Mod Rheumatol (2005) 15:97–103
DOI 10.1007/s10165-004-0373-7

ORIGINAL ARTICLE

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Infliximab acts directly on human osteoclast precursors and enhances osteoclast formation induced by receptor activator of nuclear factor κB ligand in vitro

Received: July 6, 2004 / Accepted: November 25, 2004

Abstract Infliximab is known to protect against the development of joint destruction. In the present study, we sought to determine whether Infliximab acts directly on human osteoclast precursors and influences monocyte-osteoclast differentiation induced by receptor activator of nuclear factor κB ligand (RANKL) in vitro. Peripheral blood mononuclear cells (PBMCs) isolated from rheumatoid arthritis (RA) patients and normal controls were cultured in the presence of RANKL and macrophage colony stimulating factor. Infliximab, antihuman tumor necrosis factor α (TNFα), antihuman TNF soluble receptor p55 (TNFR p55), and antihuman TNF soluble receptor p75 (TNFR p75) antibodies were added. Osteoclast formation was determined by assessing the number of tartrate-resistant acid phosphatase (TRAP) staining cells and the extent of lacunar resorption. Addition of Infliximab resulted in a marked increase in the number of TRAP-positive multinucleated cells ($TRAP^+$ MNCs) and in the extent of lacunar resorption compared with the control cultures. Antihuman TNFα antibody showed the same effect; however, the addition of neither TNFR p55 nor TNFR p75 antibody affected the extent of $TRAP^+$ MNCs and lacunar resorption. Our results suggest that infliximab acts directly on early osteoclast precursors, and stimulates osteoclast formation and lacunar resorption induced by RANKL in vitro.

Key words Bone resorption · Infliximab · Monocyte · Osteoclast

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Introduction

Rheumatoid arthritis (RA) is a severe chronic inflammatory joint disease associated with degeneration of cartilage and erosion of juxta-articular bone. The destruction of cartilage and bone is a characteristic feature of RA usually not observed in other forms of inflammatory arthritis and constitutes a major cause of progressive disability and crippling of RA patients.[1] The mechanisms leading to joint destruction still have not been fully elucidated; however, substantial evidence indicates that inflammatory cytokines play a crucial role in joint destruction in RA.[2] Among these cytokines, tumor necrosis factor α (TNFα) has been considered as the pivotal factor in the pathogenesis of RA. Mice overexpressing the proinflammatory cytokine TNF develop a destructive arthritis[3] and TNF neutralizing antibodies inhibit arthritis in murine collagen-induced arthritis.[4] Although the pathogenesis of RA remains incompletely understood, the successful effect of therapeutic blockade of TNFα clearly underline the key role of TNFα in the pathogenesis of RA.

TNFα has become an important therapeutic target and clinical trials of anti-TNFα have been undertaken.[5,6] Infliximab (Remicade; Centocor, Malvern, PA, USA) is a chimeric anti-TNFα monoclonal IgG_1 antibody that neutralizes the soluble cytokine and blocks the membrane-bound cytokine. Infliximab is very effective in reducing the chronic symptoms and indicators of inflammation in patients whose RA has failed to respond to conventional disease-modifying antirheumatic drugs,[7–12] and there is evidence that infliximab in combination with methotrexate (MTX) halts radiologic progression in RA.[11] Aside from the role of TNFα in triggering chronic synovial inflammation, it has osteoclastogenic properties and leads to increased bone resorption and bone loss in RA.[13–15] However, the mechanism of its function on bone metabolism in RA is not clear.

Osteoclasts, the principal cells responsible for bone resorption, are formed by fusion of marrow-derived mononuclear phagocyte osteoclast precursors which circulate in the monocyte fraction of peripheral blood.[16,17] Osteoclast

formation requires the presence of macrophage colony stimulating factor (M-CSF), and involves an interaction between the receptor activator of nuclear factor κB (RANK), expressed on osteoclast precursors, and RANK ligand (RANKL), which is expressed by several cell types, including osteoblasts.[18,19] Tumor necrosis factor α has been shown to regulate RANKL and osteoprotegerin (OPG) gene expression in osteoblastic cells[20–22] and is strongly synergized with RANKL.[23] It has also recently been shown that TNFα and interleukin-1α (IL-1α) are capable of inducing osteoclast formation in mouse marrow cultures by a RANKL-independent mechanism.[14,15,24]

In the present study, we investigated the role of infliximab on human monocyte–osteoclast differentiation in vitro. Interestingly, infliximab enhances human osteoclastogenesis induced by RANKL in the presence of M-CSF.

