EXTENDED REPORT

The JAK inhibitor, tofacitinib, reduces the T cell stimulatory capacity of human monocyte-derived dendritic cells

Satoshi Kubo, Kunihiro Yamaoka, Masahiro Kondo, Kaoru Yamagata, Jidong Zhao, Shigeru Iwata, Yoshiya Tanaka

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

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

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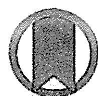

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ABSTRACT

Objective Tofacitinib, which is a Janus kinase (JAK) inhibitor, has shown clinical effects in the treatment of rheumatoid arthritis. JAKs are important kinases in lymphocyte differentiation; however, their function in dendritic cells (DCs) is unknown. In this study, the function of JAKs in DCs was investigated with tofacitinib.
Methods The effects of tofacitinib on the maturation of human monocyte-derived DCs induced by lipopolysaccharide (LPS) stimulation were investigated. In addition, its effects on T cell stimulatory capability was investigated by coculturing with naïve CD45RA-positive T cells.
Results Tofacitinib decreased expression of CD80/CD86 in a concentration-dependent manner in LPS-stimulated DCs; however, it did not affect HLA-DR expression. Tofacitinib suppressed tumour necrosis factor, interleukin (IL)-6 and IL-1β production without affecting transforming growth factor (TGF)-β and IL-10 production. Meanwhile, CD80/CD86 expression in DCs was enhanced by type I interferon (IFN) stimulation, and the LPS-induced CD80/CD86 expression was inhibited by an antibody to type I IFN receptor. Furthermore, tofacitinib suppressed production of type I IFN and activation of interferon regulatory factor (IRF)-7, which is a transcription factor involved in CD80/CD86 and type I IFN expression. Tofacitinib also decreased the T cell stimulatory capability of DCs and increased expression of indoleamine 2,3-dioxygenase (IDO)-1 and IDO-2.
Conclusions Tofacitinib, a JAK1/JAK3 inhibitor, affected the activities of human DCs. It decreased CD80/CD86 expression and T cell stimulatory capability through suppression of type I IFN signalling. These results suggest a novel mode of action for tofacitinib and a pivotal role for JAKs in the differentiation of DCs.

INTRODUCTION

Janus kinase (JAK) family members, constitutively bound to cytokine receptors, play an important role in the biological activation of cytokines through activation of the signal transducer and activator of transcription (STAT), which is a transcription factor. The JAK family consists of JAK1, JAK2, JAK3 and tyrosine kinase (TYK)2. Different JAK family members are activated by different cytokine receptors. JAK1 is activated by the class 1, class 2 and γc cytokines, while JAK3 is activated by γc cytokines. Therefore, JAK family members are not only essential for immune function, but they also play an important role in inflammation response.[1–4] Tofacitinib, which is selective for JAK1 and JAK3,[5 6] is effective for patients with rheumatoid arthritis (RA).[7–10] This finding supports the notion that JAK1 and JAK3 play an important role in autoimmune diseases. Furthermore, it is thought that elucidation of the mode of action of JAK family members in vivo will lead to a better understanding and treatment of autoimmune diseases.

Dendritic cells (DCs) play a key role in bridging natural immunity and acquired immunity. Immature DCs are potent phagocytes, and they mature through toll-like receptor (TLR) signalling. They also show antigen-specific T-cell activation abilities, which are accompanied by induction of expression of major histocompatibility complex (MHC) and costimulus molecules. Moreover, DCs play an important role in autoimmune diseases. They suppress antigen-specific responses and cause induction of immunotolerance relative to the degree of their differentiation and functional modification,[11–15] while suppression of DC apoptosis destroys immunotolerance, resulting in induction of autoimmune diseases.[16] Hence, DCs are potential targets not only for immune responses but also for autoimmune diseases.

We have previously shown that tofacitinib selectively suppresses production of cytokines and proliferation of lymphocytes.[17] These functions can be predicted to some degree by the important role that JAK family members play in the differentiation and proliferation of lymphocytes. We have reported that DCs express JAK1, JAK2 and JAK3; however, DCs derived from a JAK3-deficient mouse have been shown to overproduce interleukin (IL)-10 and exhibit anti-inflammatory activity.[18] However, how the inhibition of JAK signalling affects the phenotype, differentiation and antigen presentation of human DCs, which initiate immune responses, has not been investigated. Elucidation of the effects of tofacitinib on DC function may increase basic scientific knowledge of the clinical efficacy of tofacitinib

An increase in the number of invasive DCs has been observed in the synovitis tissues in RA, and monocyte-derived DCs (MoDCs) from patients with RA produce increased IL-6.[19] In addition, an increase in the number of DCs that express high levels of TLR4 ligands in RA synovial fluid has been reported,[20 21] suggesting activation of DCs and disruption of immunotolerance. Furthermore, JAK expression increases in synovial DCs in active

RA,[22–24] indicating its involvement in the regulation of DCs during the pathological processes. This study was conducted in order to investigate the effects of inhibition of JAK1 and JAK3 by tofacitinib and signalling mechanisms in human MoDCs.

METHODS

Inhibitors

Tofacitinib and PF956980[25] were kindly provided by Pfizer (New York, New York, USA). The following inhibitors were purchased; JAK2 kinase inhibitor, G6 (Sigma-Aldrich, St Louis, Missouri, USA), Syk inhibitor I, Syk inhibitor II, PP1, PP2 (Merck, Darmstadt, Germany), anti-IL-6 receptor α antibody, tocilizumab (Chugai Pharmaceutical Co, Tokyo, Japan).

Generation of MoDCs and cell cultures

Peripheral blood mononuclear cells were isolated with lymphocyte separation medium (ICN/Cappel Pharmaceuticals, Aurora, Ohio, USA). Monocytes were obtained by positive magnetic selection using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate immature MoDCs, we cultured monocytes at 0.5×10^6 cells/mL in the presence of IL-4 (50 ng/mL; R&D systems, Minneapolis, Minnesota, USA) and granulocyte macrophage colony-stimulating factor (50 ng/mL; Peprotech, Rocky Hill, New Jersey, USA) for 6 days. The medium was replaced with one supplemented with cytokines on day 3.

Immature MoDCs were washed and replated in fresh medium at 2.5×10^5 cells/mL and pretreated with tofacitinib (10, 100, 300, 1000 nM), PF956980 (300 nM), G6 (300 nM), PP1 (300 nM), PP2 (300 nM), cycloheximide (5 μg/mL), tocilizumab (5 μg/mL) or interferon (IFN)α/β receptor antibody (10 μg/mL) (Abcam, Cambridge, UK) for 6 h and matured with lipopolysaccharide (LPS) (*Escherichia coli*; Sigma, 100 ng/mL) for 48 h. The concentration of each drug was chosen on the basis of previous studies.[6 26–30] MoDCs were washed twice and used for coculture with T cells. Production of tumour necrosis factor (TNF)α, IL-6, IL-1β and IFNα was determined with the BD Cytometric Bead Array (CBA) human Flex Set (BD Pharmingen, Franklin Lakes, New Jersey, USA), and that of TGFβ and IFNβ was analysed by ELISA (eBioscience, San Diego, California, USA). Apoptosis was analysed with the Apoptosis Detection kit II (BD Pharmingen).

DC–T cell cocultures

CD4 T cells were negatively selected from peripheral blood mononuclear cells with the CD4 T Cell Isolation Kit II (Miltenyi Biotec), and $CD45RA^+$ naive T cells were positively isolated with anti-CD45RA microbeads (Miltenyi Biotec). MoDCs were cocultured with allogeneic human $CD45RA^+$ naive T cells at a 1:10 ratio for 6 days in Roswell Park Memorial Institute medium. IL-10 was analysed by CBA, and T cell proliferation was assessed by [^{3}H]thymidine incorporation in the last 16 h. IFNγ production was analysed after restimulation of T cells with CD3 (1 μg/mL) and CD28 (0.5 μg/mL) monoclonal antibodies (R&D Systems) for 72 h after coculture.

Flow cytometric analysis

MoDCs were incubated in blocking buffer (0.25% human globulin in phosphate-buffered saline) for 15 min and then suspended in 100 μL FACS solution (0.5% human albumin and 0.1% NaN_3 in phosphate-buffered saline) with fluorochrome-conjugated monoclonal antibodies at 4°C for 30 min and then washed with FACS solution and analysed with a FACSVerse (Becton–Dickinson, San Jose, California, USA). The following fluorochrome-conjugated mouse monoclonal antibodies were purchased from BD Pharmingen: fluorescein thiocyanate (FITC)-conjugated anti-CD80, PerCP-conjugated anti-HLA-DR, and antigen presenting cell (APC)-conjugated anti-CD86.

Quantitative real-time PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Chatsworth, California, USA). First-strand cDNA was synthesised, and quantitative real-time PCR was performed in the Step One Plus instrument (Applied Biosystems, Foster City, California, USA). TaqMan target mixes for tryptophan indoleamine-pyrrole 2,3-dioxygenase (IDO1), IDO paralogue IDO2 (IDO2), CD80 and CD86 were purchased from Applied Biosystems. Expression levels were expressed relative to that of glyceraldehyde-3-phosphate dehydrogenase. The relative quantity was calculated using the quantification-comparative cycle threshold formula–referenced sample of immature DCs.

Western blot analysis

MoDCs were lysed in Nonidet P-40 buffer containing NaCl, Tris/HCl (pH 8.0), distilled water and protease inhibitor. Lysates were mixed with an equal volume of sample buffer solution (2-mercaptoethanol; Wako Pure Chemical Industries) and boiled for 5 min. Proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, transferred on to nitrocellulose membranes (Whatman, Tokyo, Japan), blocked with 5% skimmed milk, and immunoblotted with antibodies to human phospho-NF-κB p65, human PU.1, human IFN regulatory factor 7 (IRF7), human phospho-STAT1, STAT1, phospho-STAT2 and STAT2 (Cell Signaling Technology, Tokyo, Japan) and horseradish peroxidase-labelled anti-secondary antibodies (NA931V and NA934V; GE Healthcare, Osaka, Japan), using immunoreaction enhancer solution (Can Get Signal, Toyobo, Osaka). Blots were developed with ECL Western Blotting Detection Reagents (GE Healthcare) and visualised with a light-capture instrument (ATTO, Tokyo, Japan).

Statistical analysis

Differences were examined using the Mann–Whitney test. $p<0.05$ denoted the presence of a significant difference.

RESULTS

Tofacitinib inhibited expression of CD80/CD86 without cytotoxicity in human MoDCs stimulated with LPS

The effects of tofacitinib on the expression of costimulators of human MoDCs were investigated. CD80/CD86 and HLA-DR were induced 48 h after LPS stimulation. However, CD80/CD86 expression in MoDCs was suppressed in a concentration-dependent manner by the addition of tofacitinib, whereas expression of HLA-DR was not affected (figure 1A,B). Moreover, induction of CD80/CD86 expression by LPS stimulation was suppressed by PF956980, which is a different JAK1/3 inhibitor, while CD80/CD86 expression was not suppressed by PP1, PP2 or JAK2 inhibitors. These findings suggest that suppression of CD80/CD86 expression was dependent on inhibition of JAK1/3 (figure 1C). Furthermore, cluster formation was observed 24 h after LPS stimulation, which was inhibited in a concentration-dependent manner by the addition of tofacitinib (figure 2A).

