantly decreased at 8 h after stimulation with 10 ng/ml TNF-α (Fig. 4A). Correspondingly, pretreatment of RASFs by curcumin significantly reduced IL-6 mRNA expression and protein secretion after TNF-α stimulation (Fig. 4B and C). On the other hand, in curcumin-pretreated RASFs, the level of H3ac in the GAPDH promoter and GAPDH mRNA did not change after TNF-α stimulation (Fig. 4A and B). These results suggest that

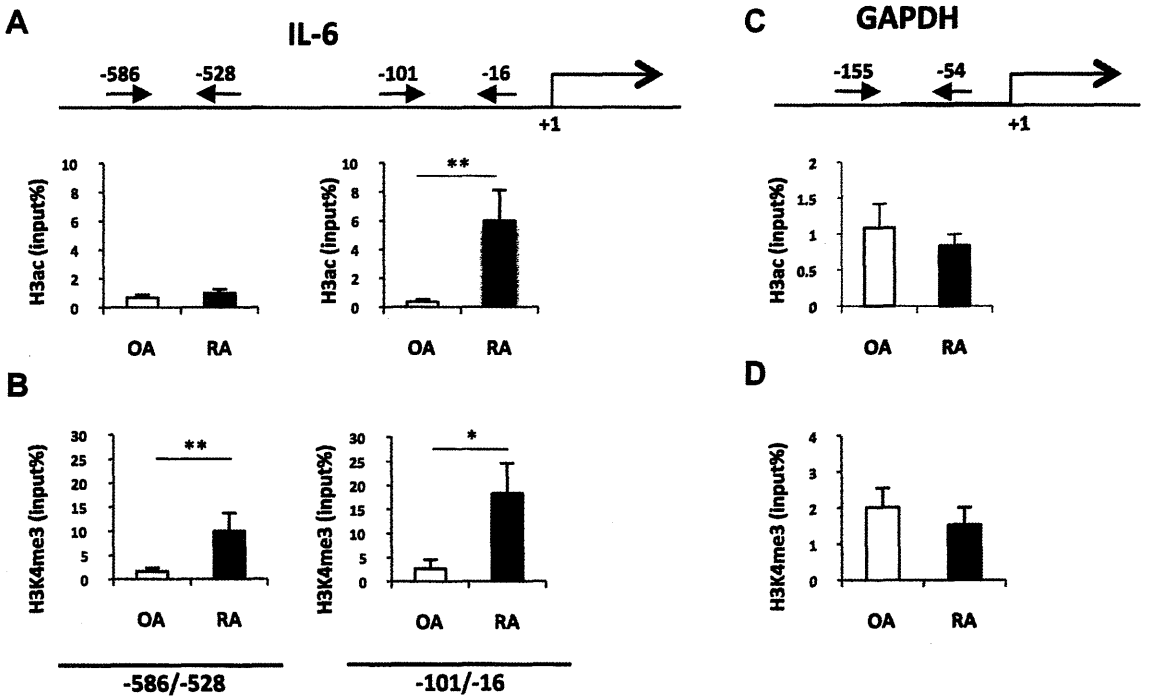

Fig. 2. The levels of H3ac and H3K4me3 increase in the IL-6 promoter in RASFs. (A and B) The levels of H3ac (A) and H3K4me3 (B) in the proximal (from −586 to −528) and distal (from −101 to −16) IL-6 promoter were analyzed by ChIP and quantitative PCR in RASFs (H3ac: $n = 11$; H3K4me3: $n = 7$) and OASFs (H3ac: $n = 11$; H3K4me3: $n = 6$). (C and D) The levels of H3ac (C) and H3K4me3 (D) in the GAPDH promoter were analyzed by ChIP and quantitative PCR in RASFs (H3ac: $n = 11$; H3K4me3: $n = 7$) and OASFs (H3ac: $n = 11$; H3K4me3: $n = 6$). The data are the means ± SEM. *$p < 0.05$, **$p < 0.01$ (Mann–Whitney U test).

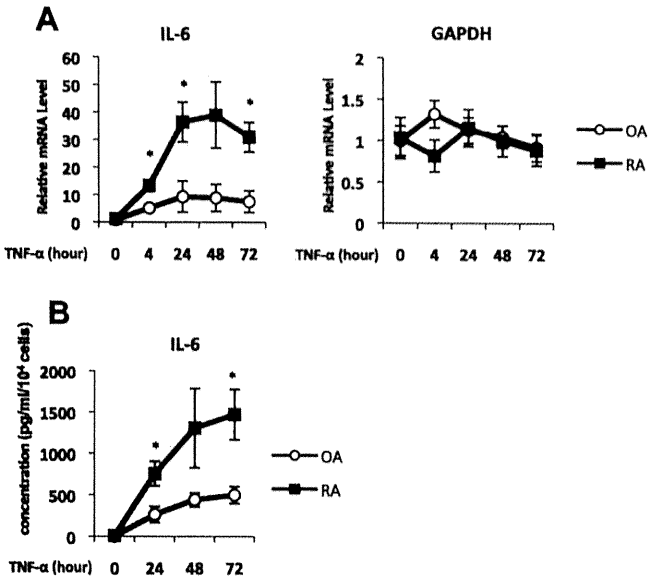

Fig. 3. TNF-α enhances IL-6 production in RASFs. (A) IL-6 and GAPDH mRNAs were examined at 0, 4, 24, 48, and 72 h after stimulation with 10 ng/ml TNF-α by quantitative RT-PCR in RASFs ($n = 8$) and OASFs ($n = 6$). The values are expressed as the fold increase versus the value in OASFs at 0 h. (B) The concentrations of the IL-6 protein in the supernatant of RASFs ($n = 7$) and OASFs ($n = 8$) were measured by ELISA at 0, 24, 48 and 72 h after stimulation with 10 ng/ml TNF-α. Values are expressed as the concentration (pg/ml) per 10^4 cells. The data are the means ± SEM. *$p < 0.05$ (Mann–Whitney U test).

high level of H3ac in the IL-6 promoter specifically increases IL-6 production in RASFs.

