

of the LCM cap.

10. Obtain the area of interest of the tissue (*see Notes 12 and 13*).

3.5 Extraction of RNA from Tissues Obtained by LCM

RNA is extracted from the harvested tissue by the following protocol. This protocol is based on the manufacturer's for the RNeasy micro kit, but we made some modifications (*see Note 14*).

1. Place 400 μ l of the RNA lysis buffer contained in the RNeasy micro kit (Buffer RLT with 2-mercaptoethanol) into a clean 1.5 ml centrifuge tube. Detach the plastic film and harvested cartilage tissue from the LCM cap and immerse them in the lysis buffer in the tube (*see Note 15*).
2. Add 20 ng of bacterial ribosomal RNA in the lysis buffer as carrier RNA, and vortex for 30 sec at the maximum speed (*see Note 16*).
3. Transfer 350 μ l of the lysis buffer to a new clean centrifuge tube.
4. Add 350 μ l of 70% ethanol to the lysis buffer, and mix well by pipetting.
5. Transfer the mixture to a spin column contained in the kit (RNeasy MinElute spin column), and centrifuge for 15 sec at ≥ 8000 g.
6. Add 350 μ l of the wash buffer (Buffer RW1) to the column and centrifuge for 15 sec at ≥ 8000 g.
7. Add 10 μ l of DNase I solution prepared as indicated by the manufacturer onto the membrane of the column, and incubate for 15 min at RT.
8. Add 350 μ l of the wash buffer and centrifuge for 15 sec at ≥ 8000 g.
9. Add 500 μ l of another wash buffer (Buffer RPE) to the spin column, and centrifuge for 2 min at ≥ 8000 g.
10. Spin the column again for 5 min at full speed to dry the membrane completely.
11. Add 16 μ l of RNase-free water onto the membrane, wait for 1 min, then spin the column for 1 min at full speed to elute the RNA (*see Note 17*).

3.6 Synthesis of cDNA from Extracted RNA

1. Transfer 12.5 μ l of the above obtained eluate to a new clean tube. Since some RNase-free water is lost during the elution, this amount of eluate contains almost all the obtained RNA.
2. Add 10x Buffer RT (2 μ l), dNTP mix (5 mM each dNTP; 2 μ l), oligo dT primer (10

μM ; 2 μl), RNase inhibitor (40 units/ μl ; 0.5 μl) and reverse transcriptase (1 μl), and mix them. Buffer RT, dNTP mix, and reverse transcriptase are contained in the Sensiscript kit (*see Note 18*).

3. Incubate the mixture for 60 min at 37°C.
4. Proceed to qPCR or store at -20°C until use (*see Note 19*).

4. Notes

1. This protocol is based on our experience with the Arcturus PexCell IIe LCM system. Although we do not have experience with other LCM models, this protocol may work reasonably well with them as long as the system uses an infrared laser and thermoplastic films. We do not have experience with microdissection by laser cutting, so another protocol should be prepared for this type of microdissection.
2. To prepare fresh cartilage samples is the first and most important step for successful RNA acquisition by LCM. For OA cartilage from an operation room, we ask the surgeons to put cartilage tissues into the cooled culture media as soon as possible during the surgery. The tissues are kept at a low temperature (4-10°C) and brought to the laboratory immediately, where it is processed upon arrival. For control cartilage from a dissection room, the condition of the sample is more critical. We ask the pathologist to harvest cartilage from donors within 24 hours after death. However, even obtained within this time period, RNA in cartilage tissues could have significantly degenerated, depending on the condition of the donor before death.
3. In our experience, OCT compound from Sakura Finetek is most suitable for the preparation of cryosections for LCM for gene expression analysis. Compounds of other brands may inhibit successful RNA acquisition, although they work fine for preparing cryosections.
4. To make tissue blocks, we use a custom-designed metal mold which measures 20 mm in length, 15 mm in width, and 20 mm in depth. It is deeper than regular molds, so that the tissue blocks would have a few millimeters of compound layer at the bottom. This compound layer will minimize elevation of tissue temperature when a block is mounted on a cryostat.
5. Although the manufacturer of the LCM device recommends that sections 5-8 μm in thickness be prepared, the amount of RNA obtained from cartilage sections of this thickness might not suffice for subsequent analyses. For this reason, we usually prepare cartilage sections 20-30 μm thick.
6. To ensure you succeed in obtaining RNA, make it a rule to complete the entire process of LCM (from the preparation of cryosections till the immersion of harvested tissue in the lysis buffer) within 30 min.
7. We do not perform tissue staining for LCM to separate cartilage zones, because such zones are easily identified by histological features without staining. Staining may be

- performed when LCM is performed for other purposes.
8. EDTA treatment of cartilage sections is essential to perform LCM. Without it, tissues may not attach firmly to the plastic film on an LCM cap, and may detach during tissue separation. EDTA treatment significantly improves the strength of tissue adhesion. From our experience, treatment with 0.5M EDTA solution for 3 min should be adequate to assure attachment.
 9. Sections must be completely dehydrated for LCM. To ensure this, we routinely treat them with 100% ethanol twice before xylene.
 10. After dehydration, cartilage sections may curl up and detach from the slide (**Fig. 3B**). Such sections must be spread out for LCM. Fine-pointed tweezers or pipette tips may be useful to do this.
 11. Cartilage tissues separated from the subchondral bone by a scalpel may contain calcified cartilage. This zone is easily recognized under a microscope, and can be removed by LCM.
 12. Tissue separation by LCM may be difficult for some cartilage samples. This is more often the case with control cartilages from non-arthritic joints, because the tissues are tougher than those from OA joints. During tissue separation, the tissue once fixed to the thermoplastic film may detach from the film, or the film may pull away from the LCM cap with the tissue. In such cases, you may have to cut out the attached area of the section using a scalpel and tweezers under a dissection microscope.
 13. Even when the area of interest is separated by LCM, the separation may be imperfect and unnecessary parts of the section may come together. We routinely examine the harvested tissue under an LCM device or a dissection microscope to remove any unnecessary parts by a scalpel and tweezers.
 14. We use the RNeasy micro kit from Qiagen, but similar kits are now available from other manufacturers, and they may work equally well.
 15. Since cell density in cartilage tissues is very low, we often cut and process 3-10 cryosections from a single block, and put them together in one tube for RNA extraction.
 16. For RNA extraction, we do not use the poly-A RNA in the RNeasy micro kit as carrier RNA since it might interfere with reverse transcription, given we use oligo-dT primers for the reaction.
 17. Although we tried several different conditions, to date, we have not yet succeeded in obtaining RNA of sufficient quality and quantity for cDNA microarray analysis. The

RNA obtained by LCM might be used for microarray analysis if it is amplified, although we have not attempted it.

18. We compared several reverse transcriptases and finally chose Sensiscript from Qiagen. In our experience, this enzyme works consistently well with small amounts of RNA or RNA of relatively low purity.
19. Due to a limited amount of RNA, for some genes, gene expression may not be evaluated reliably by qPCR. For such genes, it may be worth trying to extract RNA again using an increased number of cryosections. Alternatively, more general methods may be attempted such as altering PCR conditions, changing primer designs, and using another Taq DNA polymerase. For certain genes, it is effective to use random primers instead of, or together with, the oligo dT primer. Occasionally, denaturation of RNA before reverse transcription may improve the result of qPCR.

Acknowledgments

This work was sponsored in part by Grants-in-Aids from the Japan Society for the Promotion of Science (Nos. 15390467 and 18390424).

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Figure Captions

Fig. 1. Histology of normal adult human articular cartilage. The section is prepared in a plane vertical to the surface of cartilage. Superficial (S), middle (M), and deep (D) zones are shown together with the tide mark (TM), calcified cartilage (*10*), and subchondral bone (SCB). Safranin O/fast green staining. Scale bar, 0.5 mm.

