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ORIGINAL ARTICLE

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Scientific basis for the efficacy of combined use of antirheumatic drugs against bone destruction in rheumatoid arthritis

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Abstract Finding a means to ameliorate and prevent bone destruction is one of the urgent issues in the treatment of rheumatoid arthritis. Recent studies revealed bone-resorbing osteoclasts to be essential for arthritic bone destruction, but to date there has been scarce experimental evidence for the underlying mechanism of the bone-protective effect of antirheumatic drugs. Here we examined the effects of one or a combination of disease-modifying antirheumatic drugs (DMARDs) on osteoclast differentiation to provide a cellular and molecular basis for their efficacy against bone destruction. The effects on osteoclast precursor cells and osteoclastogenesis-supporting cells were distinguished by two in vitro osteoclast culture systems. Methotrexate (MTX), bucillamine (Buc) and salazosulphapyridine (SASP) inhibited osteoclastogenesis by acting on osteoclast precursor cells and interfering with receptor activator of NF-κB ligand (RANKL)-mediated induction of the nuclear factor of activated T cells (NFAT) c1. MTX and SASP also suppressed RANKL expression on osteoclastogenesis-supporting mesenchymal cells. Interestingly, the combination of three antirheumatic drugs exerted a marked inhibitory effect on osteoclastogenesis even at a low dose at which there was much less of an effect when administered individually. These results are consistent with the reported efficacy of combined DMARDs therapy in humans and suggest that osteoclast culture systems are useful tools to provide an experimental basis for the bone-protective effects of antirheumatic drugs.

Key words Disease-modifying antirheumatic drugs (DMARDs) · Nuclear factor of activated T cells (NFAT) c1 · Osteoclast · Receptor activator of NF-κB ligand (RANKL) · Rheumatoid arthritis (RA)

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by the chronic inflammation of synovial joints which results in severe bone destruction.[1,2] A number of anti-inflammatory and antirheumatic drugs have been clinically utilized in the treatment of RA, but there is no method to prevent bone destruction completely.[3,4] This is partly because all the antirheumatic drugs were originally developed to suppress the activation of the immune system.[5] However, a combined use of disease-modifying antirheumatic drugs (DMARDs) has considerably contributed to the amelioration of both inflammation and bone destruction, although the effects are still not fully satisfactory.[6–8] It is poorly understood how the antirheumatic drugs exert their bone-protective effects, and it has thus been extremely difficult to predict the efficacy of antirheumatic drugs against bone destruction based on in vitro experiments.

Osteoclasts are multinucleated cells of monocyte/macrophage lineage that resorb bone matrix.[9,10] The generation of osteoclasts is supported by mesenchymal cells such as osteoblasts, which provide signals essential for differentiation.[11] These signals are mediated by macrophage colony-stimulating factor (M-CSF), receptor activator of NF-κB ligand (RANKL), and costimulatory signals for RANKL.[12–16] For the evaluation of osteoclast formation, two types of in vitro osteoclast differentiation systems have been developed: osteoclast precursor cells, bone marrow-derived monocyte/macrophage lineage cells (BMMs), which are stimulated with recombinant RANKL and M-CSF (the

RANKL/M-CSF system, Fig. 1a), or are cocultured with osteoblasts in the presence of 1,25-$(OH)_2$ vitamin D_3 ($VitD_3$) and prostaglandin E_2 (PGE_2) (the coculture system, Fig. 2a). The RANKL/M-CSF system is suitable for looking at the direct effect of drugs on the osteoclast precursor cells and RANKL-induced signaling events.[17,18] The coculture system is useful in the investigation of the effect of drugs on the osteoclastogenesis-supporting cells and the expression of osteoclastogenic factors such as RANKL.[19]

Osteoclasts are abundantly observed at the bone/synovium interface in the joints of RA patients.[2,20,21] RANKL is highly expressed by synovial fibroblasts in arthritic joints and is responsible for the abnormal activation of osteoclasts.[22,23] Importantly, arthritic bone destruction is greatly reduced in *RANKL*$^{-/-}$ mice or *Fos*$^{-/-}$ mice, both of which lack osteoclasts, even though there is no significant difference in the level of inflammation between the wild type and these genetically modified mice.[24,25] Consistent with this, anti-osteoclast therapy has successfully ameliorated bone damage in models of inflammatory bone destruction.[26–28] Thus, accumulating evidence indicates that bone destruction in RA is attributable to the abnormal activation of osteoclasts and that inhibition of RANKL-mediated osteoclastogenesis may be an ideal method to control arthritic bone destruction.[1,3,12]

