IL-23 Induces Receptor Activator of NF-κB Ligand Expression on CD4+ T Cells and Promotes Osteoclastogenesis in an Autoimmune Arthritis Model


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IL-23 Induces Receptor Activator of NF-κB Ligand Expression on CD4+ T Cells and Promotes Osteoclastogenesis in an Autoimmune Arthritis Model

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IL-23, a clinically novel cytokine, targets CD4+ T cells. Recent IL-1Ra−/− mouse studies have demonstrated that IL-23 indirectly stimulates the differentiation of osteoclast precursors by enhancing IL-17 release from CD4+ T cells. IL-17, in turn, stimulates osteoclastogenesis in osteoclast precursor cells. In this study, we found that IL-23 up-regulates receptor activator of NF-κB ligand expression by CD4+ T cells, and thus contributes to osteoclastogenesis. This indirect pathway is mediated by NF-κB and STAT3. We have also demonstrated that IL-23 can influence osteoclastogenesis positively under the special conditions in the IL-1-dominant milieu of IL-1Ra−/− mice. We propose that IL-23-enhanced osteoclastogenesis is mediated mainly by CD4+ T cells. The results of this study show that IL-23 is a promising therapeutic target for the treatment of arthritis-associated bone destruction. The Journal of Immunology, 2008, 181: 1507–1518.

B one erosion in rheumatoid arthritis (RA)3 is clinically important because it results in joint dysfunction in the terminal stage (1). Excessive osteoclastogenesis is the main pathophysiology of bone erosion, and the receptor activator of NF-κB (RANK) ligand (RANKL) is a leading player in this process (2). Because RANKL is considered an important mediator of osteoclastogenesis (3), researchers have tried to determine the source of RANKL and the molecules that act in conjunction with it. Activated T cells and RA synovial fibroblasts express RANKL and have the capacity to induce osteoclast differentiation (4, 5). Several proinflammatory cytokines, such as TNF-α, IL-1β, IL-15, and IL-17, induce the multinucleation of osteoclast precursors or their commitment to the osteoclast phenotype and may act synergistically with RANKL (6).

IL-23 plays a pivotal role in the establishment and maintenance of inflammatory autoimmune diseases (7). IL-23-deficient mice are resistant to experimental autoimmune encephalitis and collagen-induced arthritis, highlighting the important role of this cytokine in autoimmune pathology. The arthritogenic potential of IL-23 is supported by the fact that IL-23 stimulates IL-17 production by CD4+ T cells (8), and that IL-17 induces IL-23 secretion from fibroblast-like synoviocytes (FLS) (9). It is assumed that IL-23 has an osteoclastogenic role in addition to its arthritogenic potential. In one recent study, the IL-23–IL-17 axis was shown to be critical for both the onset phase and the bone destruction phase of autoimmune arthritis (10). In that study, the osteoclastogenic role of IL-23 was considered to be mediated by a specialized subset of inflammatory T cells, the Th17 cells.

To clarify the link between IL-23 and T cell-mediated osteoclastogenesis, we determined the expression of RANKL on CD4+ T cells. Bone marrow cells (BMC) were cocultured with IL-23-stimulated CD4+ T cells. Tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts and quantitative assays to measure osteoclastic markers were then performed. We also investigated the in vivo effect of IL-23 on RANKL expression and bone destruction, with the intra-articular injection of adeno viral vectors encoding IL-23. We finally studied the signaling pathways involved in IL-23-induced RANKL expression in CD4+ T cells.

Materials and Methods

Animals

IL-1R antagonist-deficient mice (IL-1Ra−/− mice) in a BALB/c background were provided by Dr. Y. Iwakura (University of Tokyo, Tokyo, Japan). They were maintained under specific pathogen-free conditions at the Institute of Medical Science, the Catholic University of Korea, and given standard mouse chow (Ralston Purina) and water ad libitum. All experimental procedures were examined and approved by the Animal Research Ethics Committee of the Catholic University of Korea.

Reagents

Recombinant Abs directed against IL-23, IL-12, IL-17, IL-1β, RANKL, MCSF, and TNF-α were purchased from R&D Systems. Anti-CD3 mAb was obtained from BD Biosciences. The cell signaling inhibitors AG490, parthenolide, LY294002, SP600125, and PD98059 were obtained from Calbiochem.

Construction of replication-defective adenoviruses expressing IL-23

Recombinant replication-defective adenovirus was generated according to the AdEasy Vector System (QBiogene), as previously described (11).
IgG2a were measured in mouse sera diluted 50,000- to 400,000-fold. IgG2a ELISA quantitation kit (Bethyl Laboratories). The levels of IgG1 and were used to investigate IgG Ab subtype concentrations using the mouse IgG1/CD4+ viruses were determined by TCID50 and by plaque assays in 293 cells. CD4+ negative staining in WT mice. (red). Confocal microscopy imaging confirmed the presence of IL-17 RANKL+ cells (yellow) in the joints of the IL-1Ra−/− and WT mice.

