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*J Immunol* 2009; 182:361-370; doi: 10.4049/jimmunol.182.1.361
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IL-3 Inhibits TNF-α-Induced Bone Resorption and Prevents Inflammatory Arthritis

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IL-3, a cytokine secreted by activated T cells is well known to regulate the proliferation, differentiation, and survival of pluripotent hematopoietic stem cells. IL-3 functions as a link between the immune and the hematopoietic system. In this study, we suggest an important new role of IL-3 in inhibition of TNF-α-induced bone resorption in vitro and prevention of inflammatory arthritis in mice. We show here that IL-3 potently and irreversibly inhibits TNF-α-induced bone resorption in hematopoietic precursors of monocyte/macrophage lineage. IL-3 showed an inhibitory effect on TNF-α-induced bone resorption even in the presence of proinflammatory cytokines such as IL-1α, TGF-β1, TGF-β3, IL-6, and PGE2. We found that IL-3 prevented TNF-α-induced c-fos nuclear translocation and AP-1 DNA-binding activity. Interestingly, IL-3 pretreatment prevented the development of inflammatory arthritis in mice induced by a mixture of anti-type II collagen mAbs and LPS. Furthermore, IL-3 prevented cartilage and bone loss in the joints indirectly through inhibition of inflammation. Thus, we provide the first evidence that IL-3, a strong regulator of hematopoiesis, also plays an important role in inhibition of TNF-α-induced bone resorption and prevention of inflammatory arthritis in mice. The Journal of Immunology, 2009, 182: 361–370.

Osteoclasts, the multinucleated cells, which differentiate from hematopoietic precursors of the monocyte/macrophage lineage, are responsible for bone resorption (1). Osteoclasts that have a vital role in physiological bone remodeling also function in local bone destruction that occurs in inflammatory bone diseases. Increased bone resorption by osteoclasts is a major pathological factor in osteoporosis, rheumatoid arthritis (RA), periodontitis, and most adult skeletal diseases (2–4). The differentiation and activation of osteoclasts are under the aegis of a variety of cytokines. The crucial factor for osteoclast differentiation and bone resorption is receptor activator of NF-κB ligand (RANKL) (5, 6). TNF-α also induces osteoclast differentiation and bone resorption in vitro from M-CSF-dependent osteoclast precursors, independent of RANKL (7–11). TNF-α induces osteoclastogenesis and bone resorption by activating NF-κB and AP-1 transcription factors (8, 12). TNF-α also induces bone resorption in vivo by activation of osteoclasts (13). It is the key mediator for pathological bone loss in RA and other inflammatory diseases and, in association with accumulated proinflammatory cytokines, such as IL-1, IL-6, PGE2, and TGFs, enhances bone resorption (14–17). However, little is known about the factors that control TNF-α-induced bone resorption.

Materials and Methods

Reagents

Human M-CSF, mouse TGF-β1, TGF-β3, IL-1α, and IL-6 were obtained from R&D Systems. Osteoprotegerin (OPG) and human RANKL were from Insight Biotechnology. Mouse IL-3, TNF-α, and purified anti-CD16/32 were from BD Biosciences. Control IgG and rabbit IgG-FITC were from Insight Biotechnology. Mouse IL-3, TNF-α, RANKL, receptor activator of NF-κB ligand (RANKL), mouse TGF-β1, TGF-β3, IL-6, and PGE2 were obtained from R&D Systems. Osteoprotegerin (OPG) and human RANKL were from Insight Biotechnology. Mouse IL-3, TNF-α, and purified anti-CD16/32 were from BD Biosciences. Control IgG and rabbit IgG-FITC were from Insight Biotechnology. Mouse IL-3, TNF-α, RANKL, receptor activator of NF-κB ligand (RANKL), mouse TGF-β1, TGF-β3, IL-6, and PGE2 were obtained from R&D Systems. Osteoprotegerin (OPG) and human RANKL were from Insight Biotechnology. Mouse IL-3, TNF-α, and purified anti-CD16/32 were from BD Biosciences. Control IgG and rabbit IgG-FITC.
Abs were from Bangalore Genei. PGE2 was obtained from Sigma-Aldrich. PE-Cy5-anti-mouse CD51 was from eBioscience. Anti-p65, c-fos, c-jun, and H9252 Abs were obtained from Santa Cruz Biotechnology. Goat IgG-Cy3 and arthrogen-collagen-induced arthritis mAb mixture kit were obtained from Chemicon International. All cultures were incubated in MEM supplemented with 10% FCS, 2 mM l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Animals
BALB/c mice of 6 – 8 wk old were obtained from the Experimental Animal Facility of the National Center for Cell Science (Pune, India). Water and food were provided ad libitum. Animal protocols were approved by the institutional animal ethics committee.