Fig. 2. Preparation of cartilage tissue. (A) Cartilage tissue obtained at surgery. (B) Macroscopically intact area of the cartilage tissue (arrow) is separated from the underlying subchondral bone in full thickness. (C) Cartilage tissue is cut into a strip (arrow) which has dimensions of 20 mm x 6 mm. (D) Cross section of the cartilage strip. Arrowheads indicate surface of cartilage. (E) Cartilage tissue is embedded in OCT compound (black arrow), which is solidified in the mold (white arrow) in liquid nitrogen. (F) Close view of the solidified OCT compound containing the cartilage strip. Arrowheads indicate the cross section of the embedded cartilage tissue.

Fig. 3. Preparation of cryosections for LCM. (A) Cartilage tissue in solidified OCT compound is mounted on a cryostat. Arrowheads indicate the cross section of the cartilage tissue. (B) Cryosection is prepared and processed for LCM (Arrow). After processing, the section may curl and detach from the glass slide. (C) LCM cap may be placed directly onto the cryosection. The cap may be pressed down to flatten the section. (D) Photomicrograph of the cryosection obtained through the LCM device. S, M, and D indicate superficial, middle, and deep zones, respectively.

Figure 1

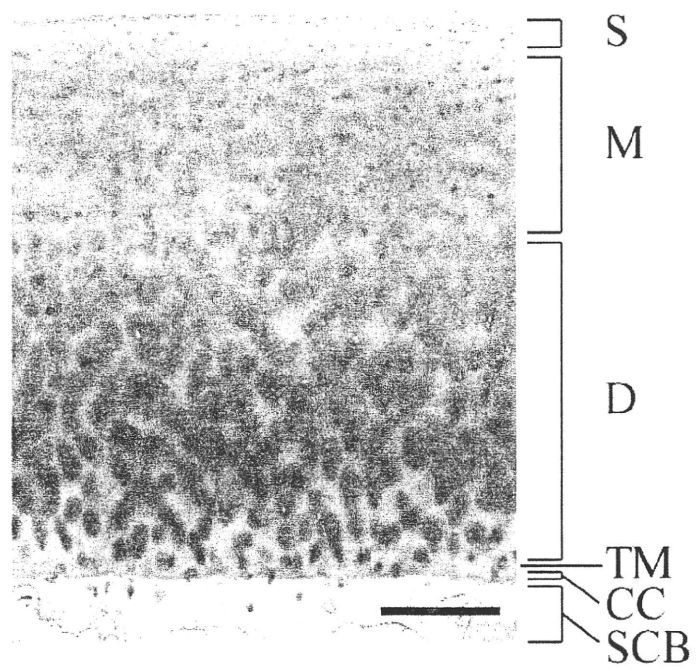


Figure 2

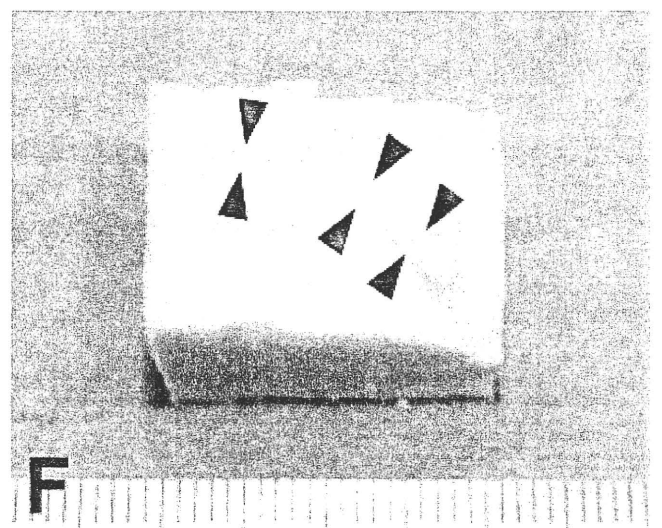
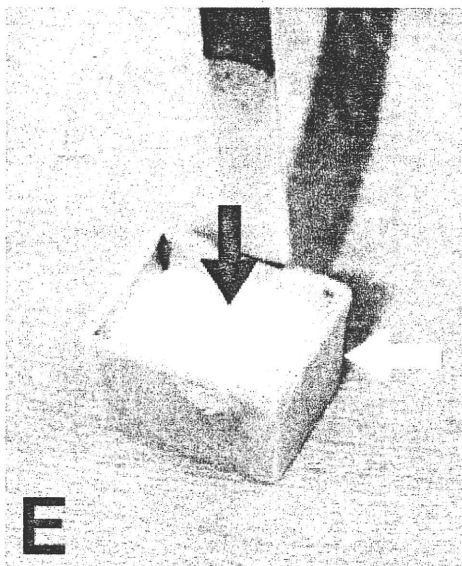
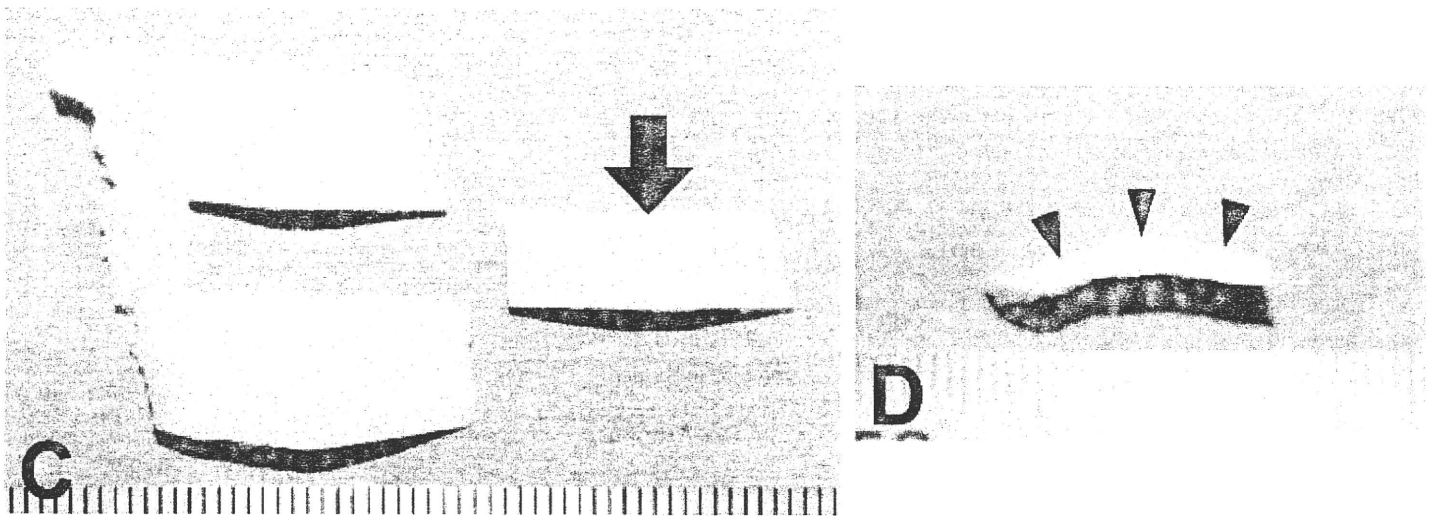
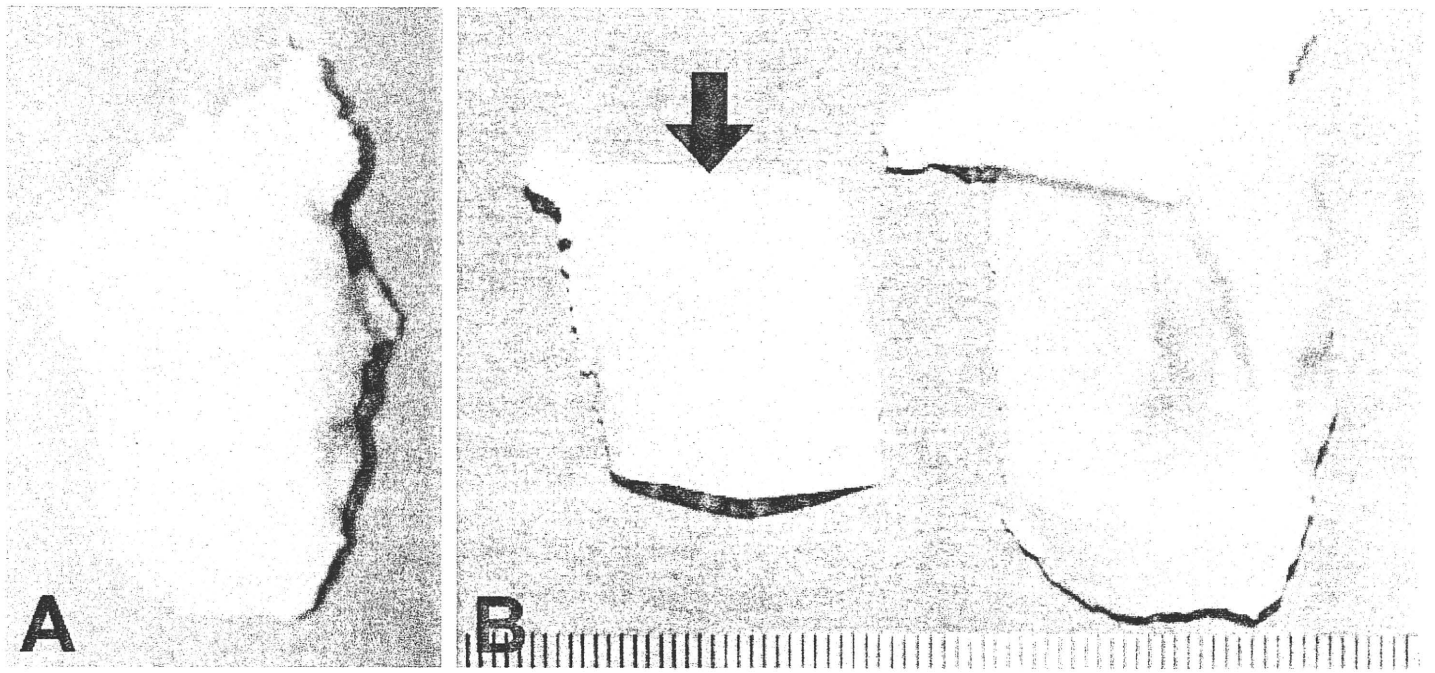
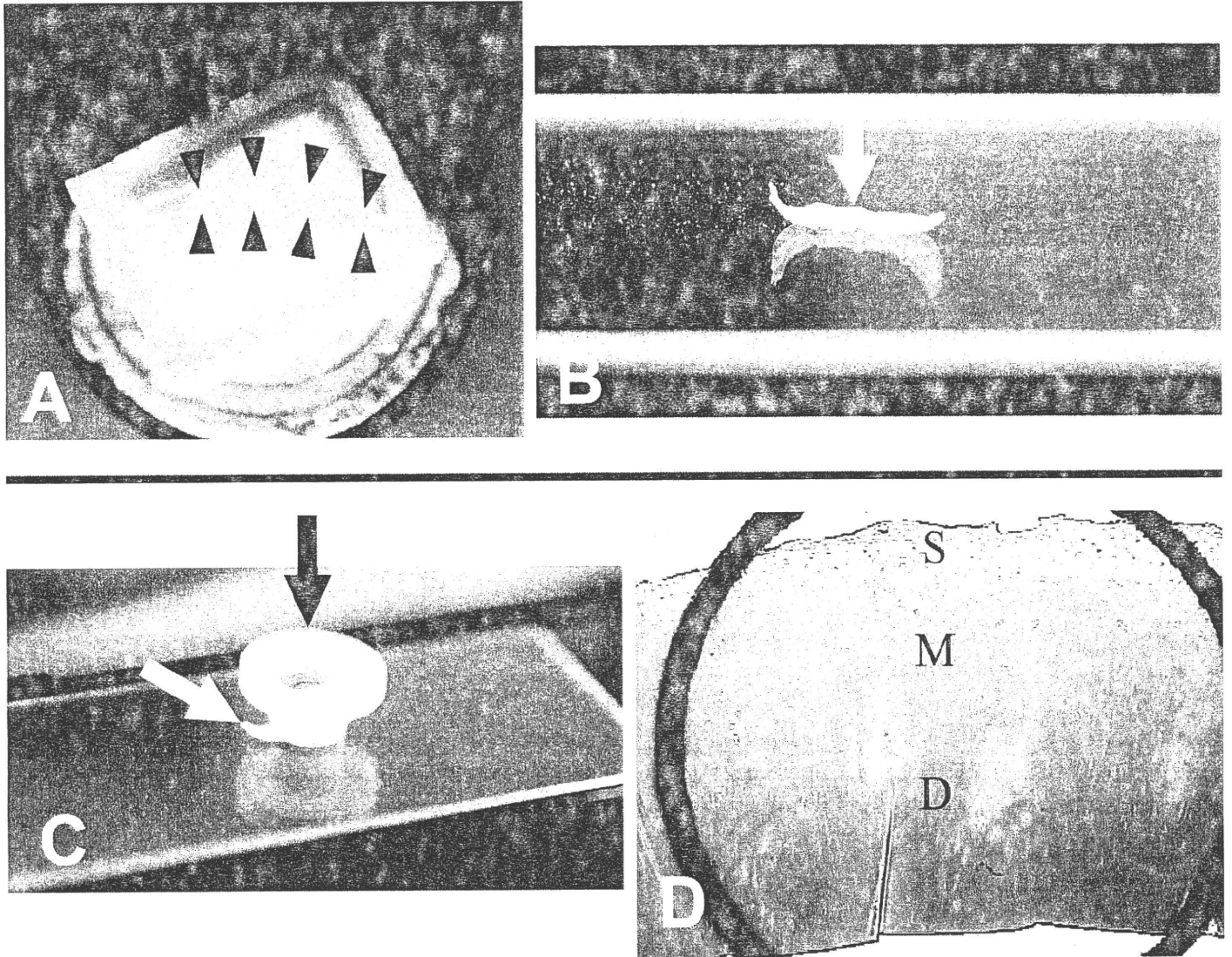


Figure 3



The interaction of monocytes with rheumatoid synovial cells is a key step in LIGHT-mediated inflammatory bone destruction

