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Activation of peroxisome proliferator-activated receptor γ inhibits TNF-α-mediated osteoclast differentiation in human peripheral monocytes in part via suppression of monocyte chemoattractant protein-1 expression

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Abstract

Tumor necrosis factor-α (TNF-α) plays critical roles in bone resorption at the site of inflammatory joints. The aim of this study is to evaluate the effect of peroxisome proliferator-activated receptor γ (PPAR-γ) agonists, a new class of anti-inflammatory compounds, on TNF-α-mediated osteoclastogenesis in human monocytes. Human monocytes were differentiated into osteoclasts in the presence of TNF-α and macrophage colony-stimulating factor. Tartrate-resistant acid phosphatase (TRAP) staining and a pit formation assay using dentin were used for the identification of activated osteoclasts. The protein and gene expressions of transcription factors were determined by immunofluorescence and real-time RT-PCR analysis, respectively. TNF-α-induced osteoclast generation from human peripheral monocytes in a dose-dependent manner, and the induction was not inhibited by osteoprotegerin, a decoy receptor for receptor activator of NF-κB ligand. The addition of PPAR-γ agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) or ciglitazone, to the culture resulted in a remarkably reduced number of generated osteoclasts. In addition, both agonists inhibited the protein and gene expressions of nuclear factor of activated T-cell isoform c1 (NFATc1), c-Fos, c-Jun and NF-κB p65, which are known to be associated with osteoclastogenesis. GW9662, an antagonist of PPAR-γ, fully rescued ciglitazone-induced inhibition, but did not affect 15d-PGJ_2-induced inhibition. Monocyte chemoattractant protein-1 (MCP-1), a CC chemokine related to osteoclastogenesis, was induced during TNF-α-mediated osteoclast differentiation, and the neutralizing antibody to MCP-1 reduced osteoclast formation by about 40%. 15d-PGJ_2 and ciglitazone blocked the induction of MCP-1 by TNF-α. Moreover, the addition of MCP-1 rescued the inhibition of TRAP-positive multinucleated cell (TRAP-MNCs) formation by 15d-PGJ_2 and ciglitazone, although generated TRAP-MNCs had no capacity to resorb dentin slices. Our data demonstrate that 15d-PGJ_2 and ciglitazone down-regulate TNF-α-mediated osteoclast differentiation in human cells, in part via suppression of the action of MCP-1. These PPAR-γ agonists may be a promising therapeutic application for rheumatoid arthritis and inflammatory bone-resorbing diseases.

Keywords: Osteoclastogenesis; TNF-α; PPARγ; MCP-1; Human monocytes

Introduction

Rheumatoid arthritis (RA) is characterized by the presence of inflammatory synovitis accompanied by the destruction of joint cartilage and bone [1]. An increasing body of evidence has demonstrated that osteoclasts are the principal cell type responsible for bone resorption in inflammatory joint diseases. Multinucleated giant cells with phenotypic features of osteoclasts

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are present at erosion sites in RA [2,3] and collagen-induced arthritis animal models [4]. Furthermore, it has been reported that mice lacking osteoclasts were resistant to arthritis-induced bone erosion [5]. These findings imply that it is important for the development of RA treatment to explore the chemical compounds inhibiting osteoclasts generated at inflammatory sites.

A number of studies suggest that a receptor activator of the NF-κB ligand (RANKL) system plays pivotal roles in inflammatory joint destruction. For example, osteoprotegerin (OPG) suppressed tumor necrosis factor-α (TNF-α)-mediated arthritis in mouse models [6,7]. In addition, RANKL-knockout mice were protected from bone erosion in a serum transfer model of arthritis [5]; however, it remains controversial how RANKL contributes to bone destruction in RA. A variety of cytokines and factors produced by synovial tissues are known to be inducers of osteoclastogenesis [8]. Among them, TNF-α has been shown to play a critical role in bone destruction in RA, and TNF-α is known to induce osteoclastogenesis in humans [9,10] and rodents [11]. Studies in mice overproducing or lacking TNF-α revealed the importance of this cytokine in articular bone loss in inflammatory arthritis [6,7,12–14]. Blockade of TNF-α activity using biologic agents, including recombinant soluble p75TNFR and an anti-human TNF-α antibody, has shown efficacy in retarding the radiographic progression of focal bone erosion [15–17]. Overall, it is essential to down-regulate TNF-α-mediated osteoclast differentiation for the treatment of RA; however, the precise regulatory mechanism of TNF-α-associated osteoclastogenesis remains unclear.

Peroxisome proliferator-activated receptor γ (PPAR-γ) is a member of ligand-activated transcription factors in the nuclear receptor superfamily, and was initially characterized as a key regulator of adipocyte differentiation and lipid metabolism [18]. Recent studies have shown that PPAR-γ agonists have anti-inflammatory and immunomodulatory therapeutic effects [19,20]. Indeed, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a natural PPAR-γ ligand, as well as synthetic PPAR-γ agonists, have been shown to ameliorate the arthritis score and bone destruction in mouse models [21–23]. Furthermore, PPAR-γ agonists have been reported to inhibit RANKL-induced osteoclastogenesis in humans *in vitro* [24]. These findings prompted us to examine the effect of PPAR-γ agonists on TNF-α-mediated osteoclastogenesis from human monocytes. In this study, we first confirmed that TNF-α could differentiate human peripheral monocytes into activated osteoclasts, and next examined whether PPAR-γ agonists inhibited the TNF-α-mediated osteoclastogenesis. In addition, the intracellular mechanism(s) through which PPAR-γ inhibited osteoclastogenesis was also studied.

Materials and methods

Reagents

Human macrophage colony-stimulating factor (M-CSF), human soluble RANKL, TNF-α, and human OPG were purchased from PeproTech EC (London, UK). Human monocyte chemoattractant protein-1 (MCP-1) and a goat polyclonal antibody to MCP-1 were purchased from R&D Systems (Minneapolis, MN). Goat polyclonal IgG was purchased from Immuno-Biological Laboratories (Gunma, Japan). 15d-PGJ_2 and ciglitazone were from Calbiochem (San Diego, CA). GW9662 was from Sigma (Saint Louis, MO).