In vitro osteoclast differentiation and bone resorption
Stromal and lymphocyte-free, M-CSF-dependent osteoclast precursors were prepared as previously described (26, 27). In brief, bone marrow cells isolated from mice were subjected to gradient purification. Cells at the gradient interface were collected, washed, and resuspended in MEM containing 10% FCS and incubated for 24 h with M-CSF (10 ng/ml). After 24 h, nonadherent cells were harvested and added to 96-well plates containing bone slices. After 1–2 h, individual bone slices were washed vigorously to remove nonadherent and loosely adherent cells. Adherent cells were further incubated with different cytokines according to each experimental condition. Concentrations of cytokines used were M-CSF (30 ng/ml), TNF-α (40 ng/ml), IL-1α (5 ng/ml), and IL-3 (10 ng/ml) except where indicated. Cultures were fed every third day and after 8 days osteoclast formation on bone slices was examined by tartrate-resistant acid phosphatase (TRAP) staining (28). Bone slices were further processed for the assessment of bone resorption.

Preparation of mouse mature osteoclasts
Osteoclast precursors were incubated on bone slices for 5 days with M-CSF (30 ng/ml), TNF-α (40 ng/ml), and IL-1α (5 ng/ml) to form mature multinuclear osteoclasts. Mature osteoclasts were depleted of mononuclear cells by incubating the cultures for 5 min in 20 mM EDTA in Ca2+- and Mg2+-free PBS. The cultures were then washed thoroughly three times with αMEM. This procedure removes the majority of mononuclear cells, while leaving multinuclear osteoclasts adherent (28). The mature osteoclasts were further incubated for 3 days with M-CSF, TNF-α, and IL-1α with or without different concentrations of IL-3. The percent bone surface resorbed was quantified.

CrossLaps ELISA
Mature osteoclasts were prepared on bone slices and purified as described above. Bone slices with mature osteoclasts were transferred to new wells and incubated for an additional 3 days with M-CSF, TNF-α, and IL-1α with or without different concentrations of IL-3. The conditioned culture medium was collected after 3 days at the end of the culture. The resorption activity in culture supernatants was determined by quantifying the degradation products of C-terminal telopeptide of type I collagen (CTX-I) using

