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G．知的所有権の取得状況

1. **特許取得**
   特記すべきことなし
2. **実用新案登録**
   特記すべきことなし
3. **その他**
   特記すべきことなし

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（免疫アレルギー疾患等実用化等研究事業　免疫アレルギー疾患実用化研究分野））
分担研究報告書

研究課題：危険因子を同定する検診制度導入によるリウマチ制圧プロジェクト（三重地区）

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研究協力者　須藤 啓広　三重大学医学部附属病院　整形外科　教授

研究要旨：抗体スクリーニング検査による関節リウマチ(RA)患者の早期発見の有無、および抗体陽性者の予後向上の有無の検討が本研究の目的である。三重県内の自治体および当科における健康診断にて、抗CCP抗体および/またはリウマトイド因子検査を行い、陽性者には専門機関への受診を勧める。定期的フォローアップを原則として行い、その後のRA発症の有無を追跡し、早期診断・早期治療に務め、抗体スクリーニング検査の有用性および抗体陽性者の予後向上の有無について検討する。

A．研究目的

抗体スクリーニング検査による関節リウマチ(RA)患者の早期発見の有無、および抗体陽性者の予後向上の有無の検討。

B．研究方法

志摩市20歳の健診受診で希望者に抗CCP抗体およびリウマトイド因子(RF)を追加で測定した。

（倫理面への配慮）

インフォームドコンセントを徹底し、対象者・対象機関が同定されないようにする必要がある場合は、匿名化により対応した。調査にあたり、「臨床研究に関する倫理指針」を遵守した。

C．研究結果

20歳の健診受診者は193人（男性62名、女性131名、平均年齢25.2歳)、RF陽性者は7人でいずれも女性であった。抗CCP抗体陽性者はいなかった。

D．考察

今年度スクリーニングによる抗CCP抗体陽性者は0%であり、RF陽性者は7人で3.6%であった。本研究は「危険因子を同定する検診制度導入によるリウマチ制圧プロジェクト」の分担研究であり、三重地区のデータとして収集される。

E．結論

抗体スクリーニング検査により抗CCP抗体陽性者は0%、RF陽性者は3.6%であった。

F．研究発表

1. 論文発表
   なし
2. 学会発表
   なし

G．知的財産権の出願・登録状況

該当なし

Ⅲ．　研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

雑誌

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ORIGINAL ARTICLE

Retreatment efficacy and safety of tocilizumab in patients with rheumatoid arthritis in recurrence (RESTORE) study

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Abstract

Objectives To evaluate the safety and efficacy of retreatment with tocilizumab (TCZ) in patients who had participated in the DREAM study (*Drug free* remission/low disease activity after cessation of tocilizumab [Actemar] monotherapy study) and had experienced loss of efficacy.

Methods Patients were retreated with TCZ or other disease modifying antirheumatic drugs (DMARDs). Disease activity was measured using the 28-joint disease activity score (DAS28) for 12 weeks.

Results A total of 164 eligible patients, including 161 who experienced loss of efficacy within 52 weeks of the DREAM study, resumed treatment: 157 with TCZ and 7 with DMARDs and/or infliximab. Of TCZ-treated patients, 88.5 % (139 patients) achieved DAS28 <2.6 within 12 weeks, whereas among patients treated with DMARDs and/or infliximab only 14.3 % (1 patient) achieved DAS28 <2.6. Adverse events were observed in 70 TCZ-treated patients (44.0 %), but no serious infusion reactions were observed.

Conclusions Retreatment with TCZ was well-tolerated and effective in patients who had responded to the preceding TCZ monotherapy but had experienced loss of efficacy after cessation of TCZ.

Keywords Interleukin 6 · Retreatment · *Drug free* · Rheumatoid arthritis · Tocilizumab

For the MRA study group for RA.

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Introduction

Tocilizumab (TCZ) treatment frequently achieves remission in patients with rheumatoid arthritis (RA) as measured by the 28-joint disease activity score (DAS28) [1–12]. We have demonstrated in the DREAM study (*Drug free* remission/low disease activity (LDA) after cessation of TCZ [Actemra] monotherapy study) [13] that in some cases the efficacy of TCZ is sustained for more than 1 year after cessation of TCZ and without the use of other disease modifying antirheumatic drugs (DMARDs). However, the majority of patients experienced loss of efficacy, and needed to restart treatment for RA. In this study we evaluate the safety and efficacy of TCZ retreatment at recurrence of disease activity after cessation of TCZ.

Methods

Patients

All patients who participated in the DREAM study and had experienced loss of efficacy were enrolled. Criteria for loss of efficacy in the DREAM study was defined as DAS28-erythrocyte sedimentation rate (ESR) >3.2 at 2 consecutive observations, initiation of additional RA treatments including increase in oral corticosteroid dose, the patient's request for retreatment, or the treating physician judging that retreatment was necessary.

Study protocol

The study protocol was approved by the Ministry of Health, Labour and Welfare of Japan and by the local ethical committees. This study is registered with http://clinicaltrials.gov (NCT00661284). Patients were treated with biologic DMARDs including TCZ and infliximab (IFX), and/or conventional synthetic DMARDs including methotrexate (MTX). If the patient received TCZ retreatment, TCZ was administered intravenously (8 mg/kg) every 4 weeks. Other biologic DMARDs and/or synthetic DMARDs were administered based on the dosage and regimen in the package insert. The concomitant use of corticosteroids and non-steroidal anti-inflammatory drugs was allowed during the study period.

Anti-tocilizumab antibodies

Serum anti-TCZ antibody levels were determined by ELISA. Serum was added to the wells coated with 100 μl of Fab fragment of TCZ (0.2 μg/ml) and incubated for 2 h. After washing, biotin-conjugated TCZ was added and developed with alkaline phosphatase conjugated to streptavidin.

IgE-type anti-TCZ antibodies were also measured by ELISA. In this case, whole TCZ was used because an antigen coated each cup, and enzyme-linked anti-IgE antibodies were used as second antibodies.

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Statistical analysis

Clinical response was measured by DAS28-ESR. Remission was defined, in accordance with the European League Against Rheumatism (EULAR) definition, as DAS28 <2.6 [14]. The rates of remission under the new EULAR/American College of Rheumatology (ACR) remission criteria (Boolean definition) were also considered [15]. Adverse events (AEs) and serious adverse events (SAEs) were tabulated after converting the verbatim event names to MedDRA Ver. 8.0 System Organ Class (SOC) terms.

The factors contributing to the resumption of DAS28-ESR remission after retreatment were estimated from univariate and multivariate logistic regression analyses using the following patient baseline data for this study: DAS28-ESR, tender joint count (TJC), swollen joint count (SJC), patient's global assessment (Pt-GA), modified health assessment questionnaire (MHAQ) score, serum C-reactive protein (CRP) concentration, erythrocyte sedimentation rate (ESR), serum IL-6 concentration, serum matrix metalloproteinase (MMP)-3 concentration, and the duration of TCZ cessation. In the multivariate logistic analysis, stepwise selection with a level of significance of 0.05 was used for entry or removal of variables. Logistic regression analysis was also conducted to analyse the relationship between the TCZ treatment interval and development of AEs during this study.

Results

Characteristics of patients

In total, 166 patients were enrolled and resumed treatments. Of the patients who received TCZ retreatment, 2 were ineligible and were excluded from the analysis of efficacy. The 164 remaining patients eligible for analysis of efficacy included 161 patients who had experienced loss of efficacy by week 52 of the DREAM study, and 3 patients who had experienced loss of efficacy after completion of the DREAM study (an interval of >1 year).

In the 164 eligible patients, 73 patients (44.5 %) resumed treatment due to DAS28-ESR >3.2 at 2 consecutive visits, 66 patients (40.2 %) to investigator's judgement, 11 patients (6.7 %) to patients' request, and 14 patients (8.5 %) to addition of RA treatments including increase in oral corticosteroid dose. The major reason investigators judged retreatment was necessary was a DAS28-ESR >3.2 score at one visit in 55/66 patients (83.3 %). Four out of eleven patients who requested treatment were also DAS28-ESR >3.2. Therefore, 146/164 patients were DAS28-ESR >3.2 at the baseline of the RESTORE study (the mean DAS28-ESR[95 %CI] was 4.6 [4.5–4.8]).

A total of 159 patients received at least 1 infusion of TCZ (including 2 ineligible patients), and 7 patients received other DMARDs, including MTX, tacrolimus, and/or IFX. In the TCZ-treated patients, 133 patients received TCZ monotherapy and 26 received TCZ therapy in combination with synthetic DMARDs (25 patients with MTX; 1 patient with salazosulfapyridine). The median treatment interval between the last TCZ infusion and restarting the TCZ treatment in this study was 13.1 weeks (min–max, 6.14–60.4 weeks). Corticosteroids were used concomitantly in 57 of the patients treated with TCZ and in 4 of the patients treated with other DMARDs. The median corticosteroid dose in TCZ-treated patients at baseline of this RESTORE study was 3.0 mg/day, which was comparable with the median dose in patients treated with other DMARDs (2.3 mg/day). Other baseline characteristics of the patients who received TCZ were comparable with those of patients treated with other DMARDs (Table 1).

