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Amelioration of bone loss in collagen-induced arthritis by neutralizing anti-RANKL monoclonal antibody ☆

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Abstract

Receptor activator of NF-κB (RANK) and its ligand (RANKL) are pivotal regulators of osteoclast differentiation. RANK and RANKL also mediate T cell/dendritic cell (DC) interaction. Previous study has shown that RANK/RANKL interaction induces prolonged DC survival and antigen presentation. In the present study, we have newly established a hybridoma which produces neutralizing anti-RANKL monoclonal antibody (IK22-5). By treating collagen-induced arthritis (CIA) mice with IK22-5, we have investigated the role of RANKL in the pathogenesis of CIA. Although IK22-5 had no effect on immune responses or inflammation, it ameliorated bone loss at the site of inflammation. Histological analyses revealed that osteoclast formation was impaired at the site of joint inflammation in IK22-5-treated CIA mice. These results suggest the utility of anti-RANKL mAb for the prevention of osteoporosis associated with joint inflammation in RA.

Keywords: Rheumatoid arthritis; TNF family; Bone remodeling; Osteoclast

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammatory synovitis, bone atrophy, and subsequent progressive destruction of articular tissue. The etiological cause of RA is not clearly understood, but cumulative evidence suggests that up-regulation of receptor activator of NF-κB (RANK) ligand (RANKL)-mediated osteoclastogenesis plays a critical role in the bone atrophy and joint destruction in RA [1].

Collagen-induced arthritis (CIA) is a well-established murine model of human RA, which can be induced by intradermal injection of type II collagen emulsified with adjuvant in DBA/1 mice [2]. CIA model has been commonly used to investigate the pathogenesis of autoimmune arthritis and to develop novel therapeutic strategy against RA [3,4].

A TNF-family molecule RANKL, also known as osteoclast differentiation factor (ODF), osteoprotegerin ligand (OPGL), TNF-related activation-induced cytokine (TRANCE), and TNFSF11, and its receptor RANK (TNFRSF11A) are known as the major regulators for the differentiation and activation of osteoclasts [5,6]. These molecules are also known to be involved in T cell/dendritic cell (DC) interactions, DC survival [7–9], and lymph node organogenesis [10–12]. It has been shown that blockade of RANK/RANKL interaction by a soluble decoy receptor osteoprotegerin (OPG, TNFRSF11B) ameliorated the crippling in a rat model of arthritis [13].

To investigate the mechanism which causes the bone erosion and joint destruction in CIA and the role of

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RANK/RANKL interaction in it, we have newly established a neutralizing monoclonal antibody (mAb) against mouse RANKL (IK22-5). In this study, we have administered CIA mice with this novel mAb specific for RANKL and examined its effect on the accelerated bone resorption caused by CIA.

Materials and methods

Mice. Male DBA/1 mice were purchased from Japan Charles River Breeding Laboratories (Kanagawa, Japan) and were used at 7–8 weeks of age. All mice were housed in animal care facilities and were used in accordance with the guidelines of Committee on Animals of Juntendo University School of Medicine.

mAbs. The anti-mouse RANKL mAbs (IK22-5, rat IgG2a and IK36-20, rat IgG2a) were generated by immunizing SD rats with recombinant mouse RANKL (kindly provided by Dr. Nobuyuki Shima, Research Institute of Life Science, Snow Brand Milk Products Co., Ltd.), fusing immune lymph node cells with P3U1 myeloma cells, and screening binding to mouse RANKL-transfected NRK cells.

The anti-mouse RANK mAb (R12-31, rat IgG2a) was generated by immunizing SD rats with mouse RANK-transfected 2PK3 cells, fusing immune lymph node cells with P3U1 myeloma cells, and screening binding to mouse RANK-transfected L5178Y cells.

Induction of CIA, antibody treatment, and clinical assessment of arthritis. Male DBA/1 mice were immunized intradermally at the tail base with 200 μg bovine type II collagen (Collagen Research Center, Tokyo, Japan) emulsified in complete Freund's adjuvant (CFA) containing 100 μg H37Ra *Mycobacterium tuberculosis* (Difco, Detroit, MI). On day 21, the mice were boosted by intradermal injection of 200 μg bovine CII in the same manner as for the initial immunization. Starting from day 21, two groups of DBA/1 mice were administered intraperitoneally with 300 μg/mouse of anti-mouse RANKL mAb (IK22-5 or IK36-20) or control rat IgG (Sigma, St. Louis, MO) three times per week for 14–21 days. Mice were inspected for the development of CIA, and inflammation of the four paws was graded from 0 to 4: grade 0, paws with no swelling; grade 1, paws with swelling of finger joints or focal redness; grade 2, paws with mild swelling of wrist or ankle joints; grade 3, paws with severe swelling of the entire paw; and grade 4, paws with deformity or ankylosis. Each paw was graded and the four scores were totaled so that the possible maximum score per mouse was 16.

Histopathology, radiological assessment of arthritis, and quantification of bone mineral density. Both hind limbs were removed at 15 days after the second immunization and fixed in buffered formalin. Radiographs of the hind limbs were obtained with a cabinet soft X-ray apparatus, and bone mineral density in the distal end of femur was measured using dual X-ray absorptiometry (DXA). For histological analyses, hind limbs were removed and fixed in buffered formalin, decalcified in 10% EDTA, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE), toluidine blue (TB), or tartrate resistant acidic phosphatase (TRAP) staining [14].