Materials and methods

Reagents

For all incubations, alpha-minimal essential medium (αMEM) culture medium (Gibco, Paisley, UK) was supplemented with 100 IU/ml penicillin, 10 μg/ml streptomycin, and 10% fetal calf serum (FCS; Gibco). Alpha-minimal essential medium alone was used for cell isolation. All cytokines and antibodies were obtained from R&D Systems (Abingdon, UK) and were used according to the manufacturer's instructions. All incubations were carried out at 37°C in 5% CO_2.

Isolation and culture of monocytes, and effect of the infliximab on osteoclast formation in RA patients and normal controls

Fifteen samples were studied: 10 seropositive RA patients and 5 age- and sex-matched normal controls. The RA population ranged in age from 34 to 67 years old and included 1 man and 9 women. The control population ranged in age from 34 to 66 years and included 1 man and 4 women. Peripheral blood of RA patients and controls was collected into heparinized tubes, diluted 1:1 in αMEM, layered over Ficoll-Hypaque (Pharmacia, Milton Keynes, UK) and centrifuged at 693 × *g* for 20 min at 4°C. The peripheral blood mononuclear cell (PBMNC) layer was removed and washed in αMEM, and the cell pellet was resuspended in αMEM/FCS. The number of PBMNCs in the cell suspension was counted in a hemocytometer after lysis of red cells with 5% v/v acetic acid solution.

Peripheral blood mononuclear cells (5×10^5 cells/well) were added to 7-mm wells of a 96-well tissue culture plate. These wells contained either dentine slices (5 mm diameter) or glass coverslips (6 mm diameter). Peripheral blood mononuclear cells were settled onto the coverslips and dentine slices for 2 h. The coverslips and dentine slices were then removed from the wells, washed vigorously in αMEM/FCS to remove nonadherent cells, and cultured in 24-well tissue culture plates containing 1 ml αMEM/FCS in the presence of RANKL (30 ng/ml) and M-CSF (25 ng/ml). All cultures were incubated for 14 and 21 days, and the culture medium containing these factors were replaced every 3–4 days. Infliximab (1 μg/ml) was added to the cultures of PBMNCs and isolated as detailed above, in the presence of RANKL (30 ng/ml) and M-CSF (25 ng/ml).

To exclude the possibility that these RANKL-expressing cells influenced macrophage–osteoclast formation in our experiments, $CD14^+$-sorted cells were also employed in experiments. Peripheral blood mononuclear cells were also cultured with CD14 magnetic MicroBeads (MACS-CD14 MicroBeads; Daiichi, Tokyo, Japan) for 20 min at 4°C. The cells were washed and passed through a MACS magnetic cell separator (Miltenyi Biotec, Tokyo, Japan). The $CD14^+$ cell fraction was washed and resuspended in MEM/FCS then counted in a hemocytometer following lysis of red blood cells using a 5% (v/v) acetic acid solution. Magnetically sorted $CD14^+$ cells were added to 96-well tissue culture plates (5×10^5 cells/well) containing glass coverslips and dentine slices.

Cytochemical assessment of osteoclast formation

All cells cultured on coverslips for 14 days were assessed histochemically for the expression of TRAP. Histochemical staining for TRAP was carried out using a commercially available kit (Sigma, St. Louis, MO, USA). Cell preparations were fixed in citrate/acetone solution and stained for acid phosphatase, using naphthol AS-BI phosphate as the substrate, in the presence of 1.0 M tartrate; the product was reacted with fast garnet GBC salt.

Functional assessment of osteoclast formation

Functional evidence of osteoclast formation was determined by a lacunar resorption assay system using cell cultures on dentine slices; the latter provide a smooth-surfaced mineralised substrate for the assessment of lacunar resorption. After cells had been cultured on dentine slices for 1 and 21 days, the slices were removed from the wells, rinsed in phosphate-buffered saline (PBS), and placed in 0.25 M ammonium hydroxide and sonicated for 5–10 min. In this way all cells are completely removed from the dentine slice, permitting examination of the surface for evidence of lacunar resorption. The slices were then washed in distilled water, stained with 0.5% (w/v) toluidine blue, and examined by light microscopy.