Efficacy of TCZ retreatment

The mean (±SD) DAS28-ESR before initial treatment using TCZ in the previous clinical studies (i.e. Japanese phase I/II open-label dose escalation study, a phase II double-blind dose finding study, a phase III open-label randomized study (SAMURAI), a phase III double-blind study (SATORI), a drug–drug interaction study, and a renal failure study) was 6.2 (±1.0) and improved with 12 weeks of TCZ treatment to 2.8 (±1.2). The mean (±SD) DAS28-ESR at the last observation point of the previous TCZ treatment studies (i.e., baseline of the DREAM study) was 1.5 (±0.7) (Fig. 1a).

In this study, the mean (±SD) DAS28-ESR in patients who restarted TCZ treatment decreased from 4.4 (±1.1) (95 % CI: 4.2–4.6) before restarting treatment to 1.8 (±0.8) (95 % CI: 1.6–1.9) after 12 weeks of treatment. In contrast, the mean (±SD) DAS28-ESR in patients treated with DMARDs and/or IFX was 4.2 (±1.1) (95 % CI: 3.2–5.2) before restarting treatment and 3.3 (±1.0) (95 % CI: 2.5–4.2) after 12 weeks of treatment (Fig. 1a).

Of the TCZ-retreated patients, 95.5 % (150/157 patients, 95 % CI: 91.0–98.2 %) achieved DAS28-ESR ≤3.2 and 88.5 % (139/157 patients, 95 % CI: 82.5–93.1 %) achieved DAS28-ESR <2.6 within 12 weeks as compared to only 28.6 % of the other DMARD-treated patients (2/7 patients, 95 % CI 3.7–71.0 %) achieving DAS28-ESR ≤3.2 and 14.3 % (1/7 patients, 95 % CI: 0.4–57.9 %) achieving DAS28-ESR <2.6.

The percentage of TCZ-retreated patients who reached DAS28-ESR <2.6 within 12 weeks in the TCZ monotherapy group (87.9 %, 116/132 patients, 95 % CI: 81.1–92.9 %) was comparable to the percentage in the TCZ plus synthetic DMARDs therapy group (92.0 %, 23/25 patients, 95 % CI: 74.0–99.0 %).

Table 1 Demographic and clinical characteristics of patients at baseline of RESTORE study

No. of patients	Total	Patients treated with TCZ	Patients treated with other DMARDs
	166	159[a]	7
Age, years (median [range])	57 (26–78)	56 (26–78)	65 (42–74)
Gender, female (%)	149 (89.8)	144 (90.6)	5 (71.4)
Disease duration, years (median [range])	7.8 (3.7–24.0)	7.7 (3.7–24.0)	8.6 (6.9–18.9)
No. (%) of patients using concomitant corticosteroids	61 (36.7)	57 (35.8)	4 (57.1)
Dose, mg/day (prednisolone equivalent) (median [range])	3.0 (0.5–10.0)	3.0 (0.5–10.0)	2.3 (2.0–7.0)
DAS28-ESR (median [range])	4.3 (0.8–7.8)	4.4 (0.8–7.8)	4.1 (2.9–5.9)
(Mean ± SD)	4.4 ± 1.1	4.4 ± 1.1	4.2 ± 1.1
Tender joint count (28-joint count) (median [range])	3.0 (0–27)	3.0 (0–27)	3.0 (1–5)
(Mean ± SD)	4.3 ± 4.3	4.4 ± 4.4	2.6 ± 1.4
Swollen joint count (28-joint count) (median [range])	2.0 (0–16)	2.0 (0–16)	2.0 (0–7)
(Mean ± SD)	3.3 ± 3.1	3.3 ± 3.2	2.4 ± 2.2
CRP, mg/dl (median [range])	0.8 (0.0–13.5)	0.9 (0.0–13.5)	0.8 (0.1–4.7)
(Mean ± SD)	1.6 ± 2.1	1.6 ± 2.1	1.2 ± 1.6
ESR, mm/h (median [range])	36 (2–115)	37 (2–115)	32 (16–113)
(Mean ± SD)	41 ± 24	40 ± 23	49 ± 39
MHAQ score (median [range])	0.3 (0.0–2.1)	0.4 (0.0–2.1)	0.0 (0.0–0.8)
(Mean ± SD)	0.5 ± 0.5	0.5 ± 0.5	0.2 ± 0.3
MMP-3, ng/ml (median [range])	95 (34–800)	96 (34–800)	77 (44–319)
(Mean ± SD)	167 ± 167	169 ± 169	129 ± 112

DAS28 28-joint disease activity score, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *MHAQ* modified health assessment questionnaire, *MMP-3* matrix metalloproteinase-3, *TCZ* tocilizumab, *DMARDs* disease modifying antirheumatic drugs

[a] Two ineligible patients who did not meet the eligible criteria of DREAM study were included

The mean (±SD) tender joint count (TJC) in 28 joints in TCZ-retreated patients improved from 4.4 (±4.4) before restarting treatment to 0.8 (±1.6) after 12 weeks. The mean (±SD) swollen joint count (SJC) in 28 joints also improved from 3.3 (±3.2) to 0.8 (±1.6) (Fig. 1b). Moreover, 63.1 % of patients (99/157) had no tender and/or swollen joints after 12 weeks retreatment with TCZ (Fig. 1c). Under the Boolean remission criteria, the remission rate by TCZ treatment was 43.9 % (69/157 patients, 95 % CI: 36.0–52.1 %) at week 12 (Fig. 1d). The mean (±SD) MMP-3 values in TCZ-retreated patients improved from 166.5 (±164.5) ng/ml at baseline in this study, i.e. prior to TCZ retreatment, to 77.4 (±64.8) ng/ml at week 12. Univariate logistic regression analysis showed the following variables to be associated with the resumption of DAS28-ESR remission: lower DAS28-ESR, lower TJC, lower SJC and lower MHAQ at baseline. On the other hand, duration of TCZ cessation in the DREAM study was not associated with resumption of DAS28-ESR remission (Fig. 2). Multivariate logistic regression analysis showed that lower DAS28-ESR at baseline was the contribution factor for resumption efficacy.

At baseline, 17 patients had DAS28-ESR $\leq$3.2. Thus, we further analysed efficacy in the 140 patients who had DAS28-ESR >3.2 at the baseline (the mean DAS28-ESR [95 % CI] was 4.6 [4.5–4.8]) and restarted TCZ in this study. Out of these patients, 87.1 % (122/140 patients, 95 % CI: 80.4–92.2 %) achieved DAS28-ESR <2.6 and 42.9 % (60/140 patients, 95 % CI: 34.5–51.5 %) achieved Boolean remission within 12 weeks. In addition, univariate and multivariate logistic regression analysis also identified lower DAS28-ESR value at baseline to be the factor contributing the resumption of DAS28-ESR remission by 12 weeks of TCZ treatment in these patients. These results are not significantly different from those including the patients with DAS28-ESR $\leq$ 3.2 at baseline.

Safety of TCZ retreatment

AEs were reported in 44.0 % (70/159) of the patients who were retreated with TCZ and in 42.9 % (3/7) of the patients treated with other DMARDs. All AEs reported in the TCZ-treated group were mild and tolerable relative to the benefit provided. The incidence rate of AEs in the TCZ monotherapy group (42.9 %, 57/133 patients, 95 % CI: 34.3–51.7) was comparable to the incidence rate in the TCZ plus synthetic DMARDs therapy group (50.0 %, 13/26 patients, 95 % CI: 29.9–70.1). There was no

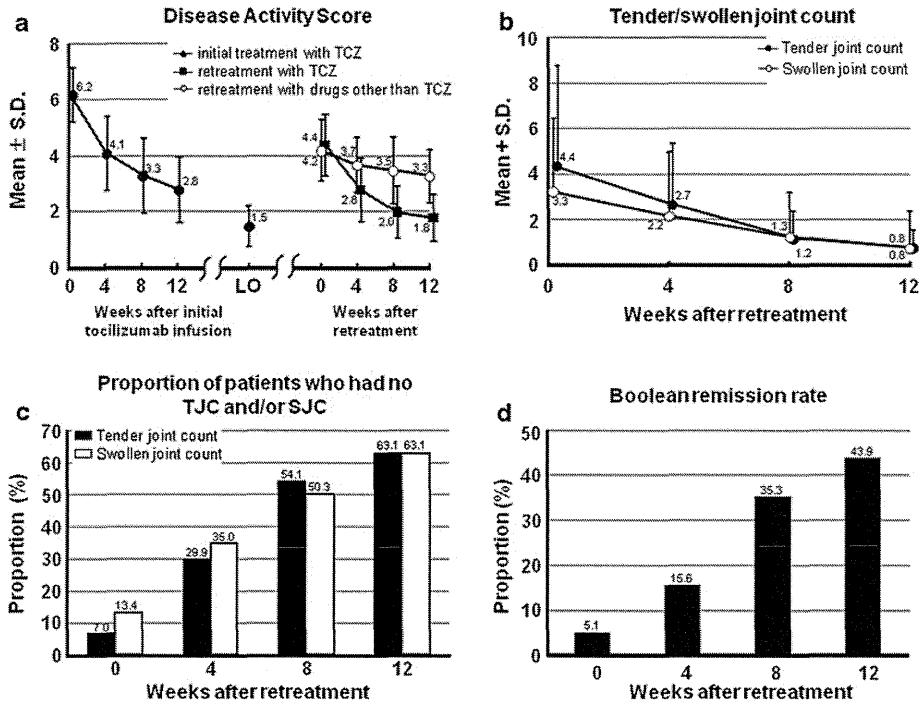

Fig. 1 Changes in DAS28-ESR, tender joint count, swollen joint count, and Boolean remission rate after resumption of treatment. **a** Mean (±SD) change in DAS28-ESR: from baseline of the initial tocilizumab (TCZ) treatment to week 12 and last observation point of the long-term extension studies (*closed circles*), and from the baseline of this study to week 12 in patients retreated with TCZ (*closed squares*) and in patients treated with other DMARDs (*open circles*). *Error bars* show SD. **b** Mean (+SD) tender joint count in 28 joints (*closed circles*), and mean (+SD) swollen joint count in 28 joints in TCZ-retreated patients (*open circles*). *Error bars* show SD. **c** Proportion of TCZ-retreated patients with no tender joints (*solid bars*) and those with no swollen joints (*open bars*). **d** Remission rates under the new EULAR/ACR remission criteria in the TCZ-retreated patients. *TJC* tender joint count, *SJC* swollen joint count, *LO* last observation point