Methotrexate (MTX), a folate antagonist, is an antirheumatic drug widely used in the world, often in combination with other drugs such as salazosulphapyridine (SASP).[6,7,29] Bucillamine (Buc), *N*-(2-mercapto-2-methylpropionyl)-L-cysteine, is used clinically in Japan and Korea to treat patients with RA.[30–32] Buc has structural similarities to D-penicillamine, but contains two free sulfhydryl groups, resulting in molecular and therapeutic effects significantly different from D-penicillamine.[32] Here we examined the effect of MTX, Buc and SASP on the osteoclast differentiation using two mouse in vitro osteoclast formation systems and found that the three drugs exerted inhibitory effects on osteoclastogenesis by differentially acting on osteoclast precursor and osteoclastogenesis-supporting cells. Even though the effect of a low dose of the drugs administered individually was small, in combination they had a marked inhibitory effect on osteoclastogenesis through a downregulation of nuclear factor of activated T cells (NFAT) c1 induction. Thus, the analysis of the effect of antirheumatic drugs on in vitro osteoclast differentiation may provide a crucial clue on their capacity to protect against bone destruction.

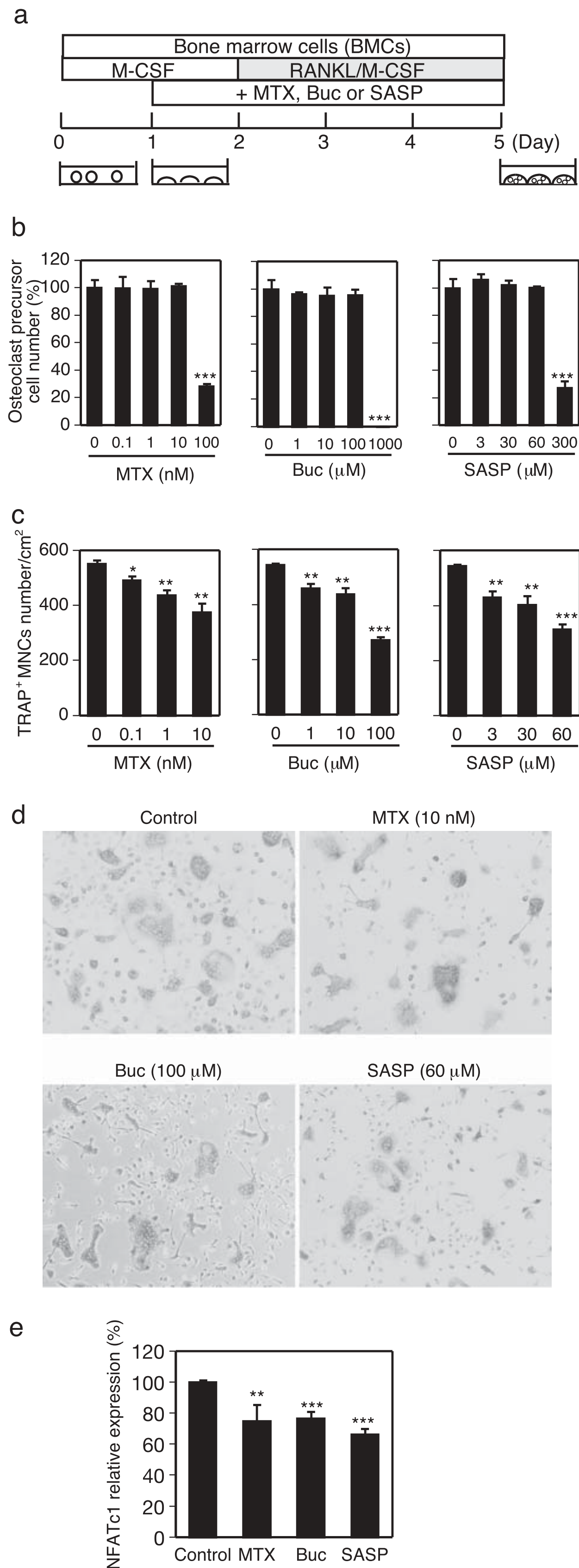

Fig. 1. Effects of methotrexate (*MTX*), bucillamine (*Buc*) and salazosulphapyridine (*SASP*) on receptor activator of NF-κB ligand (*RANKL*)-induced osteoclastogenesis. **a** A schematic of the osteoclast formation system induced by RANKL and macrophage-colony stimulating factor (*M-CSF*) (the RANKL/M-CSF system). **b** Effects of MTX, Buc, and SASP on the cell number of osteoclast precursor cells. Bone marrow-derived monocyte/macrophage lineage cells (BMMs) were treated with MTX, Buc, or SASP in the presence of M-CSF. Four days later, the number of osteoclast precursor cells was estimated microscopically. **c** Effect of MTX, Buc, or SASP on osteoclastogenesis in the RANKL/M-CSF system. **d** Microscopic photographs of RANKL-induced osteoclastogenesis in the presence of MTX, Buc or SASP (TRAP staining). **e** Expression of *NFATc1* protein in RANKL-stimulated BMMs treated with MTX (10 nM), Buc (100 μM) or SASP (60 μM)

