tissue could clarify many novel aspects of cartilage pathology, which cannot be known by any other methods.

2. Materials and Labware

2.1 Preparation of Cartilage Tissue Blocks

1. DMEM/F-12 (Life Technologies, Carlsbad, CA).
2. Scalpel blade (No. 15; Feather, Osaka, Japan).
3. Scalpel holder (No. 3; Feather).
4. Optimum Cutting Temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan).
5. Metal mold for tissue blocks.
6. Liquid nitrogen.
7. Deep freezer (-80°C).

2.2 Sectioning

1. Cryostat (CM1900; Leica Microsystems, Houston, TX).
2. Disposable microtome blades (818; Leica Microsystems).
3. Precleaned glass slide (S2444; Matsunami, Osaka, Japan).
4. RNAse-free water (Nippon Gene, Tokyo, Japan).
5. Ethylenediaminetetraacetic acid disodium salt (EDTA) solution, 0.5M (E7889; Sigma, St. Louis, MO).
6. Ethanol (054-07225; Wako Pure Chemical, Osaka, Japan).
7. Xylene (320579; Sigma).
8. RNase-free pipet tips and pipettes (1000, 200, 20 μl).
9. Lint-free paper towels.
10. Fume hood.

2.3 LCM

1. Laser Capture Microdissection System (Arcturus PixCell Ile; Molecular Devices, Sunnyvale, CA).
2. LCM caps (CapSure LCM caps; Molecular Devices).
3. Fine-pointed tweezers.
4. (Optional) Dissection microscope.

2.4 RNA Extraction

1. RNeasy Micro kit (74004; Qiagen, Hilden, Germany).
2. 2-mercaptoethanol (M7154; Sigma).

3. Ribosomal RNA from E. coli (206938; Roche Diagnostics, Bazel, Switzerland). Dissolve in RNase-free water at a concentration of 10 μM.
4. Tabletop centrifuge.
5. Clean, autoclaved 1.5 ml centrifuge tubes.

2.5 cDNA Synthesis

6. Sensiscript RT kit (205211; Qiagen).
7. Oligo dT primer (18418-020; Life Technologies).
8. RNAse inhibitor (3335399; Roche Diagnostics).
9. Heat block for 1.5 ml centrifuge tubes (37°C).

3. Methods

Due to the ubiquitous presence of RNases, RNA is extremely unstable. To minimize RNA degradation, all reagents and instruments used for the experiment should be RNase-free. Disposable experimental gloves should be worn throughout the procedure, and the experiment should be performed as quickly as possible (*see* **Note 1**).

3.1 Preparation of Cartilage Tissues

1. Cartilage tissue obtained at surgery or dissection is transferred to the laboratory without delay in chilled DMEM/F-12 medium (*see* **Note 2**).
2. Cartilage tissue should be processed immediately after arrival to minimize RNA degradation.
3. Cartilage tissues are separated from the subchondral bone in full thickness with a sharp scalpel (**Fig. 2, A** and **B**). We prefer to use a small crescent-shaped blade to do this.
4. The cartilage tissue is cut into an appropriate size (**Fig. 2, C** and **D**).
5. OCT compound is poured in a mold, and the tissue is placed within it with the cross section of the tissue parallel to the surface (*see* **Notes 3** and **4**). The mold is immediately dipped in liquid nitrogen to solidify the compound (**Fig. 2E**).
6. Remove the compound block from the mold (**Fig. 2F**), put it in an appropriate container, and store it at -80°C until use. In this condition, RNA in the tissue can be preserved for at least 3 years, as long as it is maintained strictly at -80°C.

3.2 Preparation of Cryosections

1. Cryostat is prepared for sectioning. Set a new microtome blade and cool down the chamber and specimen disc holder. We usually set the temperatures of the chamber and the holder at -20°C and -30°C, respectively, but the optimum temperature may differ among cartilage tissues, and may need further optimization. A lower temperature may be better for control cartilage.
2. A tissue block is mounted on a specimen disc and attached to the cryostat. Adjust the orientation of the specimen discs so that the sections will be cut parallel to the cross section of the cartilage tissue (**Fig. 3A**).
3. After waiting for 5-10 min to equilibrate the block temperature, cut cryosections into 20-30 μm thicknesses and mount on a glass slide (*see* **Note 5**). Place three to five sections on one slide in view of possible loss during processing.