Analysis of infliximab on human osteoclast formation

To determine the optimal concentration of infliximab that supports human monocyte–osteoclast differentiation, various concentrations of infliximab (0.001–10 μg/ml) were added to cultures at every change of culture medium (i.e., twice per week over the 21-day incubation period) in the presence of M-CSF and RANKL.

Effect of timing of the agents addition to the cultures

To determine the manner in which infliximab influences human osteoclast differentiation, the culture period of 21 days was divided into time points, which corresponded to each medium change. Cultures were treated with infliximab at various time points during the period of incubation to identify the stage at which infliximab influenced osteoclast formation and/or activity.

Effect of anti-TNF receptors p55 and p75 and monoclonal antihuman TNF antibody on human osteoclast formation

To confirm whether other antibodies can have an effect on osteoclast formation, the following factors were added in the presence of RANKL (30 ng/ml) and M-CSF (25 ng/ml): monoclonal antihuman TNF antibody (mhTNFab) 10 μg/ml, monoclonal antihuman TNF soluble receptor p55 (TNFR p55) 10 μg/ml, and monoclonal antihuman TNF soluble receptor p75 (TNFR p75) 10 μg/ml.

Statistical analysis

Each series of experiments was done in triplicate and repeated three times. All data were pooled and, where appropriate, expressed as a percentage of control cultures. Significant differences were determined using Student's *t*-test.

Results

The effect of infliximab on human monocyte-osteoclast differentiation in RA patients

After 24 h incubation, PBMNCs from RA patients cultured on glass coverslips with M-CSF, RANKL, and with or without infliximab (1 μg/ml) were shown by histochemistry to be negative for the osteoclast markers TRAP. There was no evidence of lacunar resorption pits on dentine slices in these 24-h cultures.

After 14 days' incubation, cultures of human monocytes with M-CSF and RANKL alone contained $TRAP^+$ MNCs (Fig. 1A). The addition of infliximab to those cell cultures increased the extent of $TRAP^+$ MNCs (Fig. 1B). There was no difference in the number of TRAP-positive mononuclear cells or in the size of $TRAP^+$ MNCs.

Evidence of lacunar resorption pits formation was seen when human monocytes were cultured on dentine slices for 21 days in the presence of M-CSF and RANKL (Fig. 2A). The addition of infliximab to those cell cultures increased the extent of lacunar resorption pits compared with cultures incubated with M-CSF and RANKL alone (Fig. 2B).

When human monocytes from ten RA patients were cultured in the presence of M-CSF and RANKL, the mean number of $TRAP^+$ MNCs formed in cultures treated with infliximab (105 ± 43.2) was noted to be higher than in cultures with M-CSF and RANKL alone (32.5 ± 14.4) (Fig. 3A). The mean percentage of the extent of lacunar resorption pits in cultures treated with M-CSF and RANKL was 20.4% ± 13.7%; the addition of infliximab to these cultures resulted in a significant increase in the mean percentage of the extent of lacunar resorption pits (59.7% ± 24.4%) (Fig. 3B).

To confirm whether this effect was mediated via PBMNCs derived from RA patients, PBMNCs from five controls were cultured in the same manner as in the above experiment. The same effect of infliximab was found in cultures of PBMNCs from control patients. To exclude the possibility that $CD14^-$ RANKL-expressing cells (e.g., T cells) present in PBMNCs were responsible for inducing osteoclast formation in infliximab-treated cultures, cells expressing the monocyte/macrophage marker CD14 were isolated by magnetic cell sorting and cultured in the presence of M-CSF and RANKL with or without infliximab. The number of $TRAP^+$ MNCs and the extent of lacunar resorption in the cultures of $CD14^+$-sorted cells were