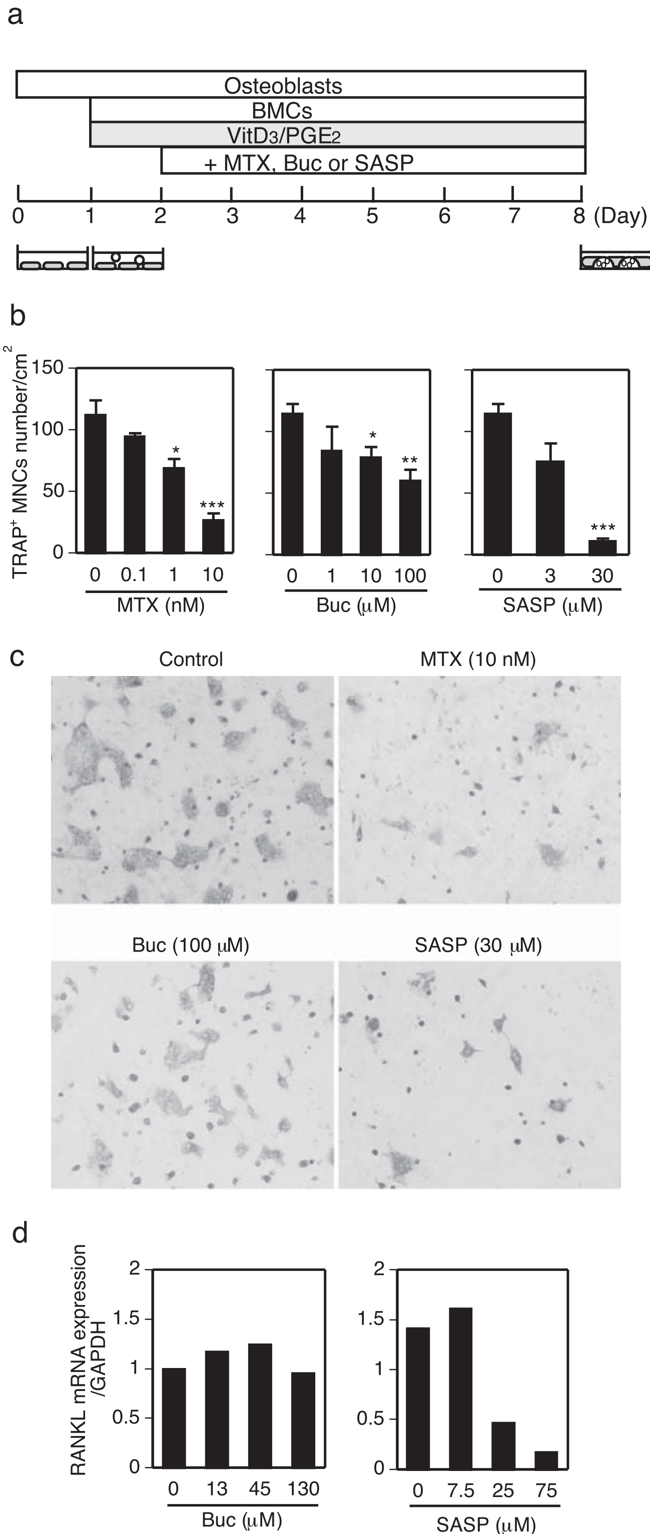

Fig. 2. Effect of methotrexate (*MTX*), bucillamine (*Buc*) and salazosulphapyridine (*SASP*) on osteoclastogenesis in the coculture system. **a** A schematic of the coculture system for osteoclastogenesis. BMCs, bone marrow cells; $VitD_3$, 1,25-$(OH)_2$ vitamin D_3; PGE_2, prostaglandin E_2. **b** Effects of MTX, Buc or SASP on osteoclastogenesis in the coculture system. **c** Microscopic photographs of osteoclastogenesis in the coculture in the presence of MTX, Buc, or SASP (TRAP staining). **d** Expression of *RANKL mRNA* in osteoblasts stimulated with Buc or SASP evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR). Mouse primary osteoblasts were stimulated with $VitD_3$ in the presence of Buc or SASP for 4 days. RT-PCR analysis was repeated several times and yielded similar results; a representative set of data is shown

Materials and methods

The RANKL/M-CSF system for in vitro osteoclast formation

The method of the RANKL/M-CSF system for osteoclastogenesis was described previously,[17,33] and is here utilized with modifications (see Fig. 1a). Nonadherent bone marrow cells (BMCs) were obtained from C57BL/6 mice (6- to 8-week-old) (CLEA Japan, Tokyo, Japan) maintained under specific pathogen-free conditions. All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University, and conformed to recognized guidelines and laws. BMCs were seeded (2×10^5 per well in a 24-well plate), and cultured in α-MEM (Gibco, Paisly, UK) containing 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 0.1 μg/ml ampicillin, 0.1 mg/ml kanamycin (Meiji Seika, Tokyo, Japan) and 10 ng/ml recombinant M-CSF (R&D Systems, Minneapolis, MN, USA) for 2 days. Adherent cells were used as BMMs after washing out the nonadherent cells including lymphocytes. These BMMs were stimulated with 