T cell stimulation in vitro. Draining lymph node (DLN) cells were isolated from mice in each group at 7 days after the booster immunization. These cells (5×10^5) were cultured in 96-well flat-bottomed microculture plates in 200 μl RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.05 mM 2-ME, and antibiotics in the presence or absence of 30 μg/ml of bovine CII. In some experiments, cultures were treated with anti-RANKL mAb (10 μg/ml), anti-B7.1 and B7.2 mAbs (10 μg/ml each) or control rat IgG (10 μg/ml). For estimating proliferative responses, the cultures were pulsed with [^{3}H]thymidine (0.5 μCi/well, Perkin-Elmer Life Sciences, Boston, MA) for the last 16 h of the 72 h culture and harvested on a Micro 96 Harvester (Skatron, Lier, Norway). Incorporated radioactivity was measured on a microplate β counter (Micro Beta Plus, Wallac, Turku, Finland). For assessment of cytokine production, culture supernatants were collected after 72 h and stored at −80 °C until the ELISA was performed.

Serum anti-CII Ab levels. Serum samples were collected at 7 days after the second immunization, and the titers of anti-CII IgG Abs were measured by ELISA. Bovine CII (1 μg/ml) was coated onto microtiter plates (Immuron 2, Dynatech, Chantilly, VA) overnight at 4 °C. After blocking with 1% BSA in PBS, serially diluted serum samples were added and incubated for 1 h at room temperature. After washing, biotin-conjugated rat anti-mouse IgG1, IgG2a, or goat anti-mouse IgG Ab was added and incubated for 2 h at 37 °C. After washing, Ab binding was visualized using Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and *o*-phenylenediamine. A standard serum composed of a mixture of sera from arthritic mice was added to each plate in serial dilutions and a standard curve was constructed. The standard serum was defined as 1 U/ml and the Ab titers of serum samples were determined by the standard curve [15].

Quantification of cytokines. Cell-free culture supernatants were assayed for IL-4 and IFN-γ contents by specific ELISA according to the protocol recommended by the manufacturer. ELISA for mouse IFN-γ and IL-4 was performed using Ready-SET-Go! ELISA kit (eBioscience, San Diego, CA) for IFN-γ and Opt-EIA kit (BD Pharmingen, San Diego, CA) for IL-4. Cytokine levels were calculated using standard curves obtained with known amounts of recombinant cytokines.

In vitro osteoclastogenesis. The co-culture of osteoclast precursor cells and osteoblasts derived from calvarial cells was performed in the presence of 10^{-8} M 1,25 $(OH)_2$ vitamin D_3 and 10^{-6} M prostaglandin E_2 in the absence of recombinant RANKL and M-CSF as described previously [1]. Three to five days later, TRAP$^+$ multinucleated (more than three nuclei) cells were counted. For RANKL-blocking experiment, anti-RANKL mAb, control rat IgG2a, or recombinant OPG was added to the culture medium at the beginning of co-culture.

Isolation of CIA synovial cells and FACS analysis. Synovial tissue was isolated from CIA mice on day 21 after the second immunization, and was incubated in α-MEM supplemented with 1 mg/ml collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.17 mg/ml DNase I (Sigma, St. Louis, MO) at 37 °C for 3 h. Resulting cell suspension was filtered to remove debris, and cells were collected by centrifugation at 1500 rpm for 5 min. These cells were stained with biotinylated anti-RANK mAb (R12-31) or biotinylated anti-RANKL mAb (IK22-5) and PE-streptavidin (CALTAG, Burlingame, CA) and then were subjected to FACS analysis on FACSCalibur and Cell Quest (BD Biosciences).

Results

Characterization of anti-mouse RANKL and RANK mAbs

We generated two mAbs (IK22-5 and IK36-20) against mouse RANKL by immunizing SD rat with recombinant murine RANKL. Similar to the staining with RANK-Ig, IK22-5 and IK36-20 specifically bound to RANKL-transfected NRK (RANKL/NRK) cells, but not to parental NRK cells (Fig. 1a). Preincubation with IK22-5, but not with IK36-20 or control rat IgG, efficiently blocked the RANK-Ig binding to RANKL/NRK cells (Fig. 1b), indicating the specific blocking of RANK/RANKL interaction by IK22-5. We also generated an anti-mouse RANK mAb (R12-31). While R12-31 specifically bound to RANK/L5178Y cells (Fig. 1c), R12-31 did not block the RANK-Ig binding to RANKL-transfected cells (data not shown).

Expression of RANKL and its receptor RANK in synovial tissue of CIA mice

To detect the expression of RANKL and RANK in synovial tissue of CIA mice, we stained synovial cells isolated from CIA mice with the newly generated