Briefly, the bicistronic cDNA of IL-23 encoding p19 and p40 (12) and the enhanced GFP (eGFP) cDNA (BD Clontech) were subcloned into the pShuttleCMV/IL-23 and pShuttleCMV/eGFP constructs were cotransformed with the adenoviral backbone vector pAdEasy into Escherichia coli strain B75183 by electroporation to achieve homologous recombination. The recombinant DNA obtained was transfected into 293 cells by the conventional calcium phosphate coprecipitation method. Recombinant adenoviruses were isolated from single plaques, expanded in 293 cells, and purified by double cesium chloride ultracentrifugation. The purified viruses were extensively dialyzed against 10 mM Tris, 5% sucrose, and 2 mM MgCl2, and stored in aliquots at −80°C. The titers of adenoviruses were determined by TCID50 and by plaque assays in 293 cells.

Delivery of adenoviral vector

IL-1Ra−/− mice were maintained until 8 wk of age. The mice were injected intraarticularly with recombinant adenovirus (rAd) expressing mouse IL-23 (rAd/mIL-23) (1 × 108 PFU per joint for each vector), rAd encoding eGFP (rAd/eGFP), or PBS. The mice were maintained under isoflurane anesthesia, their knees were swabbed with 70% ethanol, and 20 μl of the treatment solution was injected into the synovial space with a 30-gauge needle. Seven days later, the knee joints were injected again with rAd/mIL-23, rAd/eGFP, or PBS. After the two injections, the development of periarticular arthritis in the hind paw and ankle joint was monitored over a period of 4 wk. The experiment was then terminated and the mice were killed. Blood samples were collected from all treated and control mice, and the sera were stored at −80°C until use.

Assessment of arthritis

Joint swelling was monitored by inspection and given an arthritis score as follows: 0 = no joint swelling; 1 = slight edema and erythema limited to the foot or ankle; 2 = slight edema and erythema from the ankle to the tarsal bone; 3 = moderate edema and erythema from the ankle to the tarsal bone; and 4 = edema and erythema extending from the ankle to the entire leg, with severe swelling of the wrist or ankle. The final arthritis score was calculated as the sum of scores from all four legs, assessed by three independent observers with no knowledge of the experimental groups.

Analysis of IgG Ab subtypes

Blood samples collected from the IL-1Ra−/− mice and wild-type (WT) mice were used to investigate IgG Ab subtype concentrations using the mouse IgG1/ IgG2a ELISA quantitation kit (Bethyl Laboratories). The levels of IgG1 and IgG2a were measured in mouse sera diluted 50,000- to 400,000-fold.

Histological analysis

IL-1Ra−/− and WT mice were killed by cervical dislocation. Their knee joints were then dissected, fixed in 10% phosphate-buffered formalin for 1 day, decalcified in 10% EDTA for 8 h, and then embedded in paraffin. Tissue sections (7 μm) were prepared and stained with either H&E or safranin O.

Preparation of mixed arthritic joint cells

The ankle joints were removed from 10- to 12-wk-old mice and FLS were isolated. The joint tissues were homogenized and mixed joint cells were obtained from the homogenate. The mixed joint cells were maintained in RPMI 1640 medium containing 10% FBS and the FLS were maintained in DMEM containing 10% FBS. They were then incubated for 12 h in RPMI 1640 or DMEM containing insulin-transferrin-selenium A instead of FBS, in the presence of various stimuli, such as IL-23 (0.1–10 ng/ml), IL-17 (5 ng/ml), anti-mouse IL-17 Ab (10 μg/ml), or the chemical cell-signaling inhibitors LY294002 (20 μM), SP600125 (1 μM), PD98059 (20 μM), AG490 (50 μM), or parthenolide (10 μM).

Immunohistochemical analysis of the IL-23 p19 subunit, RANKL, and CD4

The ankle joints of the mice were separated and fixed in 10% neutral buffered formalin and then decalcified in EDTA and hydrochloric acid. The joints were then mounted in paraffin and cut into 7-μm sections. The paraffin sections were treated with xylene and rehydrated. Endogenous peroxidase activity was quenched for 15 min with 0.3% H2O2 in methanol (v/v) at room temperature. Nonspecific binding sites were blocked by incubation in 10% normal goat serum for 30 min at room temperature. After the removal of this solution, the sections were incubated overnight at 4°C with the primary antibody. The sections were then incubated with the appropriate biotinylated secondary Ab and a streptavidin-peroxidase conjugate, with S-(2-aminoethyl)-L-cysteine as the substrate (Histostain-SP kit; Zymed Laboratories). The samples were counterstained with hematoxylin.