In the next set of experiments, cytokine production in DCs was investigated. The production of TNFα, IL-1β and IL-6 was induced by stimulation of MoDCs with LPS for 48 h, while the production of these cytokines was suppressed by tofacitinib in a concentration-dependent manner (figure 2B). However, tofacitinib affected neither the expression of TGFβ mRNA

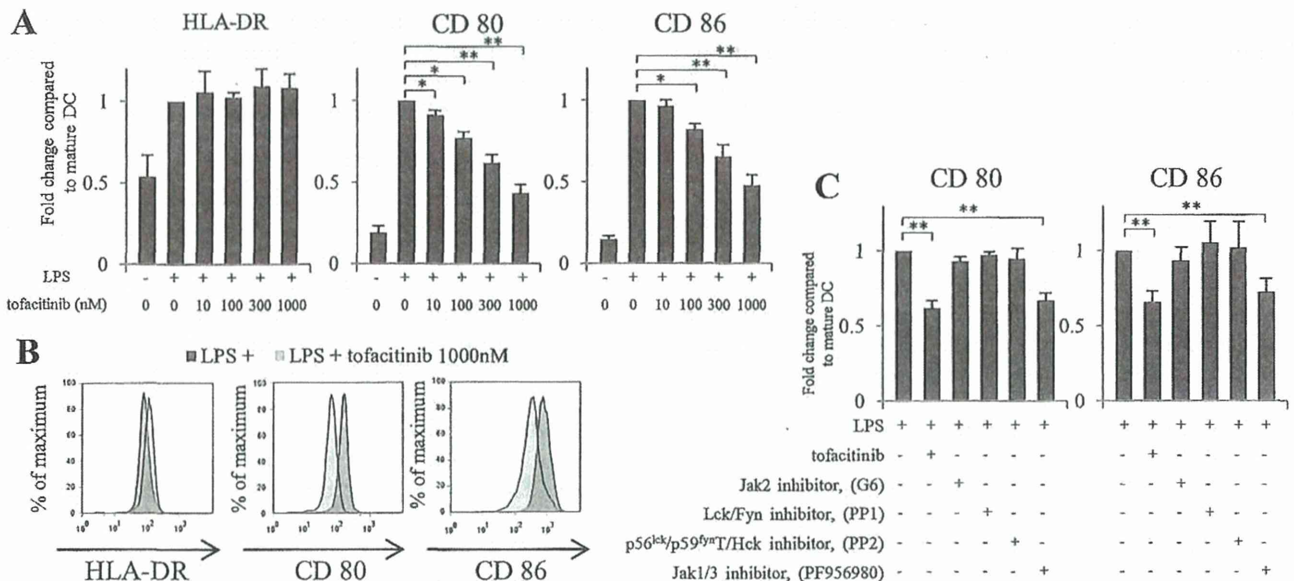

Figure 1 Tofacitinib suppresses CD80/CD86 expression. Immature monocyte-derived dendritic cells (DCs) were washed and cultured with or without tofacitinib or other tyrosine kinases during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h. The DC phenotype was evaluated using flow cytometry. (A) Expression of HLA-DR, CD80 and CD86. (B) Representative histogram data of HLA-DR, CD80 and CD86 expression. (C) Expression of HLA-DR, CD80 and CD86 in the presence of tofacitinib and other tyrosine kinase inhibitors. Representative results of three independent experiments with similar findings. Data are mean±SD. *p<0.05 and **p<0.01 (Mann–Whitney test).

(see online supplementary figure S1A) nor the production of TGFβ (figure 2C), IL-10 and IL-12p70 (see online supplementary figure S1B).

To examine whether these suppressive effects were the result of cytotoxicity of tofacitinib on DCs, the cells were stained with annexin V and propidium iodide. DCs died at a high frequency without stimulation for 48 h, while apoptosis was inhibited by LPS stimulation (figure 3A,B). Tofacitinib did not induce apoptosis, even at concentrations as high as 1000 nM, and it did not cause cytotoxicity. When DCs were pulsed with FITC-labelled albumin, tofacitinib did not affect their capability for micropinocytosis (figure 3C). These results suggest that tofacitinib suppressed cluster formation and changed the phenotype of DCs without causing their cell death.

Expression of CD80 and CD86 was inhibited by antibody to type I IFN receptor

Expression of CD80/CD86 mRNA after LPS stimulation in DCs was suppressed by cycloheximide treatment (figure 4A). After LPS activation, no JAK–STAT pathway involvement was

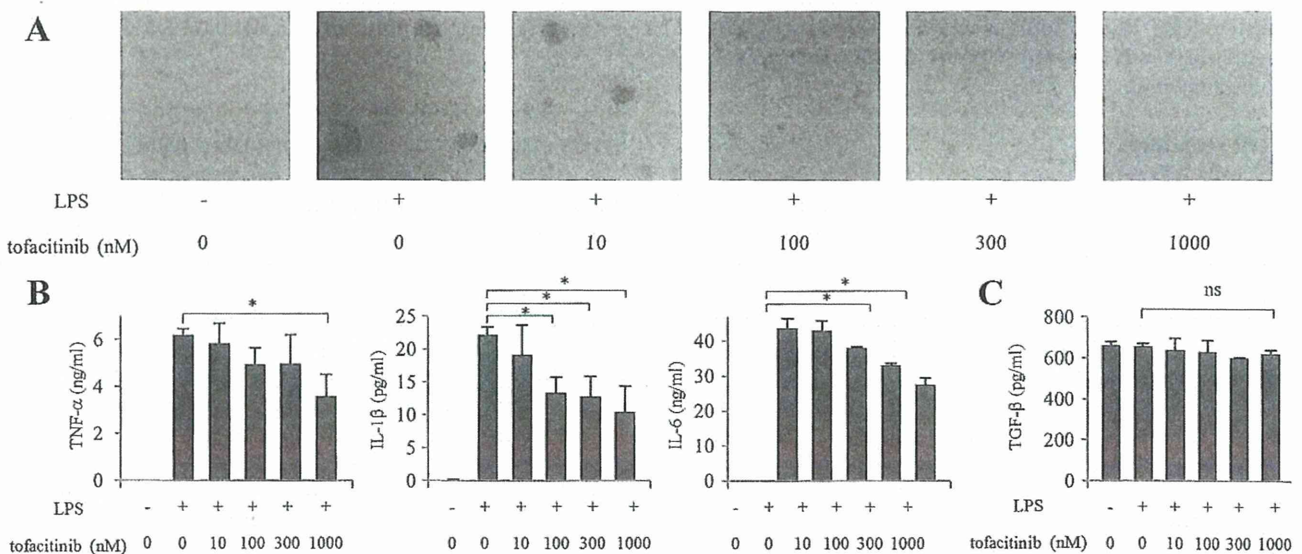

Figure 2 Tofacitinib suppresses cluster formation of dendritic cells (DCs) and inflammatory cytokine production in DCs. Immature monocyte-derived DCs were washed and cultured with or without tofacitinib during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h. (A) Morphology of DC populations as shown by phase-contrast microscopy. Representative results of three independent experiments with similar findings are shown. (B) Tumour necrosis factor (TNF)α, interleukin (IL)-1β and IL-6 concentrations in the supernatants were measured. (C) Transforming growth factor (TGF)β concentration was determined by ELISA. Data of three independent experiments are shown. Data are mean ±SD. *p<0.05 (Mann–Whitney test).

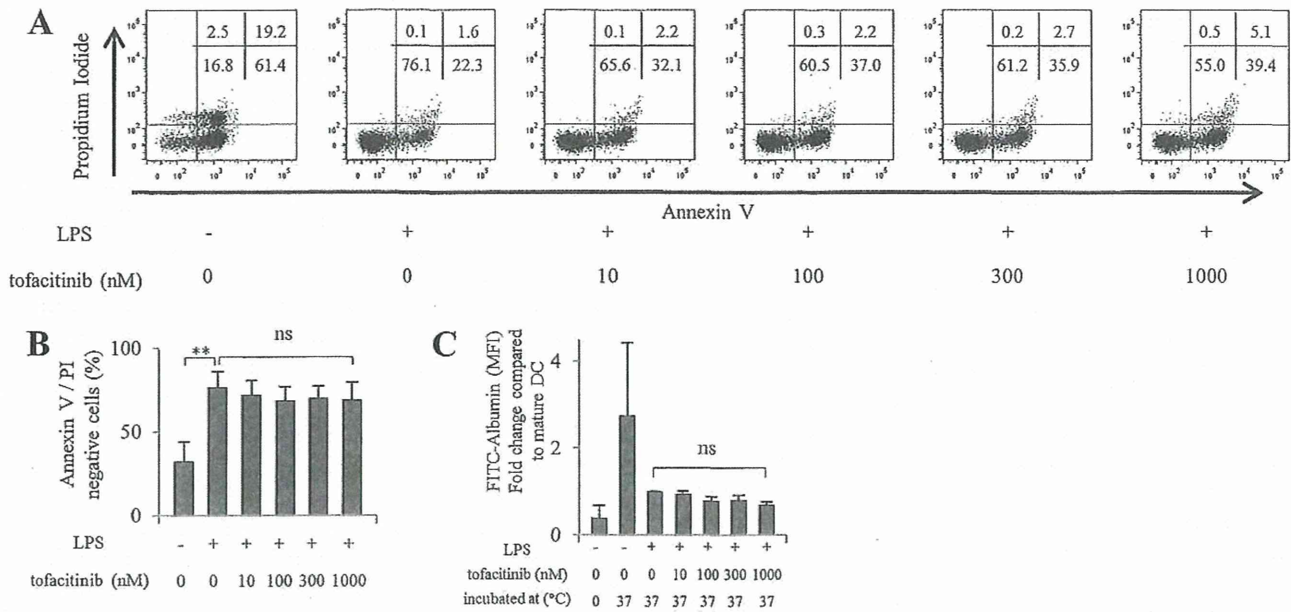

Figure 3 Tofacitinib does not induce cell death. Immature monocyte-derived dendritic cells (MoDCs) were washed and cultured with or without tofacitinib during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h. Early and late apoptosis of DCs were evaluated with flow cytometry. (A) Representative histogram data of annexin V/propidium iodide (PI) staining. (B) Rate of viable cells (annexin V_{neg}/PI_{neg}). (C) MoDCs were pulsed with 5 μg/mL fluorescein isothiocyanate (FITC)-conjugated albumin for 60 min. Cells were incubated at 0°C for background uptake and at 37°C for albumin uptake. Data indicate the ratio compared with MoDCs stimulated with LPS. Data of three independent experiments are shown. Data are mean±SD. **p<0.01 (Mann–Whitney test). MFI, mean fluorescence intensity.

indicated in the signalling pathway downstream of TLR4; therefore, an indirect mechanism was considered in which the suppression of CD80/CD86 expression by tofacitinib occurred through a proteinogenic mechanism.

We next assessed if CD80/CD86 is induced by cytokines in MoDCs. CD80/CD86 expression was not induced by IL-6, whereas it was induced by LPS stimulation. Furthermore, the CD80/CD86 expression that was induced by LPS was not affected by tocilizumab, which is an IL-6 receptor antibody (figure 4B). In contrast with IL-6, expression of CD80/CD86 was induced by type I IFN stimulation and was completely inhibited by tofacitinib. Expression of CD80/CD86, which was induced by LPS, was suppressed by a type I IFN receptor antibody (figure 4C). These results suggest that the inhibition of JAK1/3 in MoDCs partially suppressed the expression of CD80/CD86 by suppressing type I IFN signalling.

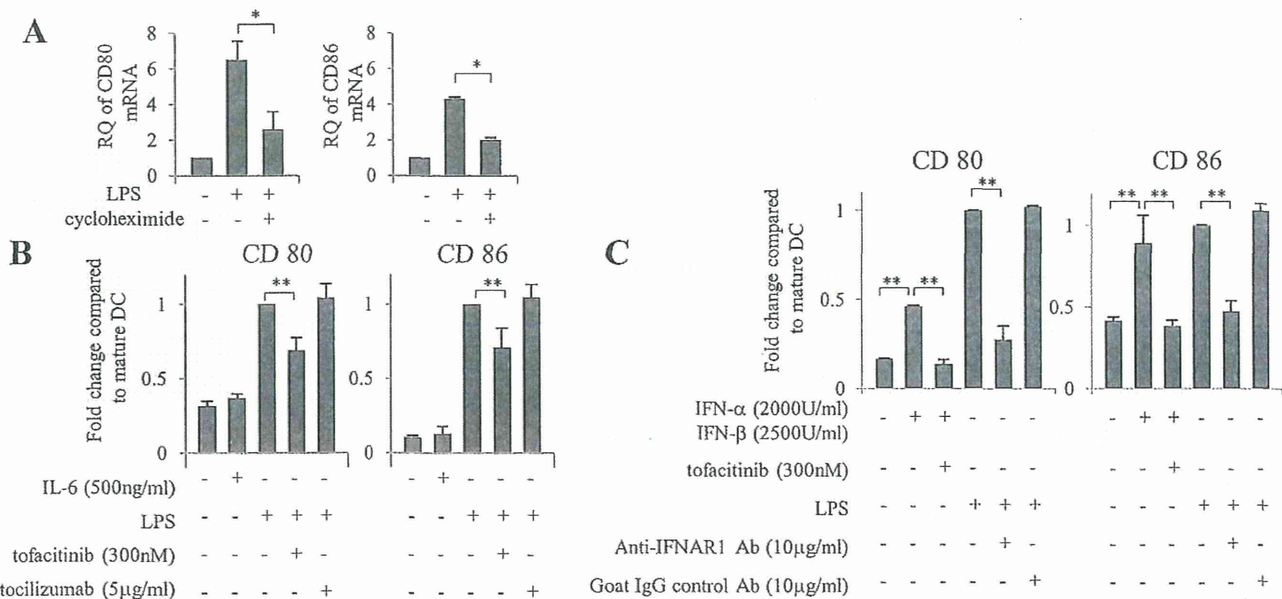

Figure 4 Lipopolysaccharide (LPS) induces the expression of CD80 and CD86 through type I interferon (IFN). Immature monocyte-derived dendritic cells (DCs) were washed and cultured with or without cycloheximide, an antibody to interleukin (IL)-6 receptor (tocilizumab) or to type I IFN receptor (IFNAR1) during LPS (100 ng/mL) stimulation for 48 h. (A) CD80 and CD86 mRNA expression were measured by quantitative real-time PCR. (B and C) Expression of HLA-DR, CD80 and CD86 determined by flow cytometry. Data are mean±SD. *p<0.05 and **p<0.01 (Mann–Whitney test). RQ, relative quantity.