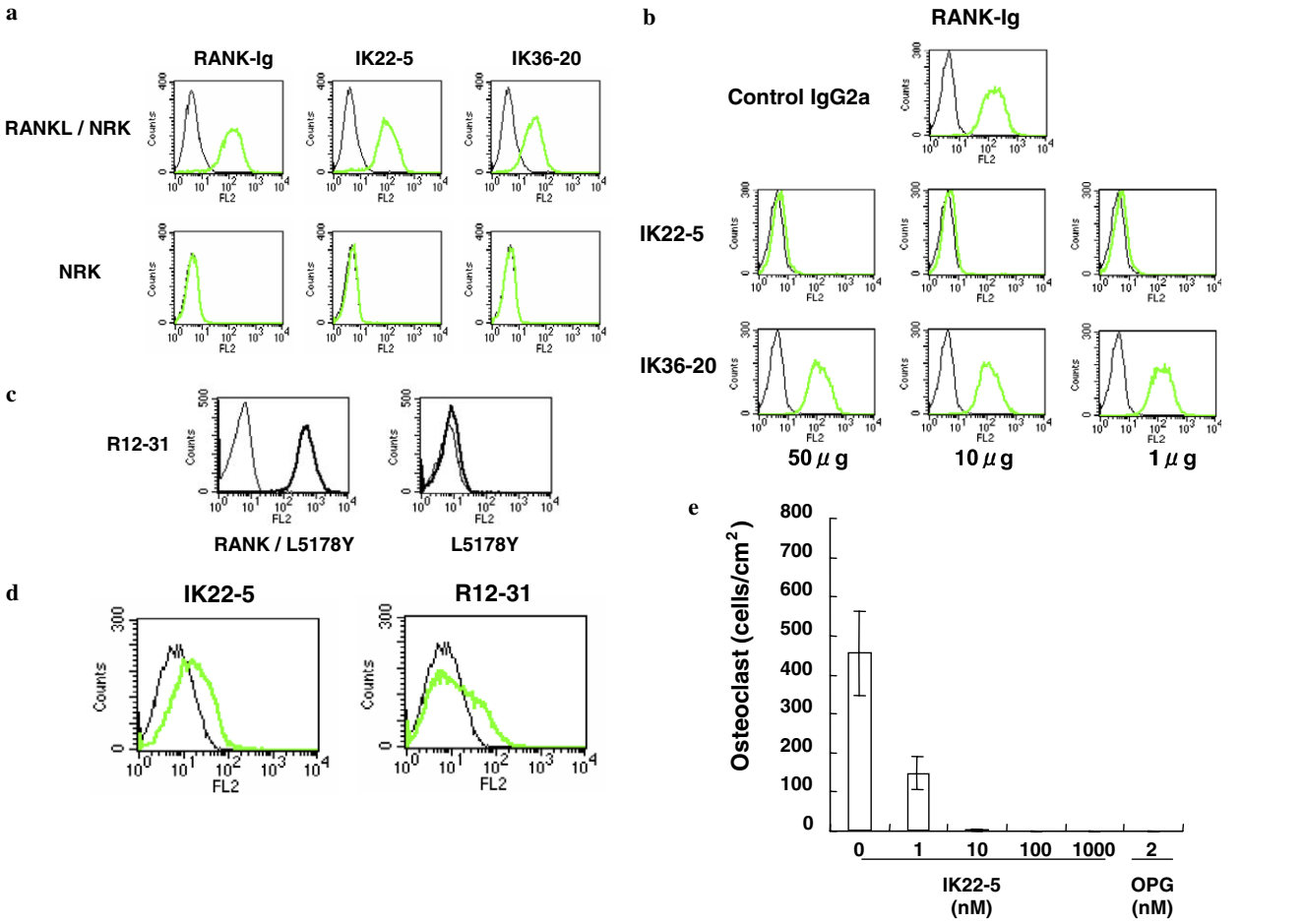

Fig. 1. Characterization of anti-RANKL mAbs (IK22-5 and IK36-20) and anti-RANK mAb (R12-31). (a) Specific binding of IK22-5 and IK36-20 to mouse RANKL. NRK and mouse RANKL-transfected NRK cells were stained with IK22-5 and IK36-20 or RANK-Ig followed by PE-anti-rat IgG Ab or PE-anti-human IgG Ab, respectively. Samples were analyzed by flow cytometry. Histograms from the cells stained with control Ig (rat IgG2a or human IgG1) are overlaid. (b) Blocking of RANK-Ig binding to RANKL/NRK cells by IK22-5. The bold line shows a histogram of the cells preincubated with graded amount of IK22-5 or IK36-20 before staining with RANK-Ig. Preincubation with control rat IgG2a did not inhibit the binding of RANK-Ig to RANKL/NRK cells (top panel). (c) L5178Y and mouse RANK-transfected L5178Y cells were stained with biotinylated R12-31 followed by PE-streptavidin. (d) Expression of RANKL and RANK on synovial cells. Synovial tissue was isolated from CIA mice 3 weeks after the booster immunization. Cell suspension was prepared from the CIA synovial tissue. These cells were stained with IK22-5 (left) or R12-31 (right) and then were subjected to FACS analysis. Both of these mAbs successfully detected the expression of RANKL and RANK in synovial tissue of CIA mice. (e) Inhibitory effects of IK22-5 on *in vitro* osteoclastogenesis. Murine BM cells and osteoblasts were co-cultured in the presence or absence of IK22-5. OPG was included as a positive control. Co-culture of BM cells and osteoblasts was performed in α-MEM supplemented with 10^{-6} M PGE_2 and 10^{-8} M 1,25-dihydroxy vitamin D_3. Three to five days later, $TRAP^+$ multinucleated (more than three nuclei) cells were counted. Data are expressed as means ± SEM of triplicate cultures. The data shown are representative of three independent experiments with similar results.

anti-RANKL mAb (IK22-5) and anti-RANK mAb (R12-31). FACS analysis of these cells revealed that both RANKL and RANK are expressed in synovial tissue of CIA mice (Fig. 1d).