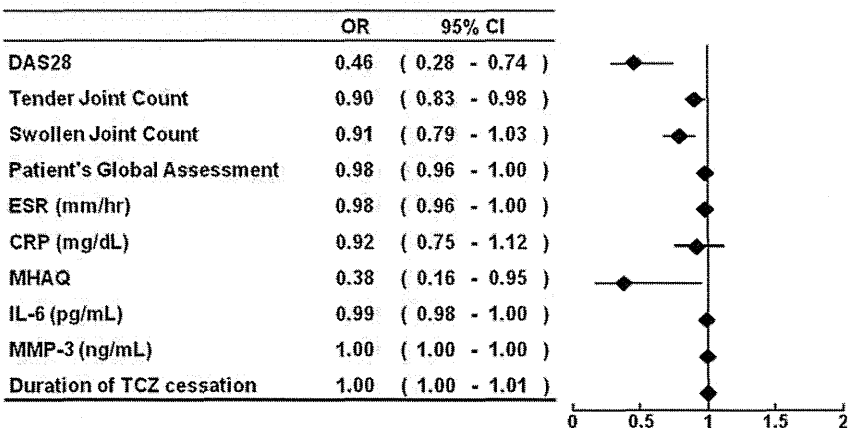

Fig. 2 Factors associated with resumption of DAS28-ESR remission by 12 weeks of TCZ retreatment after cessation of TCZ therapy. Factors contributing to the resumption of DAS28-ESR remission by 12 weeks of TCZ treatment were estimated by univariate and multivariate logistic regression analyses. *OR* odds ratio, *CI* confidence interval, *DAS28* 28-joint disease activity score, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *MHAQ* modified health assessment questionnaire, *IL-6* interleukin 6, *MMP-3* matrix metalloproteinase 3, *TCZ* tocilizumab

relationship between the development of AEs and the duration of TCZ cessation in the DREAM study. Infections were the most common AEs in the TCZ-treated group (27 patients, 17.0 %) (Table 2). None of the patients in this study were positive for anti-TCZ IgE antibodies. Only 1 patient who discontinued TCZ treatment for 35 weeks

Table 2 Adverse events observed after restarting TCZ treatment

Adverse event (SOC)	No. patients (%)
Total	70 (44.0)
Infections and infestations	27 (17.0)
Investigations	17 (10.7)
Gastrointestinal disorders	14 (8.8)
Skin and subcutaneous tissue disorders	12 (7.5)
Injury, poisoning and procedural complications	8 (5.0)
Respiratory, thoracic and mediastinal disorders	5 (3.1)
Nervous system disorders	3 (1.9)
General disorders and administration site conditions	3 (1.9)
Neoplasms benign, malignant and unspecified	2 (1.3)
Eye disorders	2 (1.3)
Vascular disorders	2 (1.3)
Musculoskeletal and connective tissue disorders	2 (1.3)
Blood and lymphatic system disorders	1 (0.6)
Immune system disorders	1 (0.6)
Ear and labyrinth disorders	1 (0.6)
Cardiac disorders	1 (0.6)
Reproductive system and breast disorders	1 (0.6)

SOC MedDRA Ver. 8.0 System Organ Class

became positive for anti-TCZ IgG antibodies 12 weeks after restarting TCZ treatment, and no decrease in the efficacy or any infusion reaction was observed in this patient. Moreover, no serious allergic reactions were reported in any patient.

One patient who discontinued TCZ treatment for 24 weeks experienced an infusion reaction 8 weeks after restarting TCZ therapy. The reactions included eruption, fatigue, and hypertension following the third infusion, but were mild and transient and did not require any treatment.

Three SAEs (1.9 %) were reported during retreatment with TCZ: appendicitis, wrist fracture, and chronic sinusitis. Causal relationships with TCZ were ruled out in the wrist fracture and chronic sinusitis.

Discussion

This study demonstrated that retreatment with TCZ was well-tolerated and effective in patients who had previously withdrawn from TCZ treatment. None of the patients in this study developed anti-TCZ IgE antibodies and only 1 patient tested positive for anti-TCZ IgG antibodies after restarting the TCZ treatment. Moreover, no serious allergic reactions were reported in any patient, including 3 patients retreated with TCZ after a long-term interval of more than 1 year. Our results confirm the results reported by Sagawa [16]. On the other hand, the development of serious infusion reactions was reported in patients who had restarted IFX treatment after long-term cessation of IFX [17]. This difference between TCZ and IFX can be attributable to the fact that, whereas IFX is a chimeric monoclonal antibody, TCZ is humanised, which reduces the content of foreign protein and thus the potential for the development of neutralising antibodies or IgE antibodies.

Regarding the efficacy of restarting TCZ at recurrence of disease activity after the cessation of TCZ treatment, the DAS28-ESR remission rate at 12 weeks after restarting TCZ was 88.5 %, which is comparable to the remission rate at the last observation point before cessation of initial TCZ treatment in the DREAM study (90.4 %). This improvement in DAS28-ESR was induced not only by improvement in acute-phase reactions, but also by improvement in TJC and SJC: over 60 % of the TCZ-retreated patients had complete improvement in terms of TJC or SJC or both (TJC or SJC or both was zero) within 12 weeks of treatment. Moreover, the Boolean remission rate as newly recommended by ACR/EULAR [15] reached 43.9 % (69/157 patients) at week 12. This value was extremely high.

The ACR/EULAR treatment recommendations state that, in patients who achieve remission with biological products, it may be possible to taper off the biological product after tapering off the corticosteroid [18]. However, in the majority of patients who discontinue treatment with biologics, it is found that efficacy cannot be sustained without use of the biologics and that disease activity may increase [16, 19]. This fact indicates that after attempting discontinuation of treatment with a biologic DMARD, it is necessary to guarantee safety and the ability to resume efficacy when restarting treatment with the same DMARD. Our results clearly indicate that TCZ was well-tolerated and effective in the patients who resumed TCZ treatment.

MMP-3 is deeply involved in cartilage destruction in RA and is also correlated with disease activity [20]. Since normalisation of the MMP-3 level is thought to reflect inhibition of excessive cartilage and bone destruction in the joints, normalisation of the MMP-3 level may indicate an improvement in the underlying cause of RA as well as synovial inflammation. In this study, we did not examine the progression of joint damage by imaging after restarting TCZ. However, since the MMP-3 levels were quickly improved after TCZ retreatment, TCZ retreatment should be considered to control disease activities and potentially prevent joint destruction once disease activity increased after the cessation of TCZ treatment. Further study of changes in radiological progression will be necessary to validate the modality of TCZ treatment investigated in the DREAM/RESTORE studies.

In conclusion, our results indicate that TCZ retreatment was effective and well tolerated in patients in whom disease activity recurred after cessation of TCZ monotherapy. Our

results also indicate that, together with the results of the DREAM study, the treatment interval of TCZ can also be adjusted flexibly without attenuation of efficacy.

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Conflict of interest N. Nishimoto has served as a consultant to and received honoraria from Chugai Pharmaceutical Co. Ltd. NN also works as a scientific advisor to F. Hoffmann-La Roche, which is developing TCZ in collaboration with Chugai Pharmaceutical Co. Ltd. NN also has received research grants from Chugai Pharmaceutical Co. Ltd., Bristol-Myers Japan, and Pfizer Japan Inc. K. Amano has received research grants from Chugai Pharmaceutical Co. Ltd., Astellas Pharm Inc., and Mitsubishi Tanabe Pharma. Y. Hirabayashi has received speakers' bureau honoraria from Chugai Pharmaceutical Co. Ltd. M. Iwamoto has received royalties from Chugai Pharmaceutical Co. Ltd. H. Kohsaka has received research grants, consultant fees, and/or speakers' bureau honoraria from Bristol-Myers Japan, Pfizer Japan Inc., and Takeda Pharmaceutical Co. Ltd. T. Mimura has received research grants from Abbott Japan, Chugai Pharmaceutical Co. Ltd., Mitsubishi Tanabe Pharma, and Takeda Pharmaceutical Co. Ltd. T. Takeuchi has received research grants, consultant fees, and/or speakers' bureau honoraria from Abbott Japan, Bristol-Myers Squibb, Chugai Pharmaceutical Co. Ltd., Eisai Co. Ltd., Janssen Pharmaceutical KK, Mitsubishi Tanabe Pharma, Novartis, Pfizer Japan Inc., and Takeda Pharmaceutical Co. Ltd. S. Tohma has received a research grant from Pfizer Japan Inc. and has received subsidies or donations from the Health and Labour Sciences Research Grants for Research on Allergic Disease and Immunology and from Chugai Pharmaceutical Co. Ltd. N. Takagi is a full-time employee of Chugai Pharmaceutical Co. Ltd. All other authors have declared no conflicts of interest.

