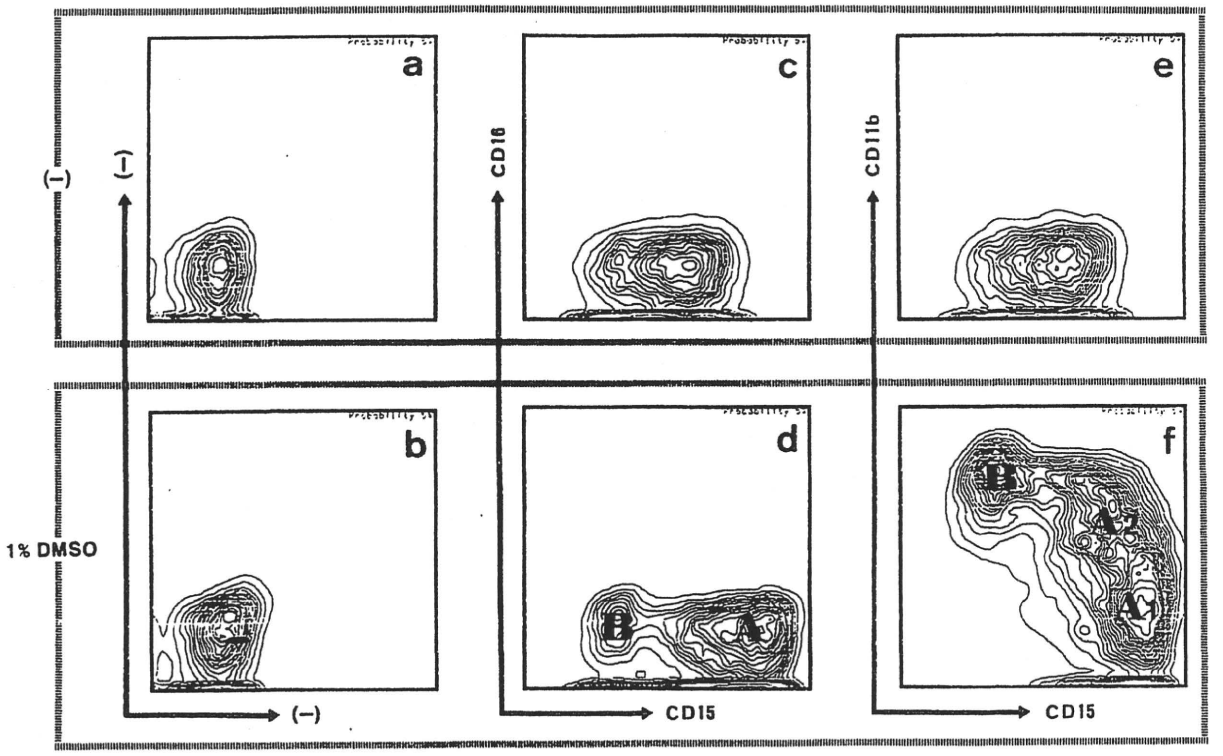

Fig. 1. Two-color FACS analysis of HL60 treated with (b, d, f) or without (a, c, e) 1% DMSO. Two-color staining was performed as follows: a and b: unstained; c and d: CD15(MX–GA) vs CD16(MG38); e and f: CD15(MX–GA) vs CD11b(Mol).

there were two cases of mutilating disease (MUD), six of the more erosive subset (MES), three of the least erosive subset (LES), and two that could not be classified because of the short duration of the disease. The controls were 8 joints from 8 patients with OA, one man and 7 women aged 48–70 (mean 58). All patients were diagnosed and treated at Oaska University Hospital.

Samples

Heparinized bone marrow blood was aspirated from the tibial proximal epiphysis during knee joint surgery, and mononuclear cell (MNC) fractions were separated by Ficoll-Paque