Inhibitory effect of IK22-5 on osteoclastogenesis in vitro

To evaluate the functional inhibitory effect of newly generated anti-RANKL mAb (IK22-5), we examined the effect of this mAb on *in vitro* osteoclastogenesis. Bone marrow (BM) cells from normal DBA/1 mice were co-cultured with calvarial cells in the presence of 1,25 $(OH)_2$ vitamin D_3 and prostaglandin E_2. Graded amount of IK22-5 or control rat IgG was added to the culture. After co-culturing for three to five days, $TRAP^+$ multinucleated (more than three nuclei) cells were counted. Similar to the effect of OPG, the addition of IK22-5, but not the addition of control rat IgG (data not shown), drastically inhibited the formation of $TRAP^+$ multinucleated cells in a dose-dependent manner (Fig. 1e). These results indicated an inhibitory effect of IK22-5 and a critical contribution of RANKL to the differentiation of osteoclast *in vitro*. On the other hand, the anti-RANK mAb (R12-31) had no inhibitory or agonistic effect upon *in vitro* osteoclastogenesis (data not shown).

Development of CIA is not affected by anti-RANKL mAb

To explore the contribution of RANKL/RANK interaction to the pathogenesis of RA, we first examined the

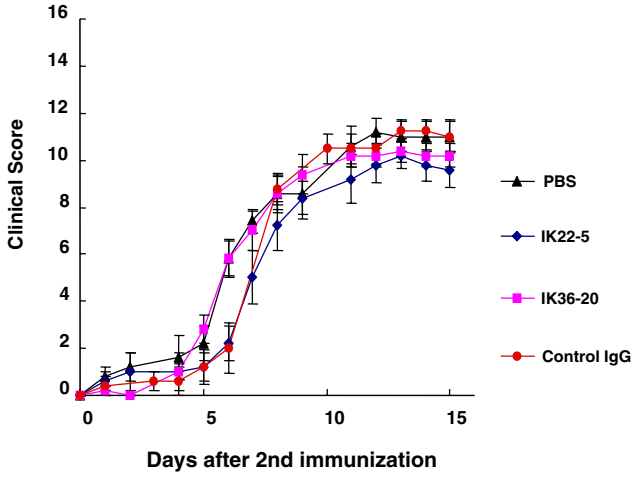

Fig. 2. Administration of anti-RANKL mAb did not affect the development of CIA. DBA/1 mice ($n = 10$ for each group) were immunized with bovine CII (200 μg/mouse) in CFA. On day 21, the mice were boosted by i.d. injection of CII in CFA. Starting from the day of booster immunization, these mice were i.p. administered with either anti-RANKL mAb (300 μg/mouse) or control IgG (300 μg/mouse) three times a week for 2 weeks. The development of CIA was evaluated as described in Materials and methods. Data are shown as means ± SEM of clinical scores at the indicated times after the booster immunization and are representative of three independent experiments with similar results.

effect of the neutralizing anti-RANKL mAb (IK22-5) on the development of CIA as an animal model for RA. DBA/1 mice were immunized twice with bovine type-II collagen (CII) in CFA to elicit CIA. Starting from day 21, two groups of DBA/1 mice were treated intraperitoneally with 300 μg/mouse of IK22-5 or control rat IgG three times per week for 14–21 days. As shown in Fig. 2, administration of IK22-5 did not significantly affect the development of CIA. In another set of experiments, the CII-immunized DBA/1 mice were treated with IK22-5 or control IgG everyday from the day of first immunization. This long-term treatment with IK22-5 also had no apparent effect on the development of CIA (data not shown). These results suggested that RANKL/RANK interaction did not play a major role in the development of inflammation in CIA mice.

Anti-RANKL mAb does not prevent the expansion of CII-specific T cells

Previous reports have suggested that the RANKL/RANK interaction may play an important role in T/DC interaction, in a manner similar to CD28/B7 and CD40L/CD40 interactions. To elucidate the effect of anti-RANKL mAb treatment on the activation of CII-specific T cells, we isolated DLN cells from the CII-immunized mice treated with IK22-5 or control rat IgG at 7 days after the second immunization, and re-stimulated the DLN cells with CII *in vitro*. As shown in Fig. 3a, no significant difference in the CII-specific proliferative response was observed between mice treated with IK22-5 and control IgG *in vivo*. Furthermore, *in vitro* treatment with IK22-5 also did not significantly affect the CII-specific proliferative response,