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doi:10.1111/j.1365-2567.2008.02965.x

Received 5 June 2008; revised 4 September 2008; accepted 15 September 2008.

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Summary

Formation of osteoclasts and consequent joint destruction are hallmarks of rheumatoid arthritis (RA). Here we show that LIGHT, a member of the tumour necrosis factor (TNF) superfamily, induced the differentiation into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) of CD14⁺ monocytes cocultured with nurse-like cells isolated from RA synovium, but not of freshly isolated CD14⁺ monocytes. Receptor activator of nuclear factor- κ B ligand (RANKL) enhanced this LIGHT-induced generation of TRAP-positive MNCs. The MNCs showed the phenotypical and functional characteristics of osteoclasts; they showed the expression of osteoclast markers such as cathepsin K, actin-ring formation, and the ability to resorb bone. Moreover, the MNCs expressed both matrix metalloproteinase 9 (MMP-9) and MMP-12, but the latter was not expressed in osteoclasts induced from CD14⁺ monocytes by RANKL. Immunohistochemical analysis showed that the MMP-12-producing MNCs were present in the erosive areas of joints in RA, but not in the affected joints of osteoarthritic patients. These findings suggested that LIGHT might be involved in the progression of inflammatory bone destruction in RA, and that osteoclast progenitors might become competent for LIGHT-mediated osteoclastogenesis via interactions with synovio-cyte-like nurse-like cells.