Tofacitinib inhibited expression of CD80 and CD86 through reduction of IRF7 signalling

The involvement of NF-κB,[31] PU.1[32] and IRF7,[33] which are transcription factors that regulate CD80/CD86 expression, was investigated. NF-κB and PU.1 were activated within 5 min, while IRF7 was activated within 3 h in DCs after LPS stimulation. The activation of NF-κB and PU.1 by LPS stimulation was not suppressed by tofacitinib, while the activation of IRF7 was suppressed by tofacitinib (figure 5A). Consistent results were achieved on analysis of nuclear and cytoplasmic fractions. LPS stimulation for 5 min induced phospho-IκBα and concurrently induced phospho-NF-κB and the subsequent translocation of phospho-NF-κB and PU.1 into the nucleus. However, tofacitinib affected the translocation of neither phospho-NF-κB nor PU.1 induced by LPS (see online supplementary figure S2). On the other hand, expression and nuclear translocation of IRF7 was induced after 3 h stimulation with LPS, and the induced translocation of IRF7 was suppressed by tofacitinib (see online supplementary figure S2). In addition, tofacitinib decreased IFNβ production (figure 5B), while IFNα production was undetectable (data not shown). Tofacitinib also suppressed phospho-STAT1/STAT2 induced by exogenous type I IFN (figure 5C). Furthermore, IFNα/β receptor was constitutively expressed and not affected by tofacitinib (data not shown). These results indicate that tofacitinib suppressed the phosphorylation of STAT1/STAT2 induced by autocrine stimulation with type I IFN, continuously suppressing IRF7 expression and the production of type I IFN, which decreased CD80/CD86 expression in MoDCs.

Tofacitinib reduced T cell stimulatory ability and induced expression of IDO in MoDCs

Finally, the T cell stimulation capability of MoDCs treated with tofacitinib was examined. MoDCs were cultured for 48 h in the presence of tofacitinib and LPS, washed, and then cocultured with allogeneic $CD4^{+}CD45RA^{+}$-naïve T cells for 6 days. MoDCs that were treated with LPS exhibited increased T cell growth capability, and IFNγ production capability was induced. However, MoDCs that were pretreated with tofacitinib exhibited decreased T cell stimulatory capability and demonstrated a concentration-dependent decrease in IFNγ production (figure 6A–C), while IL-10 production was increased (figure 6C) without any effects on regulatory T cell population (see online supplementary figure S3).

It has been reported that DCs that express IDO, which is an enzyme with catalytic activity on tryptophan, show decreased T cell stimulatory capability.[34] Therefore, the IDO mRNA in DCs was measured. Both IDO1 and IDO2 were significantly induced by tofacitinib (figure 6D). These results suggest that inhibition of JAK1/3 in DCs with tofacitinib suppressed cell maturation and induced DCs with decreased T cell stimulatory capability.

DISCUSSION

It is shown here that tofacitinib, a JAK inhibitor, promoted a tolerogenic phenotype in human DCs. The data indicate that inhibition of JAK1/JAK3 by tofacitinib regulated transcription of IRF7 by suppressing type I IFN signalling and CD80/CD86 expression. Tofacitinib was approved for treatment of RA in the USA and Japan in 2012 and 2013, respectively. The therapeutic efficacy of tofacitinib has been shown to be equivalent to TNF inhibitors,[35] and it was also found to be effective in patients who did not respond to TNF inhibitors.[36] These clinical study results indicate that JAK1/JAK3 plays an important role in inflammatory immune diseases such as RA. However, the direct suppressive effect of tofacitinib on T cells alone does not completely explain the mechanism. The results in this report suggest a novel mechanism of tofacitinib involving the induction of immunotolerance in DCs.

Tofacitinib did not affect the expression of MHC class II molecules, whereas it did suppress CD80/CD86 expression. Tofacitinib has been shown to exhibit a suppressive effect on JAK1/JAK3, while its suppressive effects on JAK2/Tyk2 are

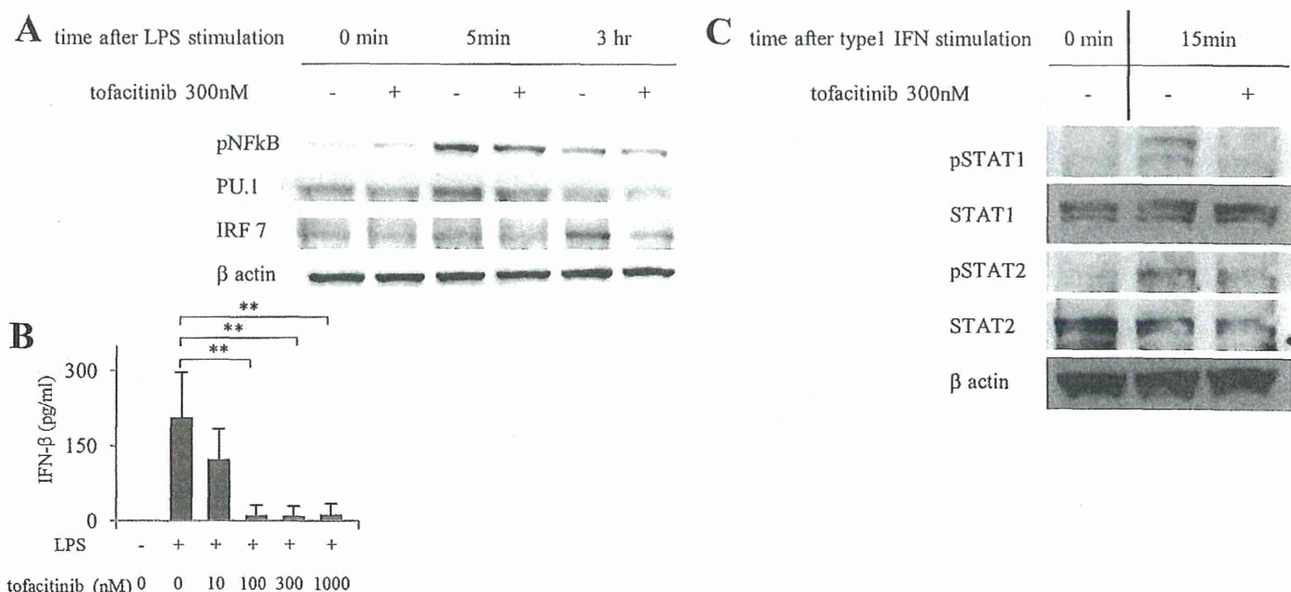

Figure 5 Tofacitinib suppresses interferon regulatory factor (IRF) activation. Immature monocyte-derived dendritic cells (MoDCs) were washed and cultured with or without tofacitinib during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h. The time course of suppression was evaluated as shown. (A) Phospho-NF-κB, PU.1, IRF7 and β-actin were detected by western blotting. (B) Interferon (IFN)β concentration in the supernatant from MoDCs cultured for 48 h was measured. (C) Phospho-signal transducer and activator of transcription 1 (pSTAT1), STAT1, phospho-STAT2 (pSTAT2), STAT2 and β-actin were detected by western blotting after stimulation by type-1 IFN for 15 min.

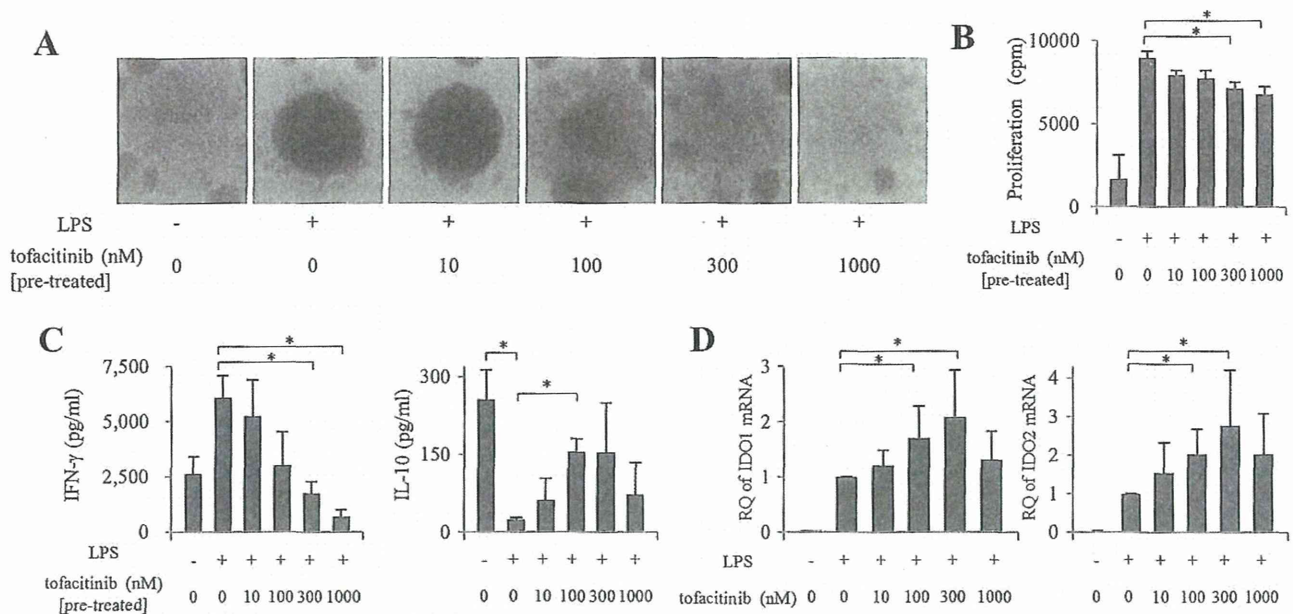

Figure 6 Tofacitinib decreased T cell stimulatory capability of dendritic cells (DCs) and increased indoleamine 2,3-dioxygenase (IDO) expression. Immature monocyte-derived DCs were cultured with or without tofacitinib during lipopolysaccharide (LPS; 100 ng/mL) stimulation for 48 h and washed. They were then cocultured with allogeneic CD4^{+}CD45RA^{+}-naïve T cells for 6 days. (A) Clustering of T cells as shown by phase-contrast microscopy. (B) [^{3}H]Thymidine incorporation was measured during the last 18 h of the 72 h culture. (C) Interferon (IFN)γ and interleukin (IL)-10 concentrations in the supernatant. (D) Indoleamine 2,3-dioxygenase (IDO)1 and IDO2 mRNA expression in DCs measured by quantitative real-time PCR. Data of three independent experiments are shown. Data are mean±SD. *$p<0.05$ (Mann–Whitney test). RQ, relative quantity.

limited.[5] Furthermore, we found that the JAK2 inhibitor (G6) did not show any effects on CD80/86 expression (figure 1), indicating that JAK1 is involved in CD80/86 induction. Recent clinical trials proved that a JAK1/JAK2 inhibitor possesses similar clinical efficacy to tofacitinib,[37] and there could be a similar inhibitory action of JAK1-mediated signalling on DCs by this JAK1/JAK2 inhibitor. Furthermore, therapies targeting suppression of CD80/CD86-mediated T cell stimulation, such as abatacept, have been successful in the treatment of autoimmune diseases, and TNF inhibitors are also able to suppress CD80/86 expression.[38] Therefore, suppression of costimulators is considered an important mechanism of action of tofacitinib.

CD80/86 expression is regulated by three transcription factors, NF-κB,[31] PU.1[32] and IRF7.[33] NF-κB and PU.1 are directly induced by TLR4 stimulation,[39 40] while IRF7 is induced through JAK1/Tyk2, which are activated by type I IFN and its downstream signals, STAT1/STAT2.[41] Furthermore, IRF7 promotes type I IFN production, which results in the formation of a positive feedback pathway.[42] The results of our study indicate that tofacitinib did not affect activation of NF-κB and PU.1, whereas it did suppress IRF7 expression. Moreover, CD80/CD86 expression was suppressed in the presence of an antibody to type I IFN receptor. According to a report by Lim *et al*,[33] IRF7 bound to the promoter lesion of CD80 and regulated its expression. Although the regulation of CD80/86 remains unclear, there may be coordinated regulatory mechanisms among NF-κB, PU.1 and IRF7, and tofacitinib may inhibit CD80/86 expression partly through IRF7.