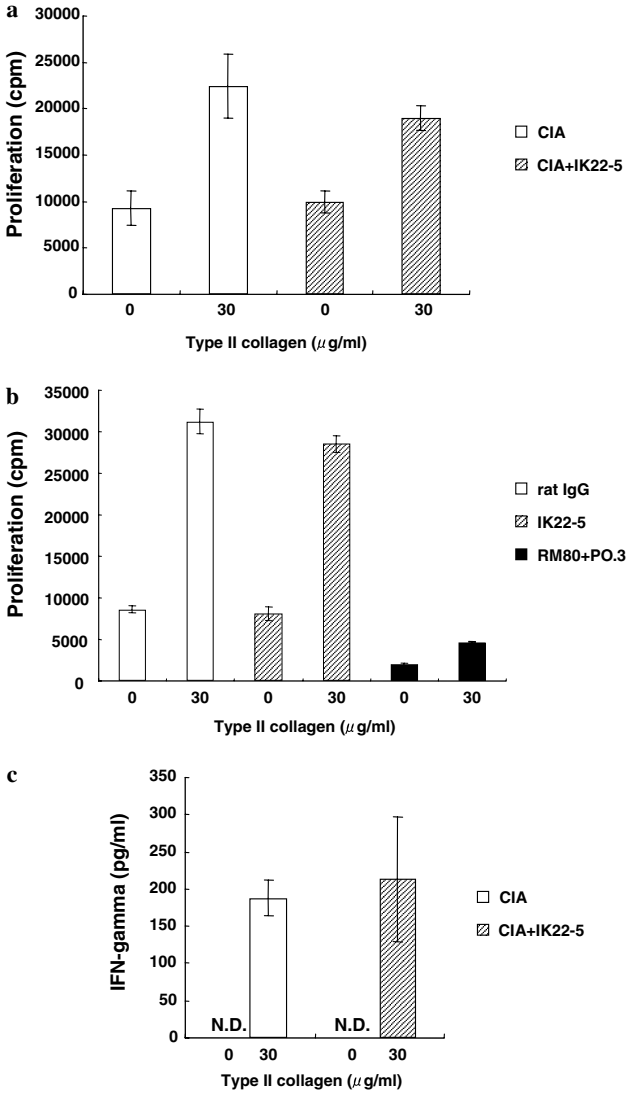

Fig. 3. Effect of anti-RANKL mAb on CII-specific T cell responses. (a) *In vivo* treatment with anti-RANKL mAb did not prevent the expansion of CII-specific T cells. DBA/1 mice were immunized with CII and treated with control IgG or anti-RANKL mAb as shown in Fig. 2 ($n = 5$). DLN cells were isolated at 7 days after the booster immunization and cultured in the presence or absence of CII (30 μg/ml) for 72 h. The cultures were pulsed with [^{3}H]thymidine for the last 16 h. (b) *In vitro* administration of anti-RANKL mAb did not affect the CII-specific proliferative response. DLN cells were isolated from DBA/1 mice immunized with CII at 7 days after the booster immunization and cultured in the presence or absence of CII (30 μg/ml) for 72 h. The indicated mAbs were added at the beginning of the cultures. Anti-B7.1 (RM80) and anti-B7.2 (PO.3) mAbs were used as a positive control. The cultures were pulsed with [^{3}H]thymidine for the last 16 h. (c) DLN cells were prepared from five mice treated with control IgG or anti-RANKL mAb at 7 days after the booster immunization, and cultured in the presence or absence of CII (30 μg/ml) for 72 h. Cell-free culture supernatants were collected and assayed for the contents of IFN-γ by specific ELISA. Data are shown as means ± SEM of triplicate cultures and are representative of three independent experiments with similar results. ND, not detectable.

while anti-B7-1 and B7-2 mAbs efficiently inhibited the response (Fig. 3b). These results indicated that the treatment with anti-RANKL mAb did not prevent the expansion of CII-specific T cells.

Anti-RANKL mAb does not affect the development of CII-specific Th1 immune response

In addition to the proliferative response against CII, we investigated the effect of anti-RANKL mAb on the cytokine production by DLN cells in response to CII. We also measured CII-specific IgG in the serum collected from IK22-5-treated or control IgG-treated CIA mice on day 7 after the booster immunization. Treatment with IK22-5 had no significant effect on the production of IFN-γ by DLN cells of CIA mice (Fig. 3c). Amount of IL-4 produced by DLN cells was below detectable level both in IK22-5-treated and control groups (data not shown). Similarly, levels of CII-specific antibodies (IgG1, IgG2a, and total IgG) in the serum of IK22-5-treated CIA mice were not significantly different from those of control IgG-treated CIA mice (Fig. 4). These results suggested that in spite of the previously reported co-stimulatory effect of RANKL/RANK interaction between T cells and DCs *in vitro*, the RANKL/RANK interaction did not play a major role in the development of both cellular and humoral auto-immune responses in CIA mice.

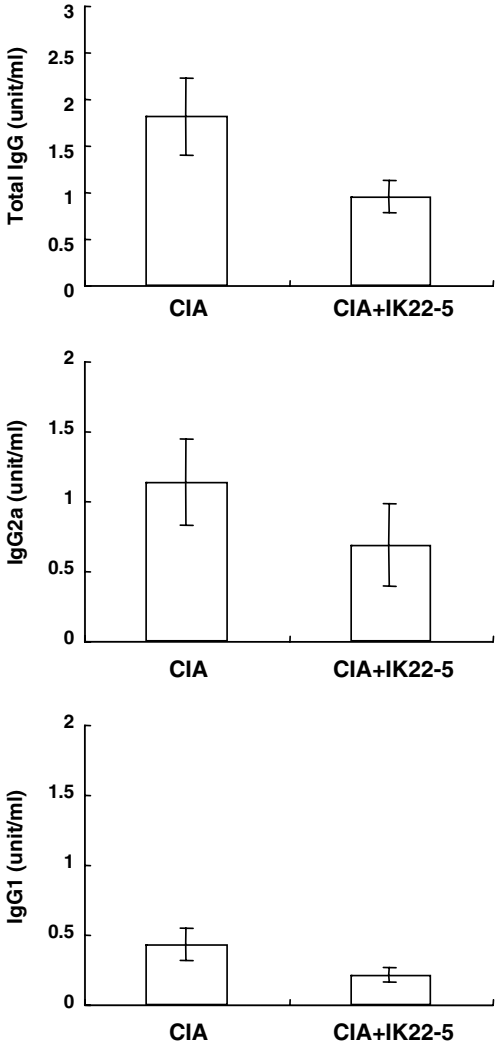

Fig. 4. Effect of anti-RANKL mAb treatment on serum CII-specific antibodies. Sera were collected from individual mice on day 7 after the second immunization. CII-specific antibody levels were measured by subclass-specific ELISA. Data are shown as means ± SEM of five mice in each group. Similar results were obtained in three independent experiments.

