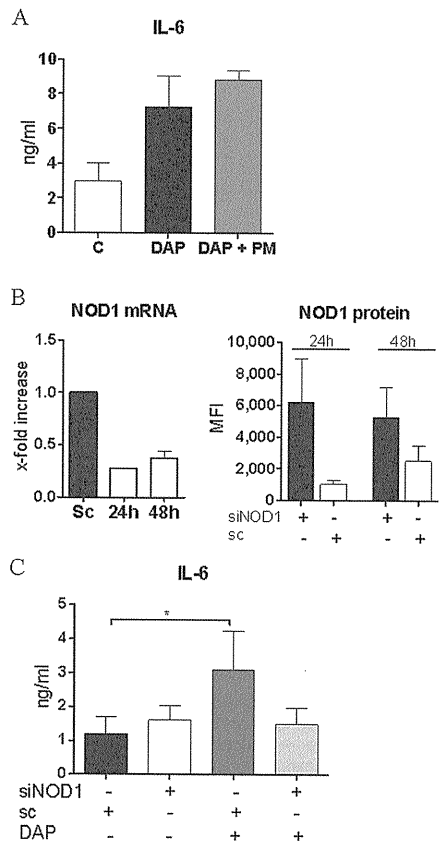

Figure 4. Tri-DAP specificity and NOD1 silencing. **A)** RASFs (n=2-6) were stimulated with Tri-DAP alone or Tri-DAP with polymyxin B for 24h and IL-6 levels in the cell supernatants were measured by ELISA. **B)** RASFs (n=3-4) were transfected with control siRNA (sc) or NOD1 targeting siRNA (siNOD1) and NOD1 mRNA and protein expression was measured 24h and 48h after transfection. **C)** 24h after transfection of RASFs (n=6) with NOD1 siRNA or scrambled control siRNA (sc), cell were stimulated for 24h with Tri-DAP and IL-6 was measured in the supernatants. Non-parametric Friedman test followed by Dunn's multiple comparison test was used. * = $p < 0.05$.

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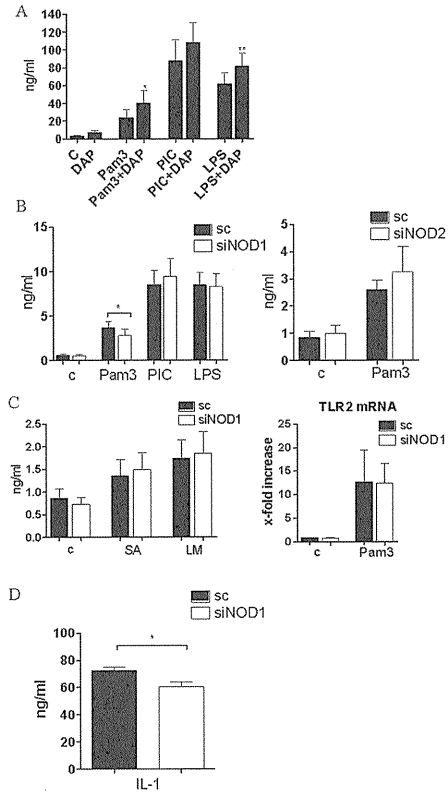

Figure 5. Silencing of NOD1 downregulates TLR2 and IL-1 induced IL-6 production. A) TwoWay ANOVA analysis showed synergistic interaction of Tri-DAP stimulation with Pam3 and LPS in the production of IL-6 in RASFs (n=6). **B)** Knock-down of NOD1 in RASFs (n=6) led to decreased levels of IL-6 after Pam3 stimulation when compared to control transfected RASFs by Wilcoxon matched pairs test (left panel). Knock-down of NOD2 did not induce any change in IL-6 levels after stimulation with Pam3 (n=4) (right panel). **C)** Stimulation with heat inactivated Staphylococcus aureus (SA) or Listeria monocytogenes (LM) resulted in similar levels of IL-6 in NOD1 siRNA and control transfected cells (n=6) (left panel). Also, mRNA levels of TLR2 did not differ between NOD1 silenced and control RASFs, but were induced by stimulation with Pam3 (n=3) (right panel). **D)** IL-6 levels were significantly lower in siNOD1 transfected cells than in control transfected cells after stimulation with IL-1 (n=6). Wilcoxon matched pairs test was used for calculations. * = $p < 0.05$; ** = $p < 0.01$.

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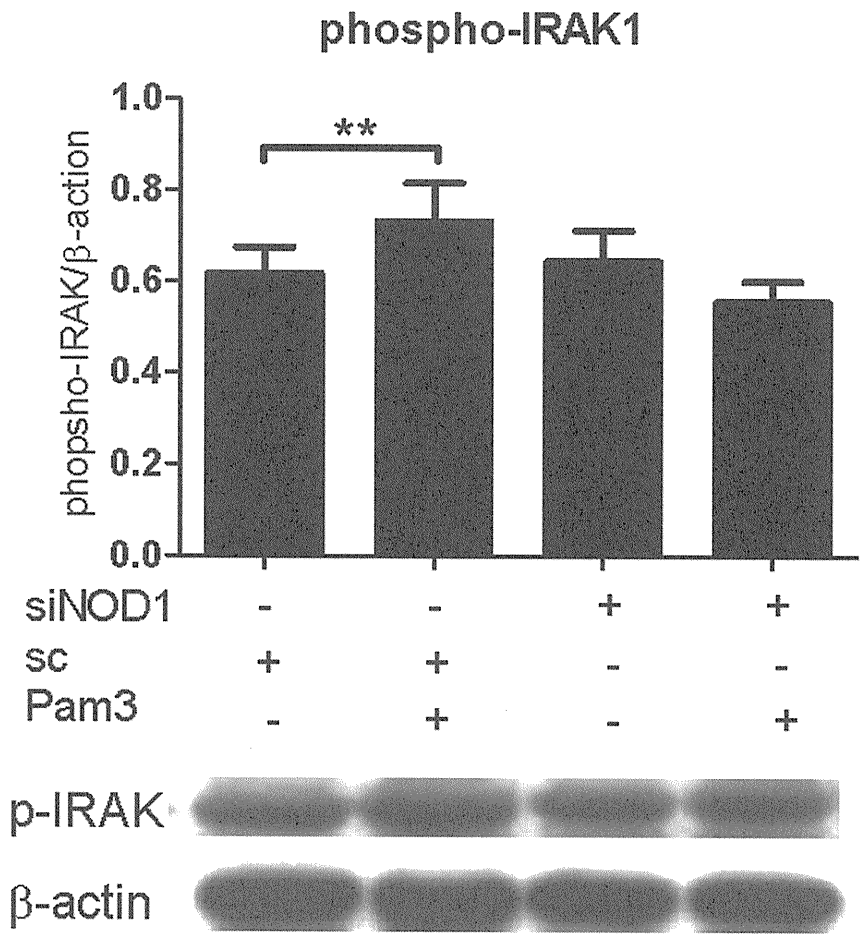

Figure 6. NOD1 influences IRAK1 phosphorylation. IRAK1 phosphorylation significantly increased after stimulation of control transfected RASFs with Pam3 for 20min, but not in NOD1 siRNA transfected cells (n=15). Wilcoxon matched pairs test was used for calculations. ** = $p < 0.01$

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INTERNAL MEDICINE

□ CASE REPORT □

Tocilizumab Improved both Clinical and Laboratory Manifestations Except for Interleukin-18 in a Case of Multiple Drug-Resistant Adult-Onset Still's Disease

Yoshihiro Yoshida, Mayuko Sakamoto, Kazuhiro Yokota, Kojiro Sato and Toshihide Mimura

Abstract

A patient with adult-onset Still's disease (AOSD) resistant to multiple drugs was treated in our hospital. Even biologics that block tumor necrosis factor (TNF) were ineffective. However, this patient responded quite well to tocilizumab, an interleukin (IL)-6 receptor blocker, suggesting that it is among the promising candidate drugs for multiple-drug resistant AOSD. Although the serum levels of most inflammatory markers such as C-reactive protein (CRP) and ferritin were reduced promptly by tocilizumab, that of IL-18 remained high. Thus, IL-18 is considered to have a further upstream position than IL-6 or to be at the same level as IL-6 in the inflammatory cascade of AOSD. This finding casts light on the pathogenesis of AOSD, and drugs that target IL-18 may prove beneficial in the treatment of this inflammatory disease.

Key words: adult-onset Still's disease, IL-6, IL-18, tocilizumab

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Introduction

Adult-onset Still's disease (AOSD) is a systemic inflammatory disease characterized by a high spiking fever, arthritis, evanescent rash, and certain laboratory findings including abnormal liver function and elevated acute-phase proteins (1). AOSD is of unknown etiology although infectious triggers have been suggested (2, 3). The mainstay of treatment is glucocorticoids and/or non-steroidal anti-inflammatory drugs (NSAIDs) (4), but in addition, immunosuppressants, such as methotrexate (MTX) (5), cyclosporine A (CyA) (6), TNF blockers (7-10) or an IL-1 blocker (11), are sometimes necessary. Some patients are refractory even to the combination of the drugs mentioned above.

Recently, an IL-6 receptor blocker has been developed and made available in the clinical field. This agent, called tocilizumab, is a humanized anti-IL-6 receptor (IL-6R) antibody; it is used for Castleman's disease (12), juvenile idiopathic arthritis (JIA) (13), and rheumatoid arthritis (14). As IL-6 is a key player in the induction of various acute-phase proteins (15) and AOSD shares some characteristics with JIA, tocilizumab is promising as an agent for multiple-drug refractory AOSD. Indeed, we successfully treated such a patient with tocilizumab, for whom even treatment with the TNF blockers, infliximab and etanercept, had not proven effective. In the course of the treatment, the levels of both CRP and ferritin decreased rapidly, but interestingly, that of IL-18 did not. We discuss the implication of this phenomenon in light of certain reports in the literature.