FIGURE 1. IL-3 irreversibly inhibits TNF-α-induced bone resorption. A, M-CSF-dependent, stromal, and lymphocyte-free osteoclast precursors were incubated for 8 days on bone slices in a 96-well plate with M-CSF (30 ng/ml) and different concentrations of TNF-α, or M-CSF, TNF-α (40 ng/ml), and anti-TNF-α Ab (1 μg/ml), or OPG (100 ng/ml), or M-CSF, TNF-α, and various concentrations of IL-1α. B and C, Osteoclast precursors were incubated with M-CSF and TNF-α with or without IL-1α (5 ng/ml) in the presence or absence of various concentrations of IL-3. The number of TRAP-positive multinuclear cells per bone slice and the percent bone surface resorbed was quantified. D, TRAP staining of osteoclasts on bone slices (original magnification, ×20), and resorption pits were examined by reflected light microscopy (original magnification, ×20). E, Osteoclast precursors were incubated on bone slices with M-CSF, TNF-α, and IL-1α with or without IL-3 (10 ng/ml). After 3 days, IL-3 was withdrawn and cells on bone slices were further cultured for 8 days with M-CSF, TNF-α, and IL-1α. Percent bone surface resorbed was quantified. Data are expressed as means ± SEM for six cultures. *, p < 0.01 vs control. Similar results were obtained in four independent experiments.
and TNF-α for 8 days with M-CSF and RANKL (30 ng/ml) or TNF-α (10 ng/ml), or M-CSF and TNF-α (10 ng/ml) and RANKL (5 ng/ml) with or without IL-3 (10 ng/ml). Percent bone surface resorbed was quantified. Results are expressed as means ± SEM for six cultures. *, p < 0.01 vs synergistic effect of RANKL and TNF-α. B and C, Osteoclast precursors were incubated on bone slices for 8 days with M-CSF (30 ng/ml), TNF-α (40 ng/ml) and IL-1α (5 ng/ml) in the absence or the presence of various concentrations of TGF-β1 or TGF-β2 or PGE2 or IL-6. *, p < 0.01 and **, p < 0.05 vs control. D, Osteoclast precursors were incubated on bone slices with M-CSF, TNF-α, IL-1α, and TGF-β1 or TGF-β2 (10 ng/ml) or PGE2 (10^{-6} M) or IL-6 (1 ng/ml) with or without IL-3 (10 ng/ml). Percent bone surface resorbed was quantified. Similar results were obtained in three independent experiments.

Assessment of bone resorption
Bone slices were immersed in 4% sodium hypochlorite for 15 min and washed thoroughly to remove the cells. After drying, bone slices were mounted onto glass slides and then sputter coated with gold. Bone slices were examined by reflected light microscopy and bone resorption was quantified using an eyepiece graticule.

FACS analysis
Cells were washed twice with FACS buffer (0.1% sodium azide and 0.5% BSA in 1× PBS) and blocked with Fc block for 30 min. Cells were treated with PE-Cy5-anti-mouse-CD51 for 1 h or goat IgG Abs for 30 min, washed three times with FACS buffer, and treated with secondary FITC or Cy3 Abs for 45 min, and blocked with Fc block for 1 h. Cells were treated with primary Abs for induction of arthritis (five mice per group). Briefly, mice were injected i.v. with the mAb blend (3 mg) on day 0, followed by 50 μg of LPS i.p. on day 3. In the treatment group, IL-3 (2 μg) was injected s.c. into the right footpad 1 day before injection of mAbs/LPS. Control mice were either un.injected or injected with PBS. Mice were examined daily by two independent, blinded examiners for development of inflammation and arthritis. Severity of the macroscopic levels of arthritis was graded in each limb up to 11 days after mAb injection on a 0–4 scale as described previously (31). The criteria for the grading were as follows: 0, normal; 1, swelling and/or redness in one joint; 2, swelling and/or redness in more than one joint; 3, swelling and/or redness in the entire paw; and 4, maximal swelling. Maximum value of the sum of the scores obtained from four limbs of each mouse was 16 (4 × 4).

Histopathology
For histopathological analysis, mice were sacrificed after 11 days and the knee joints, liver, and kidney tissues were fixed with 10% formaldehyde in a CrossLaps ELISA kit (29). One-step ELISA was performed according to the manufacturer’s instructions (Nordic Bioscience Diagnostics).

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Immunofluorescence
Cells were washed twice with PBS, fixed with 3.7% paraformaldehyde for 5 min, and blocked with Fc block for 1 h. Cells were treated with primary Abs for 2 h, washed, and treated with secondary FITC or Cy3 Abs for 45 min. Cells were permeabilized (0.1% Triton X-100 in PBS, 5 min) to stain the nuclei with 4’,6-diamidino-2-phenylindole (1 μg/ml, 5 min). Cells were washed, and mounted using FragEL mounting medium (Oncogene) and viewed with a Zeiss LSM 510 confocal microscope.

EMSA
Nuclear extracts (4 μg) isolated as described earlier (26) were used in the binding reactions (10 mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 0.5 mM MgCl₂, 2 mM DTT, 0.05 mg/ml BSA, and 0.25% Nonidet P-40) with 0.5 μg/ml poly(dI:dC) and 2 μl per reaction of end-labeled probe. Oligonucleotides used were WT-NF-κB 5’-AGTTAGGGAGCTTTCCGTGCCGGA-3’ and mutant-NF-κB 5’-AGTTAGGGACCTTTCCGTGCCGGA-3’. EMSA was assessed by measuring hind paw thickness with a caliper daily for 11 days.