4. Discussion

This study shows that increased H3ac in the IL-6 promoter was associated with elevated IL-6 production in RASFs. The chromatin structure was in an open conformation in the IL-6 promoter and IL-6 gene transcription was highly responsive to TNF-α. Curcumin decreased the level of H3ac in the IL-6 promoter and suppressed IL-6 production in RASFs. These results suggest that hyperacetylation of histone H3 in the IL-6 promoter contributes to both constitutive and TNF-α-induced IL-6 production by RASFs. Taken together, epigenetic processes are evidently involved in the pathogenesis of RA and thus may serve as potential targets for the development of RA therapeutics.

Gene transcription depends on the nuclear recruitment of transcription factors and the accessibility of transcription factors to genomic DNA. The latter is determined by epigenetic mechanisms which generate conformational changes in chromatin. NF-κB and p38 MAPK have been shown to bind to the IL-6 promoter and regulate IL-6 gene transcription in RASFs [25,26]. The present study shows that active histone modifications such as H3ac and H3K4me3 are present in the IL-6 promoter of RASFs and that IL-6 mRNA expression and protein production are increased by stimulation with TNF-α. These results imply that decondensation of the chromatin structure results in the increased recruitment of transcription factors such as NF-κB and p38 MAPK to the IL-6 promoter, resulting in an increase in transcription of the IL-6 gene.

DNA methylation and RNA interference by miRNA are well known as epigenetic mechanisms other than histone modifications. Accumulating evidence has shown that DNA methyation and miRNA are involved in the pathogenesis of RA. Global genomic DNA hypomethylation and low DNA (cytosine-5-)-methyltransferase 1 (DNMT1) expression provide RASFs with an active and aggressive phenotype [27]. DNA hypermethylation in the DR3 promoter has

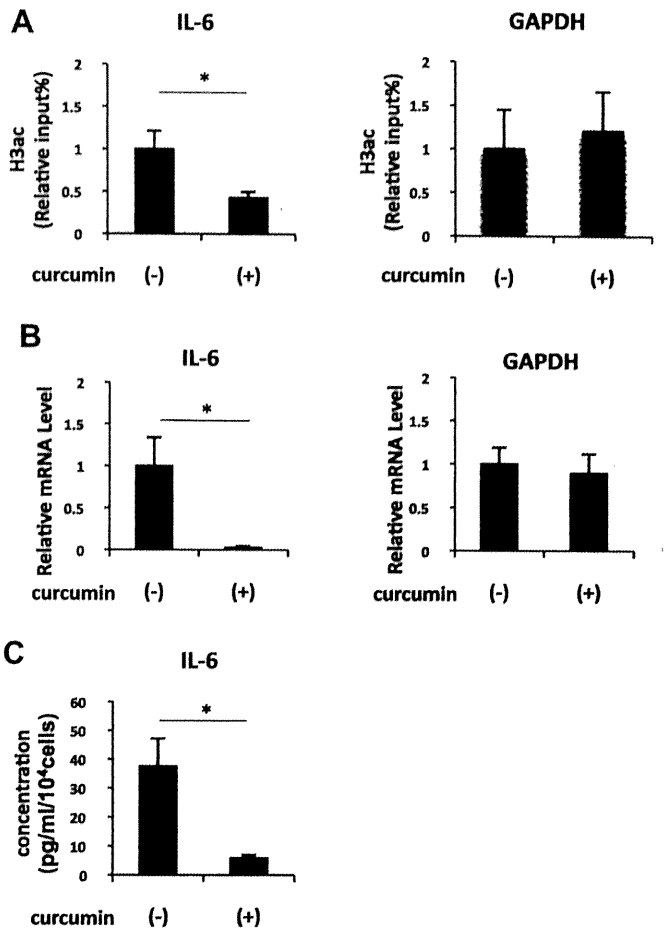

Fig. 4. Curcumin decreases the level of H3ac in the IL-6 promoter and IL-6 production in RASFs. (A and B) RASFs ($n = 5$) were pretreated with or without 20 μM curcumin, stimulated with 10 ng/ml TNF-α, and harvested at 8 h. The levels of H3ac in the IL-6 and GAPDH promoters (A) and IL-6 and GAPDH mRNA (B) were examined by quantitative PCR. Values were expressed as the fold increase versus the value in curcumin-untreated RASFs. (C) RASFs ($n = 8$) were pretreated with or without 20 μM curcumin and stimulated with 10 ng/ml TNF-α. The supernatants were replaced with DMEM containing 0.5% FBS at 8 h and collected at 24 h. The concentrations of IL-6 protein in the supernatant were measured by ELISA. Values are expressed as the concentration (pg/ml) per 10^4 cells. The data are the means ± SEM. *$p < 0.05$ (Wilcoxson's signed rank test).

been shown to result in resistance to apoptosis in RASFs [28]. Genome-wide analyses have identified differentially methylated genes that are suggested to contribute to RASF phenotypes [29,30] . In addition, miR-155 and miR-146a are also highly expressed in RASFs [31]. The same group showed that expression of miR-203 was higher in RASFs than in OASFs and that miR-203 increased MMP-1 and IL-6 production in RASFs [32]. Compared with DNA methylation and miRNA, the studies of histone modifications have been quite limited in RA. Histone methyltransferase enhancer of zeste homolog 2 (EZH2), which induces tri-methylation of histone 3 lysine 27 (H3K27me3), is overexpressed in RASFs and secreted fizzled-related protein 1 (SFRP1) has been identified as a target gene of EZH2 [33]. Here we report aberrant histone modifications in the IL-6 promoter in RASFs. This is the first report of increased active histone modification markers in the IL-6 promoter in RASFs.