Case Report

A 25-year-old man was admitted to a local hospital with fever and arthritis of the knees. As he exhibited marked leukocytosis (>20,000/μL) along with an elevated level of CRP (>20 mg/dL), infectious diseases were suspected at first. Treatment with various antibiotics did not ameliorate the symptoms, and no bacterium or virus was detected. Eventually, an evanescent salmon-colored rash appeared on his trunk. Rheumatoid factor, anti-nuclear antibody and anti-neutrophil cytoplasmic antibody (ANCA) were all negative. AOSD was suspected and the patient was treated with (i) prednisolone (PSL, 40 mg/day), (ii) intravenous methylpred-

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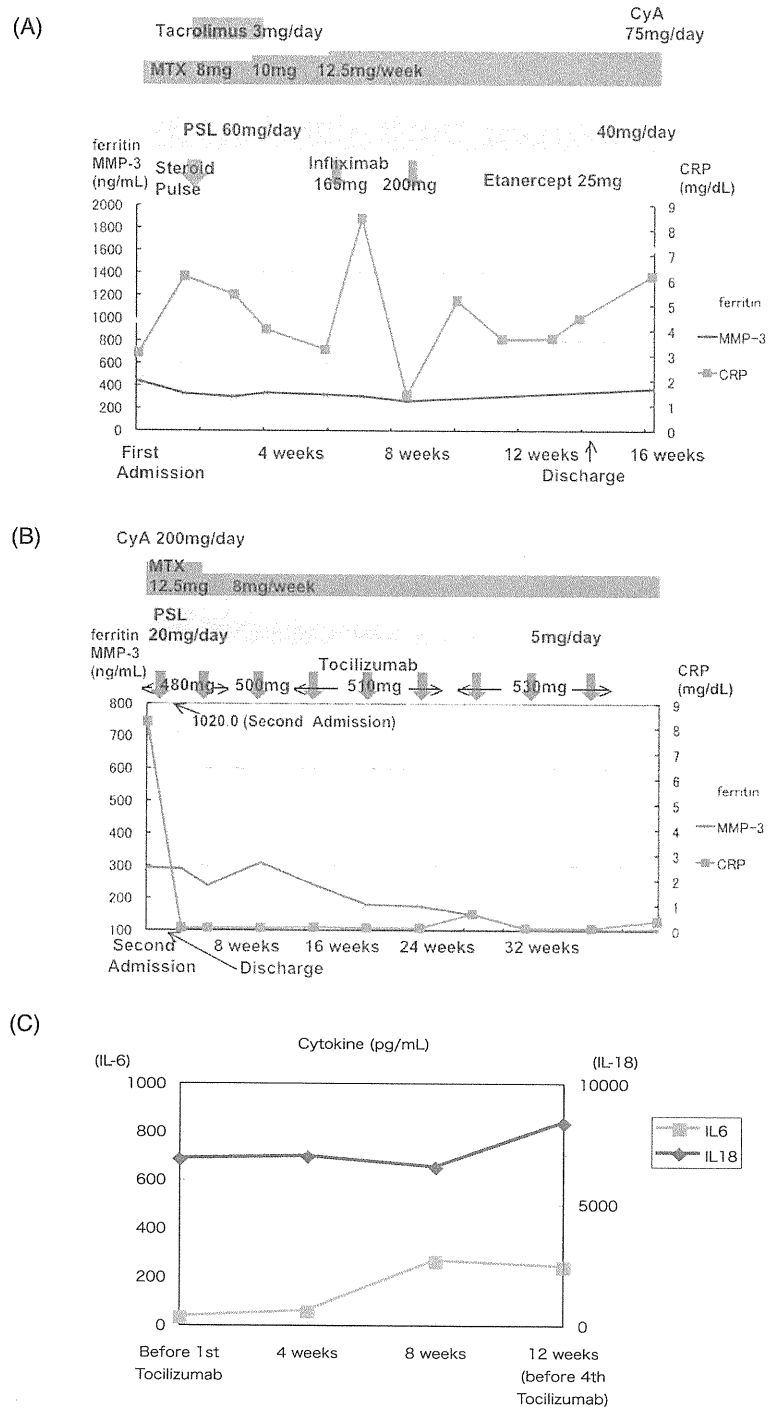

Figure 1. **Clinical course of the patient before (A) and after (B) the treatment with tocilizumab. (C) The serum levels of IL-6 and IL-18.**

nisolone 1 g/day for 3 days (steroid pulse) ×2 times, (iii) azathioprine (100 mg/day), and (iv) MTX (8 mg/week), but the levels of CRP, ferritin, and white blood cells (WBCs) did not normalize. He was referred and admitted to this hospital about 2 months after the onset of the symptoms.

On examination, the patient presented as obviously ill. His body temperature was 37.1℃, pulse 89 beats per minute, and blood pressure 125/62 mmHg. His weight was 60.0 kg and height 169 cm. Chest sounds were normal. The abdomen was flat and soft and bowel sounds were normal. The liver and spleen were not palpable. Skin rash was not observed. The leukocyte count was $25.37\times10^3/\mu L$ (92.9% neutrophil), hemoglobin level 13.8 g/dL, and platelet count $266\times10^3/\mu L$. The CRP level was 7.19 mg/dL, ferritin 1890 ng/mL, and matrix metalloproteinase-3 (MMP-3) 339.0 ng/mL. The aspartate aminotransferase level was not increased (16 IU/L, normal range 10.0-37.0) but lactate dehydrogenase (LDH) was slightly elevated (237 IU/L, normal range 107.0-220.0). No bone erosion was observed by X-ray. The patient was treated with PSL (60 mg/day) and MTX (8 mg/week), and one course of steroid pulse was performed again, followed by tacrolimus (3 mg/day). General fatigue persisted

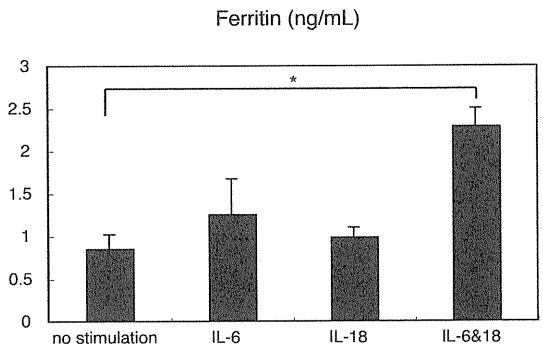

Figure 2. **Monocytes were sorted by magnetic beads(MACS, Miltenyi Biotec, Auburn, CA) from peripheral blood mononuclear cells derived from healthy controls (Institutional Review Board ID No. 06-060-1). The cells were cultured with or without the presence of IL-6 and/or IL-18 (10 ng/mL each) for 15 hours, and the level of ferritin in the supernatant was quantified using ELISA. *: $p<0.05$ by Student's t-test. The data are representative of three independent experiments.**

and the level of CRP remained high, as well as that of ferritin (Fig. 1A). The dose of MTX was increased to 10 mg/week and then to 12.5 mg/week with no evident improvement. As the blood levels of tacrolimus remained low, it was discontinued and infliximab (3 mg/kg, 165 mg in total) was begun with the result of a decrease in the CRP level. After the second infusion of infliximab (200 mg), however, the CRP level rebounded. Infliximab was switched to etanercept and the patient was discharged. However, the CRP level did not normalize in the outpatient clinic of this hospital and it was difficult to taper the dose of PSL. CyA (75 mg/day) was added and etanercept was discontinued. The dose of CyA was increased to 200 mg/day, without notable benefit. Finally, after discussion with the patient and his family, we decided to use tocilizumab. After the first treatment with tocilizumab (480 mg, 8 mg/kg), the level of CRP dropped to below 0.1 mg/dL. The patient also reported that his general fatigue disappeared. He was treated with tocilizumab every 4 weeks and the levels of ferritin and MMP-3 gradually and consecutively came into the normal range (Fig. 1B). As reported previously (16), the level of IL-6 elevated after the initiation of tocilizumab therapy, however, the level of IL-18 remained extremely high (more than 5,000 pg/mL, Fig. 1C). Almost 16 months have passed since the first treatment with tocilizumab. Now we use 560 mg (9 mg/kg) of the biologic every 5 weeks and no sign of relapse has been observed.

Discussion

In this case, multiple immunosuppressant drugs, including anti-rheumatic biologics that block TNF-α, were ineffective, but tocilizumab displayed a marked effect on both clinical symptoms and laboratory findings. Although there are reported cases in which TNF blockers were effective against AOSD, the response rate was not particularly robust. Recently, tocilizumab has been made available clinically and an increasing number of cases have been reported in which it was used successfully against the disease (17-22). Its response rate is, at present, unclear. Tacrolimus was reported to be useful against multiple-drug resistant AOSD (23), yet it was not beneficial in this case, probably because a sufficient blood concentration of the drug was not obtained.

One of the particularly interesting findings in this case is that the level of IL-18 remained high even though the levels of CRP, ferritin and MMP-3 were reduced markedly by tocilizumab (Fig. 1B and C). IL-18 was reported to be increased in AOSD patients, and it was quite high in this case. The fact that treatment with tocilizumab did not reduce the IL-18 level suggests that IL-18 is located either upstream of, or at the same level as, IL-6 in the pathogenesis of AOSD. Indeed, in the above-mentioned case in which tacrolimus was effective (23), the level of IL-18 was also reduced, suggesting that tacrolimus blocked the inflammatory cascade of AOSD at more upstream position than tocilizumab.

In order to evaluate if IL-6 and IL-18 have an additive or synergistic effect on the production of ferritin, we added IL-6 and/or IL-18 to monocytes derived from healthy controls *in vitro* and analyzed the level of ferritin in the supernatant of the culture medium (Fig. 2). Only the addition of both IL-6 and IL-18 (10 ng/mL each) led to a significant increase in the protein level of ferritin, indicating that IL-6 and IL-18 have a synergistic effect on the production of ferritin from macrophage-lineage cells. Although this does not directly prove the role IL-18 plays in AOSD, it appears to afford collateral evidence that IL-18 is involved in the pathogenesis of the disease.

This case reveals the potential of tocilizumab to be a quite effective drug for refractory AOSD. It is also possible, however, that tocilizumab may have to be continued as long as the level of serum IL-18 remains high. In such a case, drugs that target IL-18 may prove beneficial to the patients.

Author's disclosure of potential Conflicts of Interest (COI).
Toshihide Mimura, M.D.: Research funding, Chugai Pharmaceutical Co., Ltd.

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Marked Induction of c-Maf Protein during Th17 Cell Differentiation and Its Implication in Memory Th Cell Development*

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Until recently, effector T helper (Th) cells have been classified into two subsets, Th1 and Th2 cells. Since the discovery of Th17 cells, which produce IL-17, much attention has been given to Th17 cells, mainly because they have been implicated in the pathogenesis of various inflammatory diseases. We have performed transcriptome analysis combined with factor analysis and revealed that the expression level of c-Maf, which is considered to be important for Th2 differentiation, increases significantly during the course of Th17 differentiation. The IL-23 receptor (IL-23R), which is important for Th17 cells, is among putative transcriptional targets of c-Maf. Interestingly, the analysis of c-Maf transgenic Th cells revealed that the overexpression of c-Maf did not lead to the acceleration of the early stage of Th17 differentiation but rather to the expansion of memory phenotype cells, particularly with Th1 and Th17 traits. Consistently, mouse wild-type memory Th cells expressed higher mRNA levels of c-Maf, IL-23R, IL-17, and IFN-γ than control cells; in contrast, *Maf*$^{-/-}$ memory Th cells expressed lower mRNA levels of those molecules. Thus, we propose that c-Maf is important for the development of memory Th cells, particularly memory Th17 cells and Th1 cells.