4. These sections are immediately processed for LCM (*see* **Note 6**).

3.3 Processing of Tissue Sections for LCM

1. To complete the processing and LCM as quickly as possible, we usually treat one glass slide at a time (*see* **Note 7**).
2. The sections on the glass slide are washed twice briefly with 1-2 ml of ice-cold RNAse-free water to remove the OCT compound. RNAse-free water is placed on a glass slide with a pipette until the entire sections are covered. The water is pipetted up and down several times, and then removed by a pipette.
3. Treat sections with 1-2 ml of 0.5M EDTA solution for 3 min at room temperature.
4. After removing the EDTA solution, sections are rinsed once briefly with ice-cold RNAse-free water (*see* **Note 8**).
5. Sections are then treated with 75%, 95%, and 100% ethanol sequentially, each for 30 sec.
6. Treat with 100% ethanol again for 30 sec (*see* **Note 9**).
7. Finally, treat sections with xylene for 5 min. Then remove xylene with a pipette and a paper towel, and allow the sections to dry up in a fume hood. When completely dry, proceed to LCM immediately (**Fig. 3B**).

3.4 Acquisition of Cartilage Tissues by LCM

1. Place a glass slide and cryosections on the stage of an LCM device (*see* **Note 10**).
2. Identify cartilage zones through the LCM device. We usually use a 2X or 4X objective for this purpose (*see* **Note 11**).
3. The slide is fitted to the stage by a vacuum chuck equipped in the device.
4. Place an LCM cap on the section following the manufacturer's instruction.
5. Alternatively, the cap may be placed manually on the section before setting the glass slide on the stage (**Fig. 3C**).
6. Confirm that the area of interest is seen through the cap (**Fig. 3D**).
7. Pulse the laser in the area outside the section as a trial to adjust the focus. We prefer to set the spot size of the laser at 15 or 30 μm in diameter.
8. The power and duration of the laser are set at 60-100 mW and 1-3 msec, respectively. Since we use thicker sections, these parameters are greater than those for regular LCM.
9. Pulse the laser repeatedly until the area of interest is entirely attached to the plastic film

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 µl 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 µl of the lysis buffer to a new clean centrifuge tube.
4. Add 350 µl 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 µl of the wash buffer (Buffer RW1) to the column and centrifuge for 15 sec at ≥ 8000 g.
7. Add 10 µl 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 µl of the wash buffer and centrifuge for 15 sec at ≥ 8000 g.
9. Add 500 µl 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 µl 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 µl 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 µl), dNTP mix (5 mM each dNTP; 2 µl), 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 Sensiscrpt 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.

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degenerative joint disease. *Proc Natl Acad Sci U S A*, **91**, 5070-4.