Anti-collagen Ab-induced arthritis
Arthritis was induced using an arthrogen-collagen-induced arthritis mAb blend of four clones (D1, F10, A2, and D8) and LPS according to the manufacturer’s instructions (30). A total of 20 male BALB/c mice of 6–8 wk old were used for induction of arthritis (five mice per group). Briefly, mice were injected i.v. with the mAb blend (3 mg) on day 0, followed by 50 μg of LPS i.p. on day 3. In the treatment group, IL-3 (2 μg) was injected s.c. into the right footpad 1 day before injection of mAbs/LPS. Control mice were either uninjected or injected with PBS. Mice were examined daily by two independent, blinded examiners for development of inflammation and arthritis. Severity of the macroscopic levels of arthritis was graded in each limb up to 11 days after mAb injection on a 0–4 scale as described previously (31). The criteria for the grading were as follows: 0, normal; 1, swelling and/or redness in one joint; 2, swelling and/or redness in more than one joint; 3, swelling and/or redness in the entire paw; and 4, maximal swelling. Maximum value of the sum of the scores obtained from four limbs of each mouse was 16 (4 × 4).

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FIGURE 3. IL-3 inhibits TNF-α-induced α,β3 expressions. A. Osteoclast precursors were incubated for 5 days with M-CSF or M-CSF and TNF-α with or without IL-3 (10 ng/ml). Cells were stained for αv or β3 or control IgG, and the fluorescence of the cells was analyzed by FACS. M (red), M-CSF; MT (blue), M-CSF + TNF-α; MTI (green), M-CSF + TNF-α + IL-3. B. Average percent cell expression of αv,β3 from three independent experiments. *, p < 0.01 vs M-CSF + TNF-α. C. The mean fluorescent intensity (MFI) indicating the number of individual receptors expressed per cell. *, p < 0.01 vs M-CSF + TNF-α. D. Cells on bone slices were incubated with M-CSF, TNF-α, and IL-1α with or without IL-3 and analyzed for αv and β3 by immunofluorescence. Original magnification, ×63. Similar results were obtained in three independent experiments.

Bone mineral density (BMD)

BMD was measured by dual-energy x-ray absorptiometry using a pDEXA SABRE Scanner from Orthometrix. To obtain the BMD, whole knee joints were scanned and the region of interest (5 × 5 mm) was manually set for analysis (33).

Statistical analysis of data

Data are presented as mean ± SEM. Statistical differences between the mean values of control and experimental groups were analyzed using Student’s t test.

Results

IL-3 irreversibly inhibits TNF-α-induced bone resorption

To investigate the role of IL-3 on TNF-α-induced bone resorption, we first incubated stromal and lymphocyte-free osteoclast precursors on bone slices in the presence of M-CSF (30 ng/ml) and various concentrations of TNF-α. After 8 days, TRAP-positive multinuclear osteoclasts on bone slices were counted and the same bone slices were further processed for the assessment of bone resorption. In this culture system, TNF-α induced bone resorption in a dose-dependent manner, which was inhibited by anti-mouse TNF-α Ab and not by OPG, the soluble decoy receptor for RANKL, suggesting that TNF-α induces bone resorption independent of RANKL (Fig. 1A). IL-1α, which is a strong stimulator of bone resorption, enhanced TNF-α-induced bone resorption in a dose-dependent manner (Fig. 1A). IL-1α (5 ng/ml) was added in some experiments to enhance TNF-α-induced bone resorption. Cells were then incubated with M-CSF (30 ng/ml) and TNF-α (40 ng/ml) with or without IL-1α (5 ng/ml) in the absence or the presence of various concentrations of IL-3. Interestingly, IL-3 inhibited both osteoclast formation on bone surface (Fig. 1B) and bone resorption in a dose-dependent manner (Fig. 1C). Photomicrographs of bone slice in Fig. 1D show the significant inhibition of TRAP-positive multinuclear cells and bone resorption by IL-3. These results suggest that IL-3 inhibits TNF-α-induced bone resorption and osteoclast formation on bone slices. There was a highly significant correlation between the number of resorption pits and the number of osteoclasts (r² = 0.9323; data not shown). The decrease in bone resorption corresponded with a decrease in osteoclast number.