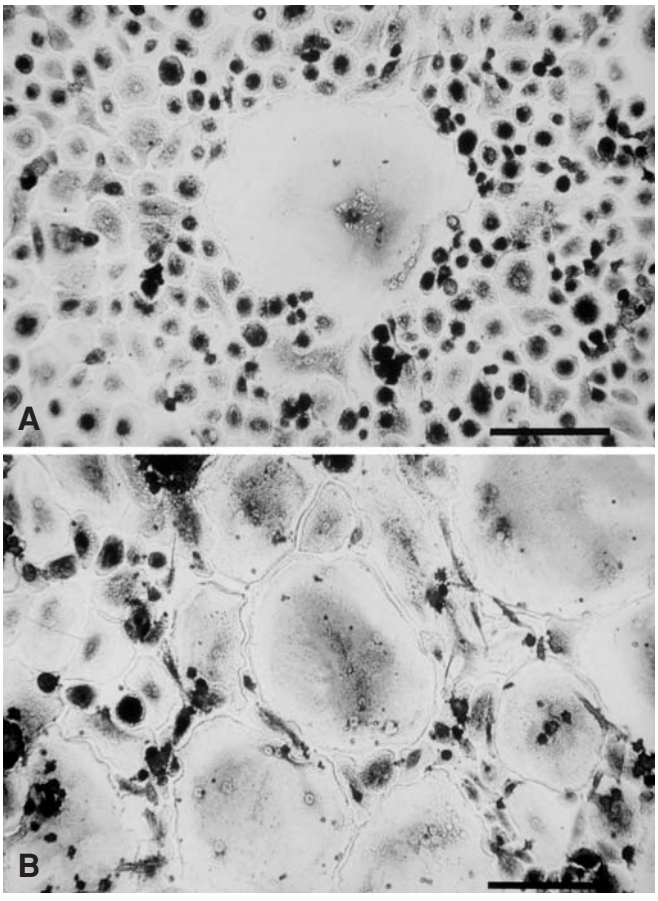


Fig. 1A,B. Human peripheral blood mononuclear cell (PBMC) cultures incubated on glass coverslip in the presence of macrophage colony stimulating factor (M-CSF) (25 ng/ml) and receptor activator of nuclear factor-κB ligand (RANKL) (30 ng/ml) for 14 days. **A** Tartrate resistant acid phosphatase-positive ($TRAP^+$) multinucleated cells (MNCs) induced by a combination of M-CSF and RANKL. *Bar* 100 μm. **B** Infliximab (1 μg/ml) enhanced $TRAP^+$ MNC formation in the presence of M-CSF and RANKL. *Bar* 100 μm

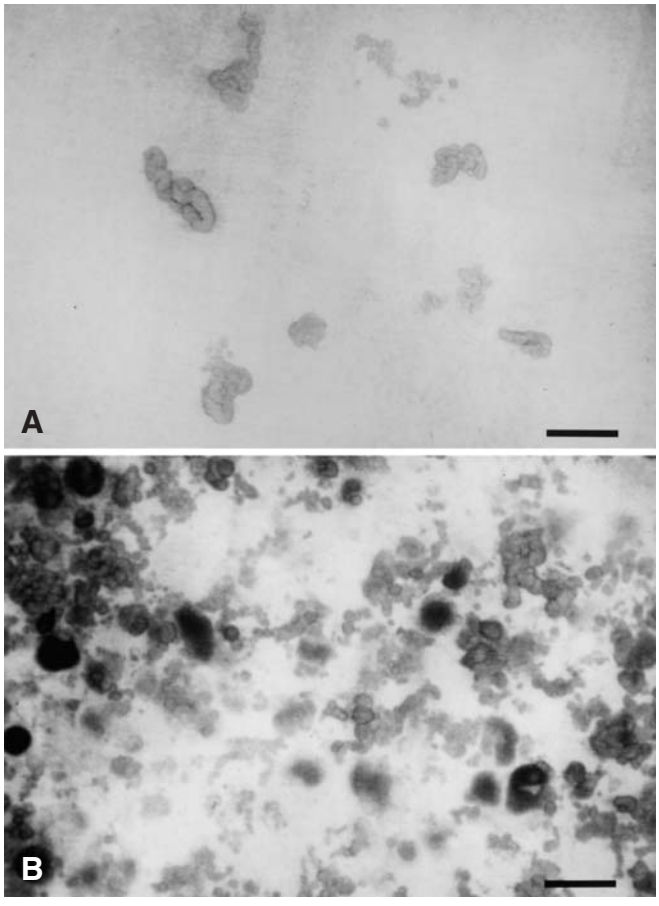


Fig. 2A,B. Human PBMC cultures incubated on dentine slice in the presence of M-CSF (25 ng/ml) and RANKL (30 ng/ml) for 21 days. **A** Lacunar resorption pits formation induced by a combination of M-CSF and RANKL. *Bar* 200 μm. **B** Infliximab (1 μg/ml) enhanced lacunar resorption pit formation in the presence of M-CSF and RANKL. *Bar* 200 μm

similar to those noted in the cultures of unsorted ($CD14^+$/$CD14^-$) cells (data not shown).