Acquired immune responses have been divided into two major categories according to the cytokine-production patterns of T helper (Th) cells. Th1 cells produce abundant IFN-γ and play important roles in cellular immune responses. On the other hand, Th2 cells produce various cytokines involved in humoral immunity, such as IL-4. It has been a predominant concept that a skewed balance of Th1/Th2 responses could lead to pathological conditions like autoimmune diseases. Recently, Th17 cells have been discovered as the third type of effector Th cells that produce large amounts of proinflammatory cytokine IL-17A (IL-17) but only minimal amounts of IFN-γ or IL-4 (1, 2). Th17 cells have been shown to play important roles in the pathogenesis of various inflammatory disease models previously considered to be Th1 diseases, such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (3, 4). Thus, Th17 cells have been receiving considerable attention from the viewpoint of the pathological basis of human inflammatory diseases.

Initially, Th17 cells were believed to be differentiated in the presence of IL-23; however, it was thereafter reported that the differentiation factors for Th17 cells are actually TGF-β and IL-6 and that IL-23 is a proliferation factor in mice (5–7). On the other hand, the possibility has been raised that IL-23 is not a mere growth factor for Th17 cells but is important for the differentiation (8–10) and/or proper function (11) of these cells. Recently, TGF-β-independent but IL-23-, IL-6-, and IL-1β-dependent Th17 differentiation has been reported (12). These nonconventional Th17 cells may be more important than conventional TGF-dependent Th17 cells in inflammatory conditions such as experimental autoimmune encephalomyelitis.

In terms of the intracellular mechanisms of Th17 differentiation, Stat3 seems to play an essential role (13, 14). This is not surprising because Stat3 is activated by phosphorylation occurring downstream of IL-6 and IL-23 signaling. In 2006, RAR-related orphan receptor (ROR)[3]γt was reported to be a master regulator transcription factor for Th17 differentiation (15); it is a nuclear receptor the ligand of which is as yet unknown. Another nuclear receptor, RORα, was also implicated to function synergistically with RORγt in Th17 differentiation (16). The entire network of transcription factors in Th17 cells, however, remains to be elucidated. Thus, we first tried to shed light on the network and encountered the transcription factor c-Maf.

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[3] The abbreviations used are: ROR, RAR-related orphan receptor; qRT-PCR, quantitative RT-PCR; FC, fold change; cRNA, complementary RNA; IL-23R, IL-23 receptor; luc, luciferase; MARE, Maf recognition element; Tg, transgenic.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 mice were purchased from CLEA Japan, Inc., and T cell-specific c-Maf Tg mice (under the control of the human CD2 promoter and locus control region) were described previously (17). All the mice were maintained under specific pathogen-free conditions. All animal experiments were performed with the approval of the Animal Study Committee of Saitama Medical University and conformed to relevant guidelines and laws.

Th1/2/17 Cell Differentiation in Vitro—Mouse naive Th cells were purified from mouse spleens using a magnetic sorter and microbeads (AutoMACS system and $CD4^+CD62L^+$ T Cell Isolation Kit II, Miltenyi Biotec). They were cultured in RPMI 1640 medium containing 10% FCS (culture medium) and stimulated with plate-bound anti-CD3 and anti-CD28 mAbs (1 μg/ml each) for 3 days. Th1 cells were cultured with 10 ng/ml IL-12 and 10 μg/ml anti-IL-4 mAb; Th2 cells with 10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ mAb; and Th17 cells with 10 ng/ml each of IL-6 and IL-23, 3 ng/ml TGF-β and 10 μg/ml each of anti-IFN-γ and anti-IL-4 mAbs. All of the antibodies were purchased from BD Biosciences, and the cytokines were purchased from R&D Systems.

GeneChip Analysis—Total RNA was used for cDNA synthesis by reverse transcription followed by the synthesis of biotinylated cRNA through *in vitro* transcription. After cRNA fragmentation, we performed hybridization with a mouse A430 GeneChip (Affymetrix). The raw data were analyzed using Affymetrix Microarray Suite (version 5.0) and normalized.

qRT-PCR and ELISA—We performed qRT-PCR analysis using an ABI PRISM 7000 Sequence Detection System with TaqMan Gene Expression Assay probes. The GAPDH expression level was used as the internal control. As to the analysis of cytokine production of Th cells, the cells were stimulated with phorbol 12-myristate 13-acetate (40 ng/ml) and ionomycin (1 μg/ml) for 5 h in the culture medium. Then, RNA was extracted from the cells, and the supernatant was subjected to ELISA. The mouse IL-17 ELISA kit was from R&D Systems.

Intracellular Staining of Transcription Factors and Cytokines—Mouse Th cells differentiated *in vitro* were preincubated with an anti-mouse CD16/CD32 (Fcγ receptor) mAb for 15 min on ice to block nonspecific staining. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm (BD Bioscience) and stained with the primary Abs (anti-c-Maf Ab, M-153; anti-GATA-3 Ab, HG3-31, Santa Cruz Biotechnology). They were then stained with appropriate secondary Abs conjugated with Alexa Fluor 488 (Invitrogen). For intracellular cytokine staining, Th cells were cultured in the culture medium in the presence of phorbol 12-myristate 13-acetate (40 ng/ml) and ionomycin (1 μg/ml) for 5 h. In the last 1 h, monensin (Golgistop) was added. The cells were then fixed and permeabilized with BD Cytofix/Cytoperm and intracellularly stained with anti-IFN-γ-FITC plus anti-IL-17-PE, or anti-IFN-γ-FITC plus anti-IL-4-PE Abs (all reagents were from BD Biosciences). Stained cells were analyzed by FACScan or FACSCanto (BD Biosciences).

Factor Analysis—Transcription factors whose expression levels (normalized signals) were higher than 100 under at least one of the Th cell differentiation conditions were selected. Forty-three probes (including overlapping probes for the same genes) were subjected to factor analysis using SPSS software (version 15.0). By the unweighted least-squares method, two principal factors were extracted, and, after rotation by the varimax method with Kaiser normalization, each gene was positioned on a plane defined by Factors 1 and 2 according to its factor loadings.

Luciferase Assay—To construct the reporter plasmid pTA-*Il23r*-luc, the mouse *Il23r* promoter region (-1440 to +110) was ligated in the NheI and XhoI gap of the pTA-luc plasmid (Clontech). The following primers were used for PCR: 5′-GCT AGC TGG AGG CAT TTC CTC AGC TG-3′ (sense) and 5′-CTC GAG CTC AGG AAT TAG GGT CTC CT-3′ (antisense). A deletion mutant of pTA-*Il23r*-luc, which lacks a putative Maf binding site (MARE-like element) was constructed as described previously (18). DNA transfection and luciferase assays were performed as described previously (19, 20). Briefly, the reporter plasmid was transfected along with a c-Maf expression vector (21), a GATA-3 expression vector (22), and/or the control vector pcDNA3.1 into HEK293T cells using FuGENE 6 (Roche Applied Science). After 24 h, the cells were harvested, and luciferase activity was measured.

Statistical Analyses—*Error bars* indicate S.D. Student's *t* test was used for statistical analyses (*, $p < 0.05$ and **, $p < 0.01$).

RESULTS

Transcriptome Analysis of Th1/2/17 Cells during Course of Their Differentiation—First, we obtained the basic profiles of Th1/2/17 cell differentiation. Mouse naive Th cells were sorted and cultured under Th1, Th2, and Th17 cell differentiation conditions, and harvested on days 1 and 3. We also harvested Th0 cells on day 1. RNA extracted from the cells was subjected to transcriptome analysis using GeneChip. The expression levels of genes encoding IFN-γ, IL-4, and IL-17, which are "signature" cytokines produced by Th1, Th2, and Th17 cells, respectively, were up-regulated significantly in each subset, indicating that the differentiation conditions were appropriate (Fig. 1*A*).

Factor Analysis of Transcription Factors—To focus on transcription factors that are specifically up-regulated during the course of Th1/2/17 differentiation, we selected transcription factor genes whose expression levels (signals) were >100 in at least one of the Th conditions that we examined. Then, we performed factor analysis using the seven sets of data (days 1 and 3 for Th1/2/17 cells and day 1 for Th0 cells). Two principal "Factors" were extracted, and each gene is plotted in the two-dimensional space defined by Factors 1 and 2, according to its factor loading (Fig. 1*B*). As expected, dots representing RORγ (one dot) and RORα (four dots, because four different probes are attributed to RORα in the chip we used) were closely located in this diagram and both were high in Factor 2 but not in Factor 1. Interestingly, all the dots representing c-Maf were plotted nearest to the dot representing *Rorc*, suggesting that c-Maf is even more closely related to RORγ than RORα is to RORγ. Histograms of the GeneChip data of *Maf*, *Rorc*, and *Rora* are shown in Fig. 1*C*. Consistent with Fig. 1*B*, *Maf* and *Rorc* demonstrated a more Th17-specific expression pattern than *Rora*.