50 ng/ml recombinant RANKL (PeproTech, Rocky Hill, NJ, USA) in the presence of 10 ng/ml M-CSF for additional 3 days. The treatment of BMMs with MTX, Buc or SASP (Santen Pharmaceutical, Tokyo, Japan) started 1 day before RANKL treatment and continued until the end of the culture. The medium was replaced completely every 2 days. Tartrate-resistant acid phosphatase-positive multinucleated cells ($TRAP^+$ MNCs) (>3 nuclei unless otherwise indicated) were counted.

The coculture system for in vitro osteoclast formation

The method of coculture system for osteoclastogenesis was described previously,[14,34] and utilized with modifications (see Fig. 2a). Primary osteoblasts were isolated from the calvaria of newborn (1–2 days old) mice by enzymatic digestion in α-MEM medium with 0.1% collagenase (Wako, Osaka, Japan) and 0.2% dispase (Sanko Junyaku, Tokyo, Japan) and cultured in α-MEM with 10% FBS. One day after these osteoblasts were reseeded (1×10^4 per well in a 24-well plate), BMCs (1×10^5 per well in a 24-well plate) were added to the culture of osteoblasts and cocultured in α-MEM with 10% FBS containing 1×10^{-8} M $VitD_3$ and 1×10^{-6} M PGE_2 (Wako) for 7 days. One day after BMCs were added, MTX, Buc or SASP was added to the coculture. $TRAP^+$ MNCs (>3 nuclei) were counted 7 days after addition of BMC.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) for 20 min, and treated with 0.2% Triton X-100 for 5 min. The cells were sequentially incubated in 5% bovine serum albumin/PBS for 30 min, 2 μg/ml anti-NFATc1 monoclonal antibody (7A6, Santa Cruz Biotech-

nology, Santa Cruz, CA, USA) for 60 min and then in Alexa Fluor 488-labeled secondary antibody (Molecular Probes, Eugene, OR, USA) for 60 min. The relative expression of NFATc1 was calculated by computational densitometry using NIH Image software.