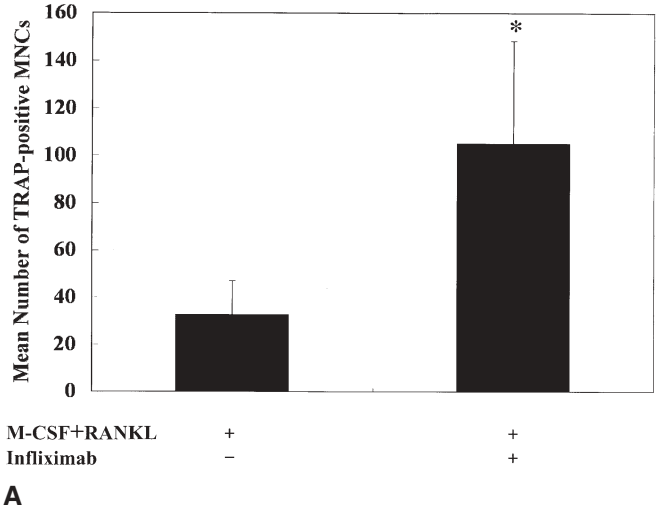
A dose-dependent increase in osteoclast formation was seen in PBMNC cultures treated with infliximab. In normal control PBMNC cultures containing 0.001–10 μg/ml infliximab, the extent of lacunar resorption were significantly increased (Fig. 4).

The effect of timing of the agent addition to culture

To determine at which stage infliximab influences human osteoclast differentiation, human monocytes were cultured with M-CSF and RANKL, with or without infliximab for different days which correspond to each medium change (Fig. 5). When infliximab was omitted for the first 3 or 6 days of culture (4–21 or 7–21 days), the extent of lacunar resorption pits was significantly reduced (28.6% ± 5.5%; 25.5% ± 6.5%) compared with that seen in the cultures in which infliximab was added throughout the culture period (0–21 days) (56.4% ± 13.8%). When infliximab was added on 0–3 days and incubated without infliximab for 4–21 days of culture, however, the extent of lacunar resorption pits in cultures was similar to that noted in the cultures in which infliximab was added throughout the culture period (0–21 days) (49% ± 10.1% vs 56.4% ± 13.8%).

The effect of mhTNFab, TNFR p55, and TNFR p75 on monocyte–osteoclast differentiation

To investigate the mechanism of stimulatory effect of infliximab on monocyte–osteoclast differentiation, the other blockers of TNFα signaling were added in these cultures in the presence of M-CSF and RANKL (Fig. 6).



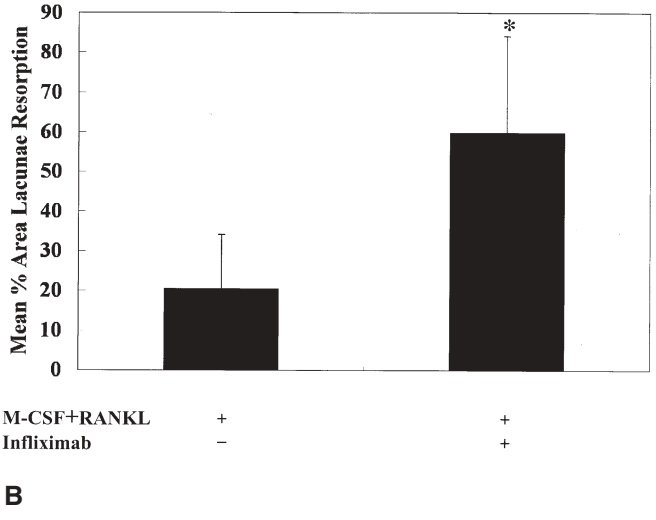


Fig. 3A,B. The effect of infliximab on human monocyte-osteoclast differentiation in rheumatoid arthritis patients. The results are expressed as the mean number of TRAP-positive MNCs per glass coverslip (**A**) and the mean percentage area of lacunar resorption pits per dentin slices (**B**). *$P < 0.01$ compared with control cultures treated with macrophage colony stimulating factor (*M-CSF*) and receptor activator of nuclear factor-κB ligand (*RANKL*) alone