Purification of peripheral blood monocytes

Human blood samples were obtained from 4 healthy donors after taking written informed consent. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden) gradient centrifugation. Highly purified peripheral blood monocytes were prepared from PBMCs by immunomagnetic selection using MACS microbeads with a cocktail of biotin-conjugated monoclonal antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and Glycophorin A with microbeads conjugated to monoclonal anti-biotin antibody (clone; Bio3-18E7. 2; mouse IgG1) for the negative selection of monocytes (human monocyte isolation kit, Miltenyi Biotec GmbH, Bergish Gladbach, Germany). All procedures were carried out according to the instruction manual. More than 92% of obtained cells were CD 14-positive, as determined by flow cytometry.

Culture system for osteoclastogenesis

Peripheral blood monocytes from one donor were cultured in a 96-well tissue culture plate at the indicated cell density with or without dentin slices (4-mm diam.). Cultures were maintained in 200 μl of α-minimal essential medium containing 10% fetal bovine serum for 14–28 days with both or either of M-CSF and TNF-α in the presence of reagents, including RANKL, 15d-PGJ_2, ciglitazone or GW9662 where needed. Half of the culture medium was replaced with fresh medium containing cytokines and chemicals every 3 days. MCP-1 and anti-MCP-1 antibodies were added to the culture medium where indicated. TNF-α, M-CSF, RANKL, 15d-PGJ_2, ciglitazone, GW9662 and MCP-1 had no effect on cell viability at concentrations used in this study.

Tartrate-resistant acid phosphatase (TRAP) staining

Cultured adherent cells were fixed with 3.7% (v/v) formaldehyde in phosphate buffered saline without calcium and magnesium (PBS) (−) for 10 min, fixed again with ethanol–acetone (50:50,v/v) for 1 min, and incubated for 10 min at room temperature with TRAP staining solution [25]. TRAP-positive cells appeared dark red and TRAP-positive multinucleated cells containing more than three nuclei (TRAP-MNCs) were counted on 96-well plates with light microscopy. Data are shown as the total cell number of TRAP-MNCs in each well, with the mean±SD of 4 determinations at each point.

Pit formation assay

Osteoclast activity was assessed by the bone resorption assay. After culturing human monocytes on dentin slices, the slices were rinsed with PBS (−) and left overnight in 1 M ammonium hydroxide to remove all cells and their debris. The slices were then washed with PBS (−) and stained with 0.5% (w/v) toluidine blue. Pit formation was analysed with light microscopy and scanning electron microscopy.

Immunofluorescence analysis

Human blood monocytes on glass culture slides were incubated with M-CSF at 37 °C for 48 h, and then treated with or without TNF-α, 15d-PGJ_2, ciglitazone or GW9662 under different experimental conditions. They were fixed with 4% paraformaldehyde in PBS (10 min), washed in PBS, permeabilized with 0.1% Triton X-100 in PBS (10 min), washed in PBS, and blocked with 3% bovine serum albumin (BSA) for 1–2 h at room temperature. The primary antibodies used were mouse monoclonal antibodies to the nuclear factor of activated T-cells isoform c1 (NFATc1, 7A6), c-Fos (D-1), and NF-κB p65 (F-6) purchased from SantaCruz Biotechnology (Santa Cruz, CA). These antibodies were diluted 1:100 in 1% BSA and applied overnight at 4 °C. The second antibody was fluorescent anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted 1:50 in HEPES buffer pH 8.2. For the localization of c-Jun, the primary antibody was

rabbit polyclonal antibody (H-79, SantaCruz Biotechnology). The antibody was diluted 1:100 in 1% BSA and applied overnight at 4 °C. The second antibody was fluorescently labeled goat anti-rabbit IgG (Texas red-conjugated goat) (Vector Laboratories, Burlingame, CA) diluted 1:50 in HEPES buffer pH 8. 2. Coverslips were mounted on slides, and fixed cells were examined with an Olympus AX 80 T fluorescence microscope.