Immunostaining for RANKL and RANK

Mixed joint cells or CD4+ T cells were attached to slides using a Cytospin cytocentrifuge (Shandon) and fixed in 4% paraformaldehyde for 20 min at room temperature. The slides were incubated overnight at 4°C with biotinylated anti-RANKL or anti-RANK Abs (Santa Cruz Biotechnology).
FIGURE 2. Expression of RANKL and RANK is increased by IL-23 in cultured mixed joint cells from IL-1Ra−/− mice. A, Mixed joint cells from IL-1Ra−/− mice were cultured with IL-23 (0.1–10 ng/ml), IL-17-neutralizing Ab (10 μg/ml), or IL-17 (5 ng/ml) for 12 h. The expression of RANKL mRNA was evaluated by real-time PCR. OD values were normalized to the HPRT band. Data represent mean ± SEM of the data from three separate experiments.

Mixed joint cells from IL-1Ra−/− mice were cultured with IL-23 (5 ng/ml), IL-23 (5 ng/ml) plus IL-17Ab, IL-17 (5 ng/ml), IL-17 (5 ng/ml) plus anti-IL-17.
The slides were then treated with avidin-peroxidase complex for 1 h at room temperature and visualized with 3,3′-diaminobenzidine (DakoCytomation) staining. The slides were counterstained with hematoxylin.

**Cell preparation and culture**

The mouse spleens were sieved through a mesh and the RBC lysed with 0.83% ammonium chloride. The remaining spleen cells were maintained in RPMI 1640 medium containing 10% FBS. To purify splenic CD4+ T cells, the splenocytes were incubated with CD4-coated magnetic beads (Miltenyi Biotec) and isolated on MACS separation columns (Miltenyi Biotec). The CD4+ T cells were cultured from 15 min to 120 h with stimuli such as IL-23 (0.1–10 ng/ml), IL-17 (5 ng/ml), or TNF-α (5 ng/ml), and chemical-cell-signaling inhibitors such as parthenolide (10 μM), SP600125 (1 μM), AG490 (50 μM), PD98059 (20 μM), or LY294002 (20 μM). The cytotoxicity of the doses of chemical inhibitors was evaluated by an MTT assay.

**Osteoclastogenesis and bone resorption**

BMCs from IL-1Ra−/− and WT mice were obtained for osteoclast progenitor cell preparation. The BMCs were isolated from the tibias and femurs of 6-wk-old IL-1Ra knockout male mice by flushing the bone marrow cavity with the α-MEM (envirogen). The cells were then centrifuged and exposed to hypotonic ACK buffer (0.15 mM NH₄Cl, 1 mM KCO₃, and 0.1 mM EDTA, (pH 7.4)) at room temperature for 30 s to remove the RBC. They were incubated with α-MEM containing antibiotics and 10% heat-inactivated FBS for 12 h to separate the floating and adherent cells. The floating cells were collected, suspended in α-MEM, counted, seeded onto 8-well chamber slides (Nalge Nunc International) at x 10⁶ cells/well, and cultured in α-MEM in the presence of 30 ng/ml M-CSF for 3 days to form macrophage-like osteoclast precursor cells. After the floating cells, including the lymphocytes, were removed by aspiration, the adherent osteoclast precursors were cocultured with activated lymphocytes in the presence of 30 ng/ml M-CSF and 100 ng/ml RANKL for 4 days to generate osteoclasts.

On day 2, the medium was replaced with fresh medium containing M-CSF and RANKL. BMCs prepared with the method described were cultured in 24-well OAS plates (Osteogenic Core Technologies). After the formation of mature osteoclasts, the cells were further incubated for 2 days with or without a redox modifier. The erosive areas were identified with microscopy.

**RT-PCR analysis of cytokine mRNA expression**

mRNA was extracted using RNazol B (Biotec Laboratories) according to the manufacturer’s instructions. Total mRNA (2 μg) was reverse transcribed at 42°C using the SuperScript Reverse Transcription system (TaKaRa). PCR amplification of cDNA aliquots was performed by adding 2.5 mM dNTPs, 2.5 U of TaqDNA polymerase (TaKaRa), and 0.25 μM sense and antisense primers. The following sense and antisense primers were used for each molecule: mouse RANKL, 5′-TGTAAGGCGGACCGCAGAGT-3′ (sense) and 5′-CCCAACTTGGTCTGCTC-3′ (antisense); mouse RANK, 5′-CCAGGAAAGTTCCACACAG-3′ (sense) and 5′-CAGTGAAGTCCACACAG-3′ (antisense); mouse STAT3, 5′-ACCAACATCGTTCCTTTGCA-3′ (sense) and 5′-ACTTGCAGCACCAGCTTCT-3′ (antisense); mouse cathepsin K, 5′-TGTTTCACTGGAACCAA-3′ (sense) and 5′-AGCAGGGTCACAGT-TCAAGC-3′ (antisense); mouse MMP-9, 5′-GTTCTCTATCTTTTTGACTCC-3′ (sense) and 5′-GGAGTTAAGGCAGGACATAGTGCG-3′ (antisense). The reaction mixtures contained 2 μl of LightCycler FastStart DNA mastermix for SYBR Green I (Roche Diagnostics), 0.5 μM of each primer, 4 μM MgCl₂, and 2 μl of template DNA. All capillaries were sealed, centrifuged at 500 g for 30 s, and then amplified in a LightCycler instrument with activation of the polymerase at 95°C for 10 min, followed by 45 cycles of 10 s at 95°C, 10 s at 55°C, and 10 s at 72°C. The temperature transition rate was 20°C/s for all steps. Double-stranded PCR products were quantified during the 72°C extension step by detection of the fluorescence associated with the binding of SYBR Green I to the product. Fluorescence curves were analyzed with LightCycler software (v. 3.0).