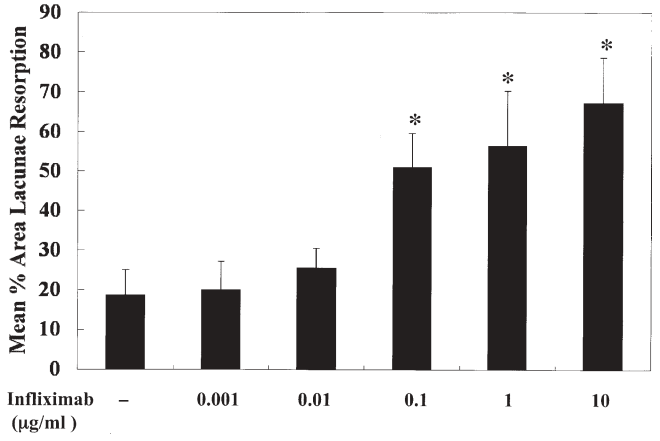


Fig. 4. The extent of lacunar resorption on human PBMC cultures in response to various concentrations of infliximab. The results are expressed as the mean percentage of lacunar resorption. *$P < 0.05$ compared with control cultures treated with M-CSF and RANKL alone

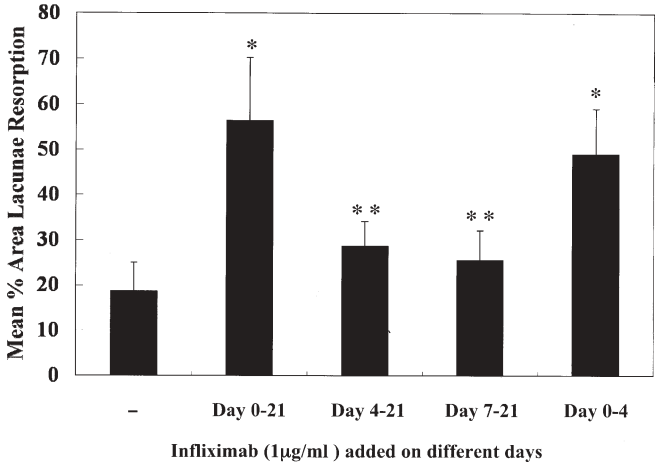


Fig. 5. Human PBMCs were cultured in the presence of M-CSF and RANKL, and infliximab was added to the cultures for 0–21, 4–21, 7–21, and 0–4 days, respectively. *$P < 0.05$ compared with control cultures treated with M-CSF and RANKL alone; **$P < 0.05$ compared with cultures treated with 0–21 days infliximab in the presence of M-CSF and RANKL

The addition of TNFR p55 did not affect the extent of lacunar resorption pits compared with control cultures with no added antibody (17.8% ± 7.5% vs 18.6% ± 6.4%). No significant effect on the extent of lacunar resorption pits (20.3% ± 4.4%) was noted in cultures to which TNFR p75 was added. However, the addition of mhTNFab resulted in a significant increase in the extent of lacunar resorption pits (53.2% ± 14.1%) compared with control cultures with no added antibody.

Discussion

In the present study, we have shown that infliximab directly acts on human osteoclast precursors and, surprisingly, promoted osteoclast formation and lacunar resorption induced by RANKL in vitro. Our results confirmed that infliximab enhanced osteoclast formation in cultures of $CD14^+$ monocytes, which do not contain RANKL-expressing cells.[25] Infliximab specifically influenced the early stage of human monocyte–osteoclast differentiation in a dose-dependent manner. Similar effects of infliximab were observed when PBMNCs were cultured with monoclonal antihuman TNF antibody.