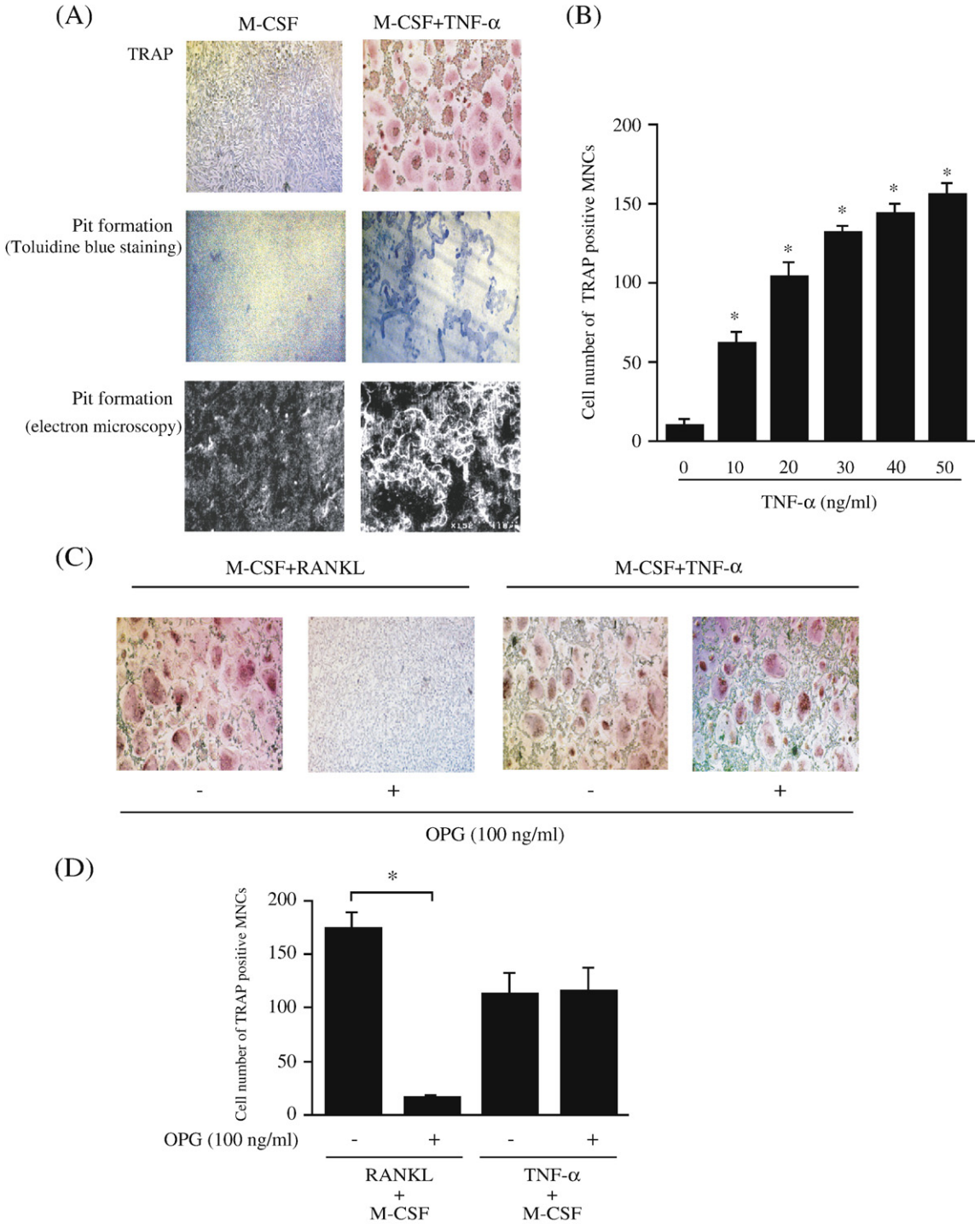


Fig. 1. TNF-α induced osteoclast differentiation in human peripheral monocytes independently of RANKL. (A) Peripheral blood monocytes (1×10^5 cells per well) were cultured either in 96-well plates for 14 days or on dentin slices for 28 days in 96-well plates in the presence of TNF-α (20 ng/ml) and M-CSF (50 ng/ml). The generated osteoclasts were subjected to tartrate-resistant acid phosphatase (TRAP) staining. The generated resorption pits on the dentin slices were determined by scanning electronic microscopy. (B) Human monocytes were cultured with various concentrations of TNF-α in the presence of M-CSF (50 ng/ml) for 14 days. The generated TRAP-MNCs were counted as osteoclasts. * $P<0.01$ versus M-CSF alone. (C, D) Human monocytes were cultured with RANKL (50 ng/ml), TNF- α (20 ng/ml) or M-CSF (50 ng/ml) in the presence or absence of OPG (100 ng/ml) for 14 days. The cultured adherent cells were subjected to TRAP staining. TRAP-MNCs containing more than three nuclei were counted as osteoclasts. Results are expressed as the mean±SD of four determinations. * Indicates a significant difference between the indicated groups (* $P<0.01$). Data are representative of three experiments with blood monocytes from different donors.