For quantitative analysis of RANKL, RANK, STAT3, TRAP, and cathepsin K, molecular masses of 134, 121, and 114 kDa, respectively, were used. The expression levels of RANKL, RANK, STAT3, TRAP, and cathepsin K were calculated and normalized to the values for the endogenously expressed control housekeeping gene (hprt1). Melting-curve analysis was performed immediately after the amplification protocol under the following conditions: 0 s (hold time) at 95°C, 15 s at 65°C, and 0 s (hold time) at 95°C. Temperature change rates were 20°C/s, except in the final step, in which it was 0.1°C/s. The melt peak generated represented the specific amplified product. The crossing point (Cₚ) was defined as the maximum of the second derivative of the fluorescence curve. Negative controls were also included and contained all the elements of the reaction mixture except the template DNA. All samples were processed in duplicate.

**Small interfering RNA (siRNA) transfection**

Mouse STAT3 siRNA was designed by Dharmaco. CD4+ T cells were plated in 24-well plates and transfected with 100 nM siRNA or 100 nM negative control siRNA conjugated with Alexa Fluor 488 (Qiagen) to monitor the efficiency of transfection with HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s protocol.

**TRAP staining**

A commercial kit (catalog no. 387-A; Sigma-Aldrich) was used according to the manufacturer’s instructions, omitting counterstaining with hematoxylin. TRAP-positive cells containing three or more nuclei were scored as osteoclasts. TRAP-positive cells were counted without knowledge of the previously counted numbers of osteoclasts.

**Statistical analysis**

Experimental values were presented as mean ± SD. Statistical significance was determined by Student’s t test using the SPSS program (v.10.0). Values of p < 0.05 were considered statistically significant.

**Results**

**Increased expression of IL-23 in mice with spontaneous arthritis**

To verify the pathogenic role of IL-23 in arthritis, the joints of 11-wk-old IL-1Ra−/− and WT mice were immunostained with specific Abs directed against IL-23 p19. IL-23 p19-positive cells were counted. **Ab, IL-23 (5 ng/ml) plus IL-17 (5 ng/ml), or anti-IL-17 Ab for 72 h or no cytokine used (Nil). RANKL expression was evaluated by immunostaining. Data represent the average value for three independent experiments. *p < 0.05 compared with Nil; **p < 0.01 compared with Nil; and #, p < 0.05 compared with 5 ng/ml IL-23. B, The expression of RANK mRNA was also evaluated by real-time quantitative PCR. RANK expression was evaluated by immunostaining. Data represent the average value for three independent experiments. *p < 0.05 compared with Nil; **p < 0.01 compared with Nil; and #, p < 0.05 compared with 5 ng/ml IL-23.**
were readily detectable in the inflamed joints of the IL-1Ra<sup>−/−</sup> mice, whereas few positive cells were observed in the joints of the WT mice (Fig. 1A). Some of our recently published data demonstrated that proinflammatory cytokines such as IL-17, IL-1β, and IL-23 may play important roles in destructive joint inflammation (8).
IL-23 is an osteoclastogenic cytokine in autoimmune disease

IL-23 mediated RANKL expression involves STAT3 and NF-κB signaling in CD4+ T cells from IL-1Ra−/− mice. A, CD4+ T cells were pretreated for 30 min with AG490, parthenolide, LY294002, SP600125, or PD98059 before exposure to IL-23 (5 ng/ml) for 12 h. Total RNAs were extracted and analyzed by real-time PCR with SYBR Green I using specific primers for the mouse RANKL cDNA sequences. HPRT mRNA was used as an internal control. *, p < 0.05 compared with Nil; **, p < 0.01 compared with Nil; #, p < 0.05 compared with IL-23; and ###, p < 0.01 compared with IL-23. B, CD4+ T cells were pretreated for 30 min with STAT3 siRNA before exposure to IL-23 (5 ng/ml) for 24 h. RANKL mRNA was analyzed by real-time PCR. C, CD4+ T cells from IL-1Ra−/− mice were pretreated for 30 min with AG490 before exposure to IL-23 (5 ng/ml) for 72 h. The secretion of RANKL was evaluated by immunostaining. Data represent the average value for three independent experiments. *, p < 0.05 compared with Nil; ***, p < 0.01 compared with Nil; #, p < 0.05 compared with IL-23; and ###, p < 0.01 compared with IL-23.