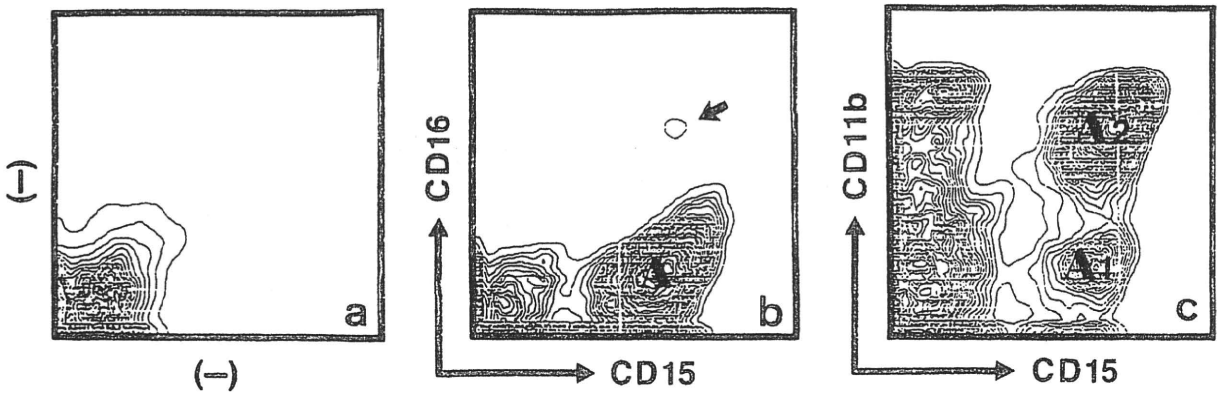

Fig. 2. Two-color FACS analysis of epiphyseal bone marrow MNCs from RA patients. a: unstained; b: CD15(MX–GA) vs CD16(MG38); c: CD15(MX–GA) vs CD11b(Mol).

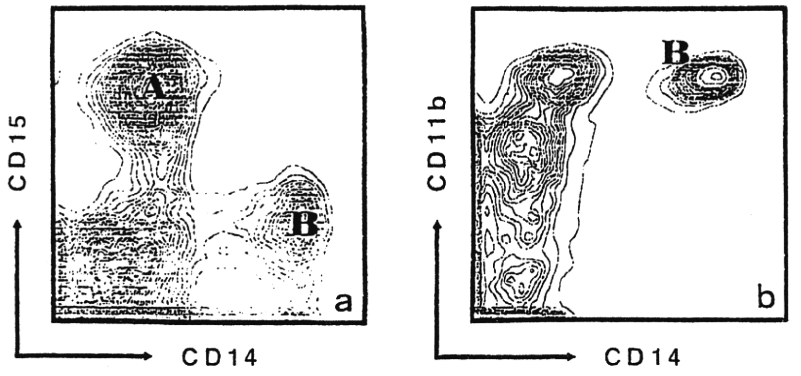

Fig. 3. Two-color FACS analysis of epiphyseal bone marrow MNCs from RA patients. a: CD14(Mo2) vs CD15(MX-GA); b: CD14(Mo2) vs CD11b(Mol).

(Pharmacia Fine Chemical, Sweden) discontinuous density gradient centrifugation (700 g, 20 min).

Cell line

HL60, a human myeloblastic leukemia cell line, was donated by Prof. Kishimoto (Institute for Molecular and Cellular Biology, Osaka University Medical School) and cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM of L-glutamine, 5×10^{-5} M of 2-mercaptoethanol, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Some cells were cultured in the same medium, but supplemented with 1% DMSO during 7 days before FACS analysis.

Monoclonal antibodies

MX-GA antibody (CD15, clone HL5), which recognizes pan-myeloids, from myeloblasts to polymorphonuclear leukocytes (PMN)(7), and also reacts with HL60(8), was purchased from Kyowa Medex (Japan). MG38 antibody (CD16), which reacts with FCγ receptors on PMN, but not on natural killer (NK)(9), was purchased from Seikagaku-Kogyo (Japan). Mol antibody (CD11b), which reacts with relatively mature myeloids, from myelocytes to PMN, and also monocytes-macrophages(10), was purchased from Coulter Clone (USA). Mo2 antibody (CD14), which is a specific marker of monocytes-macrophages(10), was purchased from Coulter Clone. If fluorescence-isothiocyanate(FITC)- or biotin-labelled antibodies were not available, labelling was performed as previously described(11).

Two-color FACS analysis

Bone marrow MNCs were washed and suspended in a staining buffer (RPMI1640 deficient biotin, riboflavin and phenol red/2% FCS/10 mM HEPES/0.02% NaN_3) at a concentration of 10^6 cells/20 μl. Appropriately diluted FITC- or biotin-labelled antibodies were simultaneously added to the cell suspension in quantities of 10 μl each, and incubated for 20 min at 4°C. After washing with the staining buffer, 20 μl of Texas-Red avidin solution was added to the cell pellets which were then suspended. Incubated for 20 min at 4°C, propidium iodide (10 μg/ml) was also incubated during the last 5 min to label dead cells. Washed 3 times, cells were applied to a FACS440 (Becton Dickinson) equipped with a dual-laser system.