RNA extraction and reverse transcriptase-polymerase chain reaction analysis

Osteoblasts isolated from mouse calvaria were reseeded (1×10^7 per well in a 10-cm dish), and cultured in α-MEM with 10% FBS containing 1×10^{-8} M $VitD_3$ in the presence of Buc (0–130 μM) or SASP (0–75 μM) for 4 days. Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) and first-strand complementary DNA (cDNA) was synthesized from total RNA using the One-Step RNA PCR kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. The primers used for the mouse RANKL were the following: 5′-TCAGAAGACAGCACTCAGTG-3′ (sense) and 5′-TCTTCACCAGCTCGGAGCTT-3′ (antisense). The amplification protocol consisted of an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. The level of mRNA expression was normalized with that of GAPDH expression. The PCR products were subjected to electrophoresis on 1.5% agarose gels and stained with ethidium bromide, and the bands were measured by computational densitometry using NIH Image software.

Statistical analysis

All data are expressed as mean ± s.e.m ($n = 5$). Statistical analysis was performed using Student's *t*-test or ANOVA followed by the Bonferroni test, if applicable (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, unless otherwise indicated). Results are representative examples of three or more independent experiments.

Results

The effect of antirheumatic drugs such as MTX and SASP on osteoclast formation have been described in previous reports.[35] However, these drugs have cell toxicity at high doses and it has been unclear whether the effects of these drugs have been investigated properly at concentrations that do not induce cell toxicity. Therefore, we first examined the effect of MTX, Buc, and SASP on the survival of osteoclast precursor cells, BMMs. BMMs were obtained by stimulating mouse BMCs with M-CSF, and the cell number of BMMs was counted after 4-day culture in the presence of various concentrations of MTX, Buc and SASP. All of these drugs had no effect on the cell number of BMMs at low concentrations, suggesting that they did not affect cell survival or proliferation. In contrast, they had severe toxic effects at high concentrations (Fig. 1b). Therefore, we used concentrations of the drugs (MTX: 0.1–10 nM, Buc: 1–100 μM, SASP: 3–60 μM) at which they exerted minimal effects on the cell number of BMMs. When these drugs were added to the RANKL/M-CSF system at these concentrations (Fig. 1a), all three drugs had statistically significant but mild suppressive effects on osteoclast differentiation in a dose-dependent manner, as revealed by the decreased formation of $TRAP^+$ MNCs (Fig. 1c, d).

NFATc1 is the essential transcription factor for osteoclastogenesis.[17,36] It has been shown that RANKL dramatically induces the expression of NFATc1 through an autoamplification mechanism and the level of NFATc1 determines the fate of cells of the osteoclast lineage.[36] Certain antirheumatic drugs that have bone-protective effects inhibit osteoclastogenesis by suppressing RANKL-induced NFATc1 expression.[17,20] To analyze the effects of the three antirheumatic drugs on the expression of NFATc1, we quantitated the protein level of NFATc1 in the RANKL-stimulated BMMs after immunostaining with a specific antibody against NFATc1. All three drugs had similar inhibitory effects on NFATc1 induction, although the effects were only partial (Fig. 1e). These results suggest that the drugs inhibited osteoclast differentiation by interfering with RANKL singling pathways upstream of NFATc1 induction.

We next examined the effects of MTX, Buc, and SASP on osteoclast differentiation in the coculture system with osteoblasts. In the coculture system, $VitD_3$ and PGE_2 are used to induce RANKL expression on the osteoblasts, and this system is useful in analyzing the effects of drugs on mesenchymal cells such as osteoblasts and synovial fibroblasts in addition to osteoclast precursor cells.[22,35] The same concentrations of MTX, Buc and SASP as in Fig. 1c were added to the coculture system (Fig. 2a). At these concentrations, there were no toxic effects of these drugs on the osteoblasts (data not shown). MTX and SASP exhibited greater inhibitory effects on the formation of $TRAP^+$ MNCs than in the RANLK/M-CSF system (Fig. 2b), suggesting that MTX and SASP exert anti-osteoclastogenic effects additionally through their effects on mesenchymal cells. In contrast, the inhibitory effects of Buc on the formation of $TRAP^+$ MNCs in the coculture system is similar to those in the RANKL/M-CSF system (Fig. 2b), suggesting that Buc mainly acts on osteoclast precursor cells to inhibit osteoclastogenesis. The photographs of $TRAP^+$ MNCs cultured in the presence of these drugs indicate that the size of the $TRAP^+$ MNCs was smaller than the non-treated cells and the number of nuclei per multinucleated cell was decreased in cells treated with the three drugs (Fig. 2c).