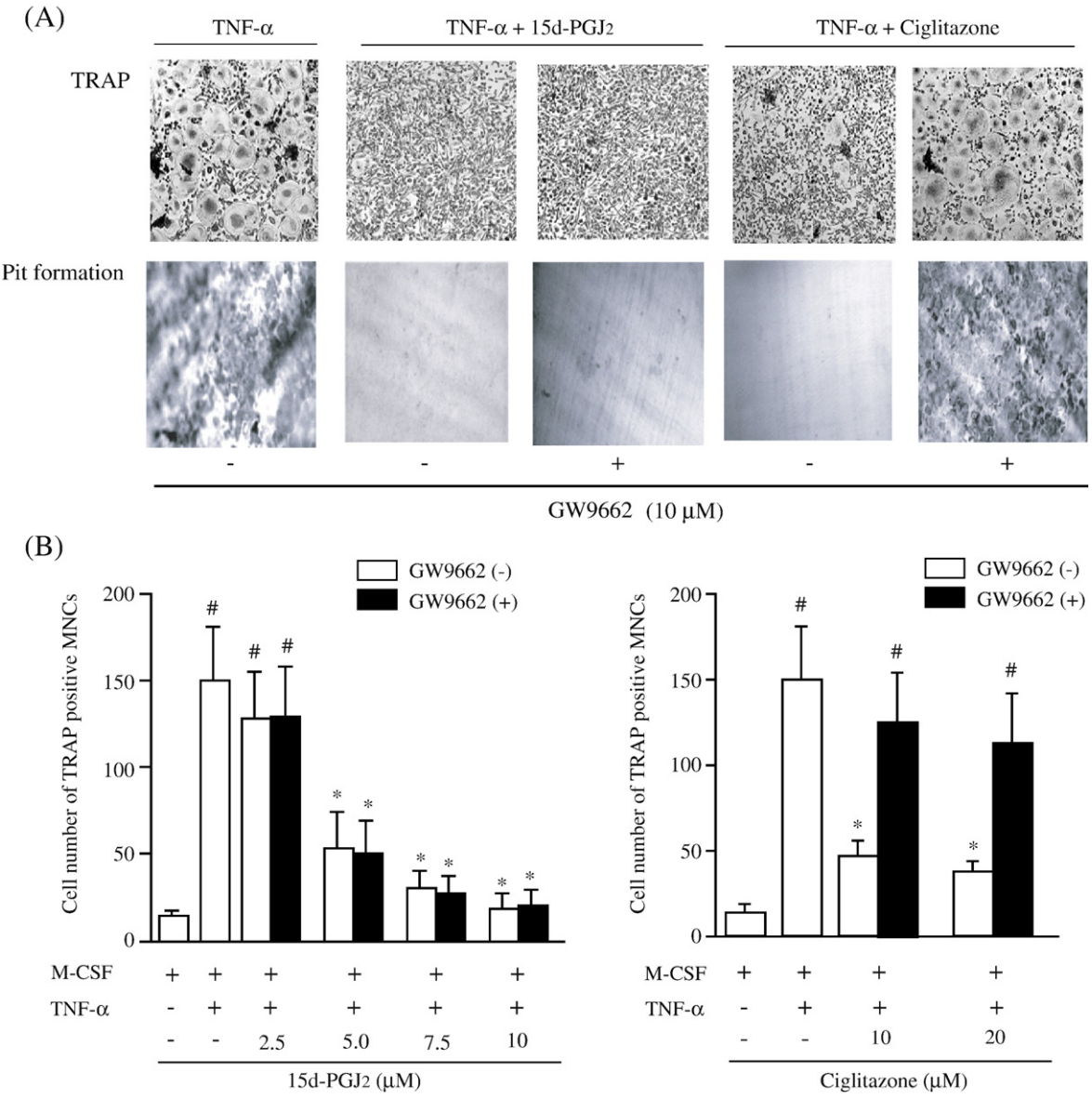
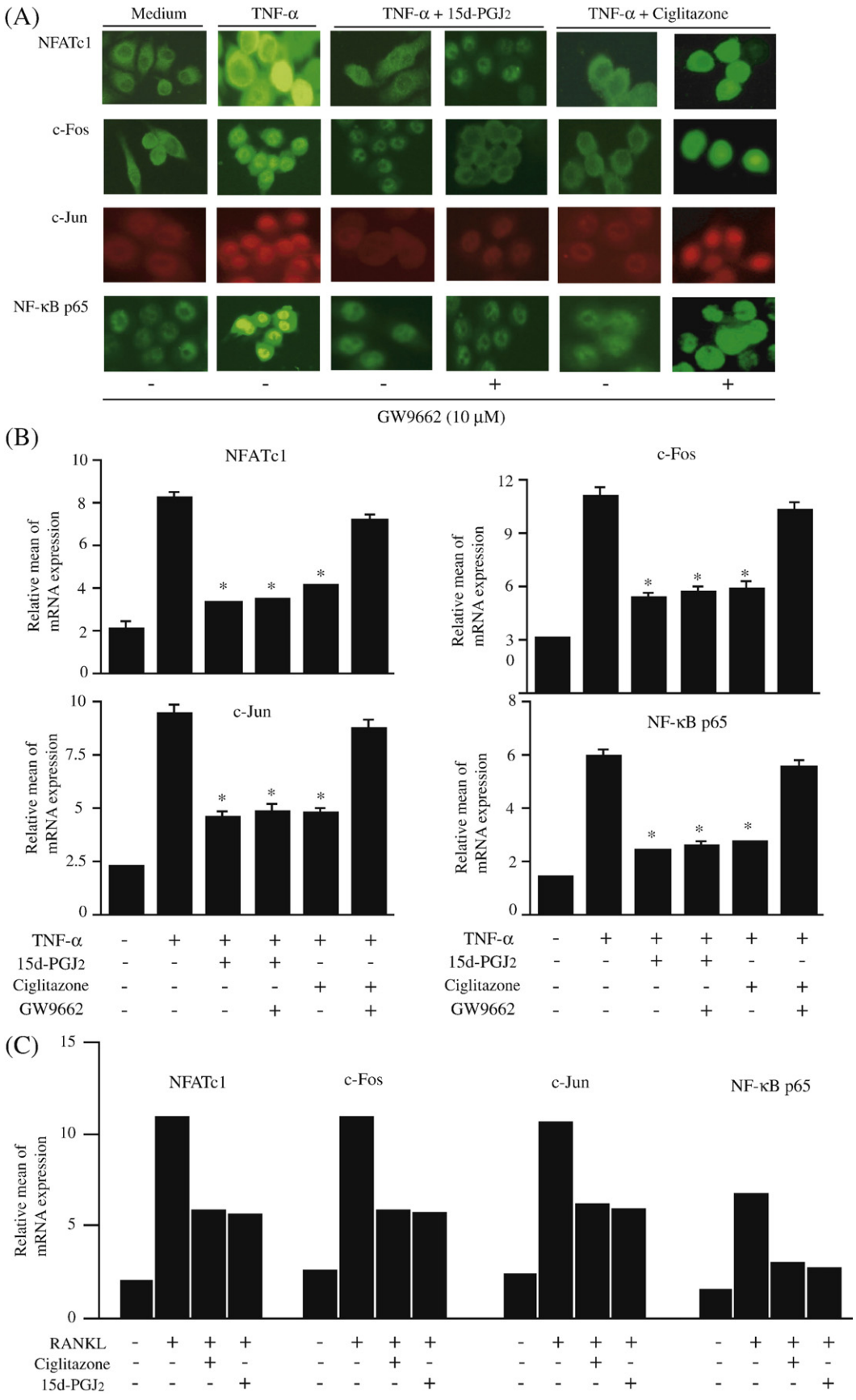


Fig. 2. PPAR-γ-activation inhibited TNF-α-induced osteoclast differentiation in human monocytes. Peripheral blood monocytes (1×10^5 cells per well in a 96-well plate) were preincubated with GW9662 (10 μM) or medium alone for 30 min. After washing, cells were cultured with 15d-PGJ_2 (10 μM) or ciglitazone (20 μM) in the presence of TNF-α (20 ng/ml) and M-CSF (50 ng/ml) for 14 days (for TRAP staining) and 28 days (for pit formation assay). (A) The generated osteoclasts were determined by TRAP staining and pit formation assay as described in Materials and methods. (B) TRAP-MNCs containing more than three nuclei were counted as osteoclasts. Results are expressed as the mean±SD of four determinations. # $P<0.01$ versus M-CSF alone. * $P<0.01$ versus TNF-α plus M-CSF. Data are representative of three experiments with blood monocytes from different donors.