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Allograft inflammatory factor-1 stimulates chemokine production and induces chemotaxis in human peripheral blood mononuclear cells

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ABSTRACT

Allograft inflammatory factor-1 (AIF-1) is expressed by macrophages, fibroblasts, endothelial cells and smooth muscle cells in immune-inflammatory disorders such as systemic sclerosis, rheumatoid arthritis and several vasculopathies. However, its molecular function is not fully understood. In this study, we examined gene expression profiles and induction of chemokines in monocytes treated with recombinant human AIF (rhAIF-1). Using the high-density oligonucleotide microarray technique, we compared mRNA expression profiles of rhAIF-1-stimulated $CD14^+$ peripheral blood mononuclear cells ($CD14^+$ PBMCs) derived from healthy volunteers. We demonstrated upregulation of genes for several CC chemokines such as CCL1, CCL2, CCL3, CCL7, and CCL20. Next, using ELISAs, we confirmed that rhAIF-1 promoted the secretion of CCL3/MIP-1α and IL-6 by $CD14^+$ PBMCs, whereas only small amounts of CCL1, CCL2/MCP-1, CCL7/MCP-3 and CCL20/MIP-3α were secreted. Conditioned media from rhAIF-1stimulated $CD14^+$ PBMCs resulted in migration of PBMCs. These findings suggest that AIF-1, which induced chemokines and enhanced chemotaxis of monocytes, may represent a molecular target for the therapy of immune-inflammatory disorders.

1. Introduction

Allograft inflammatory factor-1 (AIF-1) is a cytokine that was originally identified and cloned from rat heart allogeneic grafts undergoing chronic transplant rejection [1]. AIF-1 is a 17 kDa, interferon γ-inducible, Ca^{2+}-binding EF-hand protein that is encoded within the major histocompatibility complex (MHC) class III genomic region [1–3]. AIF-1 is thought to be involved in the regulation of cell cycle progression and cellular activation status [4].

Previously, it was reported that AIF-1 was highly upregulated in various autoimmune diseases and inflammatory disorders such as psoriasis, lichen planus, and systemic sclerosis. The main cell types expressing AIF-1 in these affected skins are macrophages and Langerhans cells [5,6].

We recently showed that mice expressed AIF in infiltrating mononuclear cells and fibroblasts in thickened skin of scleroderma-tous graft-vs.-host disease (GVHD) and in synovial tissues in rheumatoid arthritis (RA). Recombinant human AIF-1 (rhAIF-1) induced the proliferation of cultured synovial cells and the migration and proliferation of dermal fibroblasts [7]. Moreover, in patients with RA, rhAIF-1 increased IL-6 production by synovial fibroblasts and peripheral blood monocytes (PBMCs) and by dermal fibroblasts [8]. In addition, AIF-1 plays a role in the activation of macrophages, T-lymphocytes and vascular smooth muscle cells (VSMCs), and endothelial cells that participate in atherogenesis and the vascular response to injury [4,9,10].

Chemokines are small, chemoattractant cytokines that play key roles in the accumulation of inflammatory cells at the site of inflammation. Therefore, chemokines and chemokine receptors are considered to be therapeutic targets in several chronic inflammatory disorders such as RA [11]. The relationship between AIF-1 and chemokines is not clear. Recently, microarray techniques have become available that allow characterization of the mRNA

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expression pattern of a large number of genes. In this study, using the GeneChip system for comprehensive analysis, we identified the specific gene expression profiles of $CD14^+$ peripheral blood mononuclear cells ($CD14^+$ PBMCs) stimulated by rhAIF-1. Then, we examined cytokine production by ELISAs and their functions by using cell migration assay.

2. Materials and methods

2.1. Preparation of recombinant human AIF-1 (rhAIF-1)

Human *AIF1* cDNA was amplified from human peripheral blood lymphocyte cDNA (BD Bioscience Clontech, Palo Alto, CA) using PCR. The forward and reverse primers were 5′-GTG GAT CCA TGA GCC AAA CCA GGG ATT T-3′ (containing a BamHI site) and 5′-CAC TCG AGT CAG ATA GGG CTT TCT TGG CT-3′ (containing a XhoI site). The DNA fragment obtained was inserted in the BamHI/XhoI sites of pGEX-4 (Amersham Biosciences, Piscataway, NJ) in frame. To express AIF-1 as a glutathione *S*-transferase fusion protein, the protein was purified with a glutathione-*S*-transferase purification system (Amersham Biosciences) and affinity chromatography with anti-rhAIF-$1_{113-129}$ antibody.

To investigate the effect of AIF-1 on chemotaxis and cytokine induction, rhAIF-1 was treated with Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, IL, USA). Endotoxin detection was performed using *Limulus* amoebocyte lysate analysis (Wako Pure Chemical, Osaka, Japan) and treated AIF protein was confirmed to contain less than 0.1 ng/μg of endotoxin.

2.2. Preparation of anti-human AIF-1_{53-71} and AIF-$1_{113-129}$ antibodies

Two synthetic peptides that corresponded to residues 53–71 and 113–129 of human AIF-1 (AIF-1_{53-71} and AIF-$1_{113-129}$, respectively) as deduced from the nucleotide sequence of the human *AIF1* gene, were obtained with an additional cysteine residue at the N-terminus (Biologica, Nagoya, Japan). Following purification by reverse phase high-performance liquid chromatography, the synthetic peptide (purity > 90%) was coupled to keyhole limpet hemocyanin with *N*-(ε-maleimidocaproyloxy) succinimide (Sigma–Aldrich). The carrier-conjugated peptide was then emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and injected subcutaneously (0.5 mg/injection) into rabbits. The rabbits were immunized six times at ten day intervals. Blood samples were collected ten days after the last injection and the specific antibody in the sera was purified using an AIF-1 peptide-coupled cyanogen bromide-activated Sepharose affinity column. The antibodies reacted with proteins from abdominal adipose tissue and PBMCs that were identical in molecular size of purified recombinant human AIF-1.

2.3. Isolation and stimulation of $CD14^+$ PBMCs

PBMCs were isolated from healthy volunteers ($n = 5$; age: 34 ± 2) using Ficoll–Paque density gradients (GE Healthcare Bio-Sciences, Sweden). Human monocytes were purified from the cells using the MACS (Miltenyi Biotec, Germany) system, a direct magnetic labeling technique using anti-human CD14 microbeads (Miltenyi Biotec, Germany), according to the manufacturer's protocol (Daiichi Pure Chemicals Japan).

All subjects in this study provided written informed consent to participate. The study was approved by the Ethical Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan).

2.4. Preparation of biotin-labeled complementary RNA (cRNA) and hybridization to microarrays

$CD14^+$ PBMCs were seeded in 92-mm dishes at a concentration of 2×10^5 cells/mL/dish in a volume of 10 mL of serum-free RPMI1640, then incubated with PBS or rhAIF-1 (100 ng/mL). After incubation at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h, total RNA was extracted using a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). Preparation of cRNA and target hybridization was performed according to the Affymetrix GeneChip® technical protocol (Affymetrix, Santa Clara, CA, USA). Briefly, double-stranded cDNA was prepared from 1 μg of total RNA using Life Technologies Superscript Choice system (Life Technologies, Inc., Gaithersburg, MD, USA) and an oligo-(dT) 24 anchored T7 primer. Biotinylated RNA was synthesized from the double-stranded cDNA by in vitro transcription using 3′-Amplification Reagents for IVT Labeling (Affymetrix kit). Transcription products were purified using a Qiagen RNeasy column (Qiagen). After biotinylation, the in vitro transcription products were fragmented for 35 min at 94 °C in a buffer composed of 200 mmol/L Tris acetate (pH 8.1), 500 mmol/L potassium acetate and 150 mmol/L magnesium acetate. Human Genome® U133 plus 2.0 (Affymetrix, Santa Clara, CA, USA) was hybridized with the biotinylated products (0.05 μg/μL per chip) for 16 h at 45 °C using the manufacturer's hybridization buffer. After washing the arrays, the hybridized RNA was detected by staining with streptavidin–phycoerythrin SSPE, 0.01% Tween-20, pH 7.6, 2 mg/mL acetylated BSA and 10 mg/mL streptavidin–phycoerythrin (Molecular Probes, Carlsbad, CA, USA). Microarrays were scanned using a specially designed confocal scanner (GeneChip® Scanner 7G; Affymetrix).