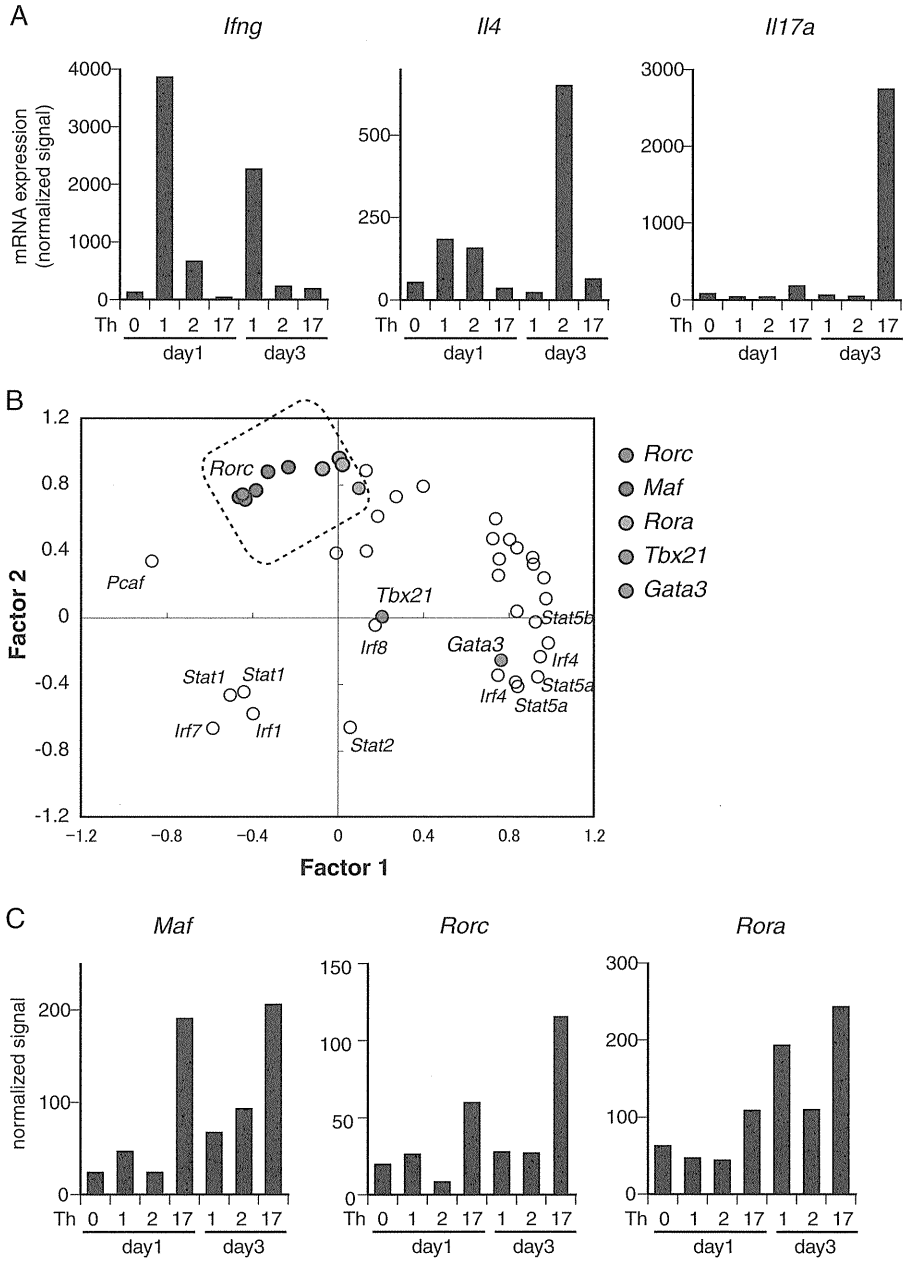

FIGURE 1. **Time series transcriptome analysis of genes representative of Th subsets at the mRNA level and factor analysis.** *A*, total RNA was obtained from Th cells cultured under the Th0, Th1, Th2, or Th17 conditions for 1 day (24 h) or 3 days (72 h). It was subjected to GeneChip analysis. As expected, IFN-γ, IL-4, and IL-17, the representative cytokines released from each Th subset, were induced in the relevant Th cells. *B*, distribution of transcription factors in a two-dimensional space defined by two factors extracted by factor analysis. Forty-three probes (including probes allotted to the same genes) were positioned in a plane defined by Factors 1 and 2. Note that all the probes representing RORγ, c-Maf, and RORα reside within a *box* with *dotted lines*. *C*, time series GeneChip data of *Maf* (data of one representative probe of five independent probes) and the known master regulator transcription factors for Th17 differentiation, namely, *Rorc* and *Rora* (data of one representative probe of four probes).

Expression of c-Maf but Not GATA-3 in Th17 Cells—The above data were rather unexpected in that c-Maf was reported to play important roles in Th2 differentiation (23). If c-Maf is highly expressed in Th17 cells, the question arises as to why they do not become Th2 cells. Indeed, similar levels of c-Maf protein expression were observed in the cells cultured under the Th2 and Th17 conditions (Fig. 2*A*, *left panel*). Interestingly, there was a large difference in GATA-3 expression level between the two subsets, whereas GATA-3 was barely detected in Th17 cells (Fig. 2*A*, *right panel*). This finding may explain why Th17 cells do not become Th2 cells. Indeed, IL-6 induces c-Maf by activating Stat3, which directly binds to the *Maf* promoter (24). GATA-3 is induced by IL-6, as well, but more indirect mechanisms are implicated (24). On the other hand, TGF-β, another important cytokine for the differentiation of mouse Th17 cells, is a potent inhibitor of Th2 development and has been shown to down-regulate GATA-3 (25). We added these cytokines to naive $CD4^+$ T cells separately or in combination and quantified the expression levels of two established markers of Th17 cells, IL-17 and IL-23R, in the screening for putative transcriptional targets of c-Maf. IL-6 alone induced *Il23r* but

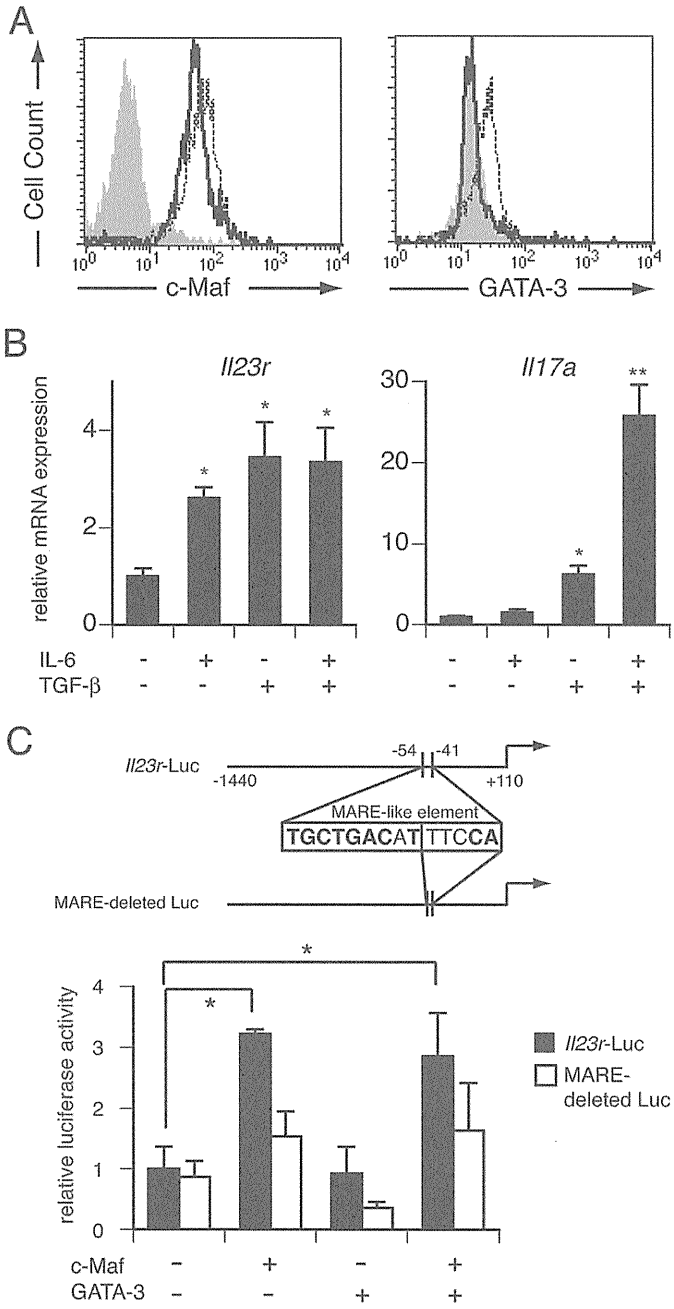

FIGURE 2. **Distinct expression patterns of c-Maf and GATA-3 in Th2 and Th17 cells and effects of these molecules on *Il23r* promoter.** *A*, flow cytometry analysis of c-Maf and GATA-3 expression in Th2 and Th17 cells. Although c-Maf expression was detected in both Th2 (*dotted line*) and Th17 (*solid line*) cells, GATA-3 expression was restricted to only Th2 cells. The *shaded areas* represent unstained Th2 cells (negative staining). *B*, qRT-PCR analysis of *Il23r* and *Il17a* expressions in naive $CD4^+$ T cells cultured for 3 days in the presence or absence of 10 ng/ml IL-6 and 3 ng/ml TGF-β. Anti-IFN-γ Ab and anti-IL-4 Ab (10 μg/ml each) were added to the culture. Either IL-6 or TGF-β is necessary for *Il23r* expression, but both cytokines are evidently necessary for significant *Il17a* induction. *C*, promoter analysis of *Il23r*. The 5′-flanking region of *Il23r* has a MARE-like sequence. (*Boldface letters* in the *box* correspond to MARE consensus sequences.) The forced expression of c-Maf had a positive effect on promoter activity, but that of GATA-3 did not. Furthermore, c-Maf did not significantly induce the luciferase activity of the deletion mutant lacking the MARE-like element, suggesting that c-Maf directly binds to this site. Data represent three independent experiments. *, $p < 0.05$.

it induced *Il17a* only slightly (Fig. 2*B*). Thus, *Il23r* is a candidate gene positively regulated by c-Maf.

Promoter Analysis of Putative c-Maf Target Genes—To obtain further insight, we performed luciferase promoter analysis using the promoters of the two genes. c-Maf overexpression induced the promoter activity of *Il23r*. c-Maf binds to a sequence called the Maf recognition element (MARE) (26). Indeed, the promoter region of *Il23r* contained a MARE-like sequence, and the deletion mutant luciferase vector that lacks the element lost responsiveness to c-Maf (Fig. 2*C*). Thus, c-Maf likely regulates Th17 proliferation via IL-23R induction. On the other hand, an ~1500-bp 5′ flanking region of *Il17a* responded to neither c-Maf nor GATA-3, suggesting that IL-17 is not a direct target of these factors (data not shown).

Ex Vivo Analysis of c-Maf Transgenic Mice—To confirm the hypothesis that c-Maf plays important role(s) in Th17 differentiation, we employed T cell-specific c-Maf Tg mice (17). As expected, the expression of IL-23R and IL-17 at the mRNA level was highly up-regulated in Tg Th cells. The expression of IFN-γ was also significantly up-regulated in Tg Th cells, but that of IL-4 was not (Fig. 3*A*). Flow cytometric analysis confirmed that the numbers of both IFN-γ-positive cells and IL-17-positive cells, but not that of IL-4-positive cells, were increased among Tg Th cells (Fig. 3*B*). To our surprise, although c-Maf Tg Th cells produced significantly more IL-17 than control cells *in vitro*, this difference disappeared when naive Th cells were sorted and stimulated under the same condition (Fig. 3*C*). This unexpected finding seems to be derived from the fact that most of the c-Maf Tg Th cells from the spleen demonstrate an effector-memory phenotype ($CD62L^{low}$ $CD44^{high}$, Fig. 3*D*). These results suggest that c-Maf does not play an essential role in the early differentiation of Th17 cells but rather in the development and/or maintenance of memory Th cells.