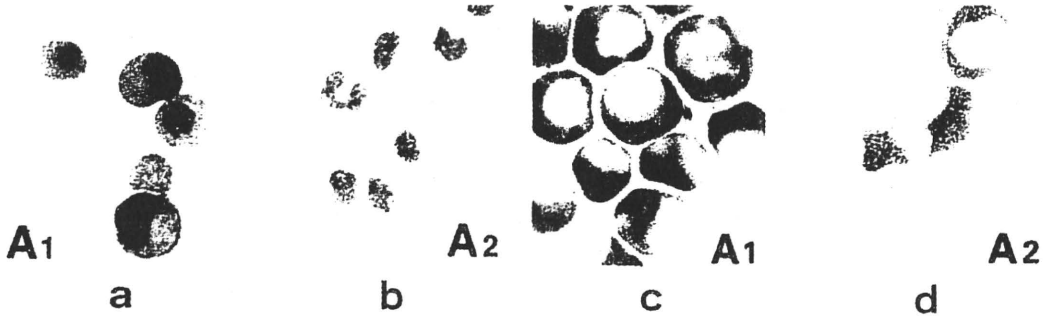

Fig. 4. Cytochemical staining of sorted cells. a and b: May-Grunwald; c and d: peroxidase staining. a and c: CD15-positive and CD11b-negative (A1) cells; b and d: CD15-positive and CD11b-positive (A2) cells.

Quantitative analysis

The gating in FACS was made on the plane of forward scatter and side scatter in each case, so as to remove the contaminated red blood cells. These could be clearly identified as small and no granular cells. The proportions of the number of each group of cells to that of whole MNCs were calculated by dividing the former by the latter.

Cytochemical staining

Sorted cells were attached to the glass slides with Cytospin (Shandon, England); some were stained with May-Grunwald and Giemsa solutions, and the others with peroxidase.

Classification of synovitis severity

The degree of synovitis was classified as follows, according to the findings at the surgical operations. (++): joint capsule was entirely covered with proliferated synovial cells; (+): about half of the capsular surface was covered with synovial proliferation; (±): synovitis was found only around the capsule-bone junction or bone-ligament junction; (−): there was no synovitis and the capsule was covered with fibrous tissue.

RESULTS

Two-color FACS analysis of HL60 cell line

The results for HL60 without chemical stimulation are shown in the upper row, and for HL60 treated with DMSO in the lower row, in Figure 1. HL60 cultured without DMSO reacted with CD15, but was not stained by CD16 nor CD11b (Figure 1 c and e). Cultured with DMSO, HL60 was not stained by CD16 either, but some cells (A) stained more brightly with CD15 (Figure 1 d). When stained with CD11b (Figure 1 f), these CD15-bright cells(A) were separated into two groups, CD11b-negative (A1) and -positive (A2).

Two-color FACS analysis of RA bone marrow MNC

One of the RA severe cases showed a FACS pattern similar to that of HL60 cultured with DMSO. There was a large number of cells (A) which reacted with CD15 (Figure 2 b), while the number of contaminated PMN was small (Figure 2 b, arrow). These CD15-positive cells

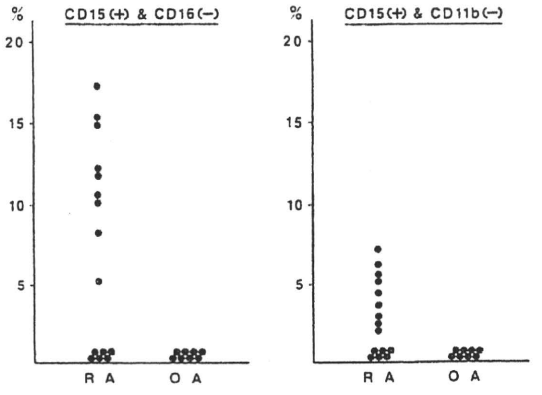

Fig. 5. Quantitative analysis of epiphyseal bone marrow myeloids from patients with RA or OA. The proportions of (A) or (A1) cells in MNCs are shown on the ordinate. (a): (A) cells = myeloids minus contaminated PMN; (b): (A1) cells = from myeloblasts to promyelocytes.

(A) could then be divided into CD11b-negative (A1) and CD11b-positive cells (A2) (Figure 2). Stained with CD14 and CD15, cells were clearly separated into three groups: CD15-positive cells (A), CD14-positive cells (B) and double-negative cells, but there existed no double-positive cells (Figure 3 a). All of the (B) cells reacted also with CD11b (Figure 3 b).

Cytochemical staining of sorted cells

The (A1) cells and (A2) cells seen in Figure 2 c were separately sorted by FACS. After being attached to glass slides, they were stained with May-Grunwald solution (Figure 4, a and b) or peroxidase (Figure 4, c and d). CD15-positive and CD11b-negative cells (A1) had a round nucleus and relatively large cytopasm (Figure 4 a). Their cytoplasmic granules were stained by peroxidase (Figure 4 c). CD15-positive and CD11b-positive cells (A2) showed a kidney-shaped nucleus (Figure 4 b), and their cytoplasmic large granules also stained with peroxidase. Consequently, (A1) cells showed the characteristics of myeloblasts-promyelocytes and (A2) cells those of myelocyte-metamyelocytes.