2.5. Induction of IL-6, CCL1, CCL2/MCP-1, CCL3/MIP-1a, CCL7/MCP-3, and CCL20 /MIP-3a, and production by $CD14^+$ PBMCs by rhAIF-1

$CD14^+$ PBMCs from healthy volunteers ($n = 5$) were incubated with serum-free RPMI-1640 medium (Nissui Pharmaceutical) containing zero, one, ten, or 100 ng/mL of rhAIF-1 or 10 ng/mL of LPS from *Escherichia coli* (Sigma–Aldrich, MO, USA). After incubation at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h, the culture supernatants were recovered and stored at −80 °C until assay. IL-6, CCL1, CCL2/MCP-1, CCL3/MIP-1α, CCL7/MCP-3 and CCL20/MIP-3α concentrations were measured using commercial ELISA kits (IL-6 and CCL2/MCP-1: eBioscience CA USA) (CCL1: Antigenix America Inc., NY, USA) (CCL3/MIP-1a, CCL7/MCP-3, and CCL20/MIP-3α: R&D systems, MN, USA) according to the manufacturer's instructions. The absorbance was measured with a microplate reader (MPRA4, TOSHO, Tokyo, Japan).

3. Cell migration assays

We prepared culture supernatants of $CD14^+$ PBMCs (1×10^6/mL, $n = 6$) that had been incubated with serum-free RPMI-1640 medium (Nissui Pharmaceutical) with or without of 1, 10, or 100 ng/mL rhAIF-1. After incubation at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 12 h, the culture supernatants were harvested, stored at −80 °C, and used as lower chamber liquids. Then, we examined human PBMC migration induced by the culture supernatants using cell culture inserts and (Control Cell Culture Inserts in two 24-well plates, pore size 3.0 μm, BD Bioscience, USA). Human PBMC suspensions (5×10^6 cells/mL) were placed in the upper chamber ($n = 6$). Culture supernatants (400 μL) were added to lower chambers filled with the culture supernatants as mentioned above or CCL3/MIP-1α (50 ng/mL) in serum-free RPMI-1640 medium. The chambers were placed in a 37 °C humidified atmosphere of 5% CO_2 in air for 90 min. Migratory PBMCs

extended protrusions towards chemoattractants and ultimately passed through the pores of the polycarbonate membrane. We assessed chemotactic response both by counting the number of migratory PBMCs under the optical microscope and by Chemotactic Index which was calculated by dividing the number of migrated cells in each chamber by that in the chamber added CCL3/MIP-1α (50 ng/mL).

3.1. Statistical analysis

Array data analysis was carried out using Affymetrix GeneChip Operating Software (GCOS) version 1.4. GCOS analyzed image data and computed an intensity value for each probe cell. Briefly, mismatched probes acted as specificity controls that allowed the direct subtraction of both background and cross-hybridization signals. To quantitatively determine RNA abundance, the average difference values (i.e., gene expression levels) representing the perfect match–mismatch for each gene-specific probe family was calculated and the fold-changes in average difference values were determined according to Affymetrix algorithms and procedures. Hierarchical clustering analysis of the gene expression profiles of 118 genes was performed using GeneSpring software 7.3.1 (Agilent Technologies, Inc., Santa Clara, CA, USA). The differences were analyzed by Wilcoxon signed-rank test in ELISA and by Mann–Whitney U and Kruskal–Wallis tests in Cell migration assay.

4. Results

4.1. Upregulated genes following stimulation of CD14$^+$ PBMC by rhAIF-1

We compared mRNA expression profiles of monocytes with and without rhAIF-1 stimulation, using CD14$^+$ PBMCs derived from five healthy volunteers. We used the Human Genome U133 plus 2.0 array (Affymetrix), which contained about 55,000 probes. Comparison of the gene expression levels from vehicle- and rhAIF-1-treated CD14$^+$ PBMCs enabled the identification of 10^5 genes demonstrating greater than twofold alterations after AIF-1 stimulation. Using hierarchical clustering analysis of the gene expression profiles, we narrowed the expression of genes to 56 gene probe sets in terms of "inflammatory diseases". That probe set contained several chemokines. They included major CC chemokine genes such as *CCL1*, *CCL2/MCP-1*, *CCL3/MIP-1α*, *CCL7/MCP-3* and *CCL20/MIP-3α*. Among them, *CCL2/MCP-1*, *CCL7/MCP-3* and *CCL20/MIP-3α* genes were strongly upregulated after rhAIF-1 stimulation (Fig. 1).

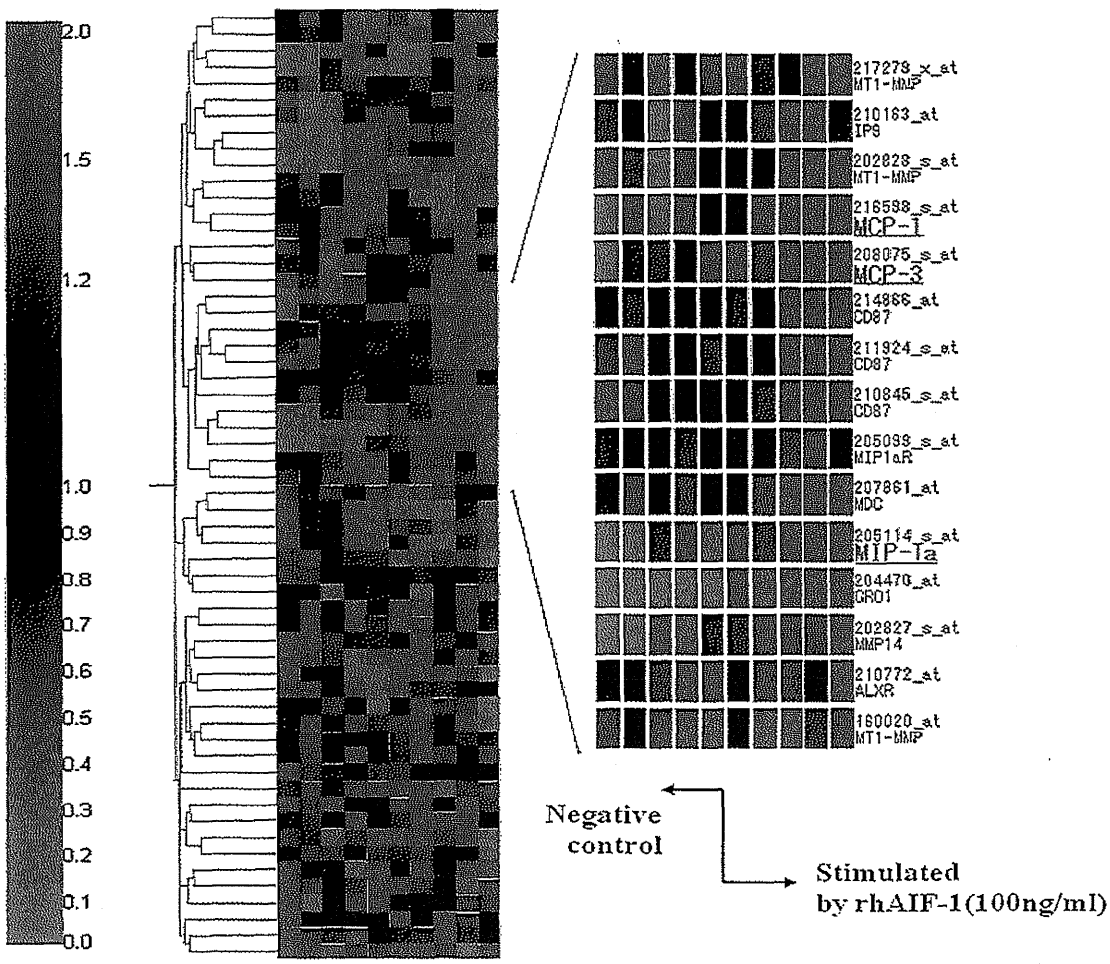

Fig. 1. Gene cluster analysis of peripheral blood CD14$^+$ mononuclear cells with and without rhAIF-1 stimulation. Starting with CD14$^+$ PBMCs stimulated by rhAIF-1 (100 ng/mL), we used hierarchical clustering analysis of the gene expression profiles of approximately 10,000 genes. We identified the expression of 58 genes associated with "proinflammatory cytokines" (at left). The data were analyzed by applying a hierarchical-tree algorithm to the normalized intensities. Upregulated genes are indicated by red shades and repressed genes by green. For one example, we picked up a region where gene expression was increased strongly after rhAIF-1 stimulation (at right). Among them, genes for CC chemokines such as CCL2/MCP-1, CCL3/MIP-1α and CCL7/MCP-3 were included in the region.

4.2. IL-6 and chemokine secretion from CD14⁺ PBMCs after rhAIF-1 stimulation

From the results of the mRNA expression profiles of CD14⁺ PBMCs stimulated by rhAIF-1 ($n = 5$), we examined the expression of IL-6, CCL1, CCL2/MCP-1, CCL3/MIP-1α, CCL7/MCP-3 and CCL20/MIP-3α proteins following rhAIF-1-stimulation. As shown in Fig. 2, the concentrations of IL-6 and CCL3/MIP-1α in the culture supernatant significantly increased after stimulation by human rAIF-1 for 24 h ($P < 0.05$). Expression of CCL1, CCL2/MCP-1, CCL7/MCP-3 and CCL20 /MIP-3 increased a very small amount (data not shown).