Anti-RANKL mAb significantly reduces osteoclasts and ameliorates focal bone erosions

To investigate the effect of anti-RANKL mAb on the focal bone erosion which follows chronic synovial inflammation caused by immunization with CII, we measured bone mineral density (BMD) in the distal end of femur. In the CIA mice treated with control IgG, BMD was significantly reduced as compared with normal DBA/1 mice of the same age (Fig. 5a) (BMD was measured on day 36 after the booster immunization using dual X-ray absorptiometry (DXA)). On the other hand, in the CIA mice treated with IK22-5, BMD was kept at the normal level in spite of severe joint inflammation (Fig. 5a).

To further confirm this protective effect of anti-RANKL mAb on bone erosion, bone resorption, and joint destruction, we evaluated soft-X ray radiographs of normal mice, CIA mice treated with control IgG, and CIA mice treated with IK22-5. As compared with normal DBA/1 mice, overall radiodensity of bones was decreased in the control CIA mice while the treatment with IK22-5 kept the radiodensity at normal level (Fig. 5b). Joint structure was well conserved in the IK22-5-treated CIA mice, while severe joint destruction was observed in the control IgG-treated CIA mice (Fig. 5b). Focal bone erosion was seen only in the CIA mice treated with control IgG (Fig. 5b).

Previous studies have verified that the multinucleated cells located at sites of bone erosion in CIA are authentic osteoclasts [16], being positive for TRAP staining. To evaluate the effect of anti-RANKL mAb on the formation of bone-resorbing cells, we performed the TRAP staining of knee joint paraffin sections to detect osteoclasts. In the CIA mice treated with IK22-5, TRAP-positive cells were markedly reduced in the juxta-articular bone and amelioration of focal bone erosion was observed (Fig. 6). This protective effect of IK22-5 treatment on focal bone erosion in CIA mice was also observed in paraffin section stained with HE (Fig. 6).

These results indicated that anti-RANKL mAb treatment has protective effect on focal bone erosion at the site of synovial inflammation and that the protective effect was due to the blockade of osteoclast formation and activation at the arthritic joint. Furthermore, bone atrophy that accompanies systemic joint inflammation could be ameliorated by administration of anti-RANKL mAb.

Discussion

RANKL is a TNF superfamily molecule known as a major differentiation factor for osteoclasts [5,6]. Previous