Quantitative analysis of myeloids in patients with RA or OA

Epiphyseal bone marrow MNCs from 15 joints with RA and 8 joints with OA were analyzed by FACS with results similar to those shown in Figure 2. The proportions of (A) or (A1) cells in epiphyseal bone marrow MNCs were calculated and are shown in Figure 5 on the ordinate. Nine of 15 joints with RA showed the existence of (A) cells (Myeloids minus contaminated PMN), with a range from 5.2% to 17.3% (mean: 11.8%) (Figure 5 a), while the others showed no myeloids (< 1%). The mean value for all RA joints was 7.1%. None of the patients with OA showed either (A) or (A1) cells. In RA patients, 9 joints with (A) Cells showed also (A1) cells (Myeloblasts-promyelocytes), with a range from 2.3% to 7.2% (mean 4.4%) (Figure 5 b). For all RA joints, the mean was 2.6%. Compared to (A) cells (Myeloids), the proportions of (B) cells (Macrophages) were more constant. In cases with RA (B) cells showed with a range from 8.7% to 16.3% (mean 12.7%), and OA showed with a range from 6.6% to 14.8% (mean 11.9%). There were no significant difference bitween RA and OA statistically.

Clinical features of RA joints

Profiles of patients with RA are listed in Table I, arranged according to the proportion of (A) cells. It was found that the group of joints with no (< 1%) (A) cells showed no synovitis, while none of the RA patients in subset LES had any (A) cells. Patients in subset MES who

Table 1. Clinical features of RA joints and proportions of myeloids in bone marrow.

case	age	sex	disease period	disease subset	operat.	synovitis	(A): CD 15 (+) & CD 16 (−)	(A1): CD 15 (+) & CD 11b (−)
M.M	71	F	2 y	(ORA)	TKR(L)	(++)	17.3%	4.4%
M.Y	30	F	12 y	MUD	TKR(R)	(±)	15.3%	7.2%
K.K	57	F	13 y	MUD	TKR(L)	(−)	15.1%	5.6%
K.O	55	F	12 y	MES	TKR(R)	(+)	12.1%	6.2%
H.M	56	M	15 y	MES	TKR(R)	(+)	12.0%	5.2%
R.K	51	F	14 y	MES	TKR(R)	(+)	10.5%	3.6%
Y.D	47	F	7 y	(N.D.)	synovectomy	(++)	10.3%	2.6%
K.M	43	F	10 y	MES	TKR(R)	(+)	8.2%	2.4%
K.O	55	F	12 y	MES	TKR(L)	(+)	5.2%	2.3%
M.Y	34	F	12 y	MES	TKR(L)	(−)	<1.0%	<1.0%
S.Y	61	F	18 y	MES	TKR(R)	(−)	<1.0%	<1.0%
N.M	53	M	11 y	LES	TKR(R)	(±)	<1.0%	<1.0%
U.O	68	F	10 y	LES	TKR(R)	(−)	<1.0%	<1.0%
U.O	68	F	10 y	LES	TKR(L)	(−)	<1.0%	<1.0%
J.H	52	F	16 y	LES	TKR(L)	(−)	<1.0%	<1.0%

ORA: Old onset RA; N. D.: Not determined; TKR: Total knee replacement; MUD: Mutilating disease; MES; More erosive subset; LES: Least erosive subset.

had (A) cells showed (+) or (++) synovitis, but patients with MUD had many (A) cells although there was no evidence of synovitis.

DISCUSSION

Myeloid cells differentiate from bi-potential stem cells to PMN finally, and they have been traditionally characterized with cytochemical staining such as May-Grunwald, Giemsa and peroxidase. Later, some cytoplasmic markers for differentiation were found, such as lactoferrin(3) and inosine mono-phosphate(12), but they are not convenient for statistical or quantitative analysis. On the other hand, some cell surface antigens are known as differentiation markers of myeloids, namely, the C3bi and Fcγ receptors(13). The monoclonal antibodies for those receptors have been produced and are classified as CD11b and CD16, respectively. However, CD11b- or CD16- positive cells are not only myeloids, but also other lineage cells, so ti is necessary to purify the myeloids with cell-biological techniques or on the image of a FACS monitor. For the latter purpose, some monoclonal antibodies belonging to CD15 are useful, that is, they recognize only myeloid lineage cells, from myeloblasts to PMN. Double staining with CD15 monoclonal and CD11b or CD16 monoclonal, showed the differentiation stages of myeloids clearly, both in HL60 cell lines treated with DMSO and human bone marrow cells. Because of the simple staining method and quantitative analysis, this method could by useful for identifying the existence of immature myeloids.