To investigate whether the inhibitory effect of IL-3 on bone resorption is irreversible, cells were incubated with M-CSF, TNF-α, and IL-1α with or without IL-3 (10 ng/ml). After 3 days, IL-3 was withdrawn by washing the bone slices thoroughly, and cells on bone slices were further stimulated with M-CSF, TNF-α, and IL-1α for 8 days. No bone resorption was induced by TNF-α and IL-1α in 8 days after removal of IL-3 (Fig. 1E). These results suggest the irreversible inhibitory effect of IL-3 on bone resorption.

Effect of IL-3 on bone resorption by mature osteoclasts and CTX-I release

We further investigated the role of IL-3 on TNF-α-induced bone resorption by mature osteoclasts. Mature multinuclear osteoclasts prepared on bone slices were purified by removing mononuclear cells by
EDTA treatment as described in Materials and Methods. Purified mature osteoclasts on bone slices were thoroughly washed and further incubated with M-CSF, TNF-α/H9251, and IL-1α/H9251 with or without different concentrations of IL-3. The percent bone surface resorbed was quantified after 3 days. We observed that IL-3 had no inhibitory effect on bone resorption by mature osteoclasts at all of the concentrations (data not shown). These results suggest that IL-3 do not inhibit bone resorption by mature osteoclasts. Thus, the negative effect of IL-3 on TNF-induced bone resorption observed in osteoclast precursors is indirect through inhibition of osteoclast differentiation.

We also examined the effect of IL-3 on bone resorption by mature osteoclasts using a more quantitative method of measurement of the collagen degradation product CTX-I. Bone slices with purified mature osteoclasts were transferred to new wells and incubated for an additional 3 days with M-CSF with or without IL-3 were stimulated by TNF-α for 30 min, and nuclear translocation of c-fos and c-jun was examined by immunofluorescence. Original magnification, ×63.

**FIGURE 4.** IL-3 inhibits TNF-α-induced AP-1 activation. A, Osteoclast precursors were incubated for 3 days with M-CSF (30 ng/ml) in the absence or the presence of IL-3 (10 ng/ml). Cells were starved for 8 h before stimulation with TNF-α (40 ng/ml) for 0, 15, and 30 min. Nuclear extracts were subjected to EMSA for AP-1-binding activity. Specificity of AP-1 binding was confirmed using cold competition with 1- and 10-fold excess wild-type and mutant AP-1 oligonucleotides. Similar results were obtained in three independent experiments. B, Cells grown on bone slices with M-CSF with or without IL-3 were stimulated by TNF-α for 30 min, and nuclear translocation of c-fos and c-jun was examined by immunofluorescence. Original magnification, ×63.

IL-3 potently inhibits TNF-α-induced bone resorption in the presence of other proinflammatory cytokines

At suboptimal concentrations, TNF-α and RANKL synergies for dramatic increase in osteoclast formation and IL-3 inhibit this synergistic effect (9, 11). Therefore, to examine whether IL-3 inhibits bone resorption in synergistic conditions, cells were incubated for 8 days with M-CSF and RANKL (30 ng/ml) or TNF-α (40 ng/ml), or M-CSF and low concentrations of RANKL (5 ng/ml) and/or TNF-α (10 ng/ml) with or without IL-3 (10 ng/ml). Many resorption pits were seen with high concentrations of either RANKL or TNF-α; however, few resorption pits were seen with low concentrations of either RANKL or TNF-α (Fig. 2A). Interestingly, at low concentrations, strong synergism between RANKL and TNF-α to enhance bone resorption was observed, and IL-3 significantly inhibited this synergistic effect.