The most significant finding of this study is that JAK1/JAK3 inhibition by tofacitinib in human DCs suppressed induction of their T cell stimulatory capability. A decrease in costimulator expression, as well as an increase in IDO expression, was observed after tofacitinib treatment. IDO is a rate-limiting enzyme in tryptophan metabolism; however, it has a strong immunomodulation effect and plays an important role in the expression of tolerogenic DC function.[34 43 44] Expression of costimulators and cytokine production capability were suppressed, and expression of IDO was increased, in MoDCs in the presence of tofacitinib. Although the mechanisms of IDO induction remain unclear, we assume that the inhibition of IL-4 played a role in IDO induction by tofacitinib for the following reasons: IL-4 is produced by DCs[45]; IL-4 activates JAK1/JAK3; tofacitinib inhibits IL-4-mediated signalling; IL-4 is known to inhibit IDO expression.[46]

To clarify the functions of IDO in DCs, MoDCs were pretreated with tofacitinib and cocultured with allogeneic CD4 T cells in the presence of 1-methyltryptophan (1-MT), an IDO inhibitor. However, the treatment of MoDCs with 1-MT did not cancel the tofacitinib-mediated suppressive effects on T cell stimulation (data not shown). Furthermore, other molecules involved in immune tolerance such as programmed death ligand (PDL)-1 and PDL-2 were not induced by tofacitinib (see online supplementary figure S4). Thus, the functional significance of IDO expression in DCs remains unclear in our studies, and we suppose that the suppressive effects of tofacitinib-treated DCs on T cell stimulation mainly depend on the inhibition of CD80/86 expression in DCs.

On oral administration of tofacitinib 5 or 10 mg twice a day, serum levels of approximately 100–300 nM are achieved, and such therapeutic levels are known to last for 4–6 h. The in vitro levels of tofacitinib used in our studies were almost comparable to the therapeutic levels achieved. Although the in vivo half-life of tofacitinib is 2–3 h, an effective concentration could be obtained in vitro by administration twice a day.

These findings suggest that the inhibition of JAK1 and JAK3 responses after LPS stimulation in human DCs was involved in the regulation of disease states through a novel mechanism. In addition to the known effects of tofacitinib on lymphocytes, we discovered novel effects on human MoDCs: tofacitinib suppressed a production and stimulation loop of type I IFN

through JAK1/JAK3, decreased CD80/CD86 expression, induced IDO expression, and suppressed T cell stimulatory capabilities. Thus, tofacitinib not only suppressed cytokine production, but also suppressed expression of costimulators by inhibiting the positive loop of type I IFN–IRF7 in DCs, which leads to immunomodulatory effects.

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

Competing interests Y Tanaka received consulting fees, lecture fees, and/or honoraria from Mitsubishi-Tanabe Pharma, Eisai, Chugai Pharma, Abbott Japan, Astellas Pharma, Daiichi-Sankyo, Abbvie, Janssen Pharma, Pfizer, Takeda Pharma, Astra-Zeneca, Eli Lilly Japan, GlaxoSmithKline, Quintiles, MSD, Asahi-Kasei Pharma, and received research grants from Bristol-Myers, Mitsubishi-Tanabe Pharma, Abbvie, MSD, Chugai Pharma, Astellas Pharma, Daiichi-Sankyo. M Kondo is an employee of the Mitsubishi Tanabe Pharma Corporation. K Yamaoka received consulting fees from Pfizer.

Ethics approval The institutional review board of the University of Occupational and Environmental Health Japan.

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Original article

IL-6-accelerated calcification by induction of ROR2 in human adipose tissue-derived mesenchymal stem cells is STAT3 dependent

Shunsuke Fukuyo[1], Kunihiro Yamaoka[1], Koshiro Sonomoto[1], Koichi Oshita[1,2], Yosuke Okada[1], Kazuyoshi Saito[1], Yasuhiro Yoshida[3], Tamotsu Kanazawa[3], Yasuhiro Minami[4] and Yoshiya Tanaka[1]

Abstract

Objective. The mechanisms of ectopic calcification in inflammatory diseases are poorly understood. We investigated the effects of inflammatory cytokines on the mechanisms of calcification in human adipose tissue-derived mesenchymal stem cells (hADSCs).

Methods. The effects of inflammatory cytokines were evaluated using hADSCs cultured in osteoblast induction medium. mRNA expression was measured by real-time PCR and protein levels were measured by western blotting. Cell mineralization was evaluated by Alizarin Red S staining.

Results. In hADSCs, administration of IL-6/soluble IL-6 receptor (sIL-6R), TNF or IL-1β accelerated calcification through enhanced expression of an osteoblast differentiation marker, runt-related transcription factor 2 (*RUNX2*). IL-6/sIL-6R had the greatest effect. The transcription of mRNA for receptor tyrosine kinase-like orphan receptor 2 (*ROR2*), involved in the non-canonical wingless-type (WNT) MMTV integration site pathway, was increased, while β-catenin expression, an essential factor in the canonical WNT signalling pathway for osteoblast differentiation, did not change. Suppression of signal transducer and activator of transcription 3 (STAT3), but not STAT1, by small interfering RNA (siRNA) exerted a strong inhibitory effect on *RUNX2* and *ROR2* expression, and inhibited accelerated calcification.

Conclusion. IL-6/sIL-6R stimulation accelerated the ROR2/WNT5A pathway in hADSCs in a STAT3-dependent manner, resulting in augmented calcification. These results suggest that the mechanisms of ectopic calcification accelerated by IL-6 in hADSCs may be involved in chronic inflammatory tissues and that IL-6 inhibitors may be beneficial in the treatment of ectopic calcification in inflammatory diseases.

Key words: IL-6, *ROR2*, ADSCs, STAT3, *WNT5A*, ectopic calcification.

BASIC SCIENCE

Introduction

Physiological calcification in living organisms involves the deposition of minerals such as calcium and phosphorus in the bone matrix during bone formation. During the development of bone, osteoblasts derived from mesenchymal stem cells (MSCs) become embedded in the bone matrix and differentiate into bone cells. In addition to physiological calcification, ectopic calcification can also occur *in vivo*. A typical example is arteriosclerosis-related vascular wall calcification, which is caused by the simple deposition of calcium in vascular walls. However, recent studies have suggested that transformation of vascular smooth muscle cells into osteoblasts may be responsible for vascular wall calcification [1].

The finding that bone matrix proteins, including osteopontin, osteonectin and bone sialoprotein (BSP), are present in calcified tissues suggests that calcification may occur via osteoblast-like differentiation [2, 3]. Fat cells,

[1]First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, [2]Pharmacology Research Laboratories I, Research Division, Mitsubishi Tanabe Pharma Corporation, Yokohama, Kanagawa, [3]Department of Immunology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu and [4]Department of Physiology and Cell Biology, Graduate School of Medicine, School of Medicine, Kobe University, Kobe, Japan.

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

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hematopoietic cells and mesenchymal cells exist in large numbers in subcutaneous adipose tissue. However, these cells are terminally differentiated and are unlikely to acquire osteoblast-specific traits or cause calcification. Alternatively, immature and undifferentiated MSCs can be induced to differentiate into osteoblasts by inflammatory cytokines and are more likely to be involved in subcutaneous calcification. Multipotential adipose tissue-derived MSCs (ADSCs) are present in subcutaneous adipose tissue. ADSCs are multipotent cells that can differentiate into MSC-specific adipocytes, chondrocytes and osteoblasts [4, 5]. MSCs isolated from patients with autoimmune diseases, such as SLE or other inflammatory diseases, are reportedly associated with abnormal function or differentiation, unlike MSCs from healthy individuals, suggesting that MSCs are involved in disease pathogenesis [6].

Multiple factors, including inflammation and oxidative stress, that are associated with diabetes, renal failure and ageing are involved in osteoblast-like differentiation [7]. In addition, autoimmune diseases are associated with inflammation-related ectopic calcification. In particular, scleroderma and JDM are complicated by ectopic subcutaneous calcification. Between 4% and 25% of patients with scleroderma and ~30% of patients with JDM develop ectopic calcification complications [8]. Scleroderma and JDM are autoimmune diseases that affect the skin and muscle tissues, respectively. These diseases are thought to be mediated by inflammatory cells such as lymphocytes and macrophages that infiltrate subcutaneous adipose tissue and muscle tissue. These cells secrete inflammatory cytokines, such as IL-6, TNF and IL-1β, which may be involved in calcification [9, 10]. This mechanism of immune-mediated calcification was supported by a study in which the administration of TNF inhibitors reduced ectopic calcification [11]. We recently demonstrated that inflammatory cytokines stimulate the differentiation of MSCs into osteoblasts by activating the non-canonical wingless-type (WNT) signalling pathway [12].

These findings suggest that inflammatory cytokines may be involved in ectopic calcification observed in the pathology of autoimmune diseases by stimulating the differentiation of ADSCs into osteoblasts. The aim of this study was to determine the ability of inflammatory cytokines to accelerate the differentiation of human ADSCs (hADSCs) into osteoblast-like cells *in vitro*.

Methods

Cells

hADSCs were purchased from Cytori (San Diego, CA, USA). ADSCs were cultured in ADSC basal medium supplemented with L-glutamine, gentamicin, amphotericin and 10% fetal bovine serum (FBS) [ADSC growth medium (ADSCGM), Lonza, Walkersville, MD, USA] at 37°C in a 5% CO_2 atmosphere and were subcultured every 6-7 days. ADSCs from passages 2-10 were used in this study. The multipotency of ADSCs was assessed by their ability to differentiate into osteoblasts, chondrocytes and adipocytes. ADSCs (1×10^4 cells) were seeded into 24-well plates (Corning, Corning, NY, USA) and cultured in osteoblast induction medium (OIM; 50 μM ascorbic acid, 10 mM β-glycerophosphoric acid and 0.1 μM dexamethasone), adipocyte induction medium [minimum essential medium (MEM) α, hydrocortisone, isobutylmethylxanthine and indomethacin] or seeded into a conical tube three-dimensional (3D) culture and cultured in chondrocyte induction medium (D-MEM, insulin, transferrin, selenious acid, BSA, linoleic acid, ascorbic acid, proline, pyruvate, TGF-β3 and dexamethasone) at 37°C in a 5% CO_2 atmosphere. All media were obtained from Lonza. Ethics approval for the experiments using hADSCs was granted by the Ethics Committee of the University of Occupational and Environmental Health, Japan.

ADSCs-conditioned medium assay

ADSCs (1×10^4 cells) were seeded into 24-well plates and cultured in OIM at 37°C in a 5% CO_2 atmosphere. Recombinant human TNF (R&D Systems, Minneapolis, MN, USA), human IL-1β (RELIATech, Wolfenbüttel, Germany) or human IL-6 (Miltenyi Biotec, Bergisch Gladbach, Germany) with human soluble IL-6 receptor (sIL-6R) (R&D Systems) were added to OIM. The medium was replaced every 2-3 days throughout the experiments. These experiments were performed with and without 1-h cycloheximide (5 μg/ml) pretreatment of hADSCs.

Measurement of mRNA expression

Total mRNA was collected with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was obtained by reverse transcription according to the manufacturer's instructions. Real-time PCR was performed using primers specific for *runt-related transcription factor 2* (*RUNX2*) (Hs-01047978-m1), *receptor tyrosine kinase-like orphan receptor 2* (*ROR2*) (Hs-00171695-m1) and *WNT MMTV integration site family member 5A* (*WNT5A*) (Hs_00998537m1), *WNT3A* (Hs-01055707-m1), *WNT7B* (Hs-00536497-m1) and *WNT10B* (Hs-00559664-m1) (Applied Biosystems, Foster City, CA, USA). *RUNX2*, *ROR2* and *WNT5A* mRNA expression levels were normalized to the levels of β-actin (TaqMan probe Hs_99999903m1) as an endogenous control and calculated using the $\Delta\Delta C_T$ method.

Mineralization assay

Cell mineralization was evaluated by Alizarin Red S (ARS) staining (Sigma-Aldrich, St Louis, MO, USA). Briefly, cells were cultured in the indicated conditions in a 24-well plate and fixed with 10% formaldehyde for 15 min and rinsed with deionized water before adding 350 μl of 1% ARS solution (pH 4.1) per well. After incubation at room temperature for 15 min, the cells were washed with deionized water.