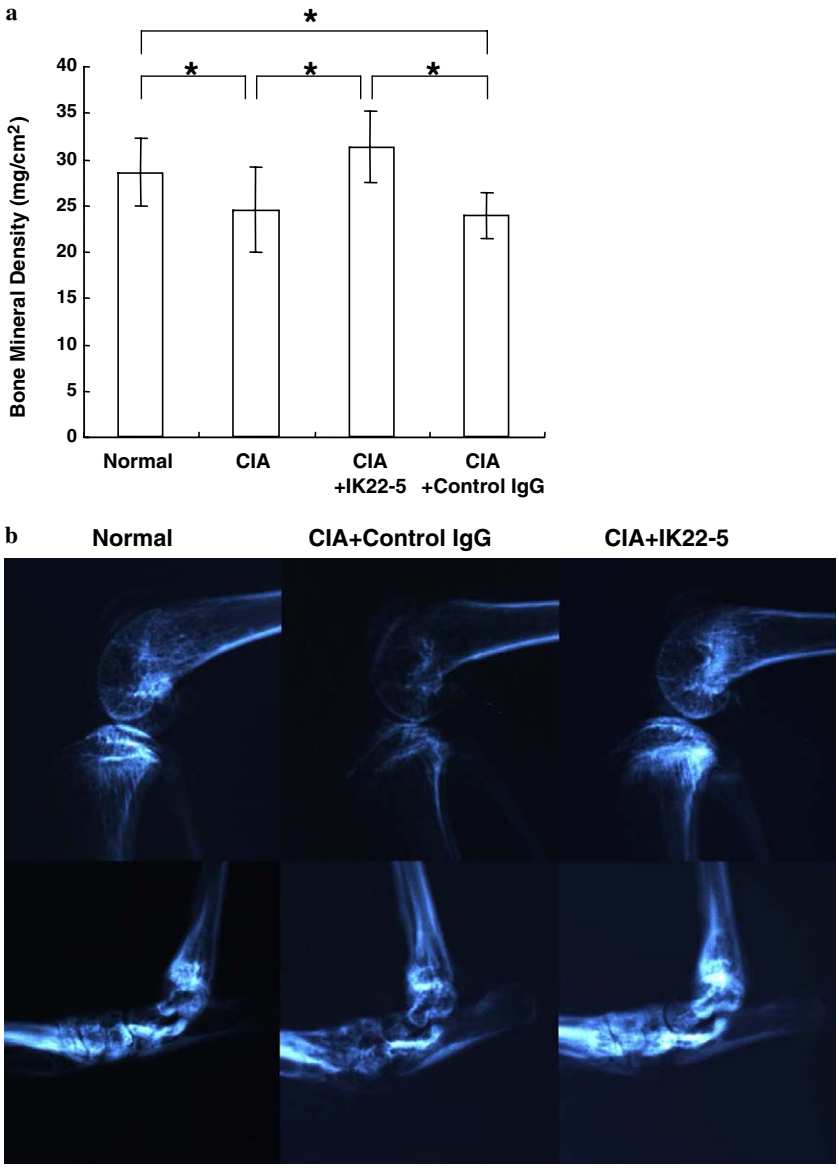

Fig. 5. Effect of anti-RANKL mAb on bone loss. (a) CIA mice were treated with IK22-5 or control IgG as shown in Fig. 2. Bone mineral density (BMD) in the distal end of femur was determined on day 15 after the second immunization. Mean value of BMD ± SD is shown ($n = 5$ per group). Unimmunized normal mice were used as a control. *$P < 0.05$ by Fisher's PLSD. (b) IK22-5 blocks bone loss in CIA. Hind limbs were removed from CIA mice treated with either IK22-5 or control rat IgG on day 15 after the second immunization. Removed hind limbs were then fixed with buffered formalin and subjected to radiographic analysis. As compared with normal mice, CIA mice treated with rat IgG showed decreased bone density, especially in the distal end of femur. This bone loss was blocked by the treatment with IK22-5.

studies have shown that blockade of RANKL/RANK interaction by some inhibitors such as recombinant osteoprotegerin (OPG) or RANK-Ig have protective effect on bone damage that is a characteristic of several arthritis models such as CIA and adjuvant-induced arthritis in rats.

CIA is a murine autoimmune model useful for investigating the pathogenic mechanism of, or possible therapeutic strategy for, joint inflammation as well as focal bone erosions, and bone atrophy in RA [16,17]. Using this model of autoimmune arthritis, we have now demonstrated therapeutic effect of a newly established neutralizing monoclonal antibody against murine RANKL on bone atrophy and focal bone erosion.

First, by flow cytometric analysis, we have shown that the novel anti-RANKL mAb (IK22-5) specifically binds murine RANKL expressed on NRK transfectants (RANKL/NRK), and that preincubation with IK22-5 blocks the binding of RANK-Ig to RANKL/NRK cells. IK22-5 also blocks osteoclast formation from murine BM cells induced by co-culture with calvarial osteoblasts *in vitro*.

Histological analysis revealed the existence of $TRAP^+$ cells at the site of pannus formation in CIA mice treated with control IgG. These osteoclast-like cells are thought to differentiate from RANK-positive monocyte/macrophage lineage cells existing in the inflamed synovial tissue

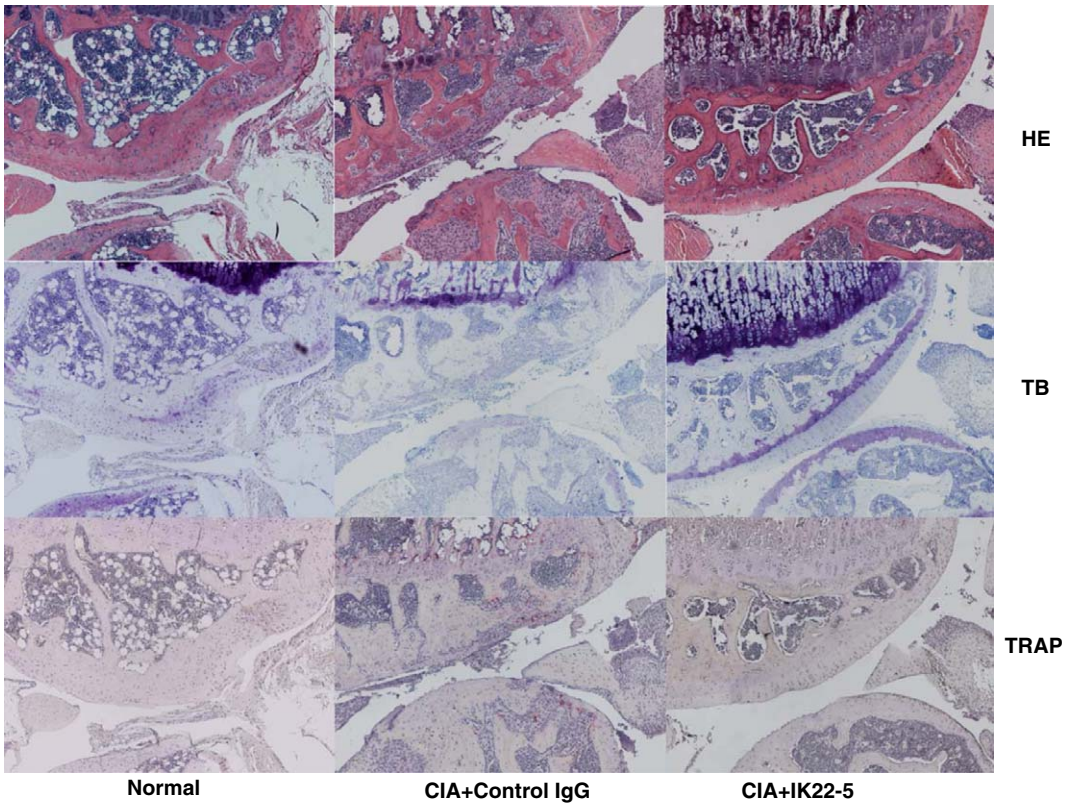

Fig. 6. Effect of IK22-5 treatment on bone, cartilage, and osteoclast differentiation. Consecutive paraffin sections show representative knee joints from normal mice (left), CIA mice treated with control rat IgG (center) or CIA mice treated with IK22-5 (right). Upper row: H&E staining. Middle row: toluidine blue (TB) staining for cartilage. Bottom row: TRAP staining for osteoclasts. Note the reduced number of $TRAP^+$ cells in marrow cavity of IK22-5-treated CIA mice.