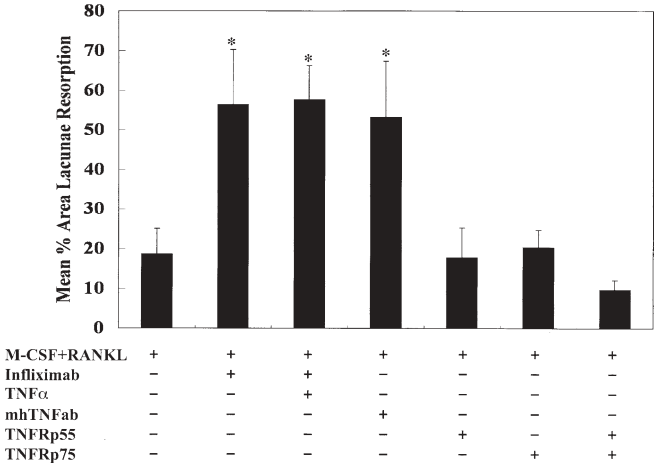


Fig. 6. The effect of monoclonal antihuman TNF antibody (*mhTNFab*) (10 ng/ml), antihuman TNF soluble receptor p55 (*TNFR p55*) (10 ng/ml), and/or TNFR p75 (10 ng/ml) on monocyte–osteoclast differentiation. The results are expressed as the mean percentage of lacunar resorption. *$P < 0.05$ compared with control cultures treated with M-CSF and RANKL alone. *TNF*, tumor necrosis factor

It is known that human osteoclast precursors circulate in the monocyte fraction,[16] and that a combination of RANKL and M-CSF is sufficient for human osteoclast formation.[26,27] Circulating monocytes in RA are known to show enhanced metabolic and phagocyte activity, and to express surface markers consistent with machrophage activation. Our results showed, however, that the stimulatory effect of infliximab on monocyte–osteoclast formation was observed not only in RA patients but also in healthy controls.

Tumor necrosis factor α is one of the most potent osteoclastgenic cytokines produced by inflammatory cells, and is crucial to the pathogenesis of bone and joint destruction that occurs in RA joints.[28] It has also been reported that TNFα mediates bone loss in estrogen deficiency.[29,30] Tumor necrosis factor α exerts the osteoclastogenic properties both indirectly via stimulation of RANKL[31,32] and directly via induction of osteoclast formation.[14,15,24] Blocking TNFα seems to inhibit RANKL-induced monocyte–osteoclast formation. However, our results showed that infliximab, a chimeric monoclonal anti-TNFα antibody with murine variable regions and human IgG_1 and κ constant regions, stimulates human monocyte–osteoclast formation induced by a RANKL signal. Our results were confirmed only in in vitro study, occurring under special conditions.

A question we needed to address was whether blocking TNFα signaling was responsible for this stimulatory effect

on human osteoclast formation. To better understand the mechanism, we also added mhTNFab, TNFR p55, and TNFR p75 to the cultures. Antihuman TNFα neutralizing antibody promotes the human monocyte–osteoclast differentiation induced by RANKL, whereas antihuman TNF receptor p55 and p75 neutralizing antibodies did not affect the human osteoclast formation and activation. Also, our experiment confirmed that adding human TNFα to the culture did not abolish the effect of infliximab. These results suggest that the stimulatory effects of infliximab on human monocyte–osteoclast differentiation were not due to the blocking of TNFα signaling.

The possible explanation of these findings is that in in vitro cultures, binding of TNFα-neutralizing antibodies to culture cells that express the transmembrane form of TNFα rather than soluble TNFα is a pivotal mechanism of action. Tumor necrosis factor α is primarily produced by activated monocytes/macrophages and there are two forms, a 26 kDa transmembrane form of pro-TNF and a 17 kDa mature TNF.[33] Several experiments have suggested that the transmembrane form of TNFα has receptor-like properties and its interaction with the receptors initiates a bidirectional signaling.[34–36] We confirmed that monocytes cultured with M-CSF and sRANKL express TNFα on their membrane (data not shown). Infliximab and mouse monoclonal antibody, but neither monoclonal antihuman TNF soluble receptor p55 nor p75, would bind to the transmembrane form of TNFα on the monocyte surface and the outside-to-inside signal might transmute through the transmembrane form of TNFα. Depending on the type and developmental stage of the target cell, TNFα can induce activation, proliferation, differentiation, or self-destruction, and it is conceivable that reverse signaling may alter the activity of different genes in diverse cell type.

The stimulatory effect of infliximab on the number of $TRAP^+$ MNCs was very similar to those on the extent of lacunae resorption pits. Infliximab promotes human monocyte–osteoclast differentiation when added to the cultures on day 0, if infliximab was not added to the cultures after day 4. However, decreased stimulation was observed when infliximab addition was delayed. The results from these experiments suggest that infliximab acts directly on human circulating osteoclast precursors and affects the early stage of monocyte–osteoclast differentiation rather than osteoclast activation.

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