Analysis of WT Memory Phenotype Th Cells—The above findings on Tg Th cells prompted us to analyze WT memory Th cells. Under specific pathogen-free conditions, nearly 80% of the Th cells from the spleens of WT mice demonstrated the naive phenotype ($CD62L^{high}$ $CD44^{low}$, Fig. 4*A*, *left panel*). To facilitate the analysis of memory phenotype WT Th cells, we utilized the system of homeostatic expansion; when naive T cells are transferred into lymphopenic mice, they proliferate vigorously and acquire the memory phenotype (27). Thus, we transferred WT Th cells into Rag-2-deficient mice. In 4 weeks, most of the splenic Th cells from the recipient mice acquired an effector-memory phenotype ($CD62L^{low}$ $CD44^{high}$, Fig. 4*A*, *right panel*). When compared with WT naive Th cells, these cells expressed significantly higher mRNA levels of *Maf* and *Il23r* (Fig. 4*B*). Similar to the results in Fig. 3, these cells also expressed higher levels of *Il17a* and *Ifng* mRNAs, but not *Il4* mRNA (Fig. 4*C*). Consistent with the data, flow cytometric analysis revealed more IFN-γ-positive and IL-17-positive cells than WT naive cells (Fig. 4*D* and data not shown). Interestingly, an IFN-γ/IL-17 double-positive population was evident (IL-17 single-positive cells and IFN-γ/IL-17 double-positive cells were present at similar levels). On the other hand, IL-4-positive cells were scarcely observed.

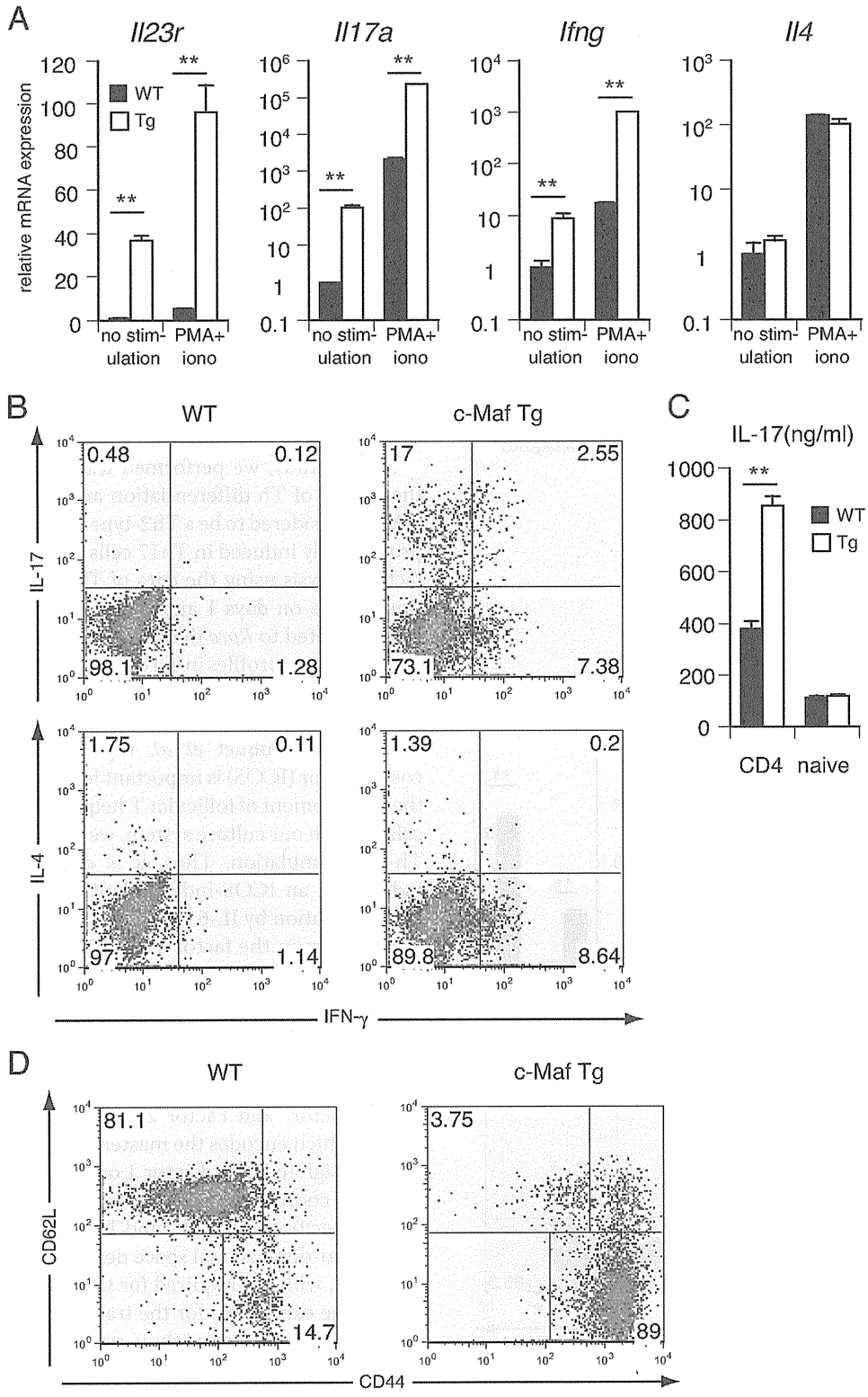

FIGURE 3. ***Ex vivo* gain-of-function analysis of c-Maf.** *A*, comparison between freshly isolated WT (*black bars*) and littermate c-Maf Tg (*white bars*) Th cells of the expression of Th differentiation markers. *B*, flow cytometric analysis of Th cells from c-Maf Tg and littermate WT mice. c-Maf Tg Th cells express more IL-17 and IFN-γ, but not IL-4, than control cells. *C*, IL-17 production from WT (*black bars*) and c-Maf Tg (*white bars*) Th cells or $CD62L^{high}$ naive Th cells stimulated *in vitro* under Th17 differentiation condition for 2 days. IL-17 released into the culture supernatant was detected by ELISA. *D*, flow cytometric analysis of splenocytes from WT and c-Maf Tg mice gated on $CD4^+$ population. Single-cell suspensions of splenocytes were stained with anti-CD4-FITC, anti-CD44-PE, and anti-CD62L-APC mAbs and analyzed by FACSCanto. 7-Amino-Actinomycin D was used to separate dead cells. Similar results were obtained in three independent experiments. **, $p < 0.01$.

Ex Vivo Analysis of c-Maf-deficient Th Cells—As c-Maf deficient mice are embryonic lethal, we generated bone marrow chimeras by injecting $Maf^{-/-}$ fetal liver cells containing hematopoietic stem cells into irradiated Rag-2-deficient mice. Six weeks later, the donor mice were sacrificed, and flow cytometric analysis of splenocytes was performed. The ratio of effector-

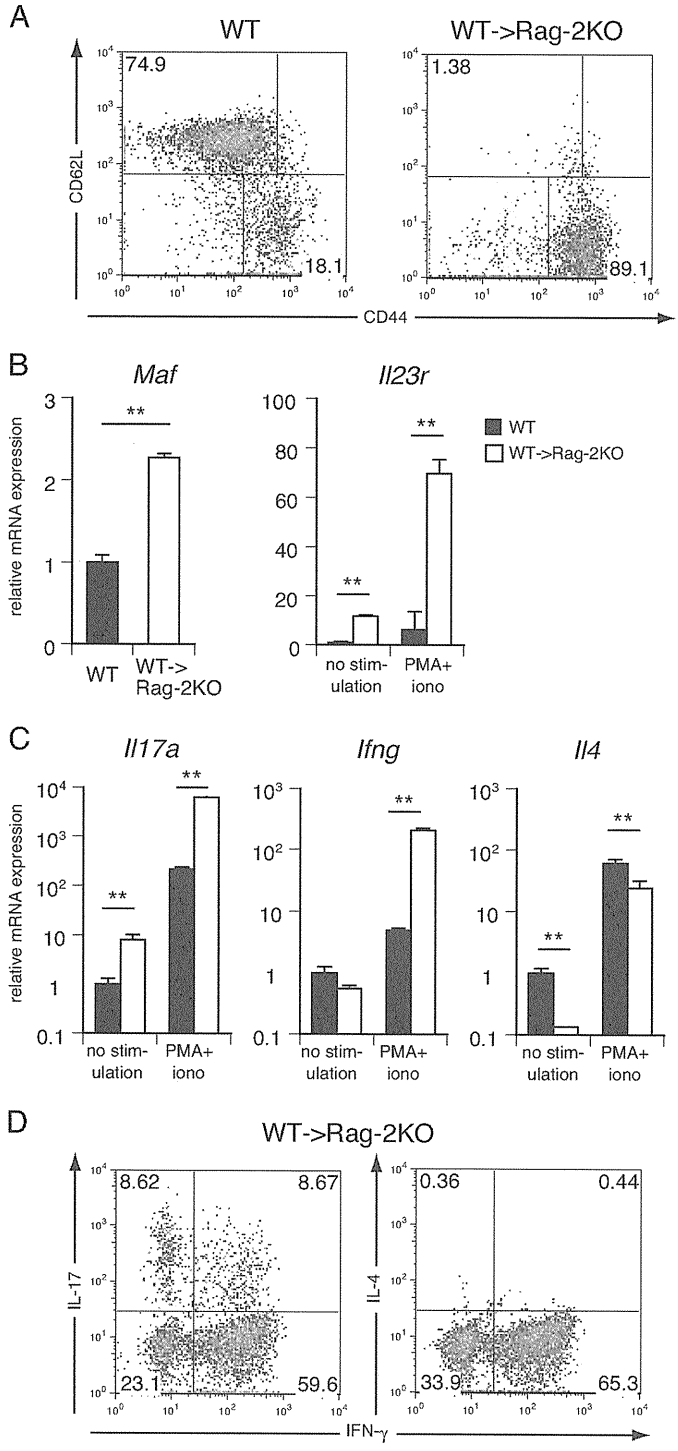

FIGURE 4. **Analysis of WT naive Th cells and memory Th cells derived from Rag-2-deficient mice transferred with WT Th cells.** *A*, flow cytometry of WT splenocytes and splenocytes from Rag-2-deficient mice transferred with WT Th cells (10^6/mouse) 4 weeks before analysis. An analysis similar to that shown in Fig. 3*D* was performed. Note that nearly 90% of the $CD4^+$ cells became $CD44^{high}$ $CD62L^{low}$, leaving only about 1% $CD44^{low}$ $CD62L^{high}$ cells. *B* and *C*, qRT-PCR analysis of the expressions of *Maf* and *Il23r* in naive (*black bars*) and memory (*white bars*) Th cells (*B*) and those of *Il17a*, *Ifng*, and *Il4* (*C*). *D*, flow cytometric analysis of memory phenotype Th cells. IL-17-positive and IFN-γ-positive populations were evident, but IL-4-positive one was not. Data represent three independent experiments. *PMA*, phorbol 12-myristate 13-acetate; *iono*, ionomycin. **, $p < 0.01$.