Western blotting

Cells were washed twice with cold PBS and dissolved with lysis buffer containing 50 mM Tris-HCl (pH 8.0),

150 mM NaCl, protease inhibitor and 10% NP-40. Equal amounts of proteins (20 μg) were electrophoresed by SDS-PAGE, transferred onto nitrocellulose membranes and blotted with antibodies against β-catenin, signal transducer and activator of transcription 1 (STAT1), STAT3, phosphorylated (p)-STAT1, p-STAT3 (Cell Signaling Technology, Beverly, MA, USA) or β-actin (Sigma-Aldrich), followed by incubation with secondary antibodies (GE Healthcare, Chalfont St Giles, UK).

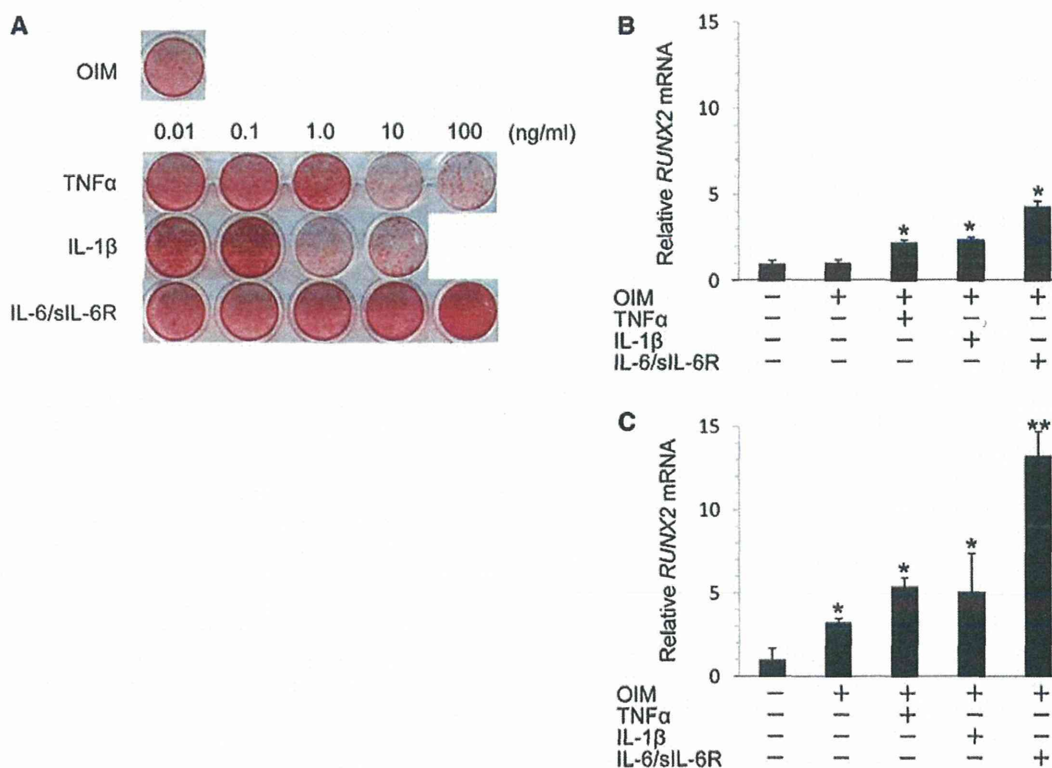
Small interfering RNA

The following small interfering RNA (siRNAs) were purchased from Invitrogen (Carlsbad, CA, USA): STAT1 siRNA-1 (5′-GCGGAGACAGCAGAGCGCCUGUAUU-3′), STAT1 siRNA-2 (5′-CCUGUCACAGCUGGAUGAUCAAUAU-3′), STAT3 siRNA-1 (5′-GCCAAUUGUGAUGCUUCCCUGAUUG-3′), STAT3 siRNA-2 (5′-UGGCCCAAUGGAAUCAGCUACAGCA-3′) and negative control siRNA (low GC: 12935-200; medium GC: 12935-112; high GC: 12935-400). Transfection was performed using lipofectamine RNAiMAX (Invitrogen). In brief, hADSCs (1×10^4 cells) were plated on a 24-well plastic plate in 500 μl of antibiotic-free ADSCGM 1 day before transfection. On the next day, transfection reagents containing 6 pmol siRNA and 1 μl lipofectamine RNAiMAX in a final volume of 100 μl with Opti-MEM I (Invitrogen) was added to each well and incubated for 24 h before adding OIM supplemented with IL-6 (100 ng/ml) and sIL-6R (100 ng/ml) for 3 or 24 h.

Immunohistochemistry

Ethics approval was obtained from the Ethics Committee of the University of Occupational and Environmental Health, Japan for the use of patient tissue. Formalin-fixed, paraffin-embedded sections (3-μm thick) from a DM patient were used for immunohistochemistry. The sections were then stained using the Histofine Simple Stain Kit (Nichirei, Tokyo, Japan) with mouse anti-human RUNX2 (ABNOVA, Taipei, Taiwan) and mouse anti-human

Fig. 1 Inflammatory cytokines accelerated mineralization and *RUNX2* mRNA expression



(**A**) hADSCs were cultured in OIM supplemented with TNF, IL-1β or IL-6/sIL-6R (0.01, 0.1, 1.0, 10 and 100 ng/ml) and Alizarin Red S staining was performed on day 8. Data are representative of three experiments with similar findings. (**B, C**) hADSCs were cultured in OIM supplemented with TNF (1.0 ng/ml), IL-1β (0.1 ng/ml) or IL-6/sIL-6R (100 ng/ml). Total RNA was isolated at 24 h (**B**) and on day 8 (**C**) and *RUNX2* mRNA expression was determined by real-time PCR. Data are shown as the mean (s.e.m.) values of three experiments. $^*P < 0.05$, $^{**}P < 0.01$ *vs* without OIM and cytokines by analysis of variance and post hoc Dunnett's test.

IL-6 (R&D Systems, Minneapolis, MN, USA). Briefly, endogenous peroxidase was inactivated in a 3% hydrogen peroxide (H_2O_2) solution after proteolytic digestion using proteinase K (Dako, Glostrup, Denmark). These sections were then blocked with serum-free protein block (Dako, Glostrup, Denmark), followed by incubation with monoclonal and polyclonal antibodies in a humid chamber for 60 min at room temperature. After incubation, all sections, including the negative control sections, were treated with peroxidase-conjugated secondary antibodies for 30 min and the colour was developed by incubating the sections in 3,3′-diaminobenzidine and H_2O_2 for 10 min, followed by counterstaining with haematoxylin solution. Negative control sections were treated with isotype-matched mouse IgG1.

Statistical analysis

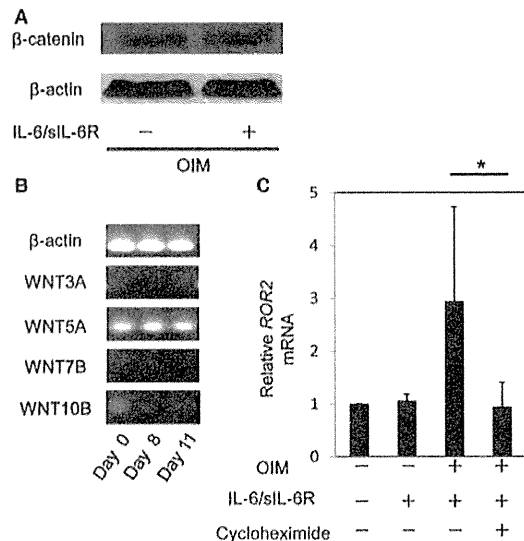
Data are expressed as mean (S.E.M.). Differences between two groups were tested for statistical significance using Student's *t*-test. Analysis of variance (ANOVA) was used to compare three or more groups. If the ANOVA was significant, Dunnett's multiple comparison test was used as a *post hoc* test. In all analyses, a *P*-value <0.05 was considered significant.

Results

Inflammatory cytokines accelerated mineralization and *RUNX2* mRNA expression in hADSCs

We first confirmed the multipotency of hADSCs, as they could differentiate into adipocytes, chondrocytes and osteoblast-like cells *in vitro* (data not shown). Previous studies demonstrated that inflammatory cytokines might be involved in osteoblast differentiation and calcification [13, 14]. Therefore we investigated the effects of different inflammatory cytokines on osteoblast-like differentiation of hADSCs. *In vitro*, hADSCs were cultured in OIM supplemented with TNF, IL-1β or IL-6/sIL-6R (0.01, 0.1, 1.0, 10 and 100 ng/ml). On day 8 of culture, calcification was assessed by ARS staining. TNF, IL-1β and IL-6/sIL-6R at concentrations of 1.0 ng/ml, 0.01 ng/ml and 100 ng/ml, respectively, accelerated the calcification compared with OIM alone (Fig. 1A). All three cytokines augmented *RUNX2* mRNA expression, an osteoblast differentiation marker, within 1 day of culture (Fig. 1B). On day 8, the enhanced expression of *RUNX2* mRNA persisted in all cytokine-supplemented cultures relative to OIM alone, with IL-6/sIL-6R showing the greatest effect (Fig. 1C). The addition of IL-6 alone did not enhance *RUNX2* mRNA expression, suggesting that functional IL-6 receptors were not expressed by hADSCs (data not shown). These results demonstrated the acceleratory effect of IL-6/sIL-6R on calcification by stimulating osteoblast-like differentiation. This indicated that the IL-6 signalling pathway had the most potent effect on the differentiation of hADSCs.

Fig. 2 IL-6 increased *ROR2* mRNA expression without affecting β-catenin expression



(A) hADSCs were cultured with or without IL-6/sIL-6R (100 ng/ml) for 4 days. β-catenin expression was detected by western blotting. (B) Total RNA was isolated from hADSCs at 0, 8 and 11 days after culture in OIM supplemented with IL-6/sIL-6R (100 ng/ml). The PCR products were separated by agarose gel. *WNT3A*, *WNT5A*, *WNT7B*, *WNT10B* and *β-actin* mRNA expression was measured by PCR. (C) PCR was performed with cDNA from hADSCs cultured for 12 h in OIM supplemented with IL-6/sIL-6R (100 ng/ml), and cycloheximide (5 μg/ml) and densimetric analysis was performed. Values are the mean (S.E.M.) of three independent experiments. *$P < 0.05$ by *t*-test.

IL-6 increased *ROR2* mRNA expression without affecting β-catenin expression in osteoblast-like differentiation

We then studied the role of the WNT signalling pathways (canonical and non-canonical) in osteoblast-like differentiation. To determine the expression level of β-catenin, a vital component of the canonical WNT pathway, hADSCs were cultured in OIM supplemented with 100 ng/ml of IL-6/sIL-6R and western blotting analysis was performed 4 days later. However, β-catenin expression was not significantly different in cultures with or without IL-6/sIL-6R (Fig. 2A).