Keywords: differentiation; LIGHT/TNFSF14; monocyte; osteoclast; rheumatoid arthritis

Introduction

Osteoclasts are large, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). Receptor activator of nuclear factor- κ B ligand (RANKL) is a key regulator of osteoclast differentiation from haematopoietic precursors of the monocyte/macrophage lineage.^{1–3} Although osteoclasts have an essential role in physiological bone remodelling, increases in their number and activity,

would lead to diseases accompanied by local bone destruction. Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by arthritis affecting multiple joints and the progressive destruction of cartilage and bone.⁴ Osteoclasts are important contributors to the joint destruction in RA. Inflammatory cytokines, such as tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1), which are upregulated in RA synovial tissues, are known to induce the differentiation and activation of

Abbreviations: CTX-I, type I collagen C-telopeptide; HVEM, herpes virus entry mediator; LT β R, lymphotoxin β receptor; MMP, matrix metalloproteinase; MNCs, multinucleated cells; NLCs, nurse-like cells; OA, osteoarthritis; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor- κ B ligand; TRAP, tartrate-resistant acid phosphatase.

osteoclasts.^{5,6} Invasive synovial tissue at sites of bone destruction, also termed pannus, plays important roles in osteoclastic bone resorption.⁷⁻⁹

We previously established nurse-like cells (NLCs) from the synovial tissues of RA patients.¹⁰ Although having the same appearance as fibroblast-like synoviocytes, NLCs have a number of distinct activities that could contribute to rheumatoid inflammation.¹⁰⁻¹⁴ Among these are their ability to promote antibody production by B cells, the capacity to protect lymphocytes from apoptosis, and the ability to secrete large amounts of cytokines and chemokines such as IL-6 and IL-8 that could promote the accumulation and activation of lymphocytes and monocytes. However, fibroblast-like synoviocytes from patients with osteoarthritis (OA) hardly show any such activities.^{10,15} Therefore, to distinguish them from general fibroblast-like synoviocytes, we have defined synovial NLCs as those that go through the active cell population from the RA synovium. The NLCs promote the survival of peripheral blood monocytes via macrophage colony-stimulating factor (M-CSF) production.^{16,17} Monocytes cocultured with NLCs for 4 weeks possessed TRAP activity and differentiated into osteoclasts in response to some cytokines, including RANKL.¹⁷ These reports have suggested that NLCs might be involved in RA-induced bone destruction by maintaining osteoclast precursors in areas of progressive synovial expansion.

LIGHT, which is homologous to lymphotoxin, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes, was recently identified as a type 2 transmembrane glycoprotein of the TNF ligand superfamily (TNFSF14).¹⁸ LIGHT is expressed on activated T lymphocytes,^{18,19} monocytes,²⁰ granulocytes²⁰ and immature dendritic cells.²¹ LIGHT signalling is transduced via two members of the TNFR family, herpes virus entry mediator (HVEM, TNFRSF14) and lymphotoxin β receptor (LT β R, TNFRSF3). The HVEM is expressed prominently on monocytes, dendritic cells and lymphocytes,^{19,22-24} whereas LT β R is expressed on many cell types with the exception of lymphocytes.^{18,20,25} LIGHT has been shown to regulate cell proliferation^{21,26,27} and apoptosis,^{20,28} to induce the secretion of various cytokines, and to augment the expression of adhesion molecules.^{26,29-31} Recently, Kim *et al.* reported that LIGHT was overexpressed in the synovial tissue of RA patients and that it induced the production of chemokines, cytokines and matrix metalloproteinase 9 (MMP-9) from macrophages in synovial fluid.³² Moreover, LIGHT contributes to the survival and activation of synovial fibroblasts in RA.^{33,34} These studies have suggested that LIGHT may be an important inflammatory cytokine in the development of RA. However, the roles of LIGHT in the bone destruction in RA have not yet been elucidated.

In this study, we compared the abilities to differentiate into osteoclasts in response to LIGHT, between fresh CD14⁺ monocytes and CD14⁺ monocytes cocultured with NLCs. We found that LIGHT induced osteoclast differentiation from CD14⁺ monocytes cocultured with NLCs, but not from freshly isolated CD14⁺ monocytes. Furthermore, LIGHT-induced osteoclasts express MMP-12, which was not expressed in osteoclasts induced by RANKL, and the MMP-12-expressing osteoclasts were observed at the erosive areas in the subchondral bones of RA patients, but not in those of OA patients. These findings suggest that CD14⁺ monocytes gain the ability to differentiate into osteoclasts in response to LIGHT through their interactions with NLCs, and that LIGHT plays a critical role in the inflammatory joint destruction in RA.

Materials and methods

Cells and cultures

Nurse-like cells were established from synovial tissues obtained from RA patients, as described previously.¹⁰ NLCs were cultured in Dulbecco's modified Eagle's minimum essential medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS). The NLCs from passages 4-9 were used for each experiment. Mononuclear cells were collected from the venous blood of healthy volunteers and CD14⁺ monocytes were prepared by further separation using anti-CD14 antibody-coated beads, as described previously.¹⁶ CD14⁺ monocytes (2.0×10^6 cells/well) were cocultured with NLCs (2.0×10^5 cells/well) in six-well plates. Half of the medium was replaced every 3 days with fresh medium. After coculture for 4 weeks, floating or weakly adherent monocytes were harvested as NLC-supported CD14⁺ monocytes (NCD14⁺ monocytes) by gently washing the culture with fresh medium. Over 97% of NCD14⁺ monocytes were TRAP positive, and their purity was confirmed cytochemically, as reported previously.¹⁶ All human specimens were obtained with written informed consent according to the study protocol, which was approved by the review board of the Sagami National Hospital.

Osteoclast formation assay

In the presence of 25 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN), freshly isolated CD14⁺ monocytes (1.0×10^5 cells/well) and NCD14⁺ monocytes (2.0×10^4 cells/well) were cultured in 96-well plates in α -minimum essential medium (Invitrogen) supplemented with 10% FCS. As indicated, the cells were further stimulated with 40 ng/ml recombinant human RANKL (Peprotech, London, UK) and/or various concentrations of recombinant human LIGHT (R&D Systems). After vari-

ous periods of time, as indicated in the Results, cells were fixed and stained for TRAP using a TRAP staining kit (Wako, Osaka, Japan). Osteoclasts were identified as TRAP-positive MNCs (more than five nuclei). AlexaFluor546-conjugated phalloidin was used to stain for F-actin (Invitrogen).

Bone resorption assay

In the presence of 25 ng/ml M-CSF, NCD14⁺ monocytes were cultured on cortical bone slices in α -minimum essential medium supplemented with 10% FCS and further stimulated with 40 ng/ml RANKL and/or 100 ng/ml LIGHT. After 21 days, the bone slices were stained with Mayer's haematoxylin solution to detect resorption pits. The concentration of the type I collagen C-telopeptide (CTX-I) in the culture supernatant was quantified using the CrossLaps for Culture kit (Nordic Biosciences Diagnostics, Herlev, Denmark), according to the manufacturer's instructions.