Proinflammatory cytokines such as IL-1, IL-6, PGE2, and TGFs have been implicated in pathological bone loss, and in vivo action of these cytokines is well interrelated with each other (14–17). Because IL-3 inhibits bone resorption induced by TNF-α and IL-1α, we believed that IL-3 might regulate the bone resorption in the presence of these cytokines. Therefore, to investigate this, osteoclast precursors were incubated for 8 days with M-CSF, TNF-α, and IL-1α in the absence or the presence of different concentrations of TGF-β1 or TGF-β3 or PGE2 or IL-6. TGF-β1, TGF-β3, and PGE2 in a dose-dependent manner increased the bone resorption induced by TNF-α (Fig. 2, B and C). IL-6 did not enhance TNF-α-induced bone resorption. To our surprise, IL-3 significantly inhibited increased bone resorption enhanced by these proinflammatory cytokines (Fig. 2D). These results indicate the potent inhibition of TNF-α-induced bone resorption by IL-3.

**Effect of IL-3 on expression of αvβ3 integrin**

Integrins αv and β3 are expressed by mature osteoclasts and mediate osteoclast function. They are also expressed by osteoclast...
precursors and needed for precursor proliferation and during osteoclast differentiation (34–37). To assess the effect of IL-3 on TNF-α-induced αv and β3 expressions, osteoclast precursors were incubated for 5 days with M-CSF or M-CSF and TNF-α in the absence or the presence of IL-3, and expression of αvβ3 was examined by FACS. IL-3 significantly down-regulated the surface expression of both αv and β3 (Fig. 3A). Fig. 3, B and C, represents the percent cell expression of αv and β3, and the number of receptors expressed per cell, respectively. Effect of IL-3 on expressions of αvβ3 on authentic bone slices was also examined by immunofluorescence. IL-3 significantly down-regulated the expression of both αv and β3 on bone matrix (Fig. 3D).

**IL-3 inhibits TNF-α-induced AP-1 activation**

To further investigate the molecular mechanism by which IL-3 inhibits TNF-α-induced bone resorption, the effect of IL-3 was examined on activation of NF-κB and AP-1 transcription factors induced by TNF-α. TNF-α is a strong activator of NF-κB that plays a functional role in bone resorption (8, 38, 39). NF-κB knockout mice are osteopetrotic because of arrest in bone resorption (40). To examine the role of IL-3 on NF-κB, osteoclast precursors were incubated with M-CSF with or without IL-3 for 3 days, and starved for 8 h before stimulation with TNF-α for 0, 15, and 30 min. Nuclear extracts were subjected to EMSA. TNF-α strongly activated NF-κB at 15 and 30 min, as assessed by DNA binding. IL-3 showed no effect on TNF-α-induced NF-κB activation (data not shown). IL-3 also did not affect TNF-α-induced nuclear translocation of p65 (data not shown). These results suggest that IL-3 inhibits TNF-α-induced bone resorption without affecting NF-κB activation.

TNF-α is released into inflamed joints and activates c-fos, an integral component of AP-1 (9, 12, 41). Also, the AP-1 complex plays a crucial role in both osteoclast formation and bone resorption (42, 43). To determine the effect of IL-3 on AP-1, osteoclast precursors were treated with IL-3 as described above and nuclear extracts were subjected to EMSA. Interestingly, IL-3 significantly inhibited the TNF-α-induced AP-1 DNA binding at all of the time points (Fig. 4A). Binding specificity was further confirmed by competing the AP-1 band with excess unlabeled wild-type
oligonucleotide, and failure of this competition was seen with mutant oligonucleotide (Fig. 4A). These results suggest that IL-3 affects TNF-α-induced bone resorption by significant inhibition of AP-1 activity. Because IL-3 inhibited AP-1, we next examined whether IL-3 inhibits nuclear translocation of c-fos and c-jun, the key players of the AP-1 complex. Cells were incubated on bone slices in the presence of M-CSF with or without IL-3 for 3 days before stimulation with TNF-α (40 ng/ml) for 30 min. As shown in Fig. 4B, IL-3 inhibited TNF-α-induced nuclear translocation of c-fos on bone slices, but showed no effect on c-jun.