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

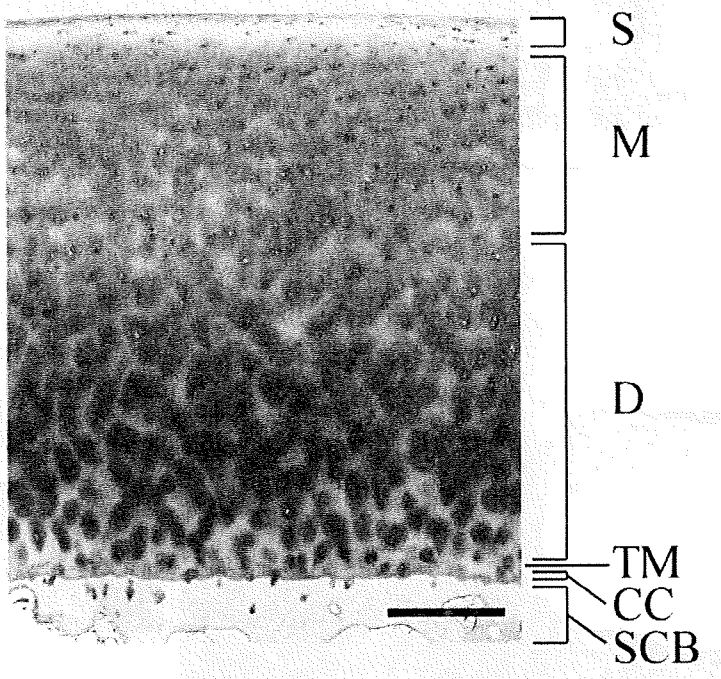


Figure 2

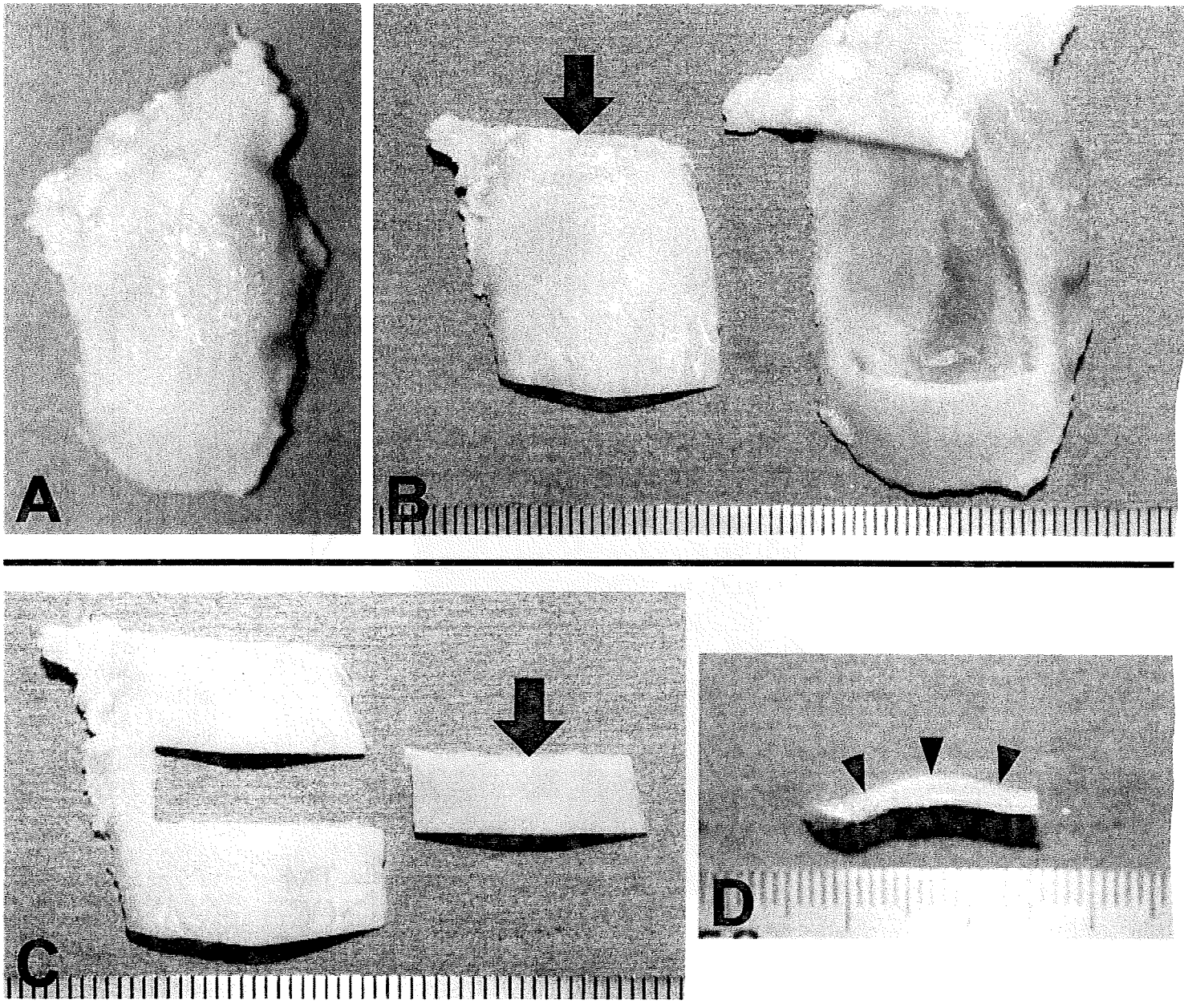
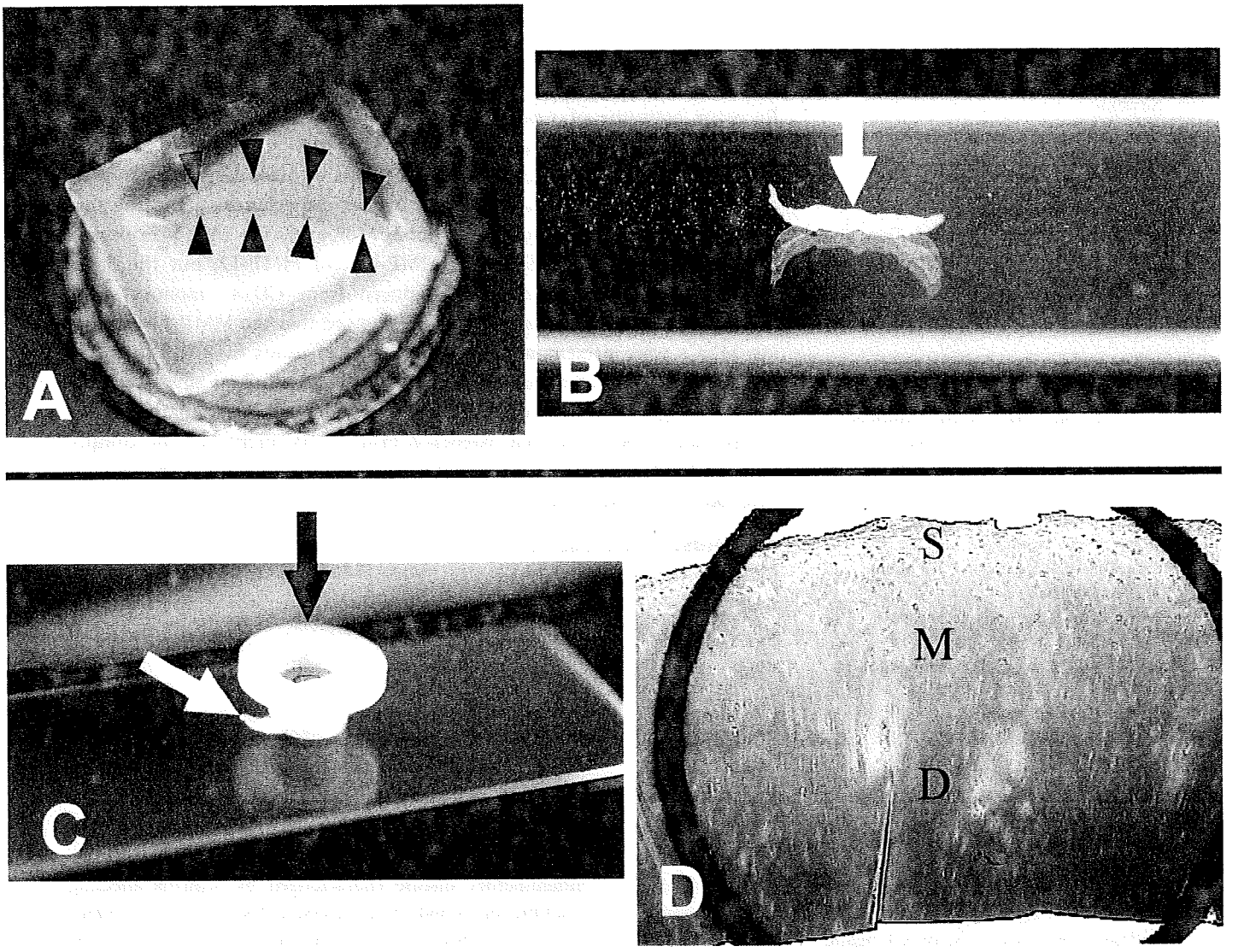