We previously reported the elevated titers of myeloid growth activity in epiphyseal bone marrow adjacent to RA joints(2), and that this could be an important factor for abnormal myelopoiesis. It remains however unclear whether relatively mature myeloids invade in this site and proliferate or immature myeloids (or stem cells) differentiate and proliferate at this site. The results of the present study show a considerable number of immature myeloids besides relative mature cells, so that the latter hypothesis should be considered more likely.

The existence of myeloids in epiphyseal bone marrow in RA, is closely related to the clinical features of RA. Furthermore, disease subsets in RA and the degree of synovitis seem

to be important factors for this phenomenon. As for MES, synovial proliferation and the existence of myeloids are well correlated, so they may have a close pathological relationship. On the other hand, in the MUD subset, many myeloids exist in epiphyseal bone marrow without being accompanied by synovitis, so it is possible that myeloids appear independently of synovial lesions in this disease subset.

The mechanism of joint destruction in MUD seems to be different from that of the other types, where, the bone destruction begins as erosion at the capsule-bone junction. The destruction in MUD is so fast that it looks like a collapse of the bone structure. In view of these findings, the epiphyseal bone marrow in MUD could be the site of a severe reaction followed by osteoporosis and myelopoiesis, although these two may not directly correlate.

The functional abnormality of myeloids accumulating in epiphyseal bone marrow adjacent to joints with RA, is not clearly understood yet. However, PMN (the final differentiation stage of myeloids) in such lesions, are functionally different from those in other lesions in RA(14), and this function is most markedly shown in MUD cases. More detailed studies are needed to determine whether myeloids in such lessions also have an abnormal function as PMN do, and to understand the relation between myeloids and joint destruction in RA.

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IMMUNOLOGY ORIGINAL ARTICLE

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.

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

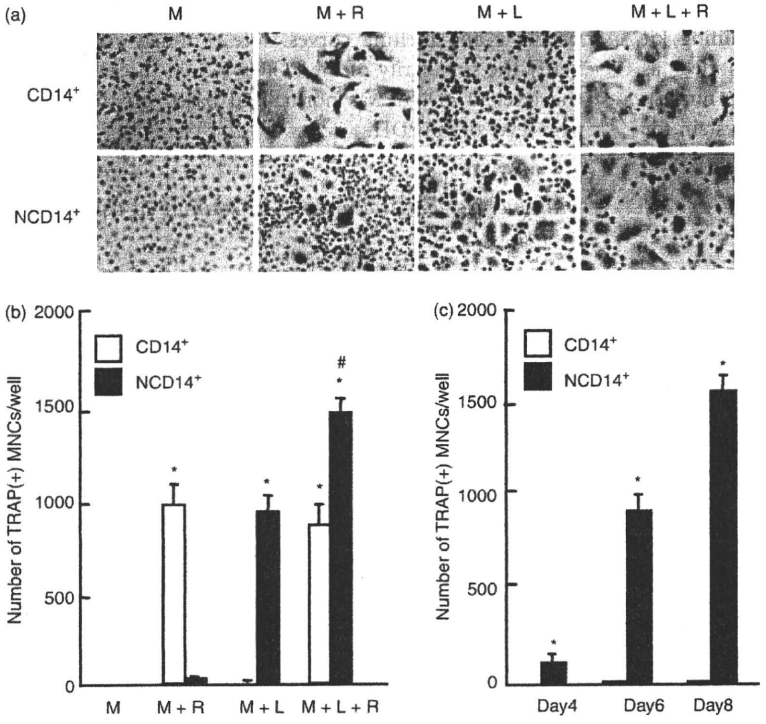

Figure 1. LIGHT induces the differentiation of NCD14$^+$ monocytes into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). (a, b) In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), CD14$^+$ or NCD14$^+$ monocytes were cultured for 6 days with 40 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). *$P < 0{\cdot}01$ versus M-CSF alone. #$P < 0{\cdot}01$ 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. *$P < 0{\cdot}01$ 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

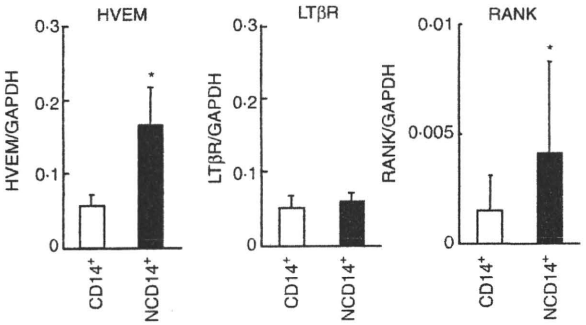

Figure 2. Expression of herpes virus entry mediator (HVEM), lymphotoxin β receptor (LTβR) and receptor activator of nuclear factor-κB (RANK) messenger RNA (mRNA) on CD14$^+$ and NCD14$^+$ monocytes. Total RNA was extracted from CD14$^+$ and NCD14$^+$ monocytes and the mRNA expression levels of HVEM, 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. *$P < 0{\cdot}01$ 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