**IL-3 prevents development of mAb/LPS-induced inflammatory arthritis**

Because IL-3 potently inhibited TNF-α-induced bone resorption even in the presence of other proinflammatory cytokines, we hypothesized that IL-3 may show in vivo anti-inflammatory effect and protect pathological bone and cartilage loss in inflammatory arthritis. To explore this, we used a well-established mouse model of mAb/LPS-induced arthritis (30). In this model, severe and consistent inflammatory arthritis is rapidly induced with marked destruction of articular cartilage and permanent ankylosis. Also, the BALB/c mice that are resistant to classic collagen-induced arthritis are highly sensitive to mAb/LPS-induced arthritis than DBA/1J mice with nearly 100% incidence of arthritis. In this study, all mice injected with mAb/LPS developed clinically evident arthritis. Arthritis was manifested by redness and swelling of the paws, including digits. In control mice, either untreated or injected with PBS, no signs of inflammation were seen. As shown in Fig. 5, A and B, joint inflammation was developed within 24–48 h after mAbs/LPS injection and inflammation peaked around day 6. By day 9, all of the mice developed clinically evident inflammatory arthritis. In contrast, mice pretreated with IL-3 for 24 h did not display severe inflammation or swelling of the hind paw during 11 days. The arthritic score was very low in IL-3-treated mice. These results indicate that IL-3-treated mice are resistant to mAb/LPS-induced arthritis. Photographs of front and side views of paws in Fig. 5C show the significant reduction of paw swelling in mice treated with IL-3. In the presence of IL-3, thickness of the inflammatory soft tissues was also decreased when examined by radiological soft x-rays (Fig. 5C).

**IL-3 indirectly prevents occurrence of cartilage and bone loss in inflammatory arthritis**

Next, by histological examination of knee joints, we checked whether IL-3 pretreatment prevented cartilage and bone loss. As shown in Fig. 6A, no infiltration of inflammatory cells and no damage to the articular cartilage were seen in the joints of control mice. The synovial region of joints in mAb/LPS-injected mice showed massive infiltration of polymorphonuclear and other inflammatory cells. There was multiple superficial cartilage erosion detected by toluidine blue staining of proteoglycans. In contrast, mice pretreated with IL-3 showed infiltration of a few inflammatory cells and also there was no erosion of articular cartilage (Fig. 6A). We next examined the development of synovitis by quantitative analysis of synovial inflammation in histological sections of knee joints. In mAb/LPS-injected mice, synovial inflammation was most prominent. Interestingly, significant reduction of synovial inflammation was seen in IL-3-treated mice (Fig. 6B).

To determine the effects of IL-3 on osteoclast formation in an in vivo model of inflammatory arthritis, we counted the number of osteoclasts present in knee joint sections. Injection of mAbs/LPS increased the number of osteoclasts. In contrast, the number of osteoclasts in knee joints of mice treated with IL-3 was
comparable to the number of osteoclasts present in knee joints of control mice that were either uninjected or injected with PBS (Fig. 6C). These findings suggest that IL-3 blocks the increased osteoclastogenesis in inflammatory joints. We next examined the effect of IL-3 on BMD of knee joints. Whole knee joints were scanned by dual-energy x-ray absorptiometry and BMD of the region of interest was manually set for analysis. In mice injected with mAbs/LPS, BMD was slightly but significantly (p < 0.05) decreased as compared with control (Fig. 6D). Interestingly, in mice pretreated with IL-3, there was no decrease in BMD, suggesting that IL-3 prevented mAb/LPS-induced bone loss. The in vivo effect of IL-3 treatment on other vital organs such as liver and kidney was also examined histologically. No inflammation or tissue damage was seen in liver and kidney sections in IL-3-treated mice (data not shown). These results suggest that IL-3 has anti-inflammatory effect and prevents development of inflammatory arthritis and indirectly protects cartilage and bone loss.