4.3. PBMC migration induced by cultured media from rhAIF-1-stimulated CD14⁺ PBMCs

CD14⁺ PBMCs were stimulated with rhAIF-1 (zero, one, ten, or 100 ng/mL) in RPMI, for 12 h. The supernatants were collected and used for cell migration assays. PBMCs ($n = 6$) were cultured for 90 min, and were attracted by the rhAIF-1-stimulated culture supernatant. The migrated cell counts were increased by culture supernatants from CD14⁺ PBMCs stimulated with 100 ng/mL rhAIF-1 compared to RPMI ($P < 0.05$, Fig. 3). There was no significant difference in the number of the migratory cells between 100 ng/mL rhAIF-1 and 50 ng/mL CCL3/MIP-1α as reference control. We confirmed that culture supernatants from rhAIF-1 stimulated CD14⁺ PBMCs induced the chemotaxis of PBMCs (Table 1).

5. Discussion

In this study, we used a high density oligonucleotide microarray technique for mRNA expression profiling of CD14⁺ PBMCs to investigate the cellular response of PBMCs to rhAIF-1 stimulation. We identified upregulated expression of several CC chemokine and cytokine genes. They included CC chemokine genes such as *CCL1*, *CCL2/MCP-1*, *CCL3/MIP-1α*, *CCL7/MCP-3* and *CCL20/MIP-3α*. Then, we used ELISAs to confirm that rhAIF-1 promoted the secretion of CCL3/MIP-1α and IL-6 by CD14⁺ PBMCs. However, secretions of CCL1, CCL2/MCP-1, CCL7/MCP-3 and CCL20/MIP-3α were at very low levels. Finally, we demonstrated that the cultured media from rhAIF-1-stimulated CD14⁺ PBMCs enhanced migration of PBMCs.

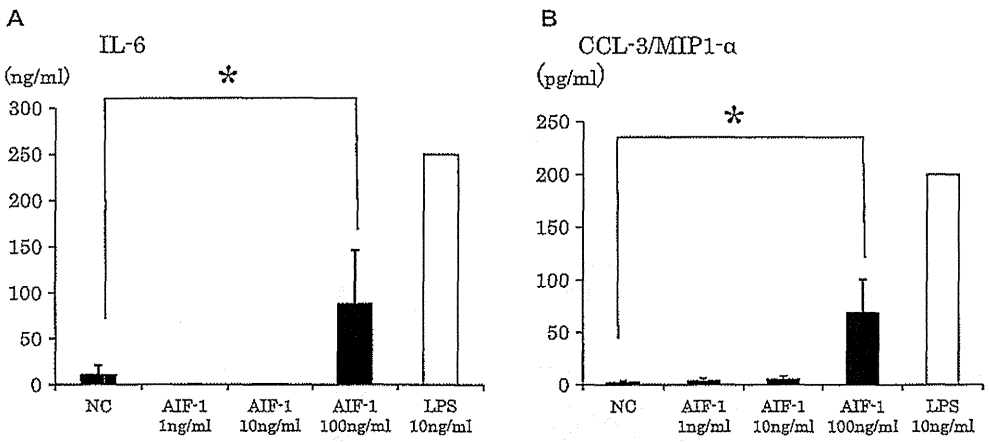

Fig. 2. Induction of IL-6 (A) and CCL3/MIP-1α (B) secretion from CD14⁺ PBMC stimulated by rhAIF-1. Human CD14⁺ PBMC ($n = 5$) were stimulated with serum-free RPMI-1640 medium containing 0, 1, 10 or 100 ng/mL rhAIF-1 or 10 ng/mL of LPS. Concentrations of IL-6 and CCL3 in the supernatant were measured with an ELISA at 24 h. Each bar represents the mean ± SE. The difference was analyzed by Wilcoxon signed-rank test. (*$P < 0.05$). NC: negative control.

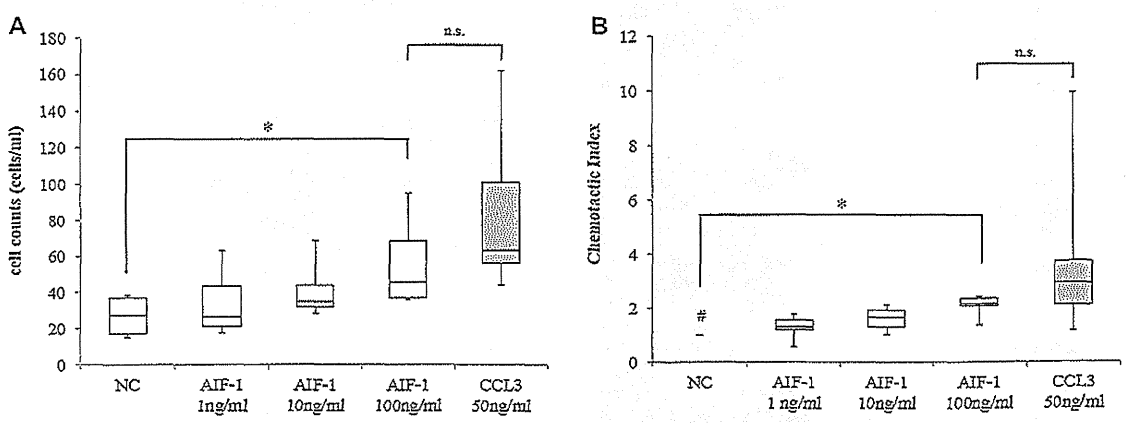

Fig. 3. PBMC migration was stimulated by culture supernatants from cells treated with rhAIF-1. Culture supernatants were prepared from human CD14⁺ PBMC ($n = 6$) that had been incubated with serum-free RPMI-1640 medium with or without of 1, 10, or 100 ng/mL rhAIF-1 for 12 h. These culture supernatants or 50 ng/mL CCL3 were added to lower chambers, and PBMCs were applied to the surface of the polycarbonate membranes at 5×10^6 cells/mL. Cell migration into the lower chamber significantly increased with culture supernatants from human CD14⁺ PBMC stimulated with 100 ng/mL of rhAIF-1 for 90 min. The difference was analyzed by Mann–Whitney U and Kruskal–Wallis tests in Cell migration assay (*$P < 0.05$). The Y-axis indicates the number of cells that migrated into the lower chamber (A) and Chemotactic Index (B). On each box, the central mark is the median, the edges of the box are the 25th and 75th percentiles. There was no significant difference in the number of the migratory cells between 100 ng/mL rhAIF-1 and 50 ng/mL CCL3/MIP-1α as reference control. NC (normal control) indicates culture supernatants incubated with serum-free RPMI-1640 medium containing no rhAIF-1. #The index of NC is 1.0. The other medians were calculated as the ratio on the basis of NC.

Table 1
Fold-changes of expression of "chemokine genes" after rhAIF-stimulation of isolated $CD14^+$ PBMCs. Signals and ratios were determined according to Affymetrix algorithms and procedures.

Probe set ID	AIF signal	NC signal	Ratio	Gene title
206365_at	499.7	27.9	13.00	CCL-1
216598_s_at	1325.1	237.0	4.00	CCL-2
205114_s_at	47689.5	5122.8	9.19	CCL-3
204103_at	48797.1	6007.6	8.00	CCL-4
1405_i_at	130.7	9.8	9.85	CCL-5
208075_s_at	195.1	69.5	2.00	CCL-7
214038_at	753.5	283.7	3.48	CCL-8
210133_at	26.5	54.8	0.57	CCL-11
216714_at	213.7	105.2	2.14	CCL-13
210390_s_at	3128.5	110.2	25.99	CCL-14, CCL-15
207354_at	122.5	56.5	2.64	CCL-16
207900_at	37.2	40.1	0.71	CCL-17
32128_at	888.7	334.4	2.14	CCL-18
210072_at	237.2	384.1	1.15	CCL-19
205476_at	7638.8	67.7	45.25	CCL-20
204606_at	92.6	416.7	0.22	CCL-21
207861_at	153.0	124.2	1.23	CCL-22
210548_at	580.0	28.5	16.00	CCL-23
221463_at	5081.6	694.9	6.50	CCL-24
206988_at	104.1	41.2	1.74	CCL-25
223710_at	64.2	128.2	0.38	CCL-26
230327_at	211.9	213.4	1.32	CCL-27
224240_s_at	358.6	629.9	0.93	CCL-28

T cells that secrete AIF-1 upregulate the proliferation of VSMCs [12]. Moreover, LPS-stimulated macrophages expressed AIF-1 and secreted interleukin IL-6, IL-10 and IL-12p40 [13]. Thus, *AIF-1* gene expression is involved in specific inflammatory signaling pathways related to T cell activation. Moreover, rhAIF itself can induce chemotaxis and proliferation as well as IL-6 production in synovial fibroblasts from patients with RA and in normal human fibroblasts [7,8]. IL-6 and AIF-1 concentrations in synovial fluid were significantly elevated in patients with RA compared with patients with osteoarthritis. There was a positive correlation between the synovial fluid levels of AIF-1 and IL-6. In this study of $CD14^+$ PBMCs, rhAIF-1 induced expression of chemokine-related genes and enhanced secretion of CCL3/MIP-1α, together with IL-6 and small amounts of other chemokines. It is reported that CCL3/MIP-1α induced chemotaxis in monocytic cells by more than 1 ng/mL [14]. Actually, we applied CCL3/MIP-1α as reference control at 0.5, 5, 50, 150 ng/mL in PBMC migration, but the chemotaxis was induced at a concentration of more than 50 ng/mL CCL3/MIP-1α (data not shown). We assume that the difference of the cells and the assay system we adopted may be a cause of this matter. As a result, these secreted molecules could induce chemotaxis of PBMCs. CCL3/MIP-1α is produced by a variety of immune cells such as monocytes and macrophage, and orchestrates acute and chronic inflammatory responses by recruiting proinflammatory cells [15]. CCL3/MIP-1α is considered one of the most important molecules in RA pathology [11,16].