Quantitative polymerase chain reaction analysis

Total RNA was prepared using an RNeasy Micro kit (Qiagen, Tokyo, Japan) and complementary DNA (cDNA) was generated from the RNA using Omniscript Reverse Transcriptase (Qiagen) following the manufacturer's instructions. The cDNA was used as a template for real-time quantitative polymerase chain reaction (PCR) in a LightCycler (Roche Diagnostics, Tokyo, Japan). The PCR was performed using SYBR Premix Ex Taq (Takara, Kyoto, Japan). The PCR primers used in this study were as follows: for NFATc1, 5'-TACCAGGTGCACCGCATCA-3' and 5'-TTTCAGGATCCGGCACAGTC-3'; for TRAP, 5'-TGCA GATCCTGGGTGCAGAC-3' and 5'-GAGTATGCAATC TGGGCAGAGACA-3'; for cathepsin K, 5'-AGCT GCAATAGCGATAATCTGAACC-3' and 5'-CGTTGTTC TTATTTTCGAGCCATGA-3'; for carbonic anhydrase II, 5'-GCGACCATGTCCCATCACTG-3' and 5'-TGGCTGTAT GAGTGTGCATGTCAA-3'; for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-GCACCGTCAAGGCTGAG AAC-3' and 5'-ATGGTGGTGAAGACGCCAGT-3'; for LIGHT, 5'-TCACGAGGTCAACCCAGCAG-3' and 5'-CC CAGCTGCACCTTGGAGTAG-3'; for HVEM, 5'-TTTG CTCCACAGTTGGCCTAATC-3' and 5'-CAATGACTGT GGCCTCACCTTC-3'; for LT β R, 5'-ATGCTGATGCTG-GCCGTTC-3' and 5'-AGGCTCCCAGCTTCCAGCTA-3'; for RANK, 5'-TTGTGCCGCCTAAGTGA-3' and 5'-ACC ACCTTGATCTGGGTAGCACATA-3'; for MMP-9, 5'-AC CTCGAACTTTGACAGCGACA-3' and 5'-GATGCCATTC ACGTCGCCTTA-3'; for MMP-12, 5'-TTGATGGCAA GGTGGAATCCTA-3' and 5'-AGGAATGGCCAATCTCGT GAAC-3'. The PCR was performed under the following conditions: initial denaturation at 95° for 10 seconds, then 40 cycles of 95° for 5 seconds and 60° for 20

Involvement of LIGHT in the bone destruction in RA

seconds. SYBR green dye was used to detect amplified products and melting curves were routinely recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized based on the expression level of GAPDH.

Immunohistochemical and TRAP staining of tissue samples

Affected knee joints were resected during joint replacement surgery from five RA and three OA patients who had given written informed consent. Serial sections of the decalcified and paraffin-embedded subchondral bone were dewaxed and reacted with anti-human MMP-12 monoclonal antibody (clone 4D2, R&D Systems). Sections were then reacted with anti-mouse immunoglobulin G-horse-radish peroxidase conjugate, chromogenic substrate and hydrogen peroxide. The neighbouring sections of those stained with anti-MMP-12 were subjected to staining with second antibody alone as a negative control or with TRAP staining as described above.

Statistical analysis

All data are expressed as means \pm SD. A non-paired Student's *t*-test was used for comparison, using the STATVIEW program (Abacus Concepts, Berkeley, CA). *P* < 0.05 was considered to be statistically significant.

Results

LIGHT induces the differentiation of NCD14⁺ monocytes into TRAP-positive MNCs

To investigate whether or not LIGHT is involved in local bone destruction, we examined the effects of LIGHT on osteoclastogenesis using established osteoclast precursors (NCD14⁺ monocytes) in addition to freshly prepared CD14⁺ monocytes, as described in the *Materials and methods* section.

In the presence of M-CSF, CD14⁺ or NCD14⁺ monocytes were cultured for 6 days with RANKL and/or LIGHT. As shown in Fig. 1(a,b), M-CSF alone did not induce TRAP-positive MNCs from either type of monocytes. CD14⁺ monocytes were differentiated into TRAP-positive MNCs by RANKL, but not by LIGHT. The combination of RANKL and LIGHT had little effect on MNC formation. Conversely, NCD14⁺ monocytes were strongly differentiated into TRAP-positive MNCs when treated with LIGHT. Although RANKL had only a slight effect on NCD14⁺ monocytes, the combination with LIGHT enhanced the formation of TRAP-positive MNCs more than LIGHT alone (Fig. 1b). The LIGHT-induced formation of MNCs was time dependent (4–8 days, Fig. 1c), and dose dependent (1–100 ng/ml, data not shown). Freshly isolated

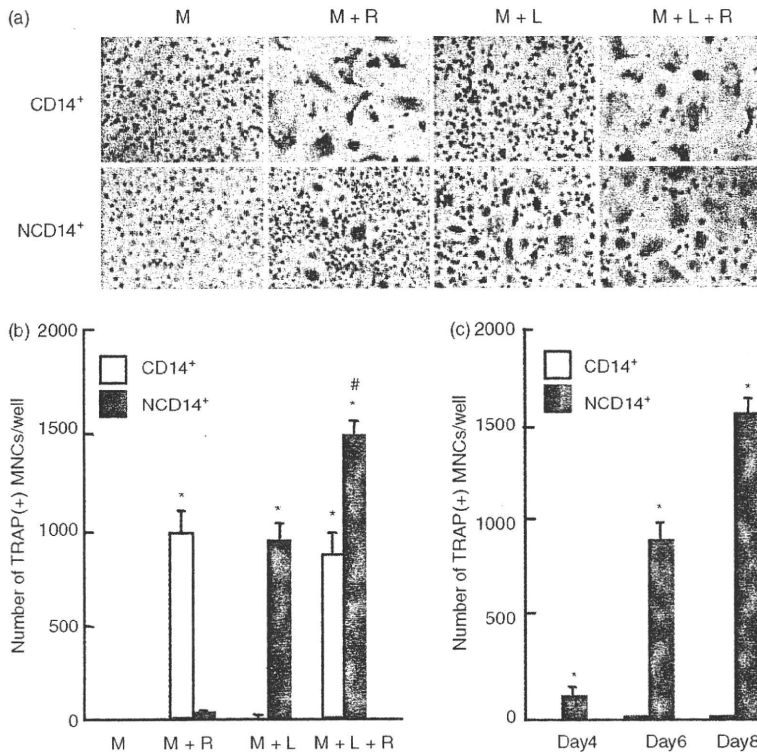


Figure 1. LIGHT induces the differentiation of NCD14⁺ monocytes into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). (a, b) In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), CD14⁺ or NCD14⁺ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor- κ B ligand (RANKL; M + R), 100 ng/ml LIGHT (M + L), or 40 ng/ml RANKL plus 100 ng/ml LIGHT (M + L + R). * P < 0.01 versus M-CSF alone. # P < 0.01 versus NCD14⁺ monocytes stimulated with M-CSF plus LIGHT. (c) CD14⁺ or NCD14⁺ monocytes were cultured for the indicated periods in the presence of 25 ng/ml M-CSF plus 100 ng/ml LIGHT. Cultured cells were fixed and stained for TRAP. The number of TRAP-positive MNCs was counted. * P < 0.01 versus CD14⁺ monocytes. Representative results of at least three independent sets of similar experiments are shown as means \pm SD of triplicate experiments.

CD14⁺ monocytes, however, did not differentiate into TRAP-positive MNCs, even after stimulation with 100 ng/ml LIGHT for 14 days (data not shown). When cultured with M-CSF for 4 weeks, CD14⁺ monocytes could not differentiate into TRAP-positive MNCs in the presence of RANKL or LIGHT (data not shown).

Increased HVEM messenger RNA expression in NCD14⁺ monocytes

Next, to clarify the reason for the difference in the efficiency of LIGHT-induced TRAP-positive MNC formation between NCD14⁺ and CD14⁺ monocytes, we analysed the messenger RNA (mRNA) expression of the LIGHT receptors, HVEM and LT β R, in both groups of monocytes. Quantitative real-time PCR analysis revealed that while the mRNA expression level of LT β R was not different between groups of monocytes, the level of HVEM mRNA was significantly higher in NCD14⁺ monocytes than in CD14⁺ monocytes (Fig. 2). Unexpectedly, the level of RANK mRNA in NCD14⁺ monocytes was higher than that in CD14⁺ monocytes (Fig. 2).