IL-23, CD4+ T cells, and RANKL colocalize in the pannus of arthritic joints

IL-23 induced in vitro RANKL expression in CD4+ T cells from IL-1Ra−/− mice. To verify this observation in vivo, immunohistochemical staining for IL-23, CD4+ T cells, and RANKL was conducted on arthritic joints from IL-1Ra−/− mice (Fig. 1B). The pannus invaded the bone at the “pannus-bone interface”. At these lesions, staining was positive for IL-23, CD4+ T cells, and RANKL, and with similar patterns of distribution. This finding suggests that a relationship among IL-23, T cells, and RANKL exists in vivo. Confocal microscopy showed that RANKL was expressed more abundantly in the tissues of IL-1Ra−/− mice than in tissues of WT mice. IL-17 is produced by T cells when they are stimulated by IL-23. A proportion of cells that were positively stained for IL-17 (green) also expressed RANKL (red), appearing as merged (yellow) cells co-expressing IL-17 and RANKL (Fig. 1C).

Expression of RANKL and RANK in mixed joint cells from IL-1Ra−/− mice is increased by IL-23

RANK and its ligand RANKL are expressed in various cells of arthritic joints, including fibroblasts, lymphocytes, osteoclasts, and endothelial cells (13). To determine whether IL-23 is responsible for up-regulating RANKL expression in vitro, we isolated mixed joint cells from IL-1Ra−/− mice. We then added various proinflammatory cytokines, including IL-23, to the mixed joint cell cultures and analyzed the mRNAs for RANKL and RANK. IL-23 increased the expression of RANKL and RANK in a dose-dependent manner. The optimal IL-23 concentrations for stimulating RANKL and RANK were 5 and 10 ng/ml, respectively (Fig. 2). IL-17 (5 ng/ml) and anti-IL-17 Ab did not cause joint cells to express RANKL or RANK as strongly as did IL-23. RANKL and RANK expression was also confirmed by immunohistochemical staining of the joint cells. RANKL- and RANK-positive cells seemed to be larger and more strongly stained in the proinflammatory cytokine-treated groups than in the untreated group. Immunohistochemical staining with anti-isotype Ab showed negatively stained mixed joint cells (Fig. 2). These results suggest that the RANKL/RANK system is activated under proinflammatory cytokine-rich arthritic conditions, and that IL-23 could up-regulate RANKL/RANK expression.

IL-23 enhances the expression of RANKL in CD4+ T cells from IL-1Ra−/− mice

Compared with the control mice, IL-1Ra−/− mice and mice injected with rAd/mIL-23 displayed a more prominent pannus (FLS, etc.) and lymphocyte infiltration (CD4+ T cells, etc.). CD4+ T cells and FLS could be responsible for the increased expression of RANKL/RANK in joint cells. Because RANKL expression is increased by TCR activation with anti-CD3 Ab or T cell stimulation with IL-7, it was necessary to assess whether the expression of both RANK and RANKL was enhanced in T cells by IL-23 in IL-1Ra−/− mice. Splenic CD4+ T cells obtained from IL-1Ra−/− mice were cultured with various concentrations of IL-23 for 72 h. A significant increase in the expression of RANKL was induced by IL-23 in the CD4+ T cells.
from the IL-1Ra−/− mice (Fig. 3A), whereas there was no change in their RANK expression. However, the results were opposite in the FLS from IL-1Ra−/−. In FLS, the expression of RANK was increased significantly by IL-23 in a dose-dependent manner, with no change in RANKL expression (data not shown). Real-time quantitative PCR revealed that the increased
expression of RANKL by CD4↑ T cells was sustained for >72 h (Fig. 3B). To block the effects of IL-17 on CD4↑ T cells, we pretreated the cells with anti-IL-17 Ab. The addition of anti-IL-17 Ab did not significantly influence IL-23-induced RANKL expression (Fig. 3A). Because a vast array of diverse cytokines are involved in the development of the arthritic joint and the expression of various cytokines have been reported, we stimulated CD4↑ T cells with IL-23 in combination with other proinflammatory cytokines. IL-23, IL-17, IL-1β, and TNF-α were combined as shown in Fig. 3C. IL-23, IL-17, IL-1β, and TNF-α increased RANKL mRNA expression. Dual combinations of cytokines had an additive effect. Interestingly, triple combinations produced the highest RANKL expression in CD4↑ T cells (Fig. 3C). This result suggests that arthritic joint environments with multiple cytokines potentially express more RANKL.