The balance of activities between HATs and histone deacetylases (HDACs) was shown to be shifted toward histone hyperacetylation in RA synovial tissues [34]. Our data also showed hyperacetylation in the IL-6 promoter in RASFs. TNF-α-induced IL-6 gene expression is regulated by a transcriptional complex which consists of CBP/p300 [35]. Therefore we stimulated RASFs with TNF-α after treatment with curcumin which specifically

inhibited CBP/p300, resulting in the decrease in the level of H3ac in the IL-6 promoter, IL-6 mRNA expression and IL-6 protein secretion. In the past few years, the introduction of anti-rheumatic biologics blocking the effects of key inflammatory cytokines, such as TNF-α or IL-6, have dramatically improved the outcomes in RA [36]. However biologics do have several problems, including limited efficacy, a high risk of infection and enormous cost. Therefore, alternative treatments have been sought and epigenetic mechanisms are promising targets. Recently HAT inhibitors have received attention to as a novel epigenetic therapeutic for RA. Curcumin has been shown to exert an anti-inflammatory effect in both murine arthritis models [37,38] and RASFs [39]. A randomized pilot study suggested the efficacy and safety of curcumin in RA patients [40]. Curcumin is thus an attractive candidate for treating RA.

In conclusion, increased histone acetylation conferred elevated IL-6 production on RASFs. Furthermore, the decrease in H3ac reduced both IL-6 mRNA expression and IL-6 protein secretion in RASFs. The results suggest that epigenetic dysregulation is involved in the pathogenesis of RA. Epigenetic mechanisms are implicated to be a promising target for RA therapeutics.

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Combination of Tumor Necrosis Factor α and Interleukin-6 Induces Mouse Osteoclast-like Cells With Bone Resorption Activity Both In Vitro and In Vivo

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Objective. **To clarify the function of osteoclast-like multinuclear cells differentiated from bone marrow–derived macrophages (BMMs) by a combination of tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), and to investigate the molecular mechanisms underlying the differentiation.**

Methods. **BMMs were stimulated by TNFα and/or IL-6. The cells were then compared with conventional osteoclasts differentiated in vitro by RANKL. An in vitro pit formation assay on dentine slices and an in vivo resorption assay of calvarial bones were performed. We also evaluated the activities and expression levels of NF-κB, c-Fos, and NF-ATc1, which are essential to the differentiation of conventional osteoclasts. Small interfering RNA was used to knock down c-Fos. The effects of genetic ablation of STAT-3 and pharmacologic inhibitors of NF-AT, JAK, and ERK were also studied.**

Results. **Osteoclast-like cell differentiation depended on TNFα and IL-6 and was not inhibited by osteoprotegerin. These differentiated cells were associated with both in vitro and in vivo bone resorption activity. TNFα and IL-6 had a synergistic effect on the activity and expression of c-Fos. Knockdown of c-Fos inhibited the expression of NF-ATc1 and the differentiation of osteoclast-like cells. All of these inhibitors blocked differentiation of the cells in vitro, but surprisingly, the conditional knockout of STAT-3 did not. Tofacitinib also inhibited the bone destruction caused by TNFα and IL-6 in vivo.**

Conclusion. **Our results demonstrate that a combination of the inflammatory cytokines TNFα and IL-6 can induce osteoclast-like cells that have in vitro and in vivo bone-resorptive activity.**

Rheumatoid arthritis (RA) is a debilitating disease that causes chronic inflammation of the joints. The periarticular bone destruction resulting from this inflammation can seriously impair the quality of life of patients. Osteoclasts are multinucleated cells of monocyte/macrophage lineage and are believed to play a major role in arthritic bone destruction. The osteoclast differentiation factor (ODF) RANKL is a member of the tumor necrosis factor (TNF) family of cytokines. Although both RANKL and TNFα stimulate similar signaling pathways, the difficulty of inducing osteoclastogenesis by TNFα alone has been reported (1,2). Nevertheless, TNFα must play an important role in the bone destruction observed in RA, because TNF blockers have been demonstrated to prevent arthritic bone destruction, particularly when administered in combination with methotrexate (MTX), which is the gold standard treatment of RA (3).

Lam et al previously reported that TNFα can promote the differentiation of osteoclasts in the presence of a small amount (i.e., "a permissive level") of RANKL (1). The order in which osteoclast precursors encounter cytokines may also be important. For in-

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stance, Ochi and colleagues reported inhibition of osteoclastogenesis when TNFα was added to cultures of precursor cells before or at the same time as the addition of RANKL. In contrast, however, osteoclastogenesis was promoted when TNFα was added after the addition of RANKL (2). Another possible role that TNFα plays is to differentiate osteoclasts in an indirect manner, via the effect of the induction of RANKL on osteoclastogenesis-supporting cells such as osteoblasts and synoviocytes. It is notable, however, that the role of MTX in this context remains unclear.