memory phenotype Th cells ($CD62L^{low}$ $CD44^{high}$) was not significantly different from that of WT Th cells (data not shown). As $Maf^{-/-}$-naive Th cells were shown to have the capacity to differentiate into Th17 cells *in vitro* (28), we decided to analyze the memory-phenotype c-Maf-deficient Th cells by transferring sorted splenic Th cells to Rag-2-deficient mice as in the experiment whose results are shown in Fig. 4 (Fig. 5*A*). In 4 weeks, most of the transferred Th cells acquired an effector-memory phenotype, and these cells were analyzed by qRT-PCR for the expressions of cytokines and *Il23r*. As expected, *Il23r*, *Il17a*, and *Ifng* expression levels were significantly lower in these cells than in the control WT cells. The expression level of *Il21*, which was reported to be a transcriptional target of c-Maf, was also lower (Fig. 5*B*).

DISCUSSION

In this study, we performed transcriptome analysis during the course of Th differentiation and unexpectedly found that c-Maf, considered to be a Th2-type transcription factor, is more prominently induced in Th17 cells than in Th2 cell. Moreover, factor analysis using the data of Th1-, Th2- and Th17-conditioned cells on days 1 and 3 demonstrated that *Maf* is more closely related to *Rorc* than *Rora* is to *Rorc*, at least in terms of the expression profiles in Th cells. As both RORγ and RORα are considered to play essential roles in Th17 differentiation (16), it occurred to us that c-Maf may also play an important role in Th17 cells. Bauquet *et al.* reported that inducible T cell costimulator (ICOS) is important for the expression of c-Maf in the development of follicular T helper cells (Tfh cells) and Th17 cells (28). In our culture system, we did not use ICOS-ligand for Th-cell stimulation. Thus, it is obvious that c-Maf can be induced in an ICOS-independent manner, probably through Stat3 activation by IL-6 (24).

As shown in the factor analysis results (Fig. 1*B*), *Maf*, *Rorc* and *Rora* were all high in Factor 2 but low in Factor 1. In contrast, *Gata3*, which encodes the master regulator transcription factor of Th2 cells, was very high in Factor 1 but low in Factor 2, and so was *Stat5a*, which is implicated in Th2 differentiation (29, 30). From these data, we may safely call Factor 1 "a Th2-related factor" and Factor 2 "a Th17-related factor". Indeed, *Tbx21*, which encodes the master regulator of Th1 cells, T-bet, was not high in either Factor 1 or Factor 2 and was positioned near the coordinate origin. Thus, these transcription factor groups specific to Th1, Th2 and Th17 cells are separately placed on the two-dimensional space defined by Factors 1 and 2.

IL-23R, which is required for the expansion of Th17 cells, is among the candidates for the transcriptional targets of c-Maf. Indeed, the promoter analysis revealed that MARE located in the 5′-prime lesion of *Il23r* is important for the luciferase activity induced by c-Maf overexpression, indicating that IL-23R is a direct target of c-Maf (Fig. 2*C*). IL-21 (28) and IL-10 (31) have also recently been reported to be targets of c-Maf in the context of Th17 differentiation. The analysis of c-Maf Tg mice, however, demonstrated that the overexpression of c-Maf did not seem to accelerate the early stage of Th17 differentiation (Fig. 3*C*). This finding is consistent with that of Bauquet *et al.*, in which c-Maf-deficient Th cells were capable of producing IL-17, although at a lower level (28). Instead, the significant

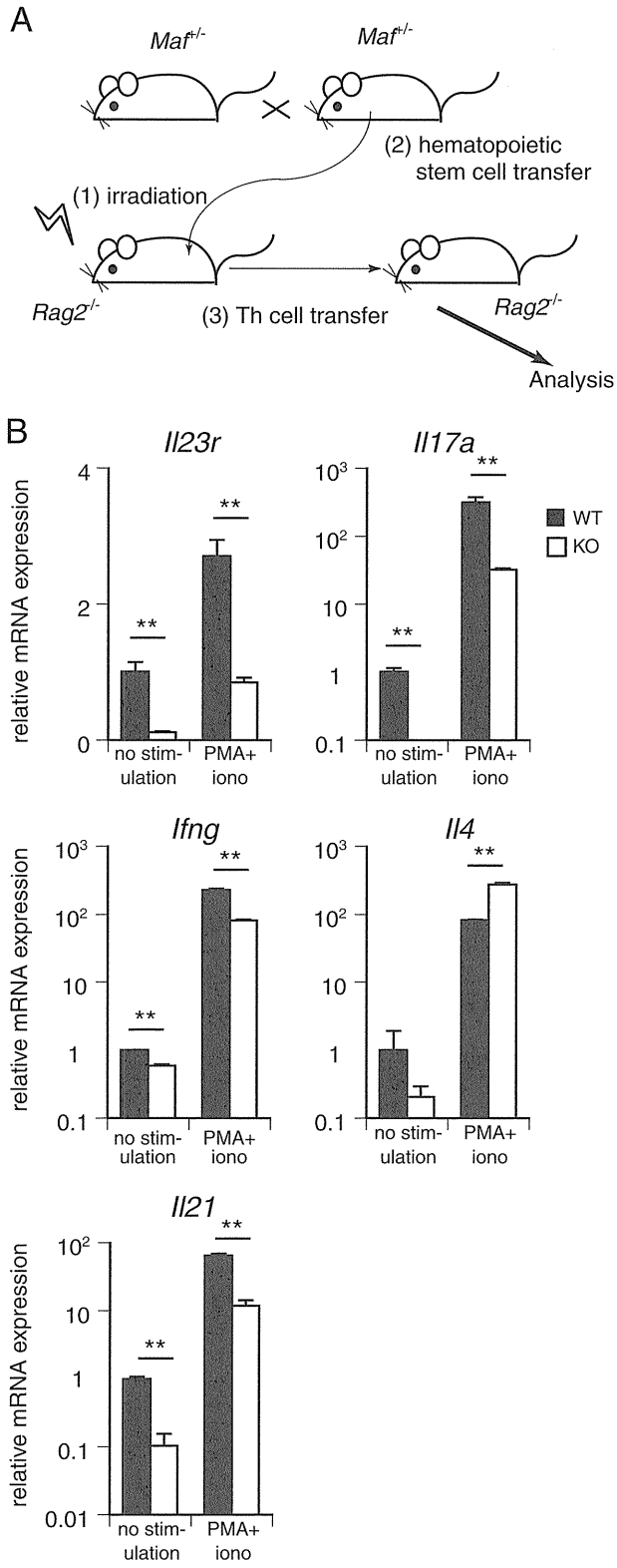

FIGURE 5. ***Ex vivo* loss-of-function analysis of c-Maf utilizing bone marrow chimera.** *A*, schematic diagram of producing bone marrow chimera and successive transfer of Th cells into Rag-2 knock-out mice. *B*, qRT-PCR analysis of the expressions of *Il23r*, *Il17a*, *Ifng*, *Il4*, and *Il21* in $Maf^{-/-}$ memory Th cells and control cells. Data represent three independent experiments. *PMA+ iono*, phorbol 12-myristate 13-acetate + ionomycin. **, $p < 0.01$.

deviation of Th cells toward the memory phenotype was observed in the Tg mice, suggesting that c-Maf may play a role in the development and/or maintenance of memory Th cells. The fact that WT mouse memory Th cells express higher mRNA levels of not only *Maf*, *Il23r* and *Il17a* but also *Ifng* than non-memory cells suggests that c-Maf indeed plays a role in memory Th17 and Th1 cells (Fig. 4*B* and *C*). In the earliest studies of Th cells and IL-17, it was memory Th cell that was shown to mainly produce the cytokine (32) particularly in response to IL-23 (33). It was not until the discovery of Th17 cells that these IL-17-producing cells were considered to be distinct from Th1 cells. Recently, however, Th cells that produce both IFN-γ and IL-17 have been gathering attention particularly in the context of inflammation, making the difference between Th1 and Th17 subsets less clear again (34–36). Although the relationship between memory Th cells and Th17 cells differentiated *in vitro* seems to be close in that both subsets express IL-23R, we have yet to determine whether Th17 cells differentiated *in vitro* are indeed the precursors of memory Th cells *in vivo*. If that is the case, c-Maf, which is expressed highly in both *de novo* Th17 cells and memory Th cell, may be a transcription factor that mediates the differentiation of the former into the latter.

In regard to this point, it is interesting that an IFN-γ/IL-17 double-positive population was evident in memory phenotype Th cells (Fig. 4*D*), which was not so apparent in Maf Tg cells. It is likely that the forced expression of c-Maf is sufficient for the expression of surface markers of memory Th cells, but not sufficient for the differentiation of IFN-γ/IL-17 double-positive Th cells.

The homeostatic expansion and induction of memory phenotype Th cells are important in a variety of clinical situations, such as during immunosuppressive therapy or chemotherapy. There is little doubt that the expansion of memory phenotype Th cells plays an important role in the defense against numerous pathogens, for example, those residing in the gut. At the same time, such expansion also bears the risk of autoinflammation, causing damage to self tissues. It has been shown that adoptive transfer of naive Th cells into lymphopenic hosts induces inflammatory bowel disease, which can be prevented by cotransfer of regulatory T (Treg) cells (37). Th1 response is implicated in the pathogenesis, whereas the role Th17 cells play in this disease model is still controversial (38). In either case, Treg cells seem to be essential for controlling the excessive response of homeostatically expanding Th cells.