Figure 3



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

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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-κB 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 synoviocyte-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-κB 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; LTβR, lymphotoxin β receptor; MMP, matrix metalloproteinase; MNCs, multinucleated cells; NLCs, nurse-like cells; OA, osteoarthritis; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase.

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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] granulocytes[20] 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 (LTβR, TNFRSF3). The HVEM is expressed prominently on monocytes, dendritic cells and lymphocytes,[19,22–24] whereas LTβR 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 ($2{\cdot}0 \times 10^6$ cells/well) were cocultured with NLCs ($2{\cdot}0 \times 10^5$ 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 Sagamihara 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 ($1{\cdot}0 \times 10^5$ cells/well) and $NCD14^+$ monocytes ($2{\cdot}0 \times 10^4$ 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′-TTTCAGGATTCCGGCACAGTC-3′; for TRAP, 5′-TGCA GATCCTGGGTGCAGAC-3′ and 5′-GAGTATGCAATC TGGGCAGAGACA-3′; for cathepsin K, 5′-AGCT GCAATAGCGATAATCTGAACC-3′ and 5′-CGTTGTTC TTATTTCGAGCCATGA-3′; for carbonic anhydrase II, 5′-GCGACCATGTCCCATCACTG-3′ and 5′-TGGCTGTAT GAGTGTCGATGTCAA-3′; for gyceraldehyde 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 LTβR, 5′-ATGCTGATGCTG-GCCGTTC-3′ and 5′-AGGCTCCCAGCTTCCAGCTA-3′; for RANK, 5′-TTGTGCCGCCTAAGTGGA-3′ and 5′-ACC ACCTTGATCTGGGTAGCACATA-3′; for MMP-9, 5′-AC CTCGAACTTTGACAGCGACA-3′ and 5′-GATGCCATTC ACGTCGTCCTTA-3′; for MMP-12, 5′-TTGATGGCAAA 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 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–horseradish 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 ± 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