In this study, working on the hypothesis that TNFα in combination with one or more factors would induce the differentiation of bone-resorbing cells, we observed that TNFα in combination with interleukin-6 (IL-6) induced osteoclast-like multinucleated cells. Next, we investigated whether these cells had bone-resorbing activity in vitro and in vivo. We also analyzed the molecular mechanisms of multinucleated cell differentiation in comparison with RANKL-mediated osteoclastogenesis.

MATERIALS AND METHODS

Mice. C57BL/6 mice (ages 6–10 weeks) were purchased from Charles River Japan. LysM-*cre*/*Stat3*$^{flox/flox}$ mice with a C57BL/6 background have been described previously (4). All mice were maintained under specific pathogen–free conditions, and all animal experiments were carried out with the approval of the Animal Study Committee of Saitama Medical University and conformed to relevant guidelines and laws.

In vitro assays for osteoclast differentiation and function. The assays used for in vitro osteoclast differentiation have been described in detail previously (5,6). Briefly, bone marrow cells (BMCs) were cultured in α-minimum essential medium (Gibco Invitrogen) supplemented with 10% fetal bovine serum, 50 units/ml penicillin/streptomycin (Gibco Invitrogen), and 10 ng/ml macrophage colony-stimulating factor (M-CSF; R&D Systems). Cells were cultured at concentrations of 2×10^5 cells/well in 24-well plates and 5×10^6 cells/dish in 6-cm dishes for 2 days.

Following the initial 2-day culture period, BMCs were then used as bone marrow–derived macrophages (BMMs), and culture continued using culture medium supplemented with RANKL (PeproTech), TNFα (PeproTech), and/or IL-6 (R&D Systems). The medium was replenished with fresh medium every other day until various assays were performed. Tartrate-resistant acid phosphatase (TRAP) was assayed with a TRAP Staining Kit (Primary Cell) according to the manufacturer's instructions. The NF-AT inhibitor tacrolimus (FK506; Sigma-Aldrich), osteoprotegerin (OPG; R&D Systems), an anti-mouse IL-1β antibody (eBioscience), the pan JAK inhibitor tofacitinib (CP-690550; Selleckchem), and the MEK inhibitors PD98059 and U0126 (Sigma-Aldrich) were added at the same time as RANKL or the proinflammatory cytokines. For pit formation assay, BMMs cultured on dentine slices (Wako) were cultured for 14 days in the presence of the cytokines. After removal of the cells, the resorption pits were examined by electron microscopy (Hitachi).

Analysis of cytokine-induced bone destruction in vivo. In vivo bone resorption assays have been described in detail previously (7,8). Briefly, phosphate buffered saline (PBS), IL-6, TNFα, or TNFα plus IL-6 (all at 0.375 μg/day) was injected into the supracalvariae of mice every day for 5 days (i.e., from day 0 to day 4). On day 5, the mice were killed, and decalcified paraffin sections of the calvarial bones were analyzed. Parameters such as the number of TRAP-positive cells per bone perimeter and the bone resorption area (eroded surface per bone surface) were determined. In one experiment, either tofacitinib (15 mg/kg body weight) or normal saline was administered intraperitoneally once daily beginning 2 days prior to the injection of PBS or TNFα plus IL-6 (i.e., from day −2 to day 4).

Immunofluorescence staining and Western blot analysis. Alexa Fluor 546–labeled phalloidin (Invitrogen), a mouse anti–NF-ATc1 monoclonal antibody (7A6; Santa Cruz Biotechnology), and an Alexa Fluor 488–labeled anti-mouse IgG antibody (Invitrogen) were used for immunofluorescence staining. Cells were observed by fluorescence microscopy (Olympus). For Western blot analysis, a rabbit anti–c-Fos monoclonal antibody, a rabbit anti–pSTAT-3 (Tyr^{705}) monoclonal antibody (both from Cell Signaling), a mouse anti–NF-ATc1 monoclonal antibody (7A6), and a mouse anti–β-actin antibody (Sigma-Aldrich) were used. The protein level was determined using a Bio-Rad calibrated densitometer.

Assay for the activity of transcription factors. For the assessment of NF-κB p65/p52 activities, the BMMs were stimulated with the proinflammatory cytokines for 12 hours, restimulated for another 30 minutes, and then harvested. To determine c-Fos activity, the cells were stimulated for 24 hours and restimulated for 30 minutes. To assess NF-ATc1 activity, the cells were stimulated for 48 hours and restimulated for 24 hours. A Nuclear Extract Kit and TransAM Transcription Factor Assay Kits (Active Motif) were used according to the manufacturer's instructions.

RNA interference. BMMs were transfected using a Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells were incubated with 100 n*M* scrambled small interfering RNA (siRNA; control) or c-Fos siRNA (Ambion), using 500 μl of a transfection solution. After 6 hours of transfection, the medium was changed, and the cells were stimulated with cytokines.

Statistical analysis. Values are presented as the mean ± SEM. The Mann-Whitney U test was used for comparisons between 2 groups. *P* values less than 0.05 were considered significant.