Chemokines expressed in joints can recruit leukocytes and stimulate both fibroblast-like synoviocytes (FLS) and chondrocytes to release inflammatory mediators, including cytokines and MMPs, leading to cartilage degradation and pannus formation. Furthermore, chemokines enhance cell proliferation and angiogenesis, leading to synovial hyperplasia. Chemokines released by leukocytes and FLS, or by the chondrocytes themselves, can induce autocrine/paracrine stimulation of these cells, leading to joint destruction [11].

IL-6 is a pleiotropic cytokine with multiple biological effects on immune regulation, haematopoiesis, inflammation, and oncogenesis [17]. These finding suggest that AIF-1 could be involved with various immune-inflammatory reactions by inducing IL-6 and chemokines in $CD14^+$ PBMCs.

In conclusion, we found that AIF-1 upregulated several CC chemokine genes, leading primarily to release of CCL3/MIP-1α that could induce PBMC migration towards inflamed tissue. Although further work is needed to clarify the molecular mechanism of action of AIF-1, we suggest that AIF-1 may represent a molecular target for the therapy of immune-inflammatory disorders.

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Inhibition of osteoclastogenesis by osteoblast-like cells genetically engineered to produce interleukin-10

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ABSTRACT

Bone destruction at inflamed joints is an important complication associated with rheumatoid arthritis (RA). Interleukin-10 (IL-10) may suppress not only inflammation but also induction of osteoclasts that play key roles in the bone destruction. If IL-10-producing osteoblast-like cells are induced from patient somatic cells and transplanted back into the destructive bone lesion, such therapy may promote bone remodeling by the cooperative effects of IL-10 and osteoblasts. We transduced mouse fibroblasts with genes for IL-10 and Runx2 that is a crucial transcription factor for osteoblast differentiation. The IL-10-producing induced osteoblast-like cells (IL-10-iOBs) strongly expressed osteoblast-specific genes and massively produced bone matrix that were mineralized by calcium phosphate in vitro and in vivo. Culture supernatant of IL-10-iOBs significantly suppressed induction of osteoclast from RANKL-stimulated Raw264.7 cells as well as LPS-induced production of inflammatory cytokine by macrophages. The IL-10-iOBs may be applicable to novel cell-based therapy against bone destruction associated with RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic disorder characterized by systemic inflammation and multiple arthritis. In addition to synovial lesions, the bones at the inflamed joints are destructed by activated osteoclasts, resulting in severe pain, deformity and disability in patients. Although the pathogenesis of RA remains to be fully understood, crucial roles are played by proinflammatory cytokines such as IL-1β, IL-6, and TNF-α that are secreted from activated T cells and macrophages [1]. These cytokines also provoke synovial fibroblasts to produce RANKL, which subsequently induces osteoclasts to cause destruction of cartilage and bone [2]. Monoclonal antibodies and soluble receptors that block the pro-inflammatory cytokine signals have been used as biological agents and drastically improved the clinical outcome of RA; however, various adverse events may be associated with the therapies such as serious infection [3]. Meanwhile, any current medication has not succeeded in healing destructive bone, and surgical intervention is required to treat patients with severe joint destruction [4,5]. Therefore, it is necessary to develop a new therapeutic approach to suppress inflammation and archive repair of the destructed bones without causing undesirable adverse events.

IL-10 is a profound immunosuppressive cytokine produced by macrophages, T cells, and certain subsets of B cells and dendritic cells (DCs) [6]. Moreover, IL-10 remarkably prevents generation of osteoclasts through the inhibition of NFATc1 expression [7]. Therefore, IL-10 is regarded as a promising cytokine applicable to anti-rheumatic therapy, due to its powerful activities to suppress inflammation as well as osteoclastogenesis.

A number of reports indicated that systemic administration of IL-10 may provide significant therapeutic benefit to animal models of experimental arthritis [8–10]. Moreover, IL-10 suppresses production of inflammatory cytokines such as IL-1β and TNF-α by

Abbreviations: RA, rheumatoid arthritis; iOBs, induced osteoblast-like cells; IL-10-iOBs, IL-10-producing induced osteoblast-like cells; OCN, osteocalcin; OPN, osteopontin; BSP, bone sialoprotein; ALP, alkaline phosphatase.

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synovial fluid macrophages of RA patients [11]. In previous clinical trials, IL-10 administration was reported safe and well tolerated, but its therapeutic efficacy for RA was unsatisfactory [12,13]. This may be due to insufficiency of distribution of IL-10 at the joint lesions after systemic administration of the cytokine. Systemic administration of a higher dose of IL-10 could potentially cause cancer development, chronic infection, and Th2-dependent autoimmune disorders that represent lupus-like symptoms [14]. Therefore, local delivery of IL-10 to the inflammatory legions is desirable. Some studies have shown that the cells genetically modified to produce IL-10 were effective in treating experimental arthritis in animals [15,16].

Osteoblasts are crucially involved in bone formation and remodeling through production of calcified bone matrix. We hypothesized that if osteoblast-like cells are engineered to produce IL-10 and transplanted into the destructed bone tissue, such a procedure may offer a great deal of therapeutic benefits to RA patients due to remodeling of bone tissue as well as suppression of articular inflammation. Osteoblast-like cells can be induced from mouse fibroblasts by transducing the Runx2 gene that plays an essential role in osteoblast differentiation [17], and this sort of technology may enable production of patient-specific, induced osteoblast-like cells (iOBs) that are suitable for transplantation therapy.

However, effect of IL-10 on osteoblast differentiation remains controversial. IL-10 gene knockout mice showed loss of alveolar bone and osteopenia-like phenotypes including bone mass reduction [18–20]. In contrast, van Vlasselaer et al. reported that administration of exogenous IL-10 inhibited the osteoblast differentiation from mouse bone marrow cells through the inhibition of TGF-β1 [21,22].

In this context, we examined whether co-transduction of Runx2 and IL-10 genes successfully induced mouse fibroblasts into IL-10-producing osteoblast-like cells with capability to produce bone matrix. We also tested whether the genetically modified cells inhibited production of pro-inflammatory cytokines by activated macrophages as well as induction of osteoclasts.

2. Materials and methods

2.1. Cells

Mouse embryonic fibroblasts (MEFs) were obtained from Balb/c embryos at the gestational age of day 13.5 by digestion with collagenase (NB4G®; Serva, Heidelberg, Germany). MEFs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM non-essential amino acids (standard medium). Raw264.7, A20, and SaOS2 were purchased from RIKEN cell bank (Tsukuba, Japan). Yac1 was maintained in our laboratory. SCC-7 was kindly gifted by Dr. Oya at the Department of Therapeutic Radiology and Oncology, Kyoto University, Japan.

2.2. Retrovirus vectors

The cDNA fragments encoding mouse Runx2 and IL-10 genes were obtained from pFLCI-mouse Runx2 and pFLCI-mouse IL-10 plasmids (Danaform, Kanagawa, Japan), respectively, and inserted into the pMX-puro with GeneArt Seamless Cloning and Assembly (Life Technologies, Carlsbad, CA). The resultant retrovirus vector plasmids, pMX-mRunx2.puro and pMX-mIL10.puro, were transfected into the Plat-E packaging cell line with X-treme Gene 9 (Roche Diagnostics, Basel, Switzerland). Twenty-four hours later, the culture medium was replaced by fresh one, and after incubation for another 24 h, culture supernatant containing retrovirus vectors was harvested.

2.3. Induction of osteoblasts

MEFs were seeded onto a 24-well plate at a density of 1.5×10^4 per well. On the next day, the retrovirus vector suspension was supplemented with 4 μg/mL polybrene (Nacalai Tesque, Kyoto, Japan) and added to the cells, which were subsequently cultured in the standard medium supplemented with 100 nM dexamethasone (Nacalai Tesque), 50 μg/mL L-ascorbic acid (Nacalai Tesque), and 10 mM β-glycerophosphate (Tokyo Chemical Industry, Tokyo, Japan)(osteogenic medium). The culture was continued for 2 to 4 weeks, while the medium was changed every 2 days.