Discussion

IL-3 secreted by activated T cell is involved in the regulation of hematopoiesis (18–20). Recently, we have shown that IL-3 inhibits osteoclast formation induced by TNF-α, and this inhibitory action of IL-3 was seen when cells were incubated on plastic (11, 26). In this study, IL-3 inhibited TNF-α-induced bone resorption in a dose-dependent manner. There was a decrease in bone resorption that corresponded with a decrease in osteoclast number on slides (38, 39). Genes activated by NF-κB include many proinflammatory cytokines, chemokines, and adhesion molecules. NF-κB-regulated genes such as TNF-α and IL-1β directly activate NF-κB to amplify the primary inflammatory response (30). IL-3 did not affect TNF-α-induced NF-κB-binding activity and nuclear translocation of p65, suggesting that IL-3 inhibits bone resorption without affecting NF-κB. IL-3 inhibited TNF-α-induced AP-1 DNA-binding activity and specificity of AP-1 DNA binding was confirmed using excess unlabeled wild-type and mutant oligonucleotides specific to AP-1. IL-3 also inhibited the TNF-α-induced nuclear translocation of c-Fos. Our results strongly suggest that IL-3 inhibits TNF-α-induced bone resorption by abrogating c-fos/AP-1 activation. c-Fos, a member of the AP-1 family, is essential for osteoclastogenesis and mice deleted of this molecule are osteopetrotic (51). c-Fos/c-jun heterodimer (AP-1) is also important in regulating the expression of IL-1, IL-6, TNF-α, and collagenase, which are essentially important in RA, and overexpression of the c-fos gene leads to joint destruction (41). Thus, inhibition of c-fos/AP-1 activation appears essentially important in arthritic joint destruction.

Although the immune response in RA is mediated by T and B cells present in the systemic circulation, tissues that are affected are located mostly within the joints (31, 52, 53). Because IL-3 inhibited both osteoclastogenesis and bone resorption induced by TNF-α and other proinflammatory cytokines, we hypothesized that IL-3 may have an in vivo anti-inflammatory effect to protect cartilage and bone damage in inflammatory arthritis. The mouse model of mAb/LPS-induced arthritis shares many similarities with human RA, and the affected mice develop swollen, red, and ankylosed joints with infiltration of inflammatory cells, pannus formation, chondrocyte death, and proteoglycan loss (54). In our studies, it is clear that IL-3 blocks the in vivo inflammatory response of mice subjected to anti-type II collagen mAbs and LPS-induced arthritis. Mice pretreated with IL-3 did not display major symptoms of inflammatory arthritis in either joints or digits, indicating that IL-3-treated mice are resistant to mAb/LPS-induced arthritis. The significant anti-inflammatory effect of IL-3 could lead to many ways to protect bone and cartilage. Presently, the mechanism of the anti-inflammatory effect of IL-3 is not fully known. Our results suggest that IL-3 prevents the occurrence of bone and cartilage damage indirectly through significant inhibition of inflammation. Also, IL-3 had no toxic effect to other tissues. IL-3 levels have been shown to decrease in patients (56). This lack of IL-3 may contribute to bone loss in arthritis.

Although many factors contribute to the pathophysiology of bone loss, TNF-α has a crucial role. A normally coupled resorption/formation system important for the homeostasis of bone is disturbed in the presence of elevated TNF-α and other proinflammatory cytokines irrespective of pathological conditions. In this study, we provide the first evidence that IL-3, a strong regulator of hematopoiesis, significantly inhibits TNF-α-induced bone resorption even in the presence of proinflammatory cytokines. Also, inhibition of c-fos/AP-1 activation offers an effective therapeutic approach for inhibiting chronic inflammatory diseases involving bone destruction. In summary,
IL-3 inhibits TNF-α-induced bone resorption through inhibition of c-fos/AP-1 activation, and our in vivo studies suggest that IL-3 has the potential to diminish the inflammatory response and indirectly arrest cartilage and bone loss in inflammatory arthritides, although further in vivo studies are required to investigate whether IL-3 has anti-arthritis activity upon therapeutic application after the onset of symptoms.

Acknowledgments

We thank Hemangini Shikhare for FACS analysis and Ashwni Atre for confocal microscopy.

Disclosures

The authors have no financial conflict of interest.

References