RESULTS

Differentiation of TRAP-positive multinucleated cells by the combination of TNFα and IL-6. Osteoclasts can be differentiated in vitro by culturing mouse BMCs with M-CSF and subsequently adding RANKL and

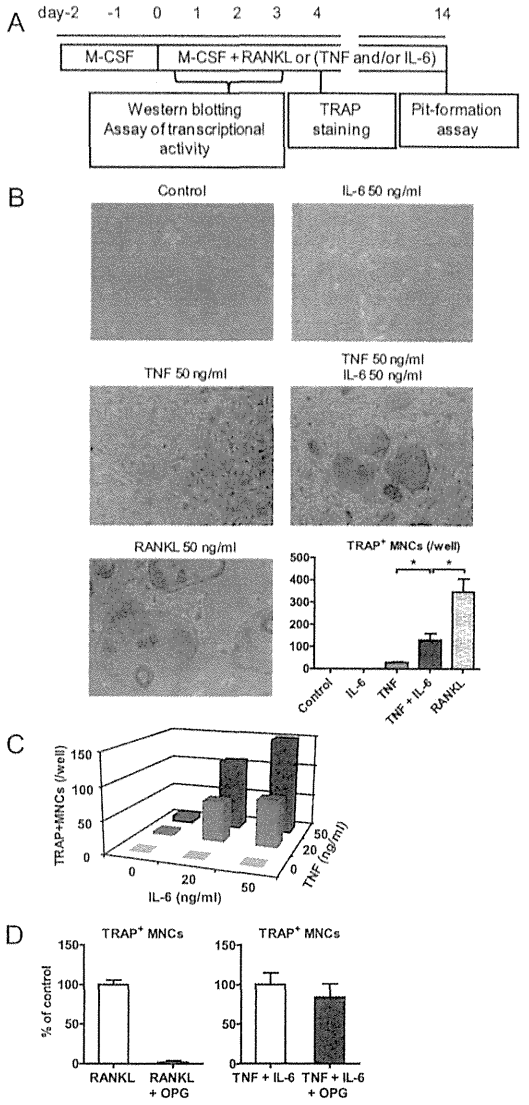

Figure 1. Tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) induce differentiation of osteoclast-like cells in a RANKL-independent manner. **A,** Schematic representation of the culture system used in the in vitro experiments. **B,** Photomicrographs and quantification of tartrate-resistant acid phosphatase (TRAP)–positive multinucleated (≥5) cells (MNCs) (n = 6 wells/experiment). Original magnification × 100. **C,** Dose-dependent response of TRAP-positive MNCs induced by TNFα and IL-6. **D,** Effect of osteoprotegerin (OPG; 1 μg/ml) on RANKL-induced osteoclastogenesis and TRAP-positive MNC differentiation induced by TNFα and IL-6 (n = 3 wells/experiment). Values in **B** (bottom right) and **D** are the mean ± SEM. * = $P < 0.05$. M-CSF = macrophage colony-stimulating factor.

M-CSF (Figures 1A and B). TRAP expression is a characteristic of osteoclasts (9). Indeed, TRAP staining revealed that these cells were positive for TRAP. When TNFα instead of RANKL was used in this culture system, multinucleated cells were barely detected, although TRAP-positive cells were abundantly present, indicating that TNFα cannot replace RANKL in the differentiation of osteoclasts (Figure 1B).

Another proinflammatory cytokine, IL-6, is also implicated in the pathogenesis of RA (10). IL-6 has been shown to trigger osteoclast formation (11) and induce bone resorption (12). However, because RANKL has been identified as the ODF, the role of IL-6 is considered to be indirect via the induction of RANKL on osteoblasts/stromal cells (13). As expected, IL-6 alone induced only a scarce number of TRAP-positive cells and almost no multinucleated cells. Interestingly, however, the combination of TNFα and IL-6 induced the formation of TRAP-positive multinucleated cells (Figure 1B). The number of TRAP-positive multinucleated cells increased with increasing concentrations of either cytokine, revealing the dose-dependent nature of the response (Figure 1C).

We next added OPG, a decoy receptor for RANKL (14), to the culture system in order to determine whether RANKL induced by IL-6 and/or by TNFα on stromal cells (which might be contained in BMCs) was involved in the differentiation of the TRAP-positive multinucleated cells. As shown in Figure 1D, OPG did not inhibit the differentiation of the TRAP-positive multinucleated cells, whereas OPG did inhibit osteoclastogenesis induced by RANKL. The combination of TNFα and IL-1β has been reported to induce osteoclastogenesis (15). In our system, we demonstrated that IL-1β was not responsible for cell fusion, because the addition of an anti–IL-1β antibody did not affect the differentiation of TRAP-positive multinucleated cells (additional information is available at http://www.saitama-med.ac.jp/uinfo/riumachi/supplementary_figure.html).

In vitro and in vivo bone-resorbing activity of TRAP-positive multinucleated cells induced by TNFα and IL-6. The pit formation assay revealed that TRAP-positive multinucleated cells had the capacity to carry out bone matrix resorption in vitro (Figure 2A). Moreover, the formation of actin rings is critically important for bone resorption (16), and indeed, these multinucleated cells formed actin rings similar to those observed in conventional osteoclasts induced by RANKL (additional information is available at http://www.saitama-med.ac.jp/uinfo/riumachi/supplementary_figure.html).