STAT3 and NF-κB signaling is required for IL-23-stimulated RANKL expression in CD4↑ T cells from IL-1Ra−/− mice

IL-23 signal transduction is activated through the JAK-STAT pathway (14). Based on this finding, we examined the signaling pathway that mediates IL-23-induced RANKL production. Splenic CD4↑ T cells isolated from IL-1Ra−/− mice were pretreated for 2 h with signaling pathway inhibitors, such as AG490 (Jak2/STAT3 inhibitor), parthenolide (NF-κB inhibitor), LY294002 (PI3K/Akt inhibitor), SP600125 (AP-1 inhibitor), and PD98059 (MEK inhibitor), and then cultured with IL-23 (5 ng/ml). The expression of RANKL was determined by real-time PCR after 12 h (Fig. 4A). The NF-κB inhibitor parthenolide and the Jak2 inhibitor AG490 suppressed IL-23-induced RANKL expression in CD4↑ T cells. However, the other signaling pathway inhibitors had no significant effect on the induction of RANKL. Therefore, the

FIGURE 6. Osteoclastogenic impact of IL-23 without CD4↑ T cells. A–C, BMCs were cultured in the presence of IL-23 (5 ng/ml) or M-CSF (10 ng/ml). The medium was changed every 3 days. After 7 days, the cells were stained for TRAP activity. The number of multinucleated TRAP-positive cells was determined. Results are the mean ± SEM of six determinations per group. M-CSF did not induce osteoclast formation. IL-23 alone induced osteoclasts, which phenomenon was more pronounced in IL-1Ra−/− mice in comparison with WT. In combination with M-CSF, IL-23 induced the same level of osteoclastogenesis as was induced with the standard medium for osteoclastogenesis. **, p < 0.01 compared with Nil; #, p < 0.05 compared with IL-23. D, IL-23-treated BMCs were cultured in the absence or presence of anti-IL-1b mAb. The medium was changed every 3 days. After 7 days, the cells were stained for TRAP activity. The number of multinucleated TRAP-positive cells was determined. Blocking IL-1 effect reduced the number of mature osteoclasts when anti-IL-1b Ab was treated in culture soup of the IL-23 plus RANKL plus M-CSF group.
FIGURE 7. Osteoclastogenic role of IL-23 in vivo. IL-23 accelerates the severity of the arthritis in IL-1Ra−/− mice with increased expression of RANKL and RANK. A. The progression of arthritis in IL-1Ra−/− mice after adenoviral gene delivery. The arthritic scores of IL-1Ra−/− mice injected with adenovirus expressing IL-23 (rAd/mIL-23) (□) or adenovirus encoding eGFP (rAd/eGFP) (□) were compared with those of mice injected with PBS (□) throughout the examination period (7–13 wk). Data represent the average value for five individual mice in each group. B. Histological sections of knee joints from IL-1Ra−/− mice treated with rAd/mIL-23, rAd/eGFP, or PBS and from WT mice. Joint photographs of safranin O staining and immunohistochemical staining for RANKL and RANK are shown. C. IgG1 and IgG2a subtype concentrations in the sera from IL-1Ra−/− mice treated with rAd/mIL-23, rAd/eGFP, or PBS and in sera from WT mice. Data represent individual values and the average value of five individual mice in each group. *, p < 0.05 compared with PBS/IL-1Ra−/−; **, p < 0.01 compared with PBS/IL-1Ra−/−. D, RANKL and RANK mRNA expression induced by IL-23. Joint cells were cultured with IL-23 (10 ng/ml) for 12 h. Total RNAs were extracted and analyzed by real-time PCR with SYBR Green I using specific primers for the mouse RANKL or RANK cDNA sequence. HPRT mRNA was used as an internal control. *, p < 0.05 compared with PBS/IL-1Ra−/−; **, p < 0.01 compared with PBS/IL-1Ra−/−; #, p < 0.05 compared with IL-23/IL-1Ra−/− in Nil; and ##, p < 0.01 compared with IL-23/IL-1Ra−/− in Nil.
expression of RANKL, mediated by IL-23, is controlled by Jak2 and NF-κB. To examine whether STAT3 is indeed a signal transduction molecule that directly regulates the expression of RANKL, T cells from IL-1Ra−/− mice were treated with STAT3 siRNA and cultured. The IL-23-induced expression of RANKL mRNA was suppressed only in cells treated with STAT3 siRNA (Fig. 4B). Immunostaining IL-23-stimulated CD4+ T cells also revealed that AG490 or parthenolide suppressed IL-23-induced RANKL expression by CD4+ T cells (Fig. 4C).

**IL-23 stimulates osteoclastogenesis indirectly via CD4+ T cells through the up-regulation of RANKL expression by CD4+ T cells**