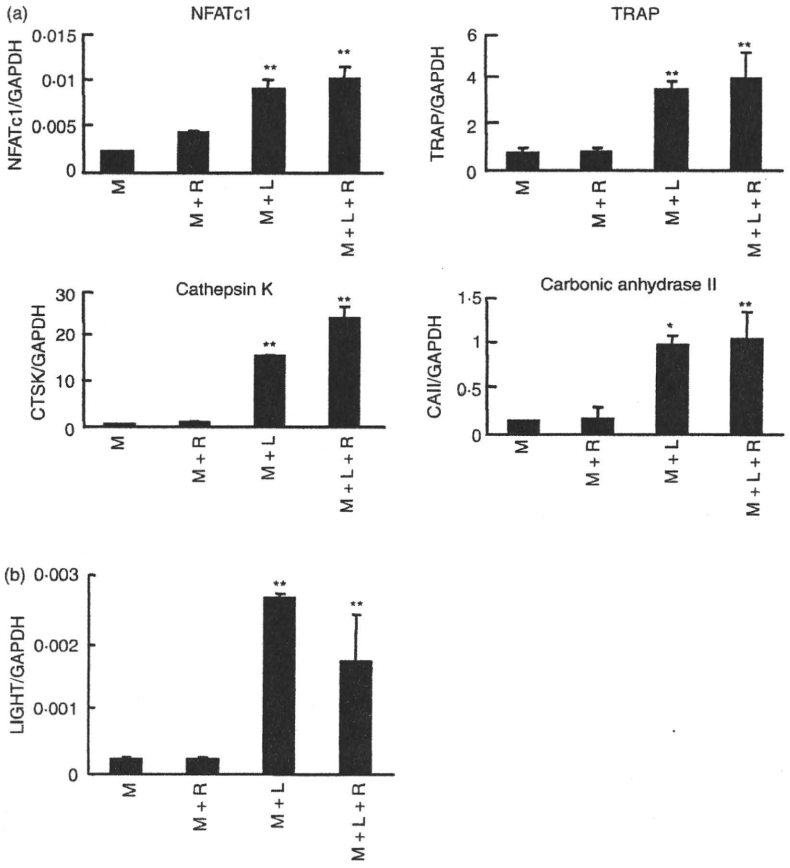

Figure 3. The molecular phenotype of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) derived from NCD14$^+$ monocytes. In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), NCD14$^+$ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor-κB 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 ± SD of triplicate experiments. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ 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

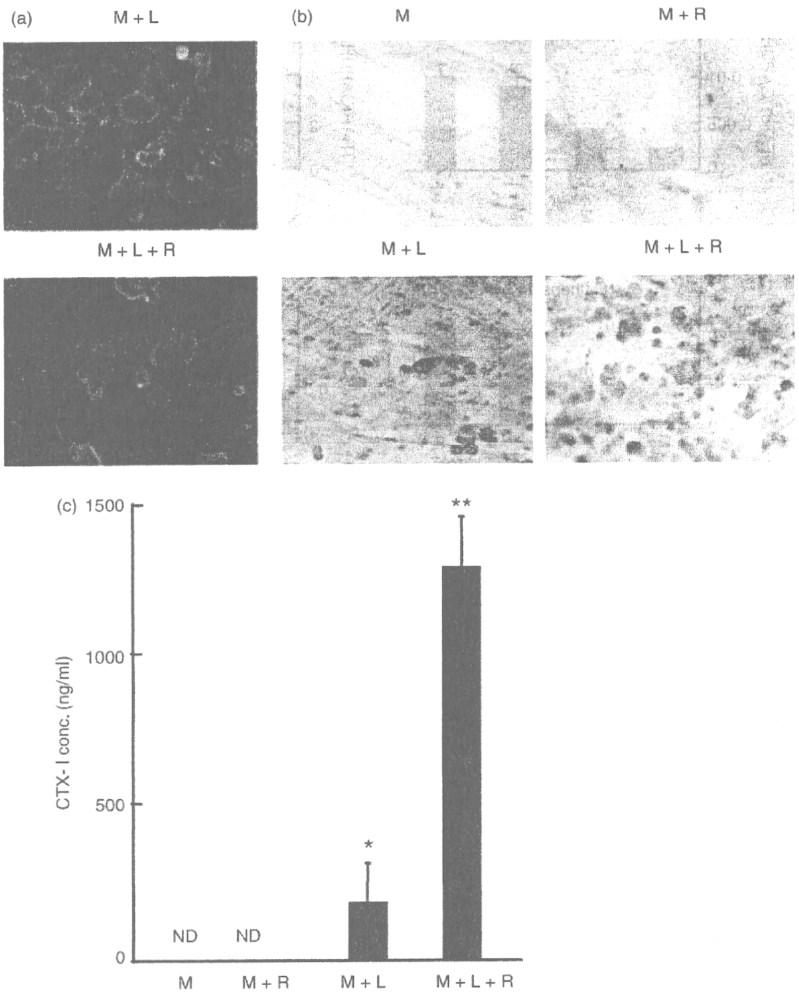

Figure 4. Osteoclastic functions of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) from NCD14$^+$ monocytes. (a) For actin ring formation, NCD14$^+$ monocytes were stimulated, in the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF), with 100 ng/ml LIGHT (M + L), or 40 ng/ml receptor activator of nuclear factor-κB 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 ± 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.