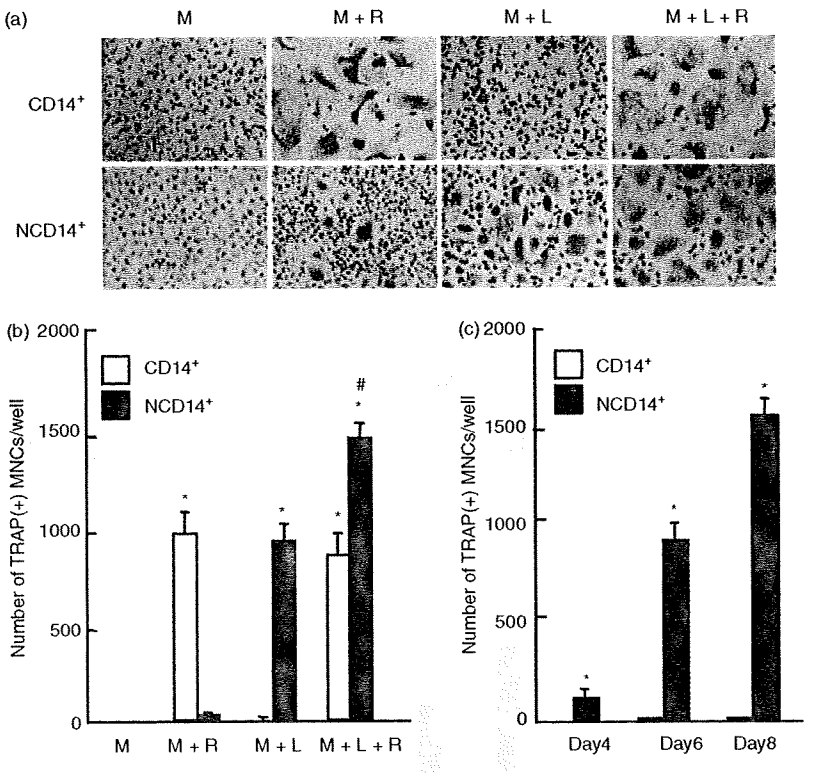

Figure 1. LIGHT induces the differentiation of NCD14$^+$ monocytes into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). (a, b) In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), CD14$^+$ or NCD14$^+$ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor-κB 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 ± 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 LTβR, in both groups of monocytes. Quantitative real-time PCR analysis revealed that while the mRNA expression level of LTβR 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

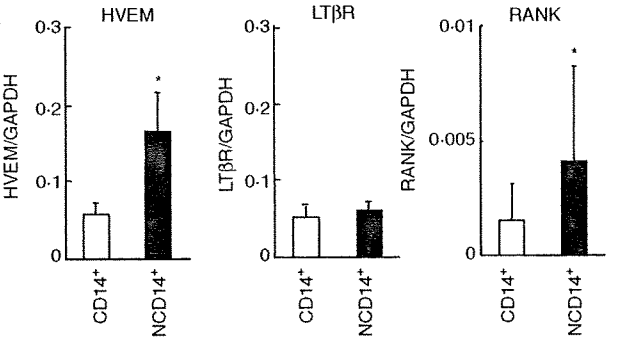

Figure 2. Expression of herpes virus entry mediator (HVEM), lymphotoxin β receptor (LTβR) and receptor activator of nuclear factor-κB (RANK) messenger RNA (mRNA) on CD14$^+$ and NCD14$^+$ monocytes. Total RNA was extracted from CD14$^+$ and NCD14$^+$ monocytes and the mRNA expression levels of HVEM, LTβR 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 ± 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