Analysis of the molecular phenotype of LIGHT-induced TRAP-positive MNCs derived from NCD14⁺ monocytes

Furthermore, we investigated the mRNA expression of major osteoclast markers, such as nuclear factor of activated T cells (NFATc1), TRAP, cathepsin K (CTSK) and

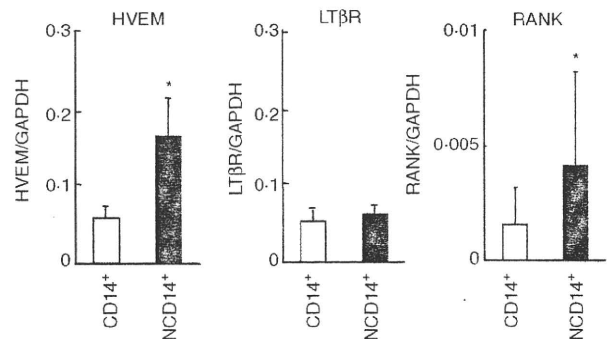


Figure 2. Expression of herpes virus entry mediator (HVEM), lymphotoxin β receptor (LT β R) and receptor activator of nuclear factor- κ B (RANK) messenger RNA (mRNA) on CD14⁺ and NCD14⁺ monocytes. Total RNA was extracted from CD14⁺ and NCD14⁺ monocytes and the mRNA expression levels of HVEM, LT β R and RANK were analysed by quantitative real-time polymerase chain reaction. Representative results of at least three independent sets of similar experiments are shown as means \pm SD of triplicate experiments. * P < 0.01 versus CD14⁺ monocytes.

carbonic anhydrase II (CAII), in LIGHT-induced TRAP-positive MNCs derived from NCD14⁺ monocytes, using quantitative real-time PCR analysis. In comparison with the control (M-CSF alone), the expression levels of all four genes were upregulated in TRAP-positive MNCs induced to differentiate by LIGHT for 6 days (Fig. 3a). The combination of LIGHT and RANKL stimulated their expression to a similar or slightly larger extent than LIGHT alone. When NCD14⁺ monocytes are stimulated

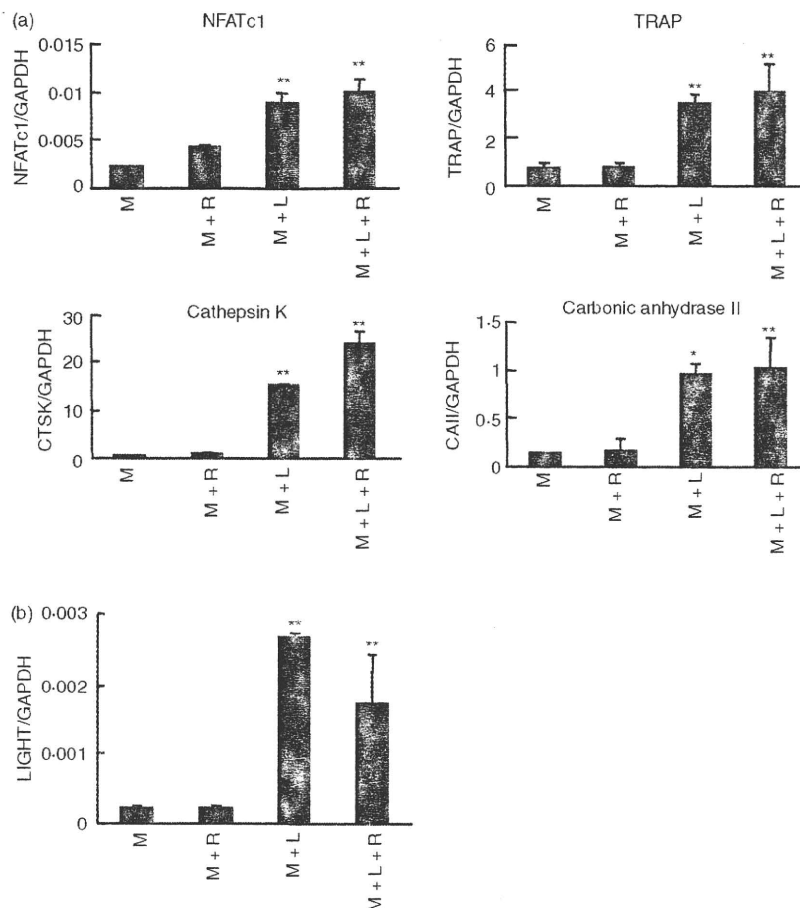


Figure 3. The molecular phenotype of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) derived from NCD14⁺ monocytes. In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), NCD14⁺ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor- κ B ligand (RANKL) (M + R), 100 ng/ml LIGHT (M + L), or both (M + L + R). The expression levels of (a) osteoclast markers and (b) LIGHT were determined by quantitative real-time polymerase chain reaction. Representative results of at least three independent sets of similar experiments are shown as means \pm SD of triplicate experiments. * P < 0.05 and ** P < 0.01 versus M-CSF alone.

by LIGHT or LIGHT plus RANKL, the CTSK and TRAP mRNA expression levels are higher in NCD14⁺ monocytes stimulated for 12 days than in those stimulated for 6 days (data not shown). These inductions suggest that prolonged stimulation with LIGHT can induce more mature osteoclasts. Moreover, LIGHT induced an increase in its own expression level in NCD14⁺ monocytes (Fig. 3b).

LIGHT-induced TRAP-positive MNCs from NCD14⁺ monocytes have bone resorption activity

Since the expression levels of genes related to bone resorption by osteoclasts were upregulated in LIGHT-induced TRAP-positive MNCs from NCD14⁺ monocytes, we assessed whether or not the MNCs could resorb bone. The formation of a ringed F-actin structure, called an actin ring, is closely related to osteoclast function.³⁵ Actin rings were recognized at the periphery of LIGHT-induced MNCs regardless of the presence of RANKL (Fig. 4a). Furthermore, resorption pits were observed on bone slices in the cultures treated with LIGHT, and the effect of LIGHT was enhanced by additional stimulation with RANKL (Fig. 4b). As expected, CTX-I release was induced in the presence of LIGHT and further increased by the

combination with RANKL (Fig. 4c). By contrast, cells treated with M-CSF alone or with a combination of M-CSF and RANKL did not form resorption pits.

LIGHT-induced TRAP-positive MNCs from NCD14⁺ monocytes express both MMP-9 mRNA and MMP-12 mRNA

In osteoclasts, MMP-9 is abundant and it is known to be a protease involved in bone resorption. We examined MMP-9 mRNA expression in both fresh CD14⁺ and NCD14⁺ monocytes stimulated by LIGHT or RANKL. Compared with RANKL, LIGHT or the combination of LIGHT and RANKL enhanced MMP-9 mRNA expression in both groups of monocytes (Fig. 5a). On the other hand, because we previously reported that MMP-12 was expressed in MNCs induced from NCD14⁺ monocytes, but not in those from CD14⁺ monocytes,³⁶ the influence of LIGHT upon the expression of MMP-12 mRNA in both monocytes was investigated. NCD14⁺ monocytes strongly upregulated MMP-12 mRNA expression in response to LIGHT or the combination of LIGHT and RANKL, compared with control (M-CSF alone) or RANKL, whereas the MMP-12 mRNA levels