Consistent with the additional inhibitory effects of SASP and MTX on the coculture system in comparison with the RANKL/M-CSF system, it has been reported that both SASP and MTX have an inhibitory effect on RANKL expression in synovial cells.[35] However, it remains unknown how Buc acts on the expression of RANKL on the osteoclastogenesis-supporting cells. Therefore, we evaluated mRNA expression of RANKL in the osteoblasts stimulated with $VitD_3$ and PGE_2 in the presence of Buc in comparison with SASP. As expected, SASP strongly inhib-

ited the mRNA expression of RANKL on osteoblasts, but Buc had only minimal effects on RANKL expression (Fig. 2d). This result further supports the notion that Buc mainly targets osteoclast precursor cells in terms of its inhibitory effect on osteoclastogenesis. Thus, antirheumatic drugs affect osteoclastogenesis through different target cells.

To gain mechanistic insight into the efficacy obtained with the combined use of antirheumatic drugs in the treatment of RA, we evaluated the effects of a combination of MTX, Buc and SASP on osteoclast differentiation in the RANKL/M-CSF system. The addition of a low dose of MTX alone had a limited suppressive effect on osteoclastogenesis, but a combined addition with Buc or SASP increased the inhibitory effects (Fig. 3a, b). The combined use of all three drugs had a marked inhibitory effect on osteoclastogenesis, even though the addition of individual drugs at the same concentration had only a slight effect (Fig. 3a, b).

In addition, we quantitated the protein level of NFATc1 in the RANKL-stimulated BMMs after immunostaining, and this revealed that RANKL-induced NFATc1 expression was significantly inhibited by treatment with a combination of the three drugs (Fig. 3c), although treatment with individual drugs at the same concentration had only a small effect on NFATc1 expression (Fig. 1e). Interestingly, the photograph of TRAP$^+$ MNCs showed that the multinucleation was severely impaired in the cells treated with a combination of the three drugs (Fig. 3b). Therefore, we investigated the number of TRAP$^+$ MNCs containing more than 20 nuclei. We found that the number of such large osteoclasts was dramatically suppressed by the combined addition of the three antirheumatic drugs (Fig. 3d). It is worth noting that even a very low dose of MTX (0.1 nM) in combination with Buc and SASP exerted a much more suppressive effect than an addition of the same concentration of MTX alone (Fig. 3d). These results suggest that the combination with other antirheumatic drugs contributes to the enhancement of the inhibitory effect of MTX in terms of differentiation into large osteoclasts with numerous nuclei.