It was unexpected that c-Maf Tg Th cells did not produce more IL-4 than WT cells (Fig. 3*A* and *B*), although c-Maf was reported to play an important role in IL-4 production (39). Consistently, the expression of *Il4* was not reduced in $Maf^{-/-}$ Th cells than in WT cells, either (Fig. 5*B*). These results may be explained by the fact that the Th1 cytokine IFN-γ strongly inhibits Th2 differentiation (40). Consistently, c-Maf Tg $CD62L^{low}$ Th cells caused Th1-mediated colitis in Rag-2-deficient mice whereas WT $CD62L^{low}$ Th cells did not (41). Thus, it is possible that the overexpression of c-Maf tips the balance of Th response toward Th1 rather than Th2 type under a neutral condition. In fact, c-Maf Tg Th cells produce more IL-4 than WT cells when cultured under a Th2 condition in the presence

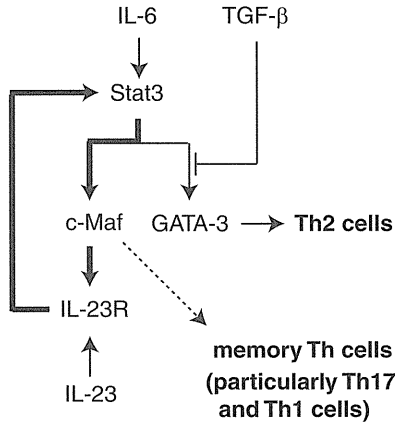

FIGURE 6. **Schematic diagram of Th cell differentiation.** IL-6 induces both c-Maf and GATA-3 via Stat3 phosphorylation and activation. GATA-3 expression can lead to Th2 differentiation, but this route is blocked in the presence of TGF-β, which inhibits GATA-3 expression. c-Maf induces IL-23R, and then IL-23 signaling activates Stat3, constituting a positive feedback loop (*boldface lines*). Although this loop does not seem to be essential to early Th17 differentiation, it may play an important role in the development and/or maintenance of memory Th cells, particularly memory Th17 cells.

of IL-4 and anti-IFN-γ antibody (in other words, in the absence of IFN-γ, data not shown). A schematic of Th cell differentiation is shown in Fig. 6; IL-6 induces c-Maf expression via Stat3 phosphorylation and is particularly important for the induction of IL-23R, which in turn augments Stat3 phosphorylation, constituting a novel positive feedback loop that leads to the differentiation of memory Th17 cells. On the other hand, TGF-β is likely involved in the inhibition of GATA-3 induction by IL-6, thereby blocking the pathway for Th cells to differentiate into Th2 cells.

c-Maf has also been implicated in the differentiation of other Th cell types, including Tfh cells (28) and regulatory type 1 (Tr1) cells (42). Our study indicates that this versatile transcription factor is also involved in the development and/or maintenance of memory Th (particularly Th17) cells. Clarification of the mechanisms of memory Th cell development in the context of c-Maf induction would be beneficial in the understanding of pathophysiology of various autoimmune inflammatory diseases.

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Geranylgeranyl-pyrophosphate regulates secretion of pentraxin 3 and monocyte chemoattractant protein-1 from rheumatoid fibroblast-like synoviocytes in distinct manners

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Abstract

Objectives

We previously reported that 10 mg/day of simvastatin significantly reduced clinical scores of rheumatoid arthritis (RA) in active RA patients with hypercholesterolemia. In this study, we have investigated the mechanism by which simvastatin inhibits the production of the mediators of inflammation, such as pentraxin 3 (PTX3) and monocyte chemoattractant protein-1 (MCP-1), from fibroblast-like synoviocytes (FLS) derived from patients with RA.

Methods

FLS from RA patients were cultured with 0-10 μM simvastatin for 24 h. ELISA and real-time PCR were used to quantitate the protein level and the mRNA level of PTX3 and MCP-1, respectively.

Results

Simvastatin both reduced the secretion of PTX3 and MCP-1 in FLS cultures and inhibited their mRNA expression in these cells. The effects of simvastatin were all completely reversed in the presence of mevalonic acid or geranylgeranyl-pyrophosphate, but not in the presence of farnesyl-pyrophosphate. The geranylgeranyl transferase inhibitor GGTI-298 and the Rho kinase inhibitor Y-27632 inhibited the production of PTX3 but not of MCP-1.

Conclusions

Although simvastatin inhibited the production of PTX3 and MCP-1 in RA FLS, the mechanisms were quite different. It inhibits PTX3 production in a Rho-dependent manner but MCP-1 production in a Rho-independent manner. These results shed light on novel aspects of the anti-inflammatory mechanisms of simvastatin and may prove its important role in the treatment of rheumatic diseases.

Key words

rheumatoid arthritis, fibroblast-like synoviocytes, 3-hydroxy-3-methylglutaryl co-enzyme A reductase inibitor, pentraxin 3, monocyte chemoattractant protein-1, small G protein

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Introduction

Pentraxins are a family of evolutionarily conserved, soluble and multifunctional pattern recognition proteins characterised by a cyclic multimeric structure (1). Pentraxins are divided into two groups: short pentraxins and long pentraxins. The short pentraxins consist of C-reactive protein (CRP) and serum amyloid P (SAP) (2). The prototype protein in the long pentraxin group is pentraxin 3 (PTX3). PTX3 has been suggested to play important roles in innate resistance to pathogens, the regulation of inflammatory reactions and the clearance of apoptotic cells (3). While short pentraxins are produced mainly in the liver (4), PTX3 is made by diverse types of cells, including endothelial cells, macrophages and fibroblasts, in response to inflammatory signals (1). Recently, PTX3 levels have been shown to be increased in synovial fluid and synovial tissue from patients with rheumatoid arthritis (RA) (5).

The 3-hydroxyl-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, statins, have shown to reduce both morbidity and mortality in many clinical trials (6, 7). Many studies have also demonstrated a wide range of their effects on cells and tissues involved in inflammation and/or autoimmunity. For example, statins attenuated the expression of interferon-γ-induced class II major histocompatibility complex molecules, via the class II transactivator protein, in a variety of cells, such as endothelial cells and monocytes/macrophages, and thus to inhibit T cell activation (8). It has been reported that lovastatin and simvastatin inhibit the interactions between leukocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 by binding to a specific recognition site on LFA-1, which is independent of their HMG-CoA reductase activity (9).

HMG-CoA reductase, a target enzyme of statins, catalyses the conversion of HMG-CoA to mevalonic acid (MVA) (10). Inhibiting MVA synthesis results in a reduced pool of isoprenoids, such as geranylgeranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP), which are involved in the post-translational modification of small GTP-binding proteins, including members of the Ras and Rho families, *i.e.*, Rho, Rac and Cdc42. Thus, MVA is considered to antagonise the whole effects of statins. On the other hand, FPP rescues the activation of Ras in the presence of statins. GGPP rescues the activation of Rho family members by way of geranylgeranyl transferase type I (GGTase I), which is inhibited by GGTI-298.

Prenylation of small GTP-binding proteins with farnesyl or geranylgeranyl groups is required for their localisation within cell membranes and hence for their function (11). Ras is farnesylated with FPP, and Rho family members are geranylgeranylated with GGPP and act as pivotal regulators of several signalling networks affecting actin cytoskeleton dynamics, transcriptional regulation, cell cycle progression and membrane trafficking (11). The key enzyme of post-translational modifications of the Rho family is GGTase I (11), which can be selectively inhibited by peptidomimetic inhibitors, such as GGTI-298 (12). When cells are stimulated, geranylgeranylated Rho binds to specific effectors to exert its biological functions, which can be specifically inhibited by Y-27632, a Rho kinase inhibitor (13).

We have previously shown that simvastatin significantly improved clinical indicators in RA patients with active disease (14, 15). We have also reported recently that pharmacological concentrations of simvastatin inhibit production of interleukin 6 (IL-6) and IL-8 and cell proliferation induced by tumour necrosis factor-α (TNF-α) in fibroblast-like synoviocytes (FLS) from patients with RA (16) and that high concentration simvastatin induces apoptosis in FLS from patients with RA (17).

In the present study, we have investigated the effect of simvastatin on the production of PTX3 and compared the mechanism with that on the production of monocyte chemoattractant protein-1 (MCP-1) by FLS derived from patients with RA.

Materials and methods

Reagents

Simvastatin was kindly provided by Merck & Co. Inc. (Rahway, NJ, USA).

A 10 mM stock solution was prepared as previously described (16). Briefly, 4 mg simvastatin was dissolved in 100 µl of ethanol and 150 µl of 0.1 N NaOH and incubated at 50°C for 2 h; the pH was adjusted to 7.0 and the volume to 1.0 ml. A control solution without simvastatin was prepared in the same way. Other chemicals and materials were purchased from the following sources: RPMI 1640 medium, fetal calf serum (FCS), penicillin and streptomycin were from Invitrogen (Carlsbad, CA, USA); trypsin/EDTA, collagenase, hyaluronidase, mevalonic acid (MVA), farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP) were from Sigma (St. Louis, MO, USA); GGTI-298 and Y-27632 were from Calbiochem (Schwalbach, Germany). We previously used GGTI-298 at the concentration of 5 to 15 µM and Y-27632 at 3 to 10 µM (17). We therefore originally chose similar concentrations. Y-27632, however, did not affect the production of MCP-1 and PTX-3 at the chosen concentrations. We therefore decided to use Y-27632 at higher concentrations (30-60 µM). Total RNA samples from cultured FLS were purified using the RNeasy mini-kit from QIAGEN GmbH (Hilden, Germany). Taqman reverse transcription reagents, Taqman universal PCR master mix, TaqMan Gene Expression Assays for PTX3 and Taqman Pre-Developed Assay Reagents for MCP-1 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were from Applied Biosystems (Foster City, CA, USA). ELISA kits for PTX3 and MCP-1 were purchased from Perseus Proteomics Inc. (Tokyo, Japan) and GE Healthcare (Buckinghamshire, UK), respectively.

Preparation and culture of FLS

Synovial tissues were obtained from 8 RA and 3 osteoarthritis (OA) patients who underwent joint replacement at Saitama Medical University. The patients with RA fulfilled the revised 1987 American College of Rheumatology differentiation criteria. A written informed consent, approved by the ethics committee at Saitama Medical University, was obtained from each patient, prior to the experiment. FLS were isolated and cultured as previously described (16). Briefly, synovial tissue was minced into small pieces and shaken for 2 h at 37°C in RPMI 1640 containing 0.15 mg/ml collagenase and 0.04% hyaluronidase. After removing tissue debris using a 70 µm nylon mesh, the cells were cultured in RPMI 1640 containing 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified 5% CO_2 incubator at 37°C. Non-adherent cells were removed the next day and adherent cells were maintained as FLS. Sub-confluent cells were sub-cultured using trypsin/EDTA and plated in 24-well plates or 100-mm culture dishes and again grown to sub-confluence. Experiments were carried out in RPMI 1640 containing 1% FCS using FLS from passages three to seven in a humidified 5% CO_2 incubator at 37°C.