2.4. Alizarin red S staining and staining by von Kossa's method

For Alizarin red S staining, cells were fixed with 95% ethanol for 10 min at room temperature. After washing with distilled water, cells were stained with Alizarin red S solution (Sigma–Aldrich, St. Louis, MO) for 30–45 min, followed by another washing with distilled water. Stained area was calculated with ImageJ [23]. For von Kossa staining, cells were fixed in 10% neutral buffered formalin for 10 min at room temperature. After 3 times washing with distilled water, 5% silver nitrate solution (ScyTek Laboratories, Logan, UT) was added to the cells, which were subsequently exposed to ultra violet for 30–60 min. Culture dishes were rinsed 3 times with distilled water, and incubated with 5% sodium thiosulfate solution (ScyTek Laboratories) for 2 min.

2.5. Alkaline phosphatase (ALP) staining

ALP activity was determined by ALP staining using a Leukocyte Alkaline Phosphatase Kit (Sigma–Aldrich) following the manufacture's instruction. Briefly, cells were fixed with 60% acetone/40% citrate. After washing with deionized water, cells were stained with a diazonium salt solution containing fast violet blue salt and 4% of naphthol AS-MX phosphate alkaline solution for 1 h under protection from light.

2.6. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde at 4 °C for 30 min, followed by washing with 0.02% Tween-20/PBS. After blocking, cells were washed and incubated with FITC-conjugated rat anti-mouse IL-10 (final concentration was 1:100) (eBioscience, San Diego, CA) and Cy5.5-conjugated rabbit anti-mouse osteocalcin (OCN) (final concentration was 1:100) (Bioss, Woburn, MA) antibodies. On the next day, cells were washed, and observed under a fluorescence microscope.

2.7. Real time RT-PCR

Cells were homogenized in Isogen 2 (Nippongene, Tokyo, Japan), and total RNA was harvested by the phenol guanidinium acid-based procedure. After reverse transcription using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan), cDNA served as template for real time PCR using Applied Biosystems 7300 Real-Time PCR System. The primers and dye probe for osteocalcin (OCN) (Bglap: Mm03413826_mH), osteopontin (OPN) (SPP1: Mm00436767_m1), bone sialoprotein (BSP) (IBSP: Mm00492555_m1), alkaline phosphatase (Alpl: Mm00492555_m1), IL-1β (Il1b: Mm00434228_m1), telomerase reverse transcriptase (Tert: Mm00436931_m1) and β-actin (Mm00607939-m1) genes were purchased from Applied Biosystems (Carlsbad, CA). The primer sequences for TNF-α and IL-6 genes were as follows: TNF-α forward, 5′-tcttctcattcctgcttgtgg-3′; TNF-α reverse, 5′-ggtctgggccatagaactga-3′; IL-6 forward, 5′-gctaccaaactggatataatcagga-3′, IL-6 reverse, 5′-ccaggtagctatggtactccagaa-3′. Corresponding proves were purchased from Roche Applied

Science (Basel, Switzerland) (Universal Probe Library: TNF-α#49, IL-6#6). Samples were incubated at 95 °C for 10 min for an initial denaturation, followed by forty PCR cycles that consisted of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. β-actin gene was regarded as endogenous standard and normalization was calculated using RQ software. All experiments were performed in triplicate.

2.8. ELISA

The concentration of IL-10 was measured using Mouse IL-10 Ready SET Go ELISA kit (eBioscience) according to the manufacturer's instruction.

2.9. Induction of osteoclasts

Raw264.7 cells obtained from the RIKEN cell bank were plated in 12-well plates at a density of 1×10^4 cells/well, and cultured in a standard medium supplemented with or without 100 ng/mL of RANKL (PeproTech, Rocky Hill, NJ). In some wells, culture supernatants that had been harvested from MEFs, induced osteoblast-like cells (iOBs), and IL-10-producing iOBs (IL-10-iOBs) were added. The culture medium was replaced by a fresh one on day 3. Six days after the initiation of the culture, cells were subjected to tartrate-resistant acid phosphatase (TRAP) staining using the TRAP kit (Primary Cell, Sapporo, Japan). Briefly, the cells were fixed with 10% neutral buffered formalin for 5 min, followed by incubation at 37 °C for 1 h in 50 mmol/L tetrate buffer containing substrate. After washing with distilled water, the TRAP-positive multi-nuclear cells were regarded as mature osteoclasts.

2.10. Activation of peritoneal macrophages

6–8 week-old female Balb/c mice were intraperitoneally injected with 2 mL of thioglycolate medium. Three days later, mice were sacrificed and 5 mL of PBS was injected into the peritoneal cavity. After gentle massage, the peritoneal exudate cells were retrieved and seeded into 12-well plates at 1.5×10^6 cells per well. After 2 h of culture, the floating cells were removed, and residual adherent cells were cultured for 24 h in the presence of supernatants of MEFs, iOBs and IL-10-iOBs. Cells were then stimulated with LPS (100 ng/mL)(Invitrogen, Carlsbad, CA) for 2 h.

2.11. Contact inhibition

SaOS2 cells and MEFs that had been transduced with Runx2 and IL-10 genes were seeded in 60 mm culture dishes at a density of 1.5×10^5 cells/dish, and cultured for 15 days without trypsinization and reseeding. Cell morphology was observed under phase-contrast microscopy.

2.12. Transplantation

Twenty-four hours after infection with the Runx2 and/or IL-10 retrovirus vectors, MEFs, iOBs and IL-10-iOBs were seeded on hydrogel scaffold (MedGel® SP; MedGEL, Tokyo, Japan) at a density of 3.5×10^4 cells/scaffold. After pre-culture for 2 days in osteogenic medium, the cells were subcutaneously transplanted into the flank of 6-week-old female Balb/c mice. Four weeks later, the graft was excised and cryosectioned. Mineralization status of the specimens was estimated by Alizarin red staining and staining by the von Kossa's method as above. To examine tumor formation, IL-10-iOBs or MEFs that had been cultured in osteogenic medium for 2 days were subcutaneously inoculated into 7-week-old male SCID/NOD mice with or without hydrogel scaffold at a dose of either 3.5×10^4 or 3.0×10^6/mouse.

2.13. Statistical Analysis

All the data were analyzed by Student's unpaired *t*-test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. IL-10 gene transduction did not prevent Runx2-mediated induction of osteoblast-like cells from fibroblasts

To generate IL-10-secreting osteoblast-like cells, we transduced primary mouse embryonic fibroblasts (MEFs) with Runx2 and IL-10 genes via retrovirus vectors, and analyzed the phenotypes of the resultant cells (IL-10-iOBs) in comparison with those of the osteoblast-like cells induced by transduction of Runx2 gene alone (iOBs). The ALP staining indicated that both IL-10-iOBs and iOBs showed high activities of ALP, an early stage marker of osteoblast differentiation, 10 days after the gene transduction (Fig. 1A, top). Alizarin red S staining unveiled that the IL-10-iOBs produced mineralized bone matrix as massively as iOBs on day 20, whereas un-transduced MEFs failed to show any significant staining (Fig. 1A, middle). Calculation of the Alizarin red S-stained areas also demonstrated comparable degrees of calcification in IL-10-iOBs and iOBs cultures (data not shown). Staining by the von Kossa's method also confirmed massive calcium deposition by IL-10-iOBs and iOBs, but not by MEFs (Fig. 1A, bottom).

To further confirm the osteoblast-like characteristics of the cells, we examined expression of osteoblast marker genes 15 days after the gene transfer (Fig. 1B). Quantitative RT-PCR analysis demonstrated that IL-10-iOBs expressed similar or even higher levels of mRNA for the osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP) and ALP genes as iOBs.

These data strongly suggested that IL-10 gene transfer did not hamper osteoblast-like phenotypic conversion of MEFs.

3.2. IL-10-iOBs produced IL-10 at a high level

To assess the IL-10 production by IL-10-iOBs, we performed quantitative RT-PCR 15 days after the initiation of the osteoblast induction. The cells expressed a high level of IL-10 mRNA, which was not the case with iOBs and MEFs (Fig. 2A). We also harvested the supernatants of the IL-10-iOBs and measured IL-10 concentrations by ELISA, which showed robust IL-10 secretion from the cells on days 1 and 2 (Fig. 2B).

Because IL-10-iOBs potentially contained heterogeneous populations, further experiments were performed to clarify whether the IL-10 was produced by the cells with osteoblast-like features. Immunofluorescence staining of IL-10-iOBs was performed to visualize OCN and IL-10 on day 14. As shown in Fig. 2C, OCN and IL-10 were co-localized in the same cells, indicating that IL-10 was produced by the osteoblast-like cells.

3.3. Conditioned medium of IL-10-iOBs suppressed induction of osteoclasts

To estimate whether the IL-10 secreted from IL-10-iOBs was capable of inhibiting osteoclastogenesis, we collected the culture supernatants of the IL-10-iOBs, iOBs and MEFs, and examined their effect on the mouse macrophage cell line, Raw264.7, that was induced to differentiate into osteoclasts by an addition of RANKL [24,7]. Six days after the induction, a number of TRAP-stained multinuclear cells (MNCs) appeared in the cultures in which the supernatant of MEFs or iOBs had been added (Fig. 3A). In contrast, the