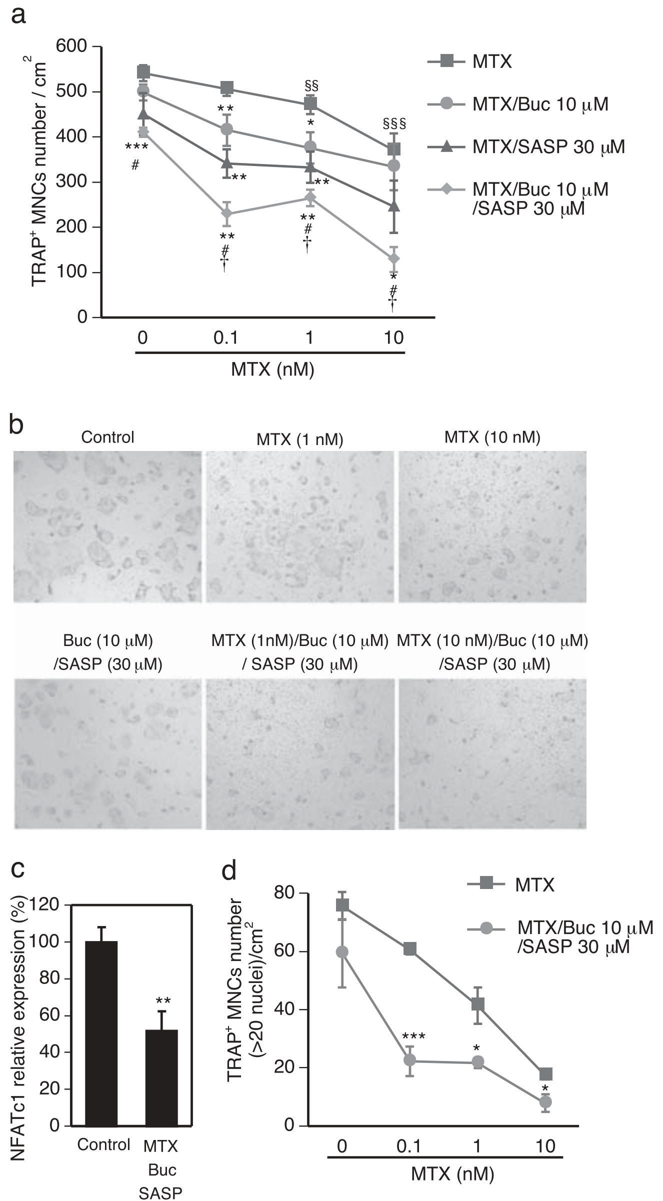

Fig. 3. Effects of methotrexate (*MTX*) alone or with the combined addition of bucillamine (*Buc*) and/or salazosulphapyridine (*SASP*) on osteoclastogenesis in the RANKL/M-CSF system. **a** Effects of MTX alone or MTX plus Buc (10 μM) and/or SASP (30 μM) on RANKL-induced osteoclastogenesis. $^{§§}P < 0.05$, $^{§§§}P < 0.001$ versus untreated BMMs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus MTX-treated BMMs. $\#P < 0.01$ versus MTX/Buc-treated BMMs. $^{\dagger}P < 0.05$ versus MTX/SASP-treated BMMs. **b** Microscopic photographs of RANKL-induced osteoclastogenesis in cells treated with MTX alone or MTX plus Buc and/or SASP (TRAP staining). **c** Expression of *NFATc1* protein in RANKL-stimulated BMMs treated with a combination of MTX (10 nM), Buc (10 μM) and SASP (30 μM). **d** Effect of the combined addition of MTX, Buc, and SASP on the formation of *TRAP$^+$ MNCs* containing more than 20 nuclei. Statistical analysis was performed in comparison with MTX-treated cells

Discussion

It has long been a challenging question as to how abnormalities of the immune system induce bone damage in RA.[1,2,37] Although the observation of giant cells at the bone destruction site dates back to about two decades ago,[38] osteoclasts have not been placed at the center of the pathogenesis of RA. After RANKL was cloned and the high RANKL expression in the synovium was brought to light, the importance of bone-resorbing osteoclasts gained general acceptance.[3,12] RANKL is abundantly expressed by RA synovial fibroblasts, possibly due to stimulation with proinflammatory cytokines.[18,22,39] In addition, RANKL is expressed in activated T cells,[27] although the effects of T cells on osteoclastogenesis are counterbalanced by negative factors such as IFN-γ.[33]

How is T cell activation linked to the enhanced expression of RANKL in RA? A recent study in our laboratory revealed that interleukin (IL)-17-producing helper T (Th) 17 cells play a distinct role in the pathogenesis of autoimmune arthritis and promote osteoclastogenesis, mostly through the production of IL-17.[37] As summarized in Fig. 4,