Next, an RT-PCR assay was performed to evaluate the expression of *WNT3A*, *WNT5A*, *WNT7B* and *WNT10B* mRNAs and assess their involvement in osteoblast-like differentiation in cultured hADSCs. mRNAs for *WNT3A*, *WNT7B* and *WNT10B* were not detected on days 0, 8 or 11 of culture. In contrast, *WNT5A* mRNA, which encodes a representative Wnt protein and is involved in the non-canonical pathway, was constantly expressed at high

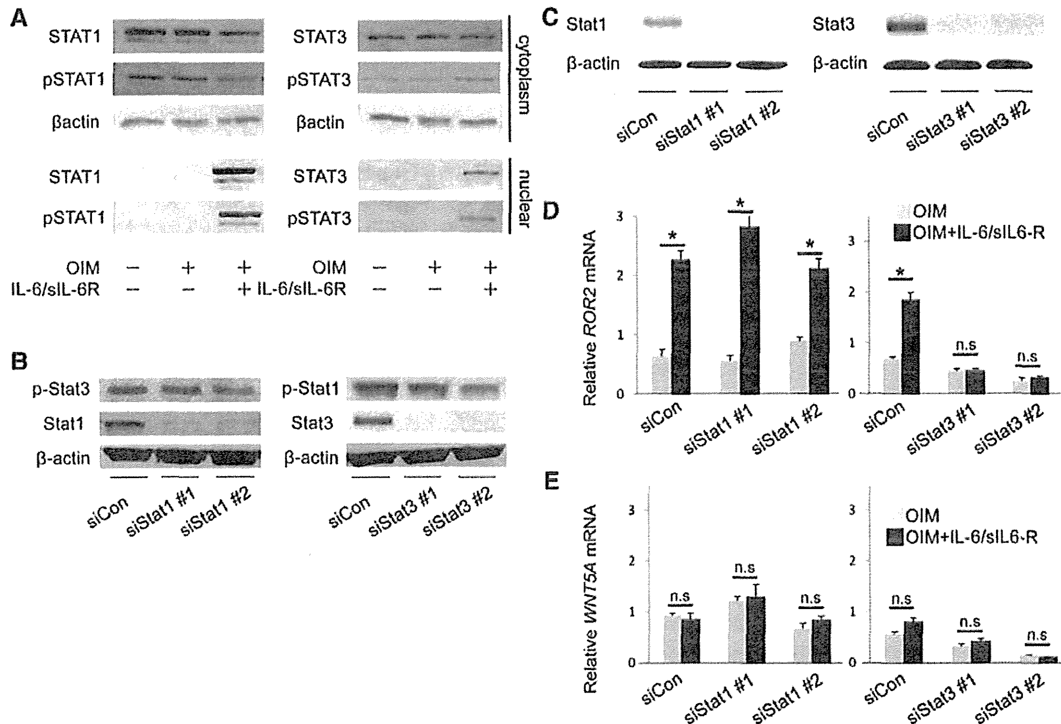
levels on days 0, 8 and 11 (Fig. 2B). The addition of IL-6/sIL-6R did not enhance this expression (data not shown). hADSC expression of *ROR2* mRNA, which encodes a cognate receptor for *WNT5A*, was enhanced 24 h after the addition of IL-6/sIL-6R compared with untreated hADSCs or hADSCs cultured in OIM alone. Pretreatment of hADSCs with cycloheximide significantly inhibited the ability of IL-6/sIL-6R to induce *ROR2* mRNA expression ($P = 0.0495$) (Fig. 2C). Taken together, these results suggest that IL-6 accelerates calcification by indirectly inducing the transcription of *ROR2* mRNAs in hADSCs.

STAT3 is involved in IL-6-mediated *ROR2* mRNA expression in hADSCs

As STAT1 and STAT3 play important roles in the IL-6 signalling pathway, we investigated their involvement in IL-6-induced *ROR2* mRNA expression in hADSCs. Phosphorylated STAT1 and STAT3 were not detected in OIM cultures. However, the administration of IL-6/sIL-6R to hADSC cultures stimulated the nuclear transport of both STAT1 and STAT3 (Fig. 3A). We then examined the effects of inhibiting STAT1 and STAT3 expression on the IL-6 signalling pathway. The expression of STAT1 and STAT3 was effectively suppressed using two different siRNA sequences for each protein (STAT1: siSTAT1#1 and siSTAT1#2; STAT3: siSTAT3#1 and siSTAT3#2). siSTAT3 did not affect IL-6/sIL-6R-induced STAT1 phosphorylation and siSTAT1 did not affect STAT3 phosphorylation (Fig. 3B). In this experiment, hADSCs were cultured in OIM for 8 days after transduction with these siRNAs and protein expression levels were assessed by western blotting. Treatment with either sequence markedly reduced the protein levels of STAT1 and STAT3 compared with administration of the control sequence (Fig. 3C). There were no apparent changes in *WNT5A* mRNA and *ROR2* mRNA expression levels following the suppression of either STATs under OIM conditions (Fig. 3E).

Next, hADSCs were cultured in OIM supplemented with 100 ng/ml of IL-6/sIL-6R after siRNA and *ROR2* mRNA expression was measured 24 h later. While inhibition of

FIG. 3 STAT3 plays an important role in IL-6-mediated *ROR2* mRNA expression



(**A**) Nuclear and cytoplasmic extract from hADSCs stimulated with IL-6/sIL-6R for 30 min were collected. Expression and phosphorylation of STAT1 and STAT3 were detected by western blotting. (**B**, **C**) hADSCs were transfected with STAT1 (siSTAT1#1, 2), STAT3 (siSTAT3#1, 2) or control siRNAs (siCon) for 2 days. hADSCs stimulated with IL-6/sIL-6R within 30 min (**B**) or cultured with OIM alone for 8 days (**C**) were collected. Expression and phosphorylation of STAT1 and STAT3 were detected by western blotting. (**D**, **E**) Transfected hADSCs were cultured in OIM with or without IL-6/sIL-6R (100 ng/ml). Total RNA was isolated from transfected hADSCs cultured in OIM with IL-6/IL-6R at 24 h and *ROR2* (**D**) and *WNT5A* (**E**) mRNA expression was determined by real-time PCR. Values are the mean (S.E.M.) of three independent experiments. $^*P < 0.05$ by *t*-test. n.s: not significant.

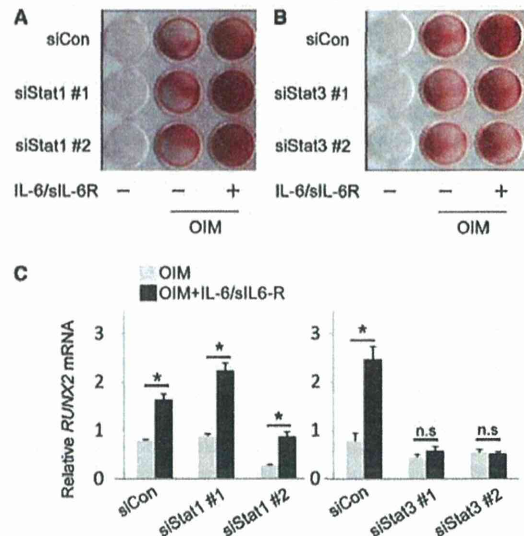
STAT1 protein expression did not show any effect, inhibition of STAT3 protein expression reduced the ability of IL-6/sIL-6R to induce *ROR2* mRNA expression (Fig. 3D). Conversely, inhibition of STAT1 or STAT3 did not affect *WNT5A* mRNA expression (Fig. 3E). These findings suggest that the nuclear transport of pSTAT3 in the IL-6 signalling pathway leads to the enhanced expression of *ROR2* mRNA.

STAT3 is essential for IL-6/sIL-6R-induced osteoblast-like differentiation and mineralization

We examined the possible involvement of STAT1 and STAT3 in the acceleration of calcification in hADSCs by IL-6/sIL-6R. hADSCs were cultured in OIM in the absence or presence of IL-6/sIL-6R and incubated with control or STAT1/3 siRNAs. hADSCs were then cultured in OIM for 8 days. Mild calcification was observed in hADSCs cultured in OIM and the addition of IL-6/sIL-6R markedly accelerated calcification (Fig. 4A). Inhibition of STAT1 protein expression in cultured hADSCs using the two different siRNA sequences hardly affected calcification after 8 days of culture in the presence or absence of IL-6/sIL-6R (Fig. 4A). In contrast, inhibition of STAT3 protein expression markedly inhibited the accelerated calcification of hADSCs induced by IL-6/sIL-6R (Fig. 4B). Although STAT3 inhibition reduced calcification of IL-6/sIL-6R-treated hADSCs to levels close to those of controls, it hardly affected the calcification of hADSCs grown in OIM alone.

Next, we measured *RUNX2* mRNA expression in hADSCs cultured in OIM in the absence or presence of IL-6/sIL-6R following transfection with control or STAT1/3 siRNAs. Incubation in OIM supplemented with IL-6/sIL-6R significantly up-regulated *RUNX2* mRNA expression compared with hADSCs cultured in OIM alone (Fig. 4C). Inhibition of STAT1 protein expression did not alter *RUNX2* mRNA expression, whereas inhibition of STAT3 reduced IL-6/sIL-6R-induced *RUNX2* mRNA expression in hADSCs (Fig. 4C). These results suggest that STAT3 is involved in calcification and osteoblast-like differentiation through a pathway independent of OIM.

Fig. 4 STAT3 is essential for IL-6/sIL-6R-induced osteoblast-like differentiation and mineralization



hADSCs were transfected with STAT1 (siSTAT1#1, 2), STAT3 (siSTAT3#1, 2) or control siRNA (siCon) for 2 days. (**A**, **B**) Transfected hADSCs were cultured in OIM with or without IL-6/sIL-6R (100 ng/ml) and Alizarin Red S staining was performed on day 8. Data are representative of three experiments with similar findings. (**C**) Total RNA was isolated from transfected hADSCs cultured in OIM with IL-6/IL-6R for 24 h and *RUNX2* mRNA expression determined by real-time PCR. Values are the mean (s.e.m.) of three independent experiments. *$P < 0.05$ by *t*-test. n.s: not significant.

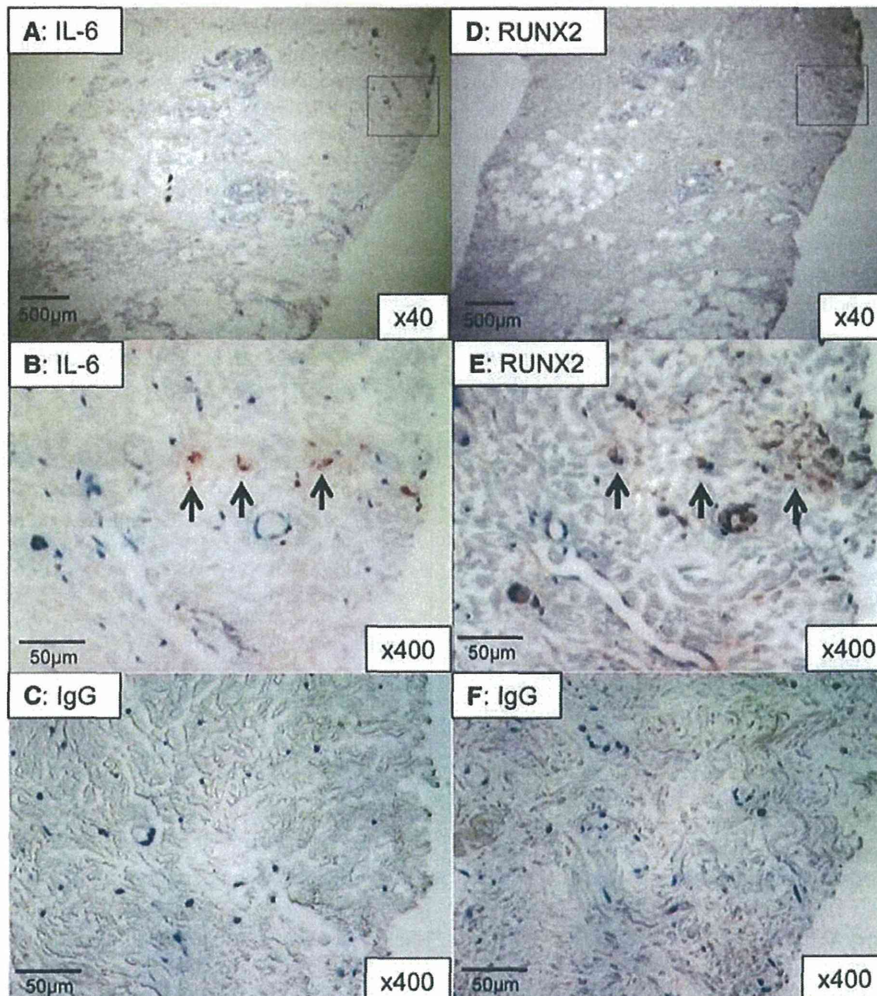
IL-6 and RUNX2 are expressed in the subcutaneous adipose tissue from a patient with DM and ectopic calcification

Finally, we examined a biopsy of a calcified lesion from a 26-year-old female suffering from DM with multiple calcifications in the subcutaneous adipose tissue. The patient also had numerous subcutaneous and/or intramuscular calcifications in the extremities, abdomen and buttocks. The biopsy sample was obtained from an osteoid mass in the left thigh. Polarization microscopy was performed after immunostaining of the epidermal, dermal and adipose tissues with anti-IL-6 (Fig. 5A and B) and anti-RUNX2 antibodies (Fig. 5D and E). Inflammatory changes were noted in the dermal and adipose tissues, and irregular calcium deposits were detected around collagen fibres in the dermis. Immunostaining revealed IL-6-positive cells in collagen fibres in the dermis and RUNX2-positive cells around these cells. Immunohistochemical staining revealed that IL-6 was produced by CD4 T cells and presumably fibroblasts (supplementary Fig. S1, available at *Rheumatology* Online). Although hADSCs could not be observed because of a technical restriction, the cellular effects of IL-6-induced osteoblast-like differentiation were observed in the patient's tissues.