RANKL and M-CSF are required for the differentiation of monocytes into mature osteoclasts. In this study, we found that CD4+ T cells can supply RANKL at the pannus and bone erosion sites in the inflammatory environment. To determine the functional potency of the RANKL expressed by CD4+ T cells in causing mouse BMCs to differentiate into mature osteoclasts, we added CD4+ T cells and IL-23-stimulated CD4+ T cells to BMC cultures. After 7 days of culture, mature osteoclasts that differentiated from the monocytes in the wells that contained either CD4+ T cells or IL-23-stimulated CD4+ T cells, as determined with a TRAP staining assay (Fig. 5A). We compared the osteoclastogenic potential of IL-23-stimulated with unstimulated CD4+ T cells. IL-23-stimulated CD4+ T cells were more potent than unstimulated CD4+ T cells in their osteoclastogenic effects. In both groups, the degree of osteoclastogenesis enhancement depended on the character of the medium, which contained either osteoclastogenic mediators or IL-23, or neither. Interestingly, the IL-23-containing medium had definite additional osteoclastogenic effects, even on IL-23-prestimulated CD4+ T cells. These results imply that IL-23 helps osteoclastogenesis, besides up-regulating the expression of RANKL in CD4+ T cells. These findings were determined by counting the number of TRAP-positive cells per well (Fig. 5B). The expression of other osteoclastogenic markers, such as carbonic anhydrase II, cathepsin K, and MMP-9, was also checked by real-time quantitative PCR (Fig. 5C). Their relative mRNA expression correlated well with the counted number of TRAP-positive osteoclasts. This result implies that the cytokine IL-23 may have a close association with osteoclastogenesis. These findings were determined by counting TRAP-positive cell numbers per well (Fig. 6, B and C). The expression of other osteoclastogenic markers, such as carbonic anhydrase II, cathepsin K, and MMP-9, was also checked by real-time quantitative PCR (data not shown). Their relative levels of mRNA expression correlated well with the counted numbers of TRAP-positive osteoclasts.

**IL-23 accelerates the progression of arthritis in IL-1Ra−/− mice and increases the expression of RANKL and RANK in destructive bone lesions**

In previous experiments on the susceptibility of IL-23 p19−/− mice to autoimmune joint inflammation, the IL-23 p19−/− mice were resistant to the development of arthritis (7). To verify the arthritogenic impact of IL-23, rAd/mIL-23 was prepared and injected into the joints of IL-1Ra−/− mice. The IL-1Ra−/− mice that were injected with a mock vector, rAd/eGFP, or PBS displayed the natural course of arthritis development usually seen in IL-1Ra−/− mice. PBS- or rAd/eGFP-injected IL-1Ra−/− mice developed typical arthritis signs, such as synovial hypertrophy, cartilage destruction, bony erosion, and pannus formation. The arthritis score increased more steeply in the IL-1Ra−/− mice injected with rAd/mIL-23 than in the other two groups and remained high throughout the experimental period, until the end of week 12 (Fig. 7A). More severe destruction of the articular joints, massive pannus formation, and RANKL and RANK expression were observed in mice injected with rAd/mIL-23 (Fig. 7B). This suggests that the expression of IL-23 accelerated the development of arthritis and maintained the arthritis in the IL-1Ra−/− mice. These data, together with the data relating to IL-17, were presented in our recently published report (8). RANKL was intensely expressed in areas of bone destruction in the joints of mice injected with rAd/mIL-23 (Fig. 7B). These results imply that an increase in exogenous IL-23 accelerates the progression of arthritis and bone destruction by up-regulating RANKL expression. RANK was also expressed in the inflamed joints. The concentration of total IgG2a, which reflects the Th1 response, and that of IgG1, which reflects the Th2 response, were higher in the IL-1Ra−/− mice than in the WT mice and were also significantly elevated after the injection of rAd/mIL-23 (Fig. 7C). The joint cells from four groups of mice were separated and the relative mRNA expression of RANKL/RANK was determined by real-time PCR. RANKL and RANK expression was higher in the IL-1Ra−/− mice than in the WT mice, and was also significantly elevated after injection with rAd/mIL-23. Interestingly, IL-23 stimulated joint cells to express increased amounts of RANKL and RANK, even when they had already been increased by rAd/mIL-23 injection (Fig. 7D).

**Discussion**

Inflammatory bone destruction is a well-known and pathognomonic phenomenon in autoimmune diseases such as RA. How inflammation causes bone destruction has been intensely studied since Kog et al. (15) found that T cells could induce osteoclastogenesis by producing RANKL. RANKL is a major ligand that induces monocytes to differentiate into mature osteoclasts and is mainly expressed by osteoblasts and stromal cells. T cells are also known to express RANKL after stimulation with anti-CD3 Ab, IL-7, or IL-18 (16–18). Therefore, RANKL is regarded as a mediator linking inflammation (by lymphocytes) to bone destruction (by osteoclasts). In this study, we have linked this observation to the novel cytokine IL-23 by showing that IL-23 can induce RANKL expression in T cells.

We used IL-1Ra−/− mice, in which IL-1 is specifically overexpressed through the deletion of IL-1Ra, as a model of spontaneous arthritis. The use of the IL-1Ra−/− mouse model could be a weak point in this study when these results are generalized to autoimmune arthritides, including RA. To overcome this weak point, we verified that IL-23 was the primary proinflammatory cytokine...
in IL-1Ra−/− mice. Immunohistochemical staining for IL-23 in IL-1Ra−/− mice showed its increased expression in arthritic joint tissues.