Fig. 3. 15d-PGJ_2 and ciglitazone inhibit TNF-α induced expressions of osteoclast-related transcription factors, NFATc1, c-Jun, c-Fos, and NF-κB in human monocytes. (A) Immunofluorescence analysis. Human monocytes on glass culture slides were preincubated with GW9662 (10 μM) or medium alone for 30 min. After washing, the cells were cultured with or without 15d-PGJ_2 (10 μM) or ciglitazone (20 μM) in the presence of M-CSF (50 ng/ml) and TNF-α (20 ng/ml) for 24 h. Cultured cells were fixed and permeabilized for immunofluorescence assays as described in Materials and methods. (B) Real-time quantitative RT-PCR analysis. Blood monocytes were preincubated with GW9662 (10 μM) or medium alone for 30 min. After washing, cells were cultured with or without 15d-PGJ_2 (10 μM) or ciglitazone (20 μM) in the presence of M-CSF (50 ng/ml) and TNF-α (20 ng/ml) for 24 h. After culture, total cellular RNA was extracted, and used for Real-Time PCR analysis for the detection of NFATc1, c-Fos, c-Jun and NF-κB (p65) mRNA levels as described in Materials and methods. Results are expressed as the relative means±SD of target gene expression. GAPDH was used as an internal control to standardize the expression of target genes. * $P<0.01$ versus TNF-α alone. Data are representative of two experiments with blood monocytes from different donors. (C) Human monocytes were cultured with or without 15d-PGJ_2 (10 μM) or ciglitazone (20 μM) in the presence of M-CSF (50 ng/ml) and RANKL (50 ng/ml) for 24 h. After culture, total cellular RNA was extracted for real-time RT-PCR analysis. Data were expressed as means of triplicate determinations.


(A)
Medium
TNF-α
TNF-α + 15d-PGJ2
TNF-α + Ciglitazone
NFATc1
c-Fos
c-Jun
NF-κB p65
- - - + - +
GW9662 (10 μM)
(B)
NFATc1
c-Fos
c-Jun
NF-κB p65
Relative mean of mRNA expression
TNF-α - + + + + +
15d-PGJ2 - - + + - -
Ciglitazone - - - - + +
GW9662 - - - + - +
(C)
NFATc1
c-Fos
c-Jun
NF-κB p65
Relative mean of mRNA expression
RANKL - + + + - + + + - + + + - + + +
Ciglitazone - - + - - - + - - - + - - - + -
15d-PGJ2 - - - + - - - + - - - + - - - +

Real-time RT-PCR analysis

Quantitative real-time RT-PCR was performed according to the manufacturer's protocol (Taqman, Applied Biosystems, Foster City, CA). Total RNA was isolated from the variously treated monocytes using ISOGEN (Nippon Gene, Tokyo, Japan) and was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and random hexamer according to the manufacturer's manual. For amplification in an ABI PRISM 7700 system, Assay-on Demand for NFATc1 (Hs00542678 ml), c-Fos (Hs00170630 ml), c-Jun (Hs00277190 s1), NF-κB p65 (Hs00153294 ml) and GAPDH (Hs99999905 m1) were used. Each cDNA was amplified with a 10 μl Taqman universal PCR master mix (Applied Biosystems) and the Assay-on-Demand mixture was diluted at 1:20, according to the manufacturer's instructions. The PCR conditions were incubation at 95 °C for 10 min for initial denaturation, followed by 40 cycles. Each cycle consisted of 92 °C for 15 s, and 60 °C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Measurement of MCP-1

The concentration of human MCP-1 in the culture medium was measured by an ELISA kit (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using either ANOVA (with Bonferroni post-hoc testing) or unpaired *t* tests. A confidence level of 95% ($P<0.05$) was chosen as statistical significance.

Results

TNF-α induced osteoclastogenesis in human peripheral monocytes independently of RANKL

The requirement of RANKL signaling in TNF-α-mediated osteoclast differentiation remains controversial. We first examined whether TNF-α directly induced the differentiation of osteoclasts from peripheral monocytes. Highly purified monocytes were cultured with or without TNF-α in the presence of M-CSF. As shown in Fig. 1-AB, TNF-α potently induced the generation of TRAP-MNCs in a dose-dependent manner. The generated cells produced resorption pits on dentin slices. To clarify the requirement of RANKL signaling in this osteoclastogenesis, we examined the effect of OPG, a decoy receptor for RANKL, on TNF-α-mediated osteoclastogenesis. Human monocytes were cultured with RANKL or TNF-α in the presence or absence of OPG for 14 days. As shown in Fig. 1-CD, OPG completely inhibited the RANKL-induced generation of osteoclasts. On the other hand, OPG did not affect TNF-α-mediated osteoclast formation.

PPAR-γ activation inhibited TNF-α-induced osteoclast differentiation in human monocytes