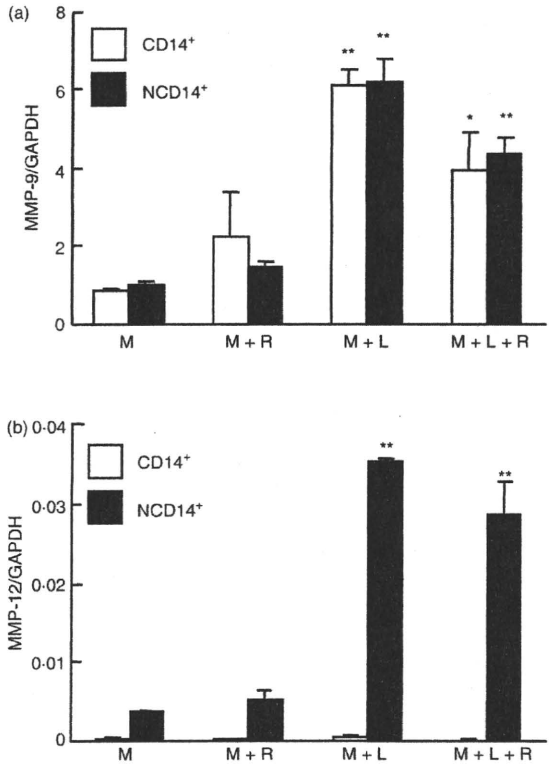

Figure 5. Comparison of matrix metalloproteinase-9 (MMP-9) and MMP-12 messenger RNA (mRNA) expression in $CD14^+$ or $NCD14^+$ monocyte-derived tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). In the presence of 25 ng/ml macrophage-colony-stimulating factor (M-CSF; M), $CD14^+$ or $NCD14^+$ monocytes were cultured for 6 days with 40 ng/ml receptor activator of nuclear factor-κB 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 ± 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 synoviocytes 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 macrophages[32] 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 LTβR.

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.

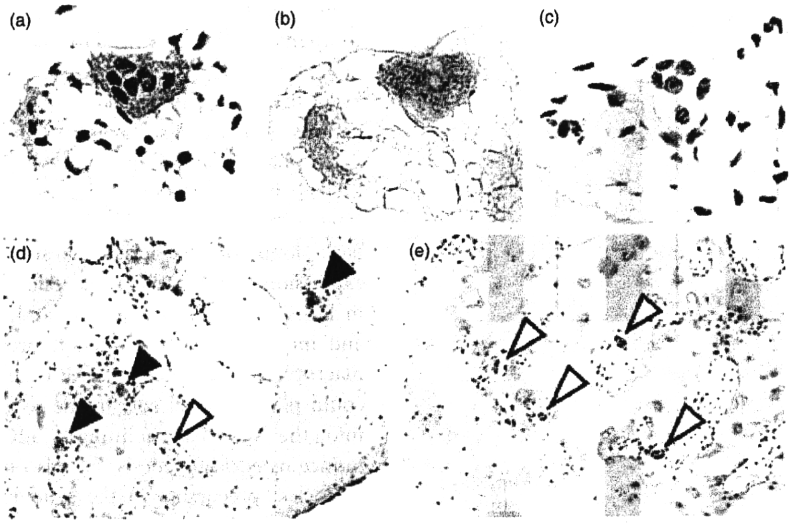

Figure 6. Detection of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) expressing matrix metalloproteinase-12 (MMP-12) in the erosive area of the rheumatoid arthritis (RA) joint. Affected subchondral bone samples were obtained from the resected knee joints of patients 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 emphysema[44] and cutaneous granulomas.[45] Rheumatoid arthritis is also a granulomatous disease with tissue hyperplasia and destruction. Osteoclasts invading uncalcified cartilage beyond the tidemark 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 activity.[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

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LIGHT Induces Cell Proliferation and Inflammatory Responses of Rheumatoid Arthritis Synovial Fibroblasts via Lymphotoxin ß Receptor

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ABSTRACT. ***Objective.*** To investigate the effects of LIGHT (lymphotoxin-like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) on the proliferation and gene expression of fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA).