Our study suggests that the increase in arthritic scores and positive RANKL expression in mice injected with rAd/IL-23 were influenced by the biological effects of IL-23 on the development of arthritis. We performed the same experiments with rAd/mIL-23 in DBA1 mice, which develop collagen-induced arthritis after the ingestion of type II collagen. These DBA1 mice showed results similar to those for the IL-1Ra−/− mice. These data support previous studies of IL-23 p19 knockout mice, in which arthritis was not induced, and confirm that IL-23 is involved in the development of arthritis. Because they express IL-23, CD4+ T cells appear to be the major pathological lymphocytes in the arthritic synovium (4). IL-23 stimulates this T cell population not only to produce IL-17, TNF, and IL-6 (19, 20), but also to increase the expression of RANKL in arthritis joint tissues. We isolated CD4+ T cells from IL-1Ra−/− mice and cocultured them with different concentrations of IL-23. RANKL expression in the CD4+ T cells increased in proportion to the IL-23 concentration applied.

A recent study demonstrated that mouse synovial fibroblasts produce RANKL and induce the development of osteoclasts from macrophages by cytokine stimulation (21, 22). To clarify whether IL-23 can induce RANKL expression in FLS, we performed the same IL-23 stimulation experiments in FLS from IL-1Ra−/− mice as we did on CD4+ T cells. IL-23 did not induce RANKL expression in FLS, but induced the expression of RANK by FLS in a dose-dependent manner. This phenomenon is interesting from the perspective of cell-cell interactions. T cells and dendritic cells can interact with each other via the RANKL/RANK system. Experiments with RANKL−/− mice showed that they do not develop lymph node organogenesis (3) and have abnormal immunological reactions (23). The ability of IL-23 to induce RANKL expression in T cells and RANK expression in FLS suggests that there may be increased interactions between T cells infiltrating the synovium and synovial fibroblasts. This finding could be clinically significant because IL-23 may both induce osteoclastogenesis and augment the cross-talk between T cells and FLS in the pannus, the well-known pathognomonic structure in RA joints.

Our group recently reported that IL-23 mediates IL-17 production via STAT3 and NF-κB signaling pathways in CD4+ T cells (8). Therefore, to assess the effect of IL-23 on CD4+ T cells, the confounding autocrine effects of IL-17 on CD4+ T cells had to be excluded. RANKL expression was not influenced by the addition of IL-17-neutralizing Ab to IL-23-stimulated CD4+ T cell cultures (Fig. 3A). IL-17 may not be a limiting step in the action of IL-23 on RANKL expression in CD4+ T cells. Although it is recognized that IL-17 is involved in the development of arthritis and the expression of RANKL (24), there have been few studies of the relationship between T cells and IL-17-induced RANKL expression. Although our data have not yet been published, they support the hypothesis that IL-17 can induce RANKL expression in CD4+ T cells, but additional experiments are required. Moreover, our previously published study revealed that IL-17 induces the production of IL-23 p19 in RA synovial fibroblasts via NF-κB- and PI3K/Akt-dependent pathways (9), which agrees with the results of the present study. An overall schema can be drawn that links CD4+ T cells and FLS in a cross-talk loop, with augmentation of RANKL expression on both sides.

STAT3 activation of stromal/osteoblastic cells is required for the induction of RANKL and the stimulation of osteoclastogenesis by the gp130-using cytokines IL-6, IL-11, and IL-1 (25). We found that STAT3 is also required in the IL-23-induced expression of RANKL by CD4+ T cells. Thus, STAT3 could be a common therapeutic target molecule in treating RANKL-stimulated osteoarticular degeneration.

Our experiments with CD4+ T cells and FLS from IL-1Ra−/− mice show that IL-23 has the potential to up-regulate RANKL expression in CD4+ T cells, with increased osteoclastogenesis in vitro. At the same time, RANKL expression by FLS is induced by IL-23, whereas RANKL expression is not affected by IL-23. Immunohistochemical staining for IL-23, RANKL, and CD4+ T cells revealed similar distribution patterns at the bone-pannus junction. This suggests that CD4+ T cells are potential providers of RANKL for osteoclastogenesis in IL-23-abundant arthritic joints. STAT3 and NF-κB are involved in the signal transduction required for RANKL expression, both in stromal cells and in CD4+ T cells.
IL-23 is an interesting cytokine because it affects osteoclastogenesis with or without the involvement of T cells. This study focuses on the osteoclastogenic effect mediated by CD4+ T cells. However, IL-23 may also have an osteoclastogenic effect without the involvement of CD4+ T cells. That IL-23 is involved in these pathways provides more concrete evidence to fortify the mechanisms of IL-23-induced osteoclastogenesis, and a pivotal point, such as IL-23 production, could be a potential therapeutic target (Fig. 8).

The results presented in this study increase our understanding of the pathogenesis of bone erosion in RA and provide an experimental basis for the development of anticytokine agents, especially IL-23 antagonists or target molecules to block common pathogenic pathways in patients who are at high risk of bone destruction or who do not respond to conventional therapy.

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Disclosures

The authors have no financial conflict of interest.

References