Discussion

The cause and mechanisms of ectopic calcification in inflammatory diseases are poorly understood. The current study investigated the effects of inflammatory cytokines on the mechanisms of calcification in hADSCs. To the best of our knowledge, this is the first study to demonstrate that inflammatory cytokines can accelerate calcification in hADSCs. Of the cytokines tested, IL-6 enhanced calcification to the greatest degree. In addition, IL-6 expression was detected in the calcified subcutaneous tissues obtained from a patient with DM and ectopic calcification, suggesting that IL-6 is involved in the pathology of the disease.

FIG. 5 IL-6 and RUNX2 are expressed in calcified subcutaneous adipose tissues from a patient with DM



(**A, B**) Immunohistochemical staining for IL-6 (arrows). (**D, E**) RUNX2 (arrows) and (**C, F**) control IgG. Original magnification 40× (**A, D**), 400× (**B, C, E, F**).

The canonical and non-canonical WNT pathways play important roles in osteoblast differentiation [15-17]. The current study suggests that IL-6 stimulation of hADSCs could also activate the non-canonical WNT pathway [18, 19]. *WNT5A* is constitutively expressed in hADSCs, and IL-6 stimulation did not increase *WNT5A* mRNA expression further. The enhanced expression of *ROR2* mRNA was detected 24 h after OIM/IL-6 administration, but not after stimulation with OIM alone, or stimulation with OIM supplemented with TNF or IL-1β (Fig. 2 and data not shown). However, pretreatment of the cells with cycloheximide suppressed the enhancement of *ROR2* mRNA. These findings suggest that indirectly enhanced *ROR2* mRNA expression through IL-6-mediated signalling may induce osteoblast-like differentiation. Furthermore, luciferase assay with the ROR2 promoter revealed that IL-6 did not directly induce *ROR2* transcription after a short period of IL-6 stimulation (data not shown).

Of all the inflammatory cytokines tested, IL-6 had the most potent effect on calcification in hADSCs. Previous studies reported that the nuclear transport of phosphorylated STAT3 is critical in IL-6-stimulated osteoblast-like differentiation [14, 20-23]. Consistent with these findings, we observed that stimulation of hADSCs with IL-6 triggered the nuclear transport of STAT3. Furthermore, STAT3-specific siRNA, but not STAT1, blocked the acceleratory effects of IL-6 on osteoblast-like differentiation of hADSCs. The pro-calcifying effects of OIM were not inhibited by STAT3 inhibition, suggesting that the induction of osteoblast-like differentiation by OIM and IL-6 may occur through different signalling pathways. Interestingly, stimulation of hADSCs with IL-6 alone did not induce

calcification, indicating that an as yet unidentified factor is required for IL-6-induced calcification. IL-6 may directly regulate the transcription of this factor, as STAT3-specific siRNA blocked IL-6-induced *ROR2* mRNA expression. Furthermore, a GAS motif, the putative *cis* element for STATs, transcription factors activated downstream of IL-6, is present in the promoter region of *ROR2*. IL-6-induced intracellular signalling can be promoted from its receptor components, gp130 and IL-6Rα or sIL-6Rα [24, 25]. Of these, IL-6 receptor components, only gp130 expression was observed in hADSCs. However, in the subcutaneous adipose tissues *in vivo*, sIL-6Rα may be present because of the shedding action of monocytes, which in turn activates IL-6 [26].

RUNX2 is a master transcription factor indispensable for the differentiation of progenitor cells into osteoblasts [27]. Treatment of hADSCs with OIM alone induced their differentiation into osteoblasts, but calcification only occurred after *RUNX2* mRNA expression. In the presence of inflammatory cytokines, calcification was accelerated. Notably this was accompanied by enhanced *RUNX2* mRNA expression, suggesting that cytokine-induced calcification involves osteoblast-like differentiation. However, in the current study, receptor activator of nuclear factor κB ligand, a late-stage osteoblast differentiation marker, and osteocalcin were not expressed by hADSCs (data not shown). This suggests that normal osteoblast differentiation did not occur in our *in vitro* culture system. This finding could be explained by the observation that peroxisome proliferator-activated receptor gamma, a master transcription factor for adipocyte differentiation, is expressed at higher levels in hADSCs (i.e. adipocyte progenitor cells) than in MSCs derived from other tissues. Thus this effect would counteract osteoblast-like differentiation [28-30]. It is important to note that the hADSCs used in the current study originated from healthy individuals and that hADSCs are largely resistant to osteoblast differentiation [31]. However, hADSCs isolated from patients with complications of ectopic calcification might have defective mechanisms of calcification inhibition.

Finally, we assessed IL-6 and RUNX2 expression in the subcutaneous adipose tissues, providing evidence of ectopic calcification from a patient with DM. IL-6-producing cells were frequently observed and cells expressing RUNX2 were prominent around the IL-6-producing cells. The relevance of serum IL-6 levels to vascular endothelial damage followed by calcification has been reported [32] and the involvement of IL-6 in the pathological processes of atherosclerosis has been reviewed [33]. However, the effect of blocking IL-6 on the pathogenesis of atherosclerosis is unknown. As some patients with JDM do not develop ectopic calcification, it seems likely that the causal factors of calcification are not only inflammatory cytokines. A recent study detected abnormal function and differentiation of MSCs in patients with SLE. Therefore abnormalities in hADSCs may also be involved in the pathogenesis of JDM. Future research is required to examine hADSCs in patients with JDM showing ectopic calcification.

In conclusion, we have developed an OIM-based system to induce osteoblast-like differentiation and observed that activation of STAT3 by IL-6/sIL-6R induced *ROR2* mRNA expression in hADSCs. Thus the expression of *ROR2* and *WNT5A* may induce activation of the non-canonical WNT pathway, leading to acceleration of calcification. IL-6-producing cells and surrounding RUNX2-expressing cells were detected in the subcutaneous adipose tissues from a patient with DM and ectopic calcification. These results suggest that inflammatory cytokine-induced ectopic calcification in hADSCs may also occur in chronic inflammatory tissues associated with other autoimmune diseases and arteriosclerosis. Therefore the development of IL-6 inhibitors may be an effective strategy for treating ectopic calcification in JDM and other inflammatory disorders.

Rheumatology key messages

- IL-6 accelerated mineralization, *RUNX2* mRNA and *ROR2* expression in adipose tissue derived-mesenchymal stem cells (ADSCs).
- IL-6 and RUNX2 were expressed in calcified subcutaneous adipose tissues from a DM patient.
- IL-6 acts on ADSCs and plays a role in ectopic calcification.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Tofacitinib, a JAK inhibitor, inhibits human B cell activation in vitro

B cells initiate and perpetuate autoimmune disease processes. Interleukin (IL)-4 and IL-21 produced by follicular helper T cells are required for B cell activation, germinal centre formation, immunoglobulin class switching and plasma cell differentiation.[1 2] The JAK inhibitor tofacitinib is approved for treatment of rheumatoid arthritis. We recently reported that tofacitinib can suppress IL-17 and interferon-γ production by CD4+ T cells[3] and inhibit the T cell stimulatory capacity of human monocyte-derived dendritic cells.[4] However, whether this action involves B cell activation remains unclear.

Here we investigated the in vitro effects of tofacitinib on the gene regulatory network that controls B cell class switching and

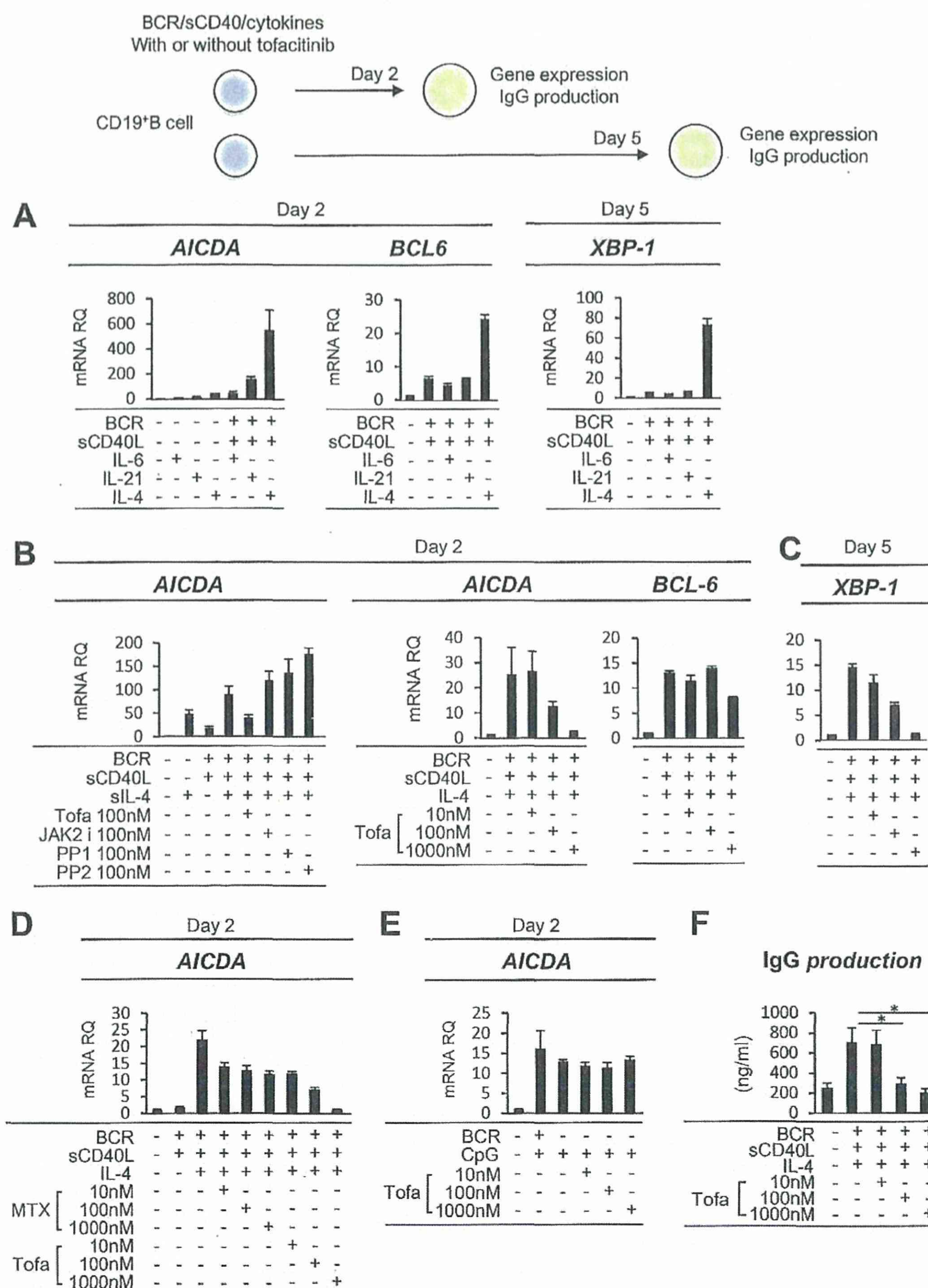

Figure 1 Effects of tofacitinib on the gene regulatory network involved in B cell class switching, differentiation and immunoglobulin (Ig) production. Peripheral blood CD19+ B cells from healthy donors were cultured with 1 μg/mL anti-B cell antigen receptor (BCR) monoclonal antibodies (mAbs), 2 μg/mL human soluble CD40 ligand (sCD40L), 2.5 μg/mL CpG oligodeoxynucleotides (CpG-ODN) 2006 and 100 ng/mL of interleukin (IL)-6, IL-21 and IL-4 for 2days or 5 days, with or without tofacitinib/PP1/PP2/JAK2 inhibitor/methotrexate (MTX), as indicated. (A–E) *AICDA*, *BCL-6* and *XBP-1* gene expression levels were detected by quantitative real-time PCR, with each sample 100 pg mRNA template/reaction. (F) IgG secretion was measured by ELISA. Data are mean±SEM of three independent experiments. *p<0.05, as determined by one-way analysis of variance with Games–Howell comparison test.

differentiation. Purified CD19+ B cells were stimulated with B cell antigen receptor (BCR), soluble CD40 ligand (sCD40L) and cytokines with/without tofacitinib. Culture medium was replenished on day 3. Cell viability tests revealed that, although B cell survival decreased considerably over time, the possibility of pharmacological toxicity by tofacitinib could be excluded (data not shown).