We next focused on PPAR-γ agonists because they exert anti-inflammatory effects on monocytes/macrophages, and have a protective effect on arthritis animal models *in vivo*. We first investigated the effect of 15d-PGJ_2 and ciglitazone as natural ligands and synthetic agonists for PPAR-γ, respectively, on TNF-α-induced osteoclast differentiation. Human monocytes were cultured with 15d-PGJ_2 or ciglitazone in the presence of TNF-α and M-CSF. 15d-PGJ_2 and ciglitazone potently inhibited the generation of osteoclasts in a dose-dependent manner, as determined by TRAP staining and Pit formation assay (Fig. 2-AB). We next tested the effect of GW9662, a PPAR-γ antagonist, on PPAR-γ agonist-induced inhibition to clarify whether the inhibition was PPAR-γ-mediated. Human monocytes were preincubated with GW9662 30 min before the addition of 15d-PGJ_2 or ciglitazone, and cultured for 14 days. As shown in Fig. 2-AB, GW9662 rescued the ciglitazone-induced inhibition of osteoclastogenesis, whereas it did not affect 15d-PGJ_2-induced inhibition. We next examined the effect of PPAR-γ agonists on the expression of osteoclastogenesis-associated transcription factors by immunofluorescence and real-time RT-PCR analyses. As shown in Fig. 3-AB, TNF-α potently enhanced the expressions of NF-κB p65, c-Jun, c-Fos and NFATc1 at protein and mRNA levels. 15d-PGJ_2 and ciglitazone inhibited the enhanced expressions of transcription factors by TNF-α. Interestingly, GW9662 rescued ciglitazone-induced inhibition, but did not affect 15d-PGJ_2-induced inhibition. Furthermore, these PPAR-γ agonists also reduced the RANKL-induced gene expressions of the transcription factors (Fig. 3-C).

MCP-1 was involved in the ciglitazone- and 15d-PGJ_2-induced inhibition of TNF-α-mediated osteoclastogenesis in human monocytes

MCP-1, which is known to be expressed in rheumatoid joint, has been shown to be involved in osteoclast differentiation in human monocytes; therefore, we first evaluated the role of MCP-1 in TNF-α-mediated osteoclast differentiation. As shown in Fig. 4-AB, TNF-α stimulated monocytes to produce substantial amounts of MCP-1. The addition of neutralizing anti-MCP-1 antibody to the culture resulted in the reduction of TNF-α-mediated osteoclast formation by about 40%, indicating that the induced MCP-1 is remarkably related to TNF-α-induced osteoclastogenesis. Ciglitazone and 15d-PGJ_2 potently inhibited the production of MCP-1 in a dose-dependent manner, and the inhibitory effect of ciglitazone was partially overcome by GW9662, whereas GW9662 did not affect 15d-PGJ_2-induced inhibition (Fig. 4-C). It is of interest to know the effect of MCP-1 on PPAR-γ activation-induced inhibition of osteoclast differentiation. As shown in Fig. 4-D, MCP-1 rescued ciglitazone and 15d-PGJ_2-induced inhibition of TRAP-MNC formation. MCP-1 induced the generation of TRAP-MNCs at a concentration of 30 ng/ml and above (Fig. 4-E); however, the generated cells were not able to resorb dentin slices (Fig. 4-F).

Discussion

We demonstrated here that TNF-α promoted osteoclastogenesis in human monocytes, and that osteoclast formation was not inhibited by OPG, a decoy receptor for RANKL. Whether TNF-α promotes osteoclastogenesis independently of RANKL or not is under debate. A number of studies demonstrated that RANK–RANKL signaling was not required for TNF-α-induced osteoclast differentiation in mouse bone marrow cells [11,26]. On the other hand, Lam, et al. reported that permissive

levels of RANKL were required for the induction [27]. Additionally, Kim, et al. demonstrated that hematopoietic precursors from TRANCE-, RANK-, or TRAF-null mice became osteoclasts with stimulation by TNF-α in the presence of cofactors such as TGF-β [28]. In the human system, TNF-α-induced osteoclast differentiation was independent of RANKL-mediated signaling in PBMC and adherent PBMC [9,10]. These findings are consistent with our results; however, our evidence was only