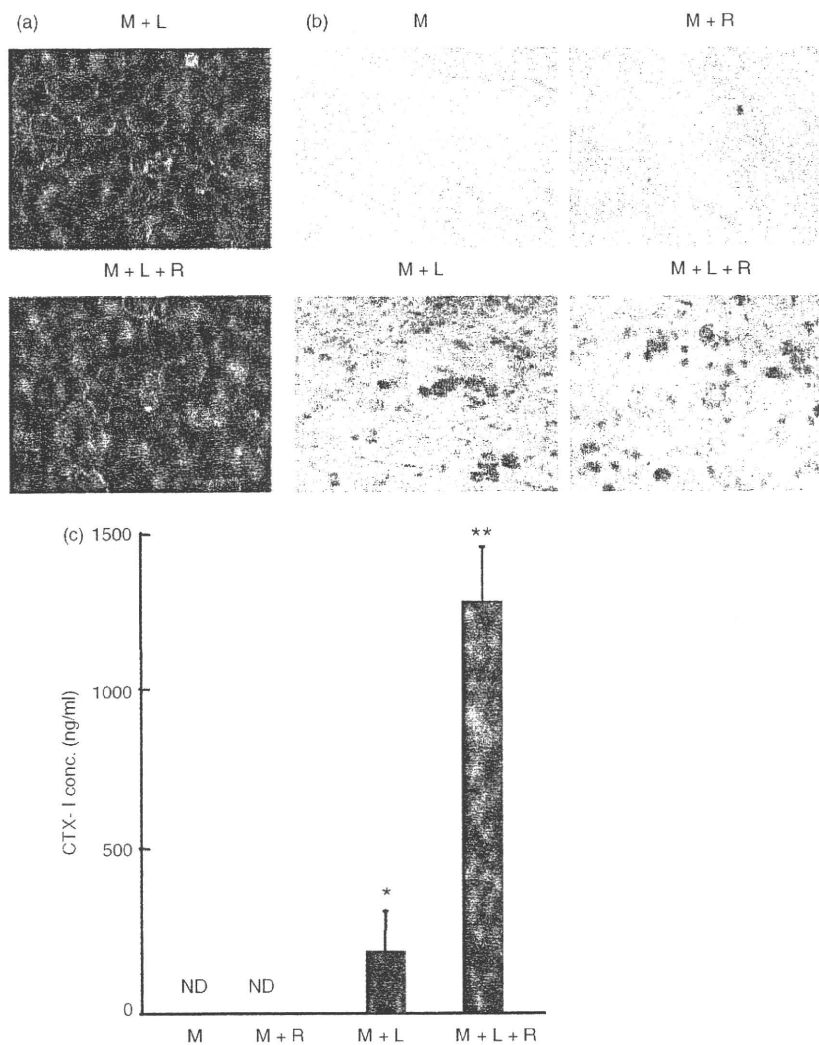


Figure 4. Osteoclastic functions of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) from NCD14⁺ monocytes. (a) For actin ring formation, NCD14⁺ monocytes were stimulated, in the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF), with 100 ng/ml LIGHT (M + L), or 40 ng/ml receptor activator of nuclear factor- κ B ligand (RANKL) plus 100 ng/ml LIGHT (M + L + R) for 6 days. Fixed cells were stained with Alexa-Fluor546-conjugated phalloidin. (b) In the presence of 25 ng/ml M-CSF (M), NCD14⁺ monocytes were cultured for 21 days on bone slices with 40 ng/ml RANKL (M + R), 100 ng/ml LIGHT (M + L), or both (M + L + R). (c) The release of type I collagen C-telopeptide (CTX-I) was quantified in the culture supernatants using enzyme-linked immunosorbent assay. Representative results of at least three independent sets of similar experiments are shown as means \pm SD of triplicate experiments. * P < 0.05 and ** P < 0.01 versus M-CSF alone.

remained low in CD14⁺ monocytes after any stimulation (Fig. 5b).

Detection of TRAP-positive MNCs expressing MMP-12 in the subchondral bone of RA patients

To confirm the presence of MMP-12-expressing MNCs in the erosive area of the RA joint, serial sections of decalcified, paraffin-embedded subchondral bone of five RA patients and three OA patients were subjected to immunostaining with an anti-MMP-12 antibody. As shown in Fig. 6(a,b), both MMP-12-positive and MMP-12-negative MNCs expressing TRAP were present in the affected bone areas of all five RA patients examined in this study. The ratios of MMP-12-positive MNCs to TRAP-positive MNCs in RA patients were 46.7% (30/63) in patient 1, 52.5% (31/59) in patient 2, 41.7% (33/79) in patient 3, 2.2% (3/135) in patient 4, and 10.0% (9/90) in patient 5. By contrast, no MMP-12-positive MNCs were observed in the OA patients (Fig. 6b).

Discussion

Formation of osteoclasts and consequent joint destruction are hallmarks of RA. We previously reported that CD14⁺ monocytes cocultured with NLCs (termed NCD14⁺ monocytes in this article) were TRAP positive and that NCD14⁺ monocytes differentiated into osteoclasts following treatment with RANKL or TNF- α . Consequently, NCD14⁺ monocytes have been thought to be osteoclast precursors.^{16,17}

In this study, to investigate whether LIGHT is involved in bone destruction in RA, we examined the effects of LIGHT on osteoclastogenesis using CD14⁺ and NCD14⁺ monocytes, and compared their abilities to differentiate into osteoclasts. When stimulated with RANKL for 6 days, a number of TRAP-positive MNCs, mature osteoclasts, were generated from CD14⁺ monocytes, but only a few MNCs were generated from NCD14⁺ monocytes. Conversely, LIGHT strongly induced MNCs from NCD14⁺ monocytes, but not from CD14⁺ monocytes.

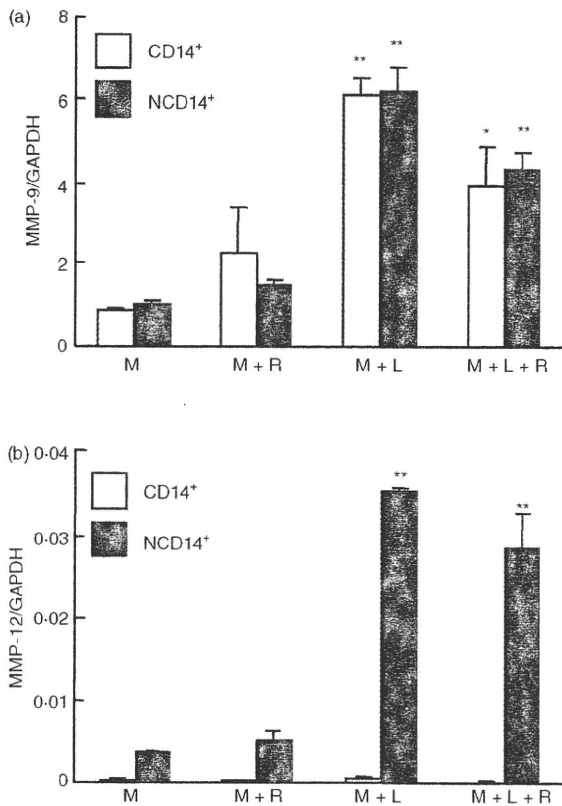


Figure 5. Comparison of matrix metalloproteinase-9 (MMP-9) and MMP-12 messenger RNA (mRNA) expression in CD14⁺ or NCD14⁺ monocyte-derived tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). In the presence of 25 ng/ml macrophage-colony-stimulating factor (M-CSF; M), CD14⁺ or NCD14⁺ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor- κ B ligand (RANKL; M + R), 100 ng/ml LIGHT (M + L), or both (M + L + R). The mRNA expression levels of (a) MMP-9 and (b) MMP-12 were determined by quantitative real-time polymerase chain reaction. Representative results of at least three independent sets of similar experiments are shown as means \pm SD of triplicate experiments. * P < 0.05 and ** P < 0.01 versus M-CSF alone.

Furthermore, LIGHT-induced MNCs derived from NCD14⁺ monocytes showed several characteristics of osteoclasts, including the expression of genes encoding NFATc1, TRAP, CTSK and CAII, actin-ring formation, and the ability to resorb bone. These results indicate that LIGHT might be involved in bone destruction by forming osteoclasts from precursors through the interaction with NLCs.