The expression of *AICDA* was slightly induced by cytokines or BCR/sCD40L alone, while costimulation with BCR, sCD40L and cytokines, especially *IL-4*, caused robust gene expression (figure 1A). *BCL6* and *XBP-1* exhibited similar expression patterns, at day 2 and day 5, respectively (figure 1A). BCR/sCD40L costimulated expression of *AICDA* and *XBP-1*was inhibited by tofacitinib in a dose-dependent manner (figure 1B, C). Tofacitinib did not inhibit *BCL6* gene expression on day 2. In contrast with tofacitinib, inhibitors of the Src-family kinase (PP1, PP2) and a JAK2 inhibitor did not affect the expression of *AICDA* (figure 1B). Methotrexate exhibited a modest suppressive effect on *AICDA* expression compared with tofacitinib (figure 1D). In addition, *AICDA* gene expression levels after CpG stimulation were comparable in the presence/absence of tofacitinib (figure 1E). Finally, tofacitinib markedly and dose-dependently abrogated IgG production by B cells stimulated with BCR, sCD40L and IL-4 (figure 1F). These data describe tofacitinib as an effective inhibitor of B cell development.

Next, the effect of tofacitinib on activated B cells was examined. Tofacitinib was added on day 2 of B cell stimulation, and

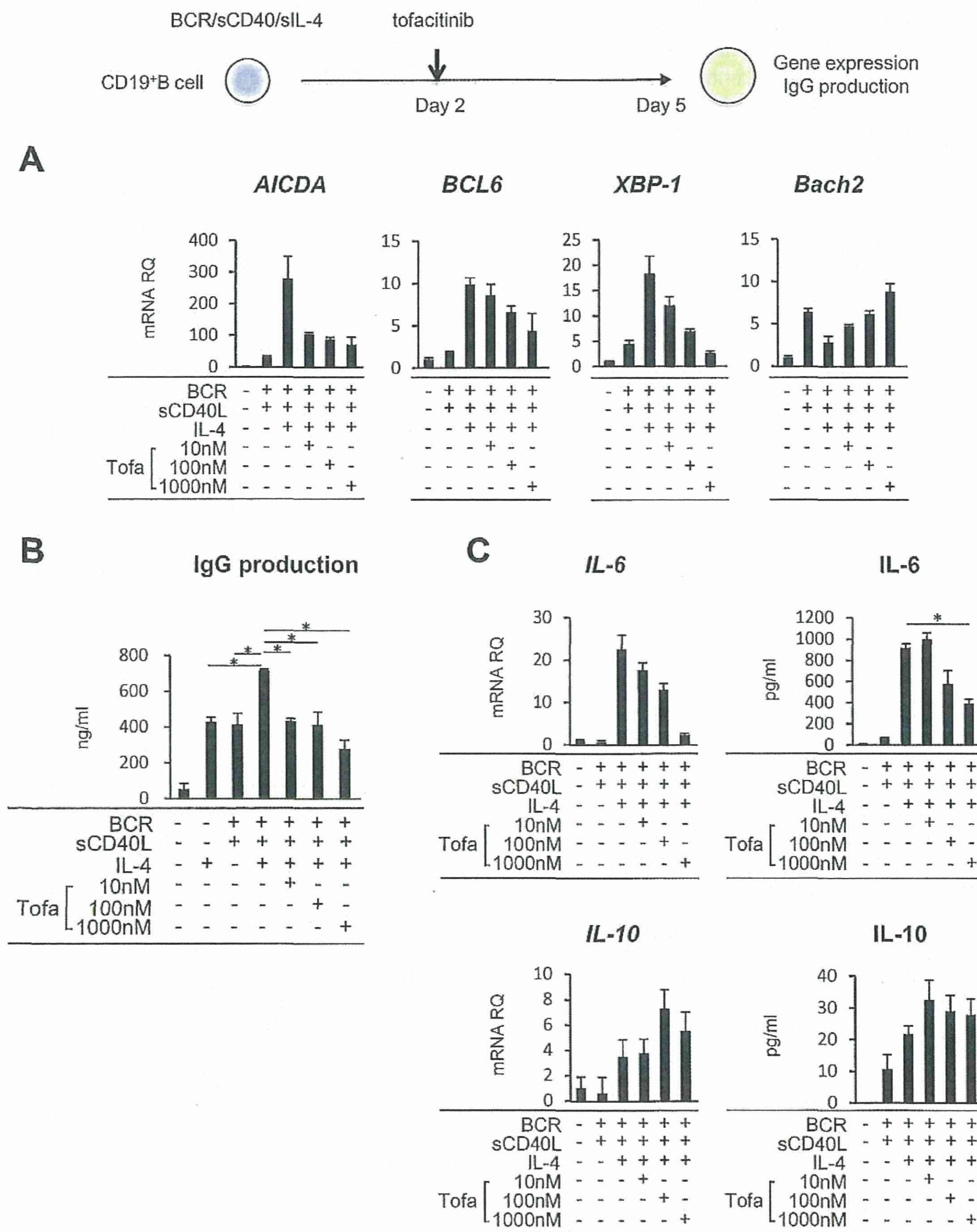

Figure 2 Effect of tofacitinib on highly activated B cells. Human PB CD19+ pan B cells from healthy subjects were cultured with 1 μg/mL anti-B cell antigen receptor (BCR) monoclonal antibodies (mAbs), 2 μg/mL human soluble CD40 ligand (sCD40L) and 100 ng/mL of interleukin (IL)-4 for 2 days, then treated with tofacitinib for another 3 days. (A) Gene expression levels were determined by quantitative real-time PCR, with each sample 100 pg mRNA template/reaction. (B) IgG secretion was measured by ELISA. (C) Cytokine production was measured by cytokine beads array. Data are mean±SEM of three independent experiments. *p<0.05, as determined by one-way analysis of variance with Games–Howell comparison test.

its effects on gene expression and immunoglobulin G (IgG) production were assessed on day 5. Tofacitinib abrogated *AICDA*, *BCL6*, *XBP-1* expression and IgG production to similar levels seen with BCR/sCD40L alone (figure 2A, B). BCL6 protein levels were also reduced by tofacitinib (data not shown). This suggested that tofacitinib may inhibit the cytokine-mediated maintenance of BCL6 at the later time point. Previous studies indicated that *Bach2* impairs germinal centre formation and class switching,[5] its downregulation enhances plasma cell differentiation from IgG1 memory B cells[6] and that *Bach2* also represses effector programmes that stabilise regulatory T cell function.[7] Uniquely, *Bach2* was induced by BCR and sCD40L, but expression was significantly inhibited by IL-4. Interestingly, the addition of tofacitinib resulted in the recovery of *Bach2* expression in a dose-dependent manner (figure 2A).

Finally, we investigated the effect of tofacitinib on the production of proinflammatory and anti-inflammatory cytokines by activated B cells. The strong induction of *IL-6* gene expression and protein production by BCR, sCD40L and IL-4 stimulation was significantly abrogated by tofacitinib in a dose-dependent manner. Meanwhile, IL-10, which is produced by regulatory B cells,[8] was not affected by tofacitinib even at the highest dose (figure 2C). These results suggest that tofacitinib suppresses B cell activation, differentiation and class switching, while maintaining B cell regulatory function.

In conclusion, our results provide new insights into the mechanisms of action of tofacitinib in the treatment of autoimmune diseases.

Sheau-Pey Wang, Shigeru Iwata, Shingo Nakayamada, Kei Sakata, Kunihiro Yamaoka, Yoshiya Tanaka

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

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

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

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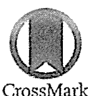

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Anakinra for the management of resistant idiopathic recurrent pericarditis. Initial experience in 10 adult cases

Recurrent idiopathic pericarditis is a common, problematic complication of acute pericarditis, occurring in approximately 30% of cases.[1] Despite appropriate management with non-steroidal anti-inflammatory drugs (NSAIDs), colchicine and corticosteroids (CS), a number of patients are either resistant to treatment requiring long-term therapy with high doses of CS or intolerant to therapy.[2–4]

This disease is currently viewed as an autoinflammatory disease based on its clinical and laboratory features (recurrent episodes of sterile serosal inflammation in the absence of specific autoreactive antibodies or T cells),[5–7] and the preliminary results showing favourable response to intereleukin-1 (IL-1) inhibition.[8–10] Anakinra, a known IL-1 receptor antagonist, has been successfully used in small series of paediatric patients[8 9] while we have recently first reported its efficacy and safety in three adult patients.[10] In this report, we extend our follow-up on these three patients, and present data on seven more adult patients treated with anakinra.

Ten patients with idiopathic, treatment-resistant recurrent pericarditis treated with anakinra were included in the study. The diagnosis of recurrent pericarditis was established by the following three criteria: (1) a documented initial episode of acute pericarditis, (2) reappearance of chest pain attributable to acute pericarditis and (3) at least one of the following findings: pericardial friction rub, fever, typical electrocardiographic changes, pericardial effusion (new appearing or worsening), and C reactive protein (CRP) elevation.[1–3] In all patients, a specific cause of pericarditis was excluded after a thorough evaluation including detailed clinical history and examination, laboratory evaluation and imaging studies. All patients signed an informed consent form prior to enrolment, and the study was approved by the institutional review board. All patients were resistant and/or intolerant to previous treatment with aspirin (ASA) and/or NSAIDs, colchicine and CS, while two (20%) had failed also azathioprine therapy (table 1). The mean number of previous recurrences was 8, the mean baseline dose of prednisolone was 14.1 mg/day (n=8, 2 patients had discontinued CS due to intolerance or side effects) and the mean baseline CRP level was 74 mg/dL.

The treatment protocol of the initial three patients has been previously reported.[10] The next seven patients (table 1, numbers 4–10), were given daily subcutaneous anakinra (100 mg) for 6 months followed by alternate day dosing for

特集 キナーゼ阻害によるリウマチ性疾患の治療 —現在と未来—

CaMKⅣ

一瀬邦弘　古賀智裕　川上　純
ICHINOSE Kunihiro, KOGA Tomohiro, KAWAKAMI Atsushi
長崎大学大学院医歯薬学総合研究科展開医療科学講座（第一内科）

Key Words ▶▶▶▶ 全身性エリテマトーデス　CaMKⅣ　KN-93　Th17

Ca^{2+}/calmodulin-dependent protein kinase Ⅳ（CaMKⅣ）はリンパ球，血球系前駆細胞，神経細胞，胸腺細胞，骨芽細胞などに広く発現しており，シグナル伝達物質として細胞の核内に移行し，さまざまな転写因子活性を有する．CaMKⅣ阻害薬であるKN-93はT細胞におけるIL-2やIL-17発現をコントロールし，全身性エリテマトーデス（SLE）などの自己免疫疾患の病態を制御する可能性がある．またT細胞だけではなく，抗原提示細胞や単球・マクロファージに類似した機能をもつ腎メサンギウム細胞，破骨細胞にも関与し，炎症や増殖を抑制することによって，さまざまな病態における治療的側面を担う可能性がある．

はじめに

Ca^{2+}/calmodulin-dependent protein kinase Ⅳ（CaMKⅣ）は，リンパ球や脳などに多く存在しており，細胞内では核内に局在していることが知られている．CaMKⅣはcAMP response element modulator（CREM）などの転写因子をリン酸化することにより，カルシウム依存的に遺伝子の発現調節をおこなっている．最近のゲノムワイド関連研究（genome-wide association study：GWAS）ではリウマチ性疾患のなかでも全身性エリテマトーデス（systemic lupus erythematosus：SLE）はほかの多因子疾患と比較して，免疫系における機能を有する関連多型が多く見出されている[1]．SLEのT細胞の核内ではCaMKⅣの発現が増加しており，Interleukin（IL）-2を介したT細胞機能の異常を引き起こすことが報告されている．正常のT細胞のSLE患者血清で刺激をおこなうと，血清中の抗CD3抗体によりT細胞レセプター（T-Cell Receptor：TCR）を介してCaMKⅣが活性化され，核内に移行することが想定されている[2]．核内に移行したCaMKⅣはIL-2プロモーター領域の－180にあるCREのsiteでCREMαをリン酸化しIL-2発現を低下させている（図❶A）．

一方，IL-17を産生するT helper（Th）17細胞は関節リウマチ，乾癬，SLEなどの自己免疫疾患の発症に関与することが知られている．そのなかでSLEではIL-17を産生するTh17細胞と制御性T細胞（Treg）のアンバランスが発症に関与していると考えられている．KogaらはCaMKⅣがCREMαを介したIL-17産生をコントロールしながらTh17細胞の分化に関与することを報告し，Th17細胞におけるCaMKⅣ-CREM pathwayの役割を明らかにした[3]（図❶B）[4]．

またCaMKⅣはSLEのモデルマウスであるMRL/*lpr*マウスのT細胞でも発現が亢進している．われわれはCaMKⅣ阻害薬である低分子化合物KN-93（図❶C，D）