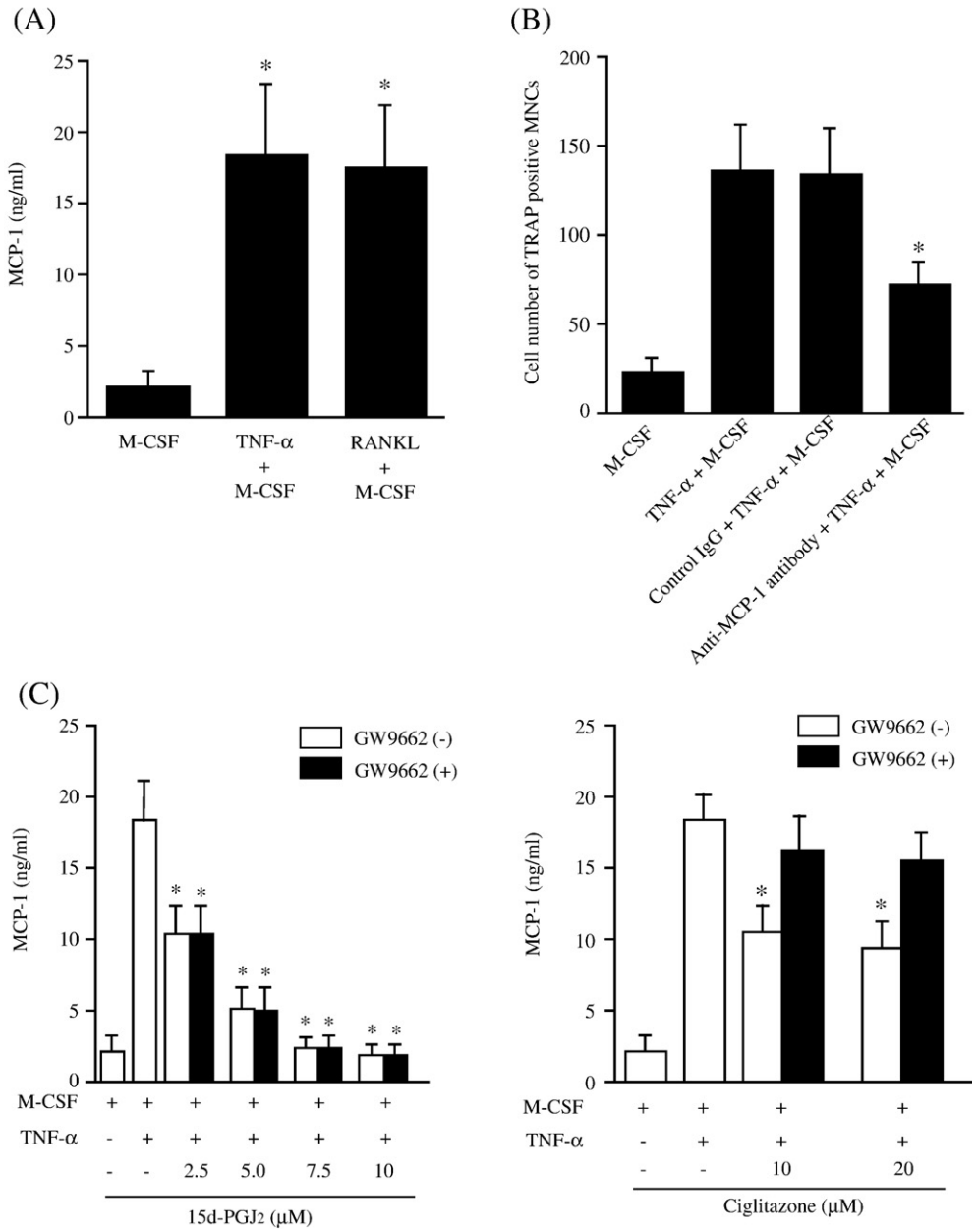


Fig. 4. MCP-1 was involved in PPAR-γ activation-induced inhibition of TNF-α mediated osteoclast differentiation. (A) Peripheral blood monocytes (1×10^5 cells per well in a 96-well plate) were cultured with TNF-α (20 ng/ml) or RANKL (50 ng/ml) in the presence of M-CSF (50 ng/ml) for 48 h. The amount of MCP-1 in the culture medium was measured by an ELISA assay. Results are expressed as the mean±SD. * $P<0.01$ versus M-CSF alone. (B) Human monocytes were cultured with neutralizing antibody to MCP-1 (1 μg/ml) or goat polyclonal IgG antibody (1 μg/ml) in the presence of TNF-α (20 ng/ml) and M-CSF (50 ng/ml) for 14 days. TRAP-MNCs with more than three nuclei were counted as osteoclasts. Results are expressed as the mean±SD. * $P<0.01$ versus TNF-α alone. (C) Human monocytes (1×10^5 cells per well in a 96-well plate) were preincubated with GW9662 (10 μM) or medium alone for 30 min. After washing, cells were cultured with M-CSF (50 ng/ml) and TNF-α (20 ng/ml) in the presence or absence of indicated concentrations of 15d-PGJ_2 and ciglitazone for 48 h. The amount of MCP-1 in the cultured supernatant was measured by an ELISA kit as described in Materials and methods. Results are expressed as the mean±SD of four determinations. * $P<0.01$ versus TNF-α alone. (D) Human monocytes were cultured with TNF-α (20 ng/ml), MCP-1 (50 ng/ml), 15d-PGJ_2 (10 μM) or ciglitazone (20 μM) in the presence of M-CSF (50 ng/ml) for 14 day. Results are expressed as the mean±SD. * Indicates a significant difference between the indicated groups (* $P<0.01$). (E) Human monocytes were cultured with TNF-α (20 ng/ml), M-CSF (50 ng/ml) or 15d-PGJ_2 (10 μM) in the presence of various concentrations of MCP-1 for 14 days. $P<0.01$ versus the culture in TNF-α and 15d-PGJ_2. (F) Human monocytes were cultured with 15d-PGJ_2 (10 μM), ciglitazone (20 μM) or MCP-1(50 ng/ml) in the presence of TNF-α (20 ng/ml) and M-CSF (50 ng/ml) for 14 days (For TRAP staining) or 28 days (For pit formation assay). All data are representative of at least two experiments with blood monocytes from different donors.