RANKL enhanced LIGHT-induced osteoclast formation and bone resorption. Recent reports have shown that synovial cells from patients with RA express a higher level of RANKL and could induce osteoclastogenesis from synovial macrophages.⁷⁻⁹ Hence, the synergistic effects of RANKL and LIGHT on osteoclastogenesis may play an important role in the bone destruction in RA. LIGHT has

been reported to be augmented in RA synovial fluids compared with those in OA patients.³⁴ Reports on the LIGHT-expressing cells in RA synovial tissue remain confused because of inconsistent immunohistochemical evaluations. These cells have been separately identified as macrophages³² and T lymphocytes.³⁴ In the present study, LIGHT induced its own expression in NCD14⁺ monocytes, suggesting that LIGHT might drive a positive feedback loop of osteoclastogenesis. LIGHT induced the expression of intercellular adhesion molecule-1 (ICAM-1) in RA synovial fibroblasts,^{33,34} and the expression of IL-8 and monocyte chemoattractant protein-1 in RA synovial macrophages.³² Since ICAM-1 and these chemokines could play a crucial role in the recruitment of monocytes into the synovial sublining,³⁷ LIGHT might not only induce osteoclastogenesis, but also increase the number of osteoclast precursors in the synovium of RA patients by recruiting monocytes. The exact mechanism by which NCD14⁺ monocytes gain the ability to differentiate into osteoclasts in response to LIGHT is unclear. In this study, we confirmed the upregulation of HVEM only at the mRNA level. If HVEM mRNA expression correlates with protein expression, augmented HVEM can explain how the cells gain the ability to differentiate into osteoclasts in response to LIGHT. On the other hand, the decreasing responsiveness of NCD14⁺ monocytes to RANKL alone contradicts the increasing expression of RANK mRNA. Because RANKL drastically enhanced LIGHT-induced osteoclastogenesis from NCD14⁺ monocytes and the bone-resorbing activity, NCD14⁺ cells could be responsive to RANKL. These findings indicate that the decreasing responsiveness to RANKL may be the result of alterations in the downstream pathways of RANK/RANKL signalling in NCD14⁺ monocytes. Whereas LIGHT could not induce osteoclast differentiation from CD14⁺ monocytes, it has been previously reported that LIGHT induces the expression of MMP-9 in monocytes.^{31,32,38} These findings suggest that CD14⁺ monocytes could somehow respond to LIGHT via HVEM or LT β R.

A recent study reported that LIGHT promotes osteoclastogenesis in RANKL-dependent and -independent manners;³⁹ however, in the present study, we could not observe LIGHT-induced osteoclastogenesis from CD14⁺ monocytes. The discrepancy might be caused by differences in culture conditions or in the monocyte preparation methods (an adherence technique versus a magnetic bead method).

In addition to MMP-9 expression, LIGHT-induced MNCs derived from NCD14⁺ monocytes expressed MMP-12 mRNA, which was not expressed in osteoclasts induced from CD14⁺ monocytes by RANKL. The TRAP-positive MNCs expressing MMP-12 were present in the erosive areas of RA joints, but were not found in the affected bones of OA joints, implying that they may be unique to RA.

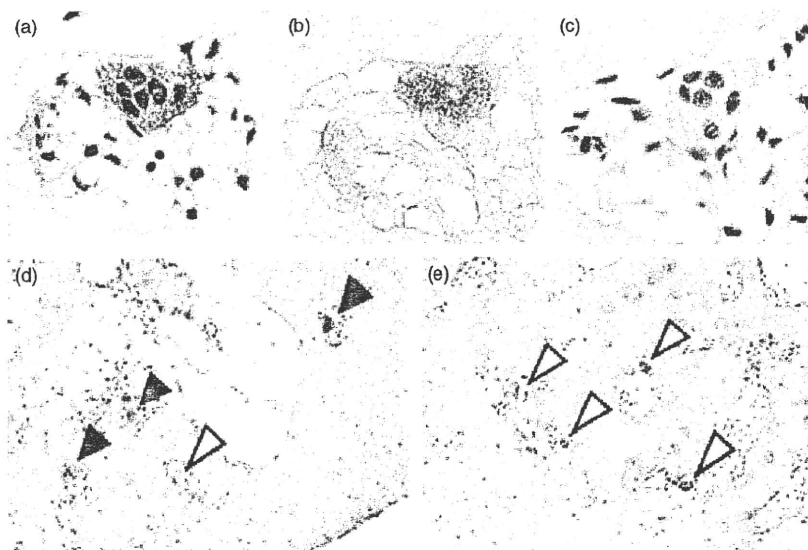


Figure 6. Detection of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) expressing matrix metalloproteinase-12 (MMP-12) in the erosive area of the rheumatoid arthritis (RA) joint. Affected subchondral bone samples were obtained from the resected knee joints of patients with RA and patients with osteoarthritis (OA). Serial sections were cut from decalcified and paraffin-embedded subchondral bone. (a) RA tissue samples were stained with anti-human MMP-12 monoclonal antibody. Neighbouring sections were stained for TRAP activity (b) or with second antibody alone as a negative control (c). All three sections were consecutive. (d) RA and (e) OA tissue samples were stained with anti-human MMP-12 monoclonal antibody. Solid arrowheads and open arrowheads show MMP-12-positive and -negative MNCs, respectively. Representative results from five RA patients and three OA patients are shown.

Although MMP-12 was expressed in rabbit and mouse osteoclasts,⁴⁰ MMP-12-expressing osteoclasts have not been identified in humans. This is the first report to demonstrate the presence of MMP-12-expressing osteoclasts in human RA joints. Increased expression of MMP-12 in RA synovial tissues and synovial fluids indicates the involvement of this enzyme in RA pathogenesis.⁴¹ A study using MMP-12-deficient mice demonstrated that MMP-12 was not critical for bone resorption or osteoclast recruitment.⁴⁰ Although MMP-12 might not be directly involved in bone resorption in humans, several reports suggest that MMP-12 could be involved in cartilage destruction.^{42,43} We previously reported that NCD14⁺ and MMP-12-producing osteoclast-like cells differentiated from NCD14⁺ monocytes could degrade the proteoglycan of bovine cartilage.³⁶ MMP-12 is critical for invasion and destruction in pathologies such as emphysema⁴⁴ and cutaneous granulomas.⁴⁵ Rheumatoid arthritis is also a granulomatous disease with tissue hyperplasia and destruction. Osteoclasts invading uncalcified cartilage beyond the tide-mark are frequently found in the erosive areas of RA joints. Consequently, MMP-12-producing osteoclasts might play important roles in inflammation or cartilage destruction as well as in the bone destruction in RA.

Synovial fluid macrophages from RA patients could differentiate into osteoclasts via both RANKL and TNF- α signalling pathways, whereas OA macrophages differentiated into osteoclasts only through the RANKL pathway.⁴⁶ Moreover, osteoclasts derived from circulating precursors in RA patients have an increased bone-resorbing activ-

ity.⁴⁷ Osteoclast precursors responsive to inflammatory cytokines such as LIGHT would be present in RA patients, and the cytokine-induced osteoclastogenesis might be involved in enhanced bone destruction in RA. Although it should become clear from further investigations whether or not LIGHT-induced MNCs are identical to the MMP-12-expressing osteoclasts of RA joints, our *in vitro* differentiation system is robust and reproducible. All monocytes become LIGHT-responsive precursors of osteoclast-like cells via their interactions with RA-NLCs. We believe that our coculture system should be useful for elucidating the interactions between synovial cells and infiltrating monocytes in the pathogenic condition of RA, and the mechanism underlying inflammation-associated osteoclastogenesis.

The present study strongly suggested that LIGHT might be involved in the progression of bone destruction in RA, and implied that the blocking of LIGHT signalling may be a therapeutic target for the enhanced bone destruction in RA.

Disclosures

The authors have no financial conflict of interest.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan, and the Japan Society for the Promotion of Science.

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