The in vivo effects of the cytokines were assessed by application into the subcutaneous tissue overlying the calvariae of mice. Administration of TNFα alone induced some TRAP-positive cells and slight bone de-

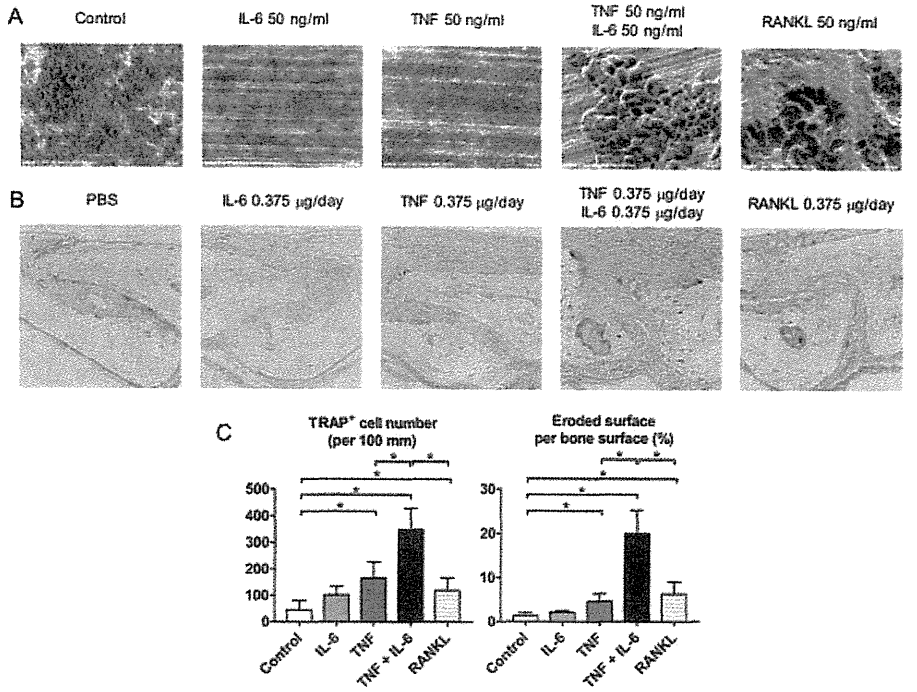

Figure 2. TNFα and IL-6 induce bone-resorbing activity of TRAP-positive multinucleated cells, in vitro and in vivo. **A,** Electron microscopic images of resorption pits on dentine slices. Original magnification × 500. **B,** Histologic findings in the calvarial bones excised from mice after daily supracalvarial administration of phosphate buffered saline (PBS), IL-6, TNFα, TNFα plus IL-6, and RANKL for 5 days. Original magnification × 200. **C,** Histomorphometric analysis of calvariae, showing the number of TRAP-positive cells per bone perimeter and eroded surface per bone surface. Values are the mean ± SEM. $* = P < 0.05$. See Figure 1 for other definitions.

struction, but when IL-6 was also administered, the degree of bone destruction was substantially increased (Figure 2B). The administration of IL-6 alone did not induce bone destruction or TRAP positivity. Interestingly, compared with administration of TNFα plus IL-6, administration of RANKL resulted in fewer numbers of TRAP-positive cells and a smaller bone resorption area (eroded surface/bone surface) (Figure 2C). It was therefore clear that this cytokine combination induced TRAP-positive cells with both in vitro and in vivo bone-resorption activity.

Analysis of activated intracellular signaling molecules in osteoclast-like cells. We next attempted to elucidate the mechanisms underlying differentiation of osteoclast-like cells induced by TNFα and IL-6. The master regulatory transcription factor for osteoclast differentiation is believed to be NF-ATc1 (17). During the course of the differentiation of osteoclast-like cells, the NF-ATc1 protein level increased, and NF-ATc1 was translocated into the nucleus (Figures 3A and B). Accordingly, the transcriptional activity of NF-ATc1, as quantified by an enzyme-linked immunosorbent assay–based method, was elevated (additional information is available at http://www.saitama-med.ac.jp/uinfo/riumachi/supplementary_figure.html). Translocation of NF-ATc1 into the nucleus depends on its dephosphorylation by calcineurin. As expected, the calcineurin inhibitor tacrolimus (FK506) inhibited the differentiation of osteoclast-like cells (Figure 3C). Thus, we demonstrated that NF-AT activity is necessary for the differentiation of osteoclast-like cells, although the elevation in the NF-ATc1 level was not as marked as that observed during the differentiation of conventional osteoclasts (17).

The transcriptional activities of NF-κB and activator protein 1 (AP-1) are also considered to be important for the induction of NF-ATc1 during osteoclast differentiation. Adaptor proteins known as TNF receptor (TNFR)–associated factors (TRAFs) are activated downstream of RANK or TNFRs. TRAFs in turn activate NF-κB and MAPKs, and MAPKs then activate AP-1. Mice doubly deficient in NF-κB1 and NF-κB2 were rendered osteopetrotic due to the lack of osteoclasts (18,19). Mice deficient in c-Fos, which is a critical component of AP-1, had a similarly osteopetrotic phenotype (20,21).