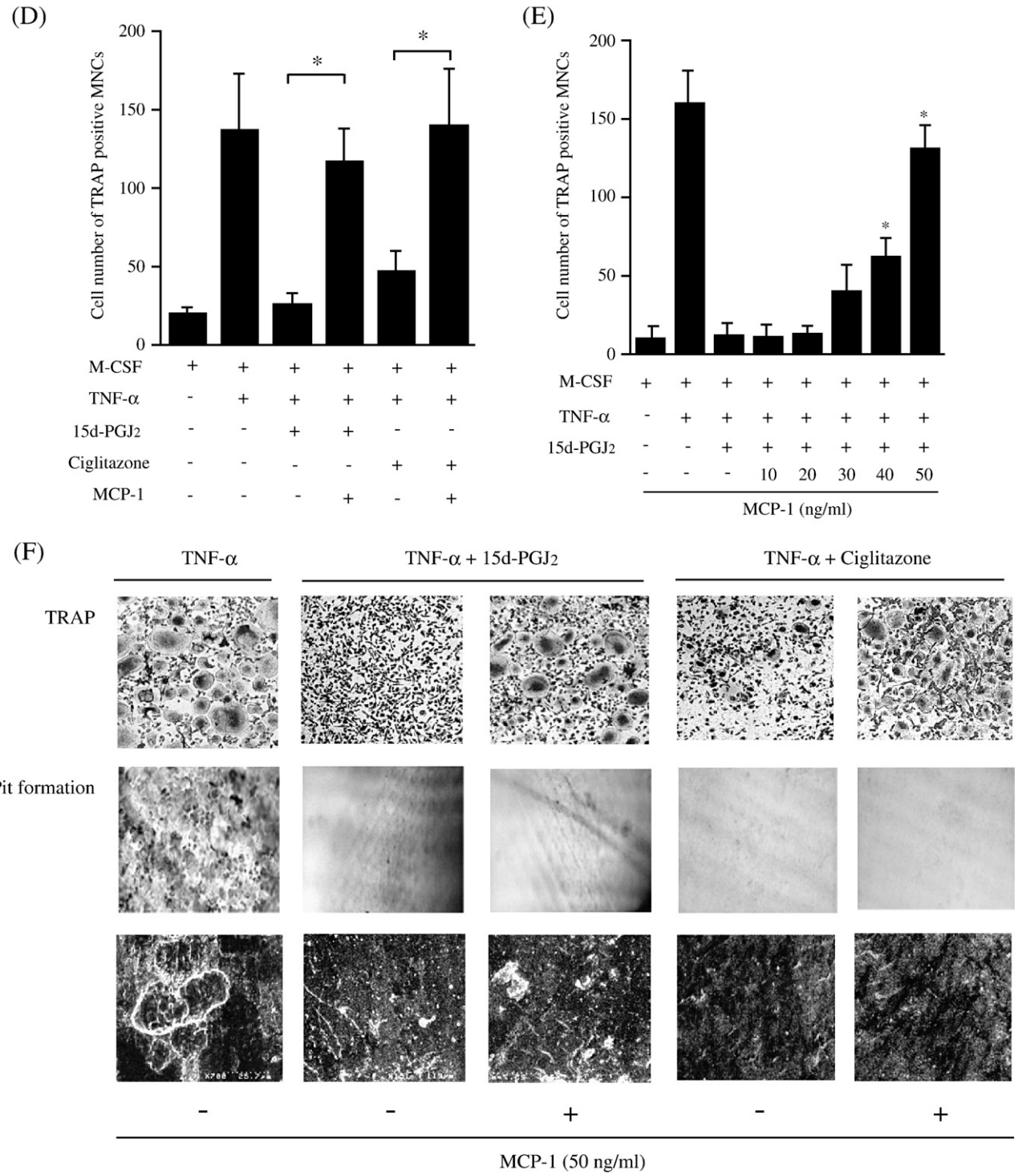


Fig. 4 (*continued*).

based on rescue by OPG (Fig. 1-CD). Further study is needed to resolve the question. Considering the critical roles of TNF-α in bone erosion, our *in vitro* system may become a useful tool for screening chemical compounds effective in inflammatory bone resorption.

We demonstrated here that TNF-α-induced osteoclast generation was potently inhibited by PPAR-γ agonists, ciglitazone and 15d-PGJ_2. Inhibition by these agonists was also observed with RANKL stimulation (data not shown). In addition, they also inhibited the expressions of the osteoclastogenesis-associated transcription factors including NF-κB, c-Fos, c-Jun and NFATc1. To our knowledge, this is the first report showing that PPAR-γ activation inhibits TNF-α-induced osteoclastogenesis in human cells, suggesting that PPAR-γ agonists would be effective in

TNF-α-induced inflammatory bone loss. Under physiological conditions, PPAR-γ is known to negatively control bone metabolism via a decreased number of osteoblasts *in vivo* [29,30], although RANKL-mediated osteoclastogenesis was inhibited by the activation of PPAR-γ *in vitro* [24]. At the site of inflammation, PPAR-γ agonists have been shown to down-regulate the inflammatory process and become a class of anti-inflammatory compounds. Indeed, the agonists inhibit macrophage-derived osteoclastogenic cytokines or enzymes such as IL-1, TNF-α, IL-6, nitric oxide and cycloxygenase-2 [19,31]. Recently, these agonists have been shown to ameliorate arthritis and bone destruction in rodent models *in vivo* [21–23].

We show here that 15d-PGJ_2-induced inhibition of osteoclastogenesis was not rescued by a PPAR-γ antagonist, GW9662, and that 15d-PGJ_2 inhibited the expression of c-Fos, c-Jun and NFATc1 in a PPAR-γ-independent manner. This finding is consistent with previous reports that 15d-PGJ_2 acts through PPAR-γ-independent pathways [32–34]. 15d-PGJ_2 directly suppresses NF-κB activation by inhibition of IκB kinase complex activity [35,36], and reduction of NF-κB binding by alkylating p50/p65 dimers [37]. On the other hand, ciglitazone-induced inhibition was completely rescued by GW9662. The anti-inflammatory effect of PPAR-γ agonist was shown to be mediated by antagonizing the activation of NF-κB, AP-1, and STAT [19,38,39]. Moreover, these PPAR-γ effects were mediated by the transrepression of NF-κB and AP-1 through the sequestration of essential, shared co-activators [40]. Although NFATc1 has been established as a master gene for osteoclastogenesis [41,42], little is known about the effect of PPAR-γ agonists on NFATc1 activation. Yang, et al. showed that PPAR-γ-induced inhibition in T-cells is mediated by the physical interaction between NFAT and PPAR-γ [43]; however, there is no report showing the inhibitory effect of PPAR-γ agonists on the gene expression of NFATc1.

MCP-1 is a CC chemokine commonly found at the site of tooth eruption, bone degradation in RA [44], and bacterially induced bone loss [45]. MCP-1 is expressed in mature osteoclasts, and induced by RANKL [46, 47]. In addition, MCP-1 was reported to rescue the GM-CSF-induced inhibition of osteoclast differentiation, and the generated TRAP-MNCs were capable of resorbing bone [48]. Furthermore, in the absence of RANKL, MCP-1 strongly induced NFATc1, and generated $TRAP^+CTR^+$ MNCs; however, these cells are negative for bone resorption [49]. We demonstrate here that antibody-induced deletion of MCP-1 reduced TNF-α-mediated osteoclast formation by about 40%. Furthermore, PPAR-γ agonists potently inhibited the production of MCP-1, and the addition of MCP-1 rescued PPAR-γ-mediated inhibition of generation of multinucleated cells (MNCs), while the generated MNCs were unable to resorb dentin slices. Previous study [49] and our results indicate that MCP-1 is important in the cell fusion step during osteoclast differentiation, and that another signal is needed for bone-resorbing activity.

In conclusion, PPAR-γ activation down-regulates TNF-α-mediated osteoclast differentiation in part via suppression of MCP-1. Taking into consideration the critical role of TNF-α at inflamed joints, PPAR-γ agonists seem to be a promising therapeutic application for inflammatory bone-resorbing diseases such as RA.

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