[18]. As for the source of RANKL in this context, there are several different hypotheses. Kong et al. reported that RANKL expressed on activated T cells induced osteoclast differentiation both *in vitro* and *in vivo* [13], while Takayanagi et al. suggested that RANKL expressed on synovial fibroblasts, upon stimulation with inflammatory cytokines such as TNF-α and IL-1, played the essential role in osteoclastogenesis *in vivo*. By immunohistochemistry, Lubberts et al. reported that RANK/RANKL expression in inflamed synovial tissue of CIA mice was correlated with the development of CIA [19]. As shown in Fig. 1d, FACS staining of synovial cells by the novel mAbs against RANK and RANKL (R12-31 and IK22-5, respectively) further confirmed the expression of RANK/RANKL in the inflamed synovial tissue of CIA mice. However, on which cell population RANK and RANKL are expressed is yet to be investigated.

In the present study, administration of IK22-5 markedly suppressed the formation of $TRAP^+$ cells at the site of pannus formation. The likely mode of action of IK22-5 is that intraperitoneally injected IK22-5 is delivered to the site of pannus formation on the blood stream and binds RANKL expressed on T cells or synovial fibroblasts, thus preventing the engagement of RANK on osteoclast progenitor cells by RANKL. In agreement with previous studies using OPG in adjuvant-induced arthritis in rats [20], the prevention of RANKL/RANK interaction causes suppression of osteoclast formation, resulting in the amelioration of focal bone erosion.

One of the symptoms characteristic of RA (as well as CIA) is osteoporosis. In chronic inflammatory states of RA or CIA, RANKL, and pro-inflammatory cytokines such as TNF-α and IL-1 are produced by activated T cells and macrophages. These molecules can induce RANKL expression in osteoblasts and bone marrow stromal cells [21]. The enhanced expression of RANKL would result in imbalance between bone resorption by osteoclasts and bone formation by osteoblasts. This imbalance of bone turnover may be responsible for the osteoporosis in RA. In present study, soft X-ray radiography has shown that bone atrophy observed in the hind limbs of CIA mice can be prevented by the treatment with IK22-5. We have also shown that CIA-associated decrease of BMD in the area around epiphysis, where the bone turnover is most active, can be inhibited by the IK22-5 administration. These suppressive effects against osteoporosis may be due to the decreased number of osteoclasts in this area. IK22-5 treatment blocks the engagement of RANK by RANKL, and thus mitigates the imbalance between bone formation and bone resorption by preventing over-formation of osteoclasts. Supportive evidence is shown by the histopathological analysis of TRAP-stained paraffin

sections, in which increased osteoclast formation seen in CIA mice is drastically inhibited by the IK22-5 treatment. In short, the IK22-5 treatment can mitigate the osteoporotic symptom of CIA by modifying the balance between bone formation and bone resorption.

In addition to the function as a mediator of osteoclast differentiation, it has been suggested that RANKL also functions in the interaction between DCs and T cells. Previous studies demonstrated that RANKL could mediate DC survival via Bcl-X_L induction [7–9]. It was also demonstrated that soluble RANKL treatment of antigen-pulsed mature DCs *in vitro* enhanced the number and persistence of antigen-presenting DCs in the draining lymph nodes *in vivo* [8]. Furthermore, it was reported that vaccination with RANKL-treated DCs increased antigen-specific primary T cell response as well as memory T cell response [8]. Since the T/DC interaction is thought to play an important role in the pathogenesis of CIA, we assessed the effect of anti-RANKL mAb treatment on the autoimmune T cell response against CII. However, CII-specific proliferative response of DLN cells was not affected by the treatment with anti-RANKL mAb. Moreover, profile of cytokine (IFN-γ and IL-4) production by DLN cells in response to CII was not altered by the treatment with anti-RANKL mAb. In accordance with these *in vitro* results, there was no significant difference in the clinical arthritis score between the IK22-5-treated group and the control IgG-treated group. These results suggest that RANKL/RANK signaling in the context of T/DC interaction does not have a major role in the pathogenesis of CIA. Probably CD40L/CD40 interaction, which has functional similarity to RANKL/RANK interaction, may substitute the function of RANKL/RANK as a mediator of DC activation and survival.

One of the most critical symptoms in RA is osteoporosis and bone erosion, especially at the sites of inflammation. Usually, bisphosphonate is used to ameliorate these symptoms caused by RA [22], but the mechanism by which bisphosphonate blocks osteoclast activity is not sufficiently elucidated yet. In accordance with the results of previous studies using OPG or RANK-Ig in rat arthritis model, we have now demonstrated that blockade of RANKL/RANK interaction by neutralizing anti-RANKL mAb can specifically block the osteoclast formation/activation, and thus prevent focal bone erosion and osteoporosis caused by chronic inflammation in CIA. Moreover, OPG is known to bind to another TNF family molecule, TRAIL, which is reported to have inhibitory effect upon autoimmune inflammation [23]. Therefore, RANKL-blockade by OPG can also cause blockade of TRAIL, possibly leading to aggravation of autoimmune inflammation. On this aspect, specific blockade of RANKL by a neutralizing mAb may be advantageous over OPG. As represented by the success in clinical use of anti-TNFα mAbs, humanized neutralizing mAbs against RANKL would be clinically useful for the prevention of bone erosion and osteoporosis in RA patients.

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