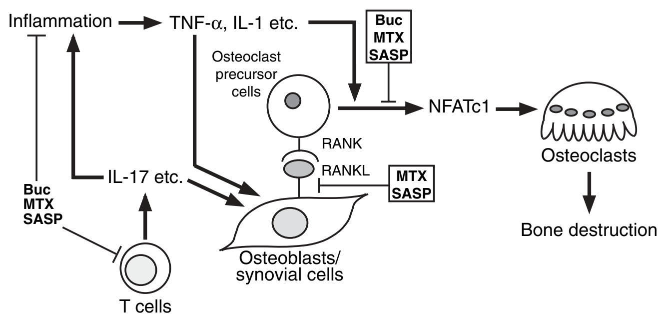

Fig. 4. How antirheumatic drugs, including methotrexate (*MTX*), bucillamine (*Buc*) and salazosulphapyridine (*SASP*) inhibit the pathways promoting bone destruction in rheumatoid arthritis (RA). Among the T-cell-derived mediators of inflammation, interleukin *(IL)-17* has emerged as one of the most important factors in the pathogenesis of RA. IL-17 not only induces *RANKL* on osteoblasts/synovial fibroblasts of mesenchymal origin, but also activates local inflammation, leading to the upregulation of inflammatory cytokines such as TNF-α and IL-1. These cytokines also induce RANKL on mesenchymal cells and additionally act directly on the osteoclast precursor cells to greatly enhance the RANKL activity. RANKL activates the differentiation of osteoclasts by inducing the expression of *NFATc1*, the master transcription factor for osteoclastogenesis. In this study, we showed that MTX, Buc and SASP suppress RANKL-induced NFATc1 expression, and that MTX and SASP inhibit RANKL expression on mesenchymal cells (shown in *boxes*). It has been previously reported that these three drugs exert inhibitory effects on the activation of T cells and inflammatory responses of synovial cells (shown in the *left lower section*)

IL-17 induces RANKL on mesenchymal cells (synovial fibroblasts/osteoblasts) and also stimulates local inflammation by inducing proinflammatory cytokines such as IL-1 and TNF-α, which in turn induces RANKL expression on mesenchymal cells.[3,40] RANKL then acts on osteoclast precursor cells of monocyte/macrophage lineage and stimulates osteoclast differentiation via the induction of NFATc1.[17,36]

This study demonstrates the mechanisms underlying the bone-protective effects of antirheumatic drugs (summarized in Fig. 4): MTX, Buc, and SASP had a suppressive effect on osteoclast differentiation by acting on osteoclast precursor cells and inhibiting RANKL-mediated expression of NFATc1 on the one hand. On the other hand, MTX and SASP had inhibitory effects on the RANKL expression in mesenchymal cells. It has been reported that MTX, Buc, and SASP inhibit the activation of T cells and inflammatory responses of synovial cells (which is also indicated in Fig. 4).[29,41–43] This may explain the anti-inflammatory effects of these drugs, but does not provide insight into how these drugs are related to the regulation of bone cells, which question we addressed experimentally in this study.

In keeping with the observation that the administration of these drugs individually has only limited clinical efficacy against bone destruction,[3,44] they have only a small inhibitory effect on osteoclast differentiation. However, such in vitro inhibitory effects is enhanced if the drugs are administered in combination. This is consistent with the efficacy of combined DMARDs therapy in comparison with the treatment with a single DMARD.[6,7,31] It was recently reported that the combination of MTX and Buc has more beneficial effects than MTX alone in treating RA patients.[45]

It is likely that a combination of antirheumatic drugs that inhibit osteoclastogenesis through different mechanisms will be helpful in ameliorating or preventing bone destruction in RA.

There are a number of drugs available for the treatment for RA, but most of them were developed to modulate immune reactions.[1,5] Therefore, antirheumatic drugs, effective in treating pain and inflammation, do not always have bone-protective effects: patients still fairly frequently have to undergo joint replacement surgery because of the progressive bone damage that develops despite treatment with antirheumatic drugs.[3,46] This study provides a beneficial method to screen antirheumatic drugs for their efficacy against bone destruction. Despite the remarkable impact of anti-cytokine therapies on the treatment of RA,[47] it scarcely needs saying that antirheumatic drugs will continue to occupy a substantially important position in the foreseeable future.[5,8] The mechanism of action of antirheumatic drugs in the context of bone destruction has been poorly understood, but the identification of an effective set of existing antirheumatic drugs by means of in vitro osteoclast culture systems is a promising strategy for improving the future treatment of RA.

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