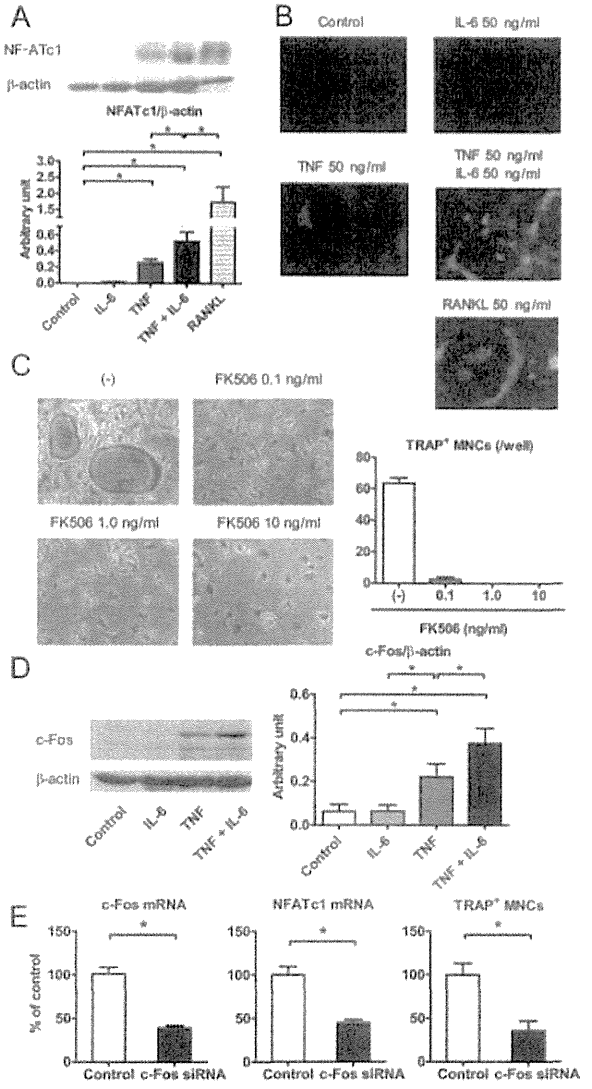

Figure 3. Mechanisms involved in TNFα- and IL-6–induced osteoclast-like cell differentiation. **A,** Top, Western blot analysis of NF-ATc1 after 72-hour stimulation of osteoclast precursors with IL-6, TNFα, TNFα plus IL-6, and RANKL. Bottom, Quantification of the data. **B,** Immunofluorescence staining for NF-ATc1 after 96-hour stimulation of osteoclast precursors. The expression level of NF-ATc1 increased in the cells stimulated by TNFα plus IL-6, and NF-ATc1 accumulated in the nuclei. Original magnification × 200. **C,** Effect of the calcineurin inhibitor tacrolimus (FK506) on the differentiation of TRAP-positive multinucleated cells. Original magnification × 200. **D,** Left, Western blot analysis showing the level of c-Fos induced by stimulation with IL-6, TNFα, and IL-6 plus TNFα for 24 hours and restimulation with the cytokines for 30 minutes. Right, Quantification of the data. **E,** Effect of *Fos* knockdown on *Nfatc1* expression and differentiation of TRAP-positive multinucleated cells. Values are the mean ± SEM (n = 6 samples/experiment). * = $P < 0.05$. See Figure 1 for definitions.

First, we measured the activation levels of both the canonical and noncanonical NF-κB pathways and observed little difference between stimulation with TNFα alone and stimulation with TNFα plus IL-6 (additional information is available at http://www.saitama-med.ac.jp/uinfo/riumachi/supplementary_figure.html). Next, we investigated the expression and activity of c-Fos at the protein level. Previous studies (22) have detected c-Fos as several distinct bands, suggesting extensive posttranslational modification. In our study, we observed that administration of TNFα plus IL-6 induced a higher level of c-Fos than the level induced by TNFα alone, and that the slower migrating band was clearly dominant (Figure 3D). The activity of c-Fos also showed a similar pattern (additional information is available at http://www.saitama-med.ac.jp/uinfo/riumachi/supplementary_figure.html). We attempted to knock down *Fos* by introducing siRNA into BMCs and observed that siRNA against *Fos* significantly down-regulated the expression level of not only *Fos* but also *Nfatc1* relative to the scrambled siRNA control (Figure 3E). Consistent with this result, the differentiation of TRAP-positive multinucleated cells by TNFα plus IL-6 was also significantly inhibited, demonstrating the importance of c-Fos in the differentiation of these cells.

Dependence of osteoclast-like cells, but not conventional osteoclasts, on JAK. Intracellular signaling by IL-6 is largely transmitted via the JAK/STAT pathway, in which STAT-3 plays an important role (10). Thus, we sought to determine whether the addition of the pan JAK inhibitor tofacitinib (23) would inhibit the differentiation of osteoclast-like cells. Tofacitinib was recently shown to be effective in the treatment of RA (24). In fact, we demonstrated that the in vitro addition of 10–100 n*M* tofacitinib inhibited the multinucleation of precursor cells (Figure 4A). In this instance, TRAP positivity was not affected. Consistent with a previous report (13), the same concentrations of tofacitinib did not inhibit RANKL-induced osteoclastogenesis (data not shown).

Our next step was to use LysM-*cre*/*Stat3*$^{flox/flox}$ mice in which *Stat3* is knocked out in macrophages (4). Consistent with the very high deletion efficiency (~90%) achieved in the mature macrophages of the LysM-*cre* mice (25) and the dominant-negative effect of the truncated STAT-3 protein (26), phosphorylation of STAT-3 (Tyr^{705}) after IL-6 stimulation was substantially reduced in the BMMs derived from the LysM-*cre*/*Stat3*$^{flox/flox}$ mice compared with those derived from the control *Stat3*$^{flox/flox}$ mice (additional information is available at http://www.saitama-med.ac.jp/uinfo/riumachi/supplementary_figure.html). As expected, BMMs from the conditional knockout mice and those from control