Quantitative PCR

Total RNA was extracted from FLS, using RNeasy Mini Kits according to the manufacturer's instructions. Total RNA samples were reverse transcribed using Taqman Reverse Transcription Reagents. The mRNA expression level was determined using a Taqman PCR system and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). PTX3 and MCP-1 mRNAs were detected using TaqMan Gene Expression Assays and Taqman Pre-Developed Assay Reagent kits, respectively. GAPDH was amplified simultaneously and used for standardisation. PCR reactions consisted of 25 µl 2x Taqman Universal PCR Master Mix, 2.5 µl 20x target primers and probe, 2.5 µl 20x control primers and probe and 300 ng cDNA, and were made up to 50 µl with RNase-free water. The conditions for quantitative PCR were; 95°C for 10 min, followed by 40 cycles of 2-step PCR, including denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. For quantitative mRNA analysis for PTX3 and MCP-1, changes in the reporter fluorescence from each reaction well were evaluated. For each gene, the threshold cycle (Ct) was defined as the PCR cycle at which fluorescence rose above baseline. The difference between the threshold cycle of the target gene and that of the control GAPDH gene gave the standardised expression level, ΔCt. The difference between ΔCt values for nontreated and simvastatin-treated FLS gave the ΔΔCt value, which was used to calculate relative expression level in simvastatin-treated FLS as $2^{-\Delta\Delta Ct}$. The expression level of each gene was interpreted as fold-increase in simvastatin-treated FLS compared with that in nontreated controls (16).

ELISA for PTX3 and MCP-1

The detection of PTX3 and MCP-1 proteins in cell supernatants was performed with PTX3 and MCP-1 ELISA kits according to the manufacturer's instructions.

Statistical analysis

All data were expressed as means ± standard error (SEM). Statistical analysis used the Mann-Whitney U-test with p-values <0.05 considered as significant.

Results

Inhibitory effects of simvastatin on PTX3 production in FLS

As shown in Figure 1A, the levels of PTX3 secreted by RA FLS after 24 h-culture in the absence of simvastatin were significantly higher than those with OA. When FLS from RA patients were incubated for 24 h with simvastatin, PTX3 in culture supernatants was reduced significantly, to 70.0±2.0% of control in the presence of 1.0 µM simvastatin and 62.0±6.7% of control in the presence of 10 µM simvastatin (Fig. 1B). The expression of PTX3 mRNA was also reduced in cultures treated with simvastatin, to 62.3±11.0%, 38.8±5.0% and 14.3±1.2%, compared with control, in the presence of 0.1 µM, 1.0 µM and 10 µM simvastatin, respectively (Fig. 1C).

MVA and GGPP restore the production of PTX3 in FLS in the presence of simvastatin

As shown in Figure 2A, the inhibitory effect of 1.0 µM simvastatin on PTX3 production in RA FLS was suppressed when the cells were simultaneously treated with 100 µM MVA or 10 µM GGPP, but unaffected with FPP. MVA, GGPP and FPP had minimal effects

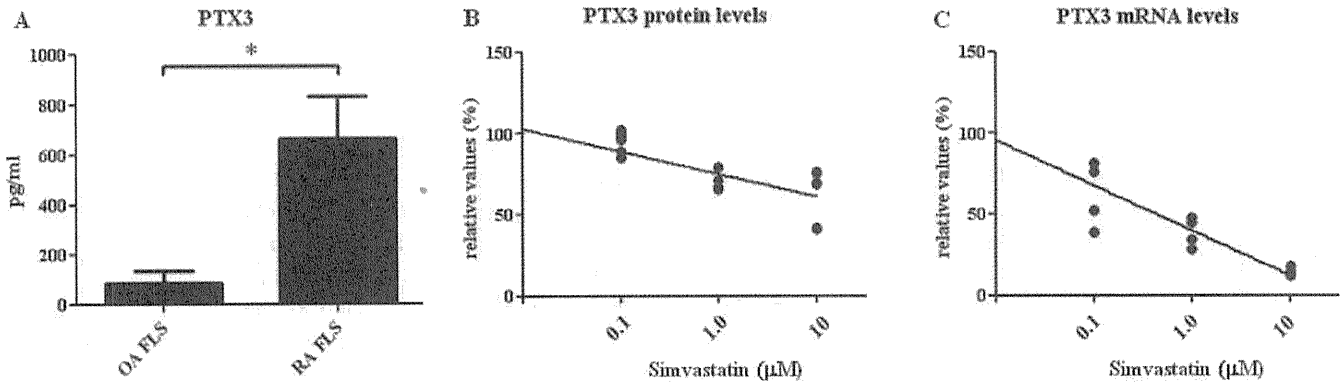

Fig. 1. RA FLS specifically produced PTX3, which was suppressed by simvastatin.
A. FLS from patients with RA or OA were incubated without simvastatin for 24 h. PTX3 levels in culture supernatants were determined by ELISA. Data are expressed as means ± SEM of duplicate experiments (n=6); $^{*}p<0.05$ versus control. **B, C.** The expression of PTX3 was reduced by simvastatin in a dose-dependent manner both at the protein level (**B**, n=6) and at the mRNA level (**C**, n=4). FLS from RA patients were incubated with 0.1–10 μM simvastatin for 24 h. Data shown are values relative to control cells cultured in the absence of simvastatin. Tendency lines are also shown.

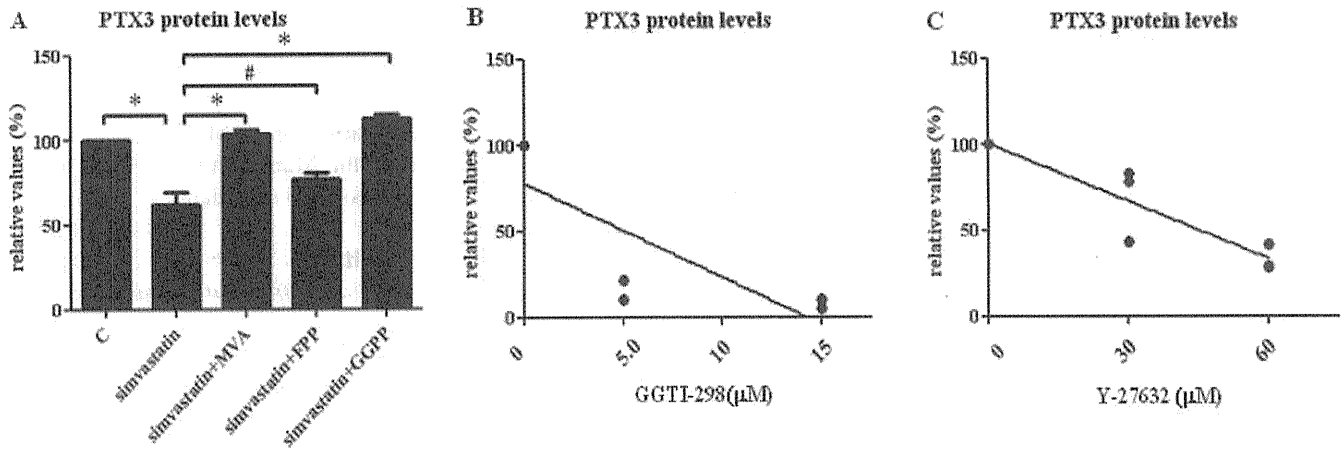

Fig. 2. Effects of MVA and isoprenoids on the simvastatin-mediated suppression of PTX3 production by FLS.
A. MVA and GGPP attenuated suppression of PTX3 production by simvastatin in RA FLS. FLS were incubated with 0.1 - 10 μM simvastatin for 24 h. PTX3 levels in culture supernatants were determined by ELISA (n=6); $^{*}p<0.05$ versus control, $^{\#}$ not significant. **B, C.** An inhibitor of geranylgeranylation, GGTI-298 (**B**), and a Rho kinase inhibitor, Y-27632 (**C**), suppressed PTX3 production in RA FLS in a dose-dependent manner. FLS were incubated with 5.0 or 15 μM GGTI-298 or with 30 or 60 μM Y-27632 for 24 h. Tendency lines are shown.

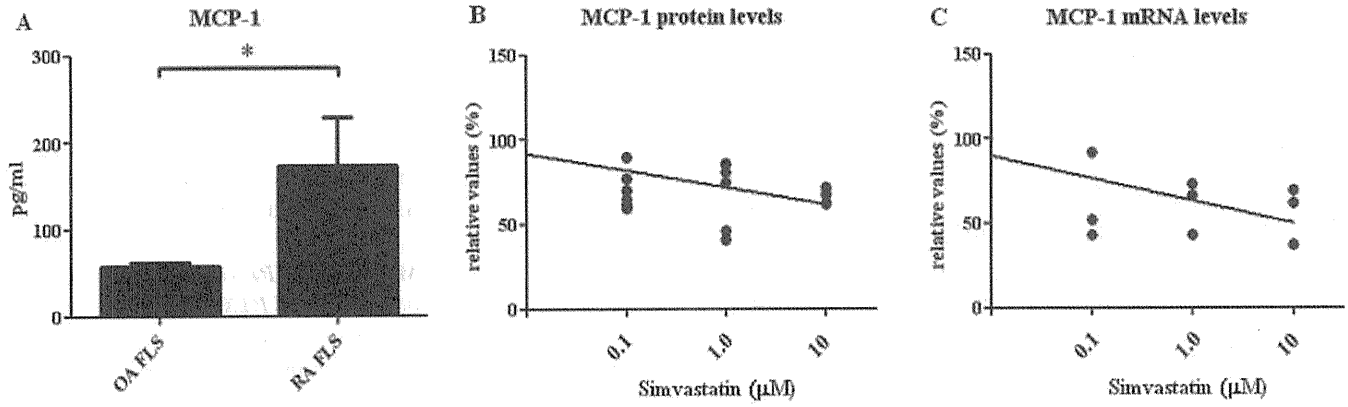

Fig. 3. RA FLS specifically produced MCP-1, which was suppressed by simvastatin.
A. FLS from patients with RA or OA were incubated without simvastatin for 24 h. MCP-1 levels in culture supernatants were determined by ELISA (n = 6); $^{*}p<0.05$ versus control. **B, C.** The expression of MCP-1 was suppressed by simvastatin in a dose-dependent manner both at the protein level (**B**, n=6) and at the mRNA level (**C**, n=3). FLS from RA patients were incubated with 0.1 - 10 μM simvastatin for 24 h. Data shown are values relative to control cells cultured in the absence of simvastatin. Tendency lines are shown.