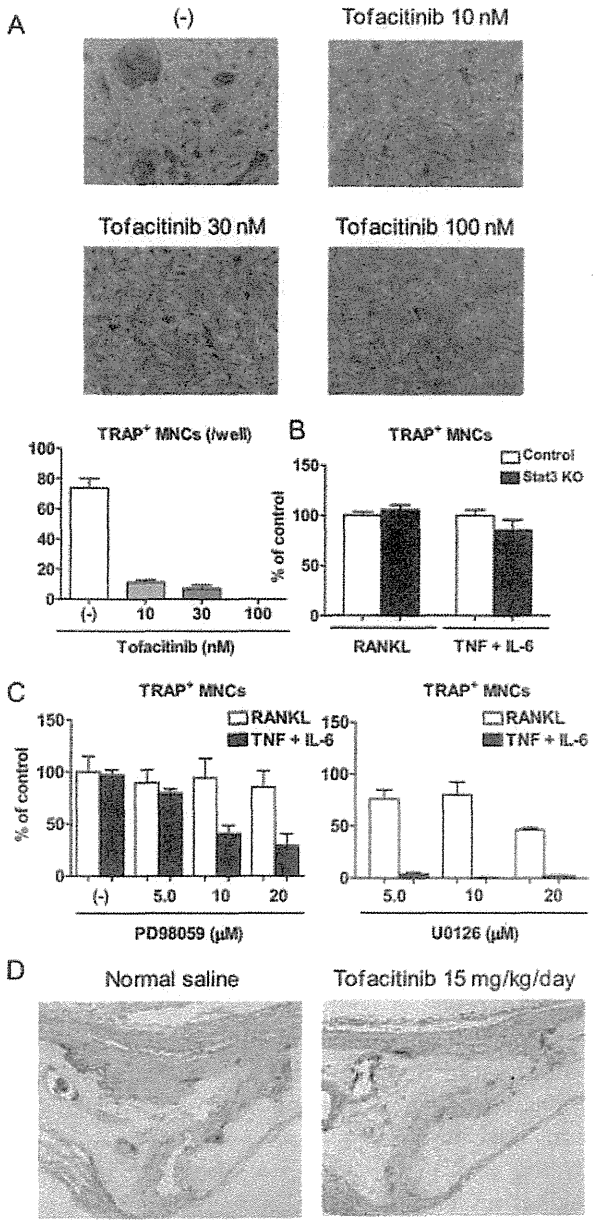

Figure 4. TRAP-positive multinucleated cell differentiation depends on JAK and is inhibited by tofacitinib both in vitro and in vivo. **A,** Effect of tofacitinib on the differentiation of TRAP-positive multinucleated cells in vitro (n = 3 wells/experiment). Original magnification × 100. **B,** Differentiation levels of TRAP-positive multinucleated cells. Bone marrow–derived macrophages from LysM-*cre*/*Stat3*$^{flox/flox}$ mice (STAT-3–knockout) and control *Stat3*$^{flox/flox}$ mice (n = 4 independent experiments) were stimulated in vitro with either RANKL or TNFα plus IL-6. **C,** Effect of the specific MEK inhibitors PD98059 and U0126 on in vitro differentiation of TRAP-positive multinucleated cells (n = 4 wells/experiment). **D,** Effect of systemically administered tofacitinib on calvarial bone destruction induced by TNFα and IL-6. Values are the mean ± SEM and are representative of 3 independent experiments. Original magnification × 200. See Figure 1 for definitions.

mice were differentiated into conventional osteoclasts by RANKL at similar levels. Unexpectedly, we observed little difference in the ability of the 2 types of BMMs to differentiate into TRAP-positive multinucleated cells following stimulation with TNFα plus IL-6 (Figure 4B), indicating that their differentiation is not dependent on STAT-3.

This result focused our attention on another important IL-6 signaling pathway. Activation of the MAPK pathway has also been reported to depend on JAK (27). In particular, ERKs, one subgroup of MAPKs, have been implicated in the activation and stabilization of c-Fos in IL-6–mediated signaling (28). We applied the MEK-specific inhibitors PD98059 and U0126 at concentrations that did not significantly affect the number of TRAP-positive multinucleated cells induced by RANKL. Overall, we observed that these inhibitors, especially U0126, significantly blocked differentiation of TRAP-positive multinucleated cells by TNFα plus IL-6 (Figure 4C). Finally, we questioned whether tofacitinib was also effective in the treatment of the bone destruction induced by TNFα plus IL-6 in vivo. This was indeed the case. Systemic administration of tofacitinib significantly ameliorated bone erosion at the outer periosteal surface (mean ± SEM 17.0 ± 1.1% in mice treated with tofacitinib versus 28.6 ± 6.6% in untreated mice), whereas it did not decrease the number of TRAP-positive cells (mean ± SEM 518 ± 55 and 266 ± 179, respectively) (n = 3 independent experiments) (Figure 4D and results not shown).

DISCUSSION

In this study, we demonstrated that the combination of TNFα and IL-6 differentiates TRAP-positive multinucleated cells, which resemble osteoclasts. Indeed, these osteoclast-like cells have the capacity to absorb bone matrix in vitro, and the same combination of proinflammatory cytokines induced in vivo erosion of the calvarial bones. This finding is surprising, because Duplomb et al reported that IL-6 inhibited osteoclast differentiation in vitro (29). Although proinflammatory cytokines are believed to induce RANKL on stromal cells, the fact that the addition of OPG did not inhibit the differentiation of osteoclast-like cells indicates that TNFα and IL-6 do not differentiate the cells via the induction of RANKL. We cannot rule out the possibility, however, that a trace amount of RANKL that escaped blocking by OPG was necessary for their differentiation. The apparent discrepancy between our results and those of the previous study (29) may arise because