Methods. We measured LIGHT levels in RA synovial fluids (SF) by ELISA, and compared them with those in osteoarthritis (OA) SF. Levels of LIGHT and its receptors in RA-FLS and synovium were assessed using real-time quantitative polymerase chain reaction (PCR). RA-FLS proliferation was examined by a bromodeoxyuridine assay. Expression of intercellular adhesion molecule-1 (ICAM-1) and several chemokines, such as interleukin 8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1α (MIP-1α), was examined by real-time quantitative PCR, ELISA, and flow cytometry. The effects of LIGHT on nuclear factor-κB (NF-κB) activation were investigated using immunofluorescence and Western blotting.

Results. LIGHT was upregulated in both SF and synovium of RA patients compared with OA patients. Herpes virus entry mediator (HVEM) and lymphotoxin ß receptor (LTßR), but not LIGHT, were detected in RA-FLS. LIGHT significantly promoted RA-FLS proliferation and induced expression of MCP-1, IL-8, MIP-1α, and ICAM-1 by RA-FLS. As well, LTßR small interfering RNA (siRNA), but not HVEM siRNA, inhibited these effects of LIGHT. LIGHT induced IκBa degradation and NF-κB translocation, and a NF-κB inhibitor suppressed the effects of LIGHT on RA-FLS.

Conclusion. Our findings suggest that LIGHT signaling via LTßR plays an important role in the pathogenesis of RA by affecting key processes such as the proliferation and activation of RA-FLS. Regulation of LIGHT-LTßR signaling may represent a new therapeutic target for RA treatment. (J Rheumatol First Release April 15 2008)

Key Indexing Terms:
RHEUMATOID ARTHRITIS INFLAMMATION SYNOVIOCYTES

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia and progressive destruction of cartilage and bone. Fibroblast-like synoviocytes (FLS), an important component of the synovial lining in joints, proliferate aggressively to form a pannus causing irreversible joint damage. In RA synovial tissue, activated FLS and infiltrating macrophages and lymphocytes produce inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin 1ß (IL-1ß), and IL-6, that play important roles in the pathogenesis of RA[1,2]. These cytokines have been shown to not only directly promote FLS proliferation leading to pannus formation[3], but also to induce the expression of inflammatory cytokines, chemokines, and adhesion molecules, which further recruit inflammatory leukocytes and perpetuate inflammatory responses.

LIGHT (lymphotoxin-like, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes) is a recently identified type-2 transmembrane glycoprotein of the TNF ligand superfamily (TNFSF14)[4]. LIGHT is expressed on activated T lymphocytes[4,5], monocytes[6], granulocytes[6], and immature dendritic cells[7]. LIGHT signaling is transduced via 2 members of the TNFR family,

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herpes virus entry mediator (HVEM, TNFRSF14) and lymphotoxin ß receptor (LTßR, TNFRSF3). HVEM is expressed prominently on monocytes, dendritic cells, and lymphocytes[5,8-10], whereas LTßR is expressed on many cell types with the exception of lymphocytes[4,6,11]. LIGHT has been shown to regulate cell proliferation[7,12,13] and apoptosis[6,14] to induce the secretion of various cytokines, and to augment the expression of adhesion molecules[12,15-17]. Recently, Fava, *et al* reported that LTßR-Ig protein blocked the induction of experimental arthritis in mice[18]. Moreover, LIGHT induced the expression of inflammatory cytokines in macrophages from RA synovial fluid (SF)[19]. These studies suggest that LIGHT may be an important inflammatory cytokine in the development of RA. However, the effect of LIGHT on RA-FLS has not yet been analyzed.

Our aim was to clarify the role of LIGHT in the proliferation and inflammatory response of RA-FLS. We observed that the concentrations of LIGHT in both SF and synovium were higher in patients with RA than in those with osteoarthritis (OA). In addition, LIGHT signaling via LTßR, but not HVEM, enhanced RA-FLS proliferation and induced the expression of inflammatory cytokines, chemokines, and adhesion molecules in RA-FLS through a nuclear factor-κB (NF-κB)-dependent signal transduction pathway. We suggest that activation of RA-FLS by LIGHT/LTßR signaling may play an important role in the pathogenesis of RA.

MATERIALS AND METHODS

Chemicals. Recombinant human LIGHT and platelet-derived growth factor (PDGF)-AB were obtained from R&D Systems (Minneapolis, MN, USA). Monoclonal antibodies (mAb) against human actin and NF-κB p65 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and BD Biosciences (Palo Alto, CA, USA), respectively. The mAb against IκBa was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Pyrrolidine dithiocarbamate (PDTC) was purchased from Calbiochem (La Jolla, CA, USA).

Patients and tissue samples. All patients with RA fulfilled the 1987 American College of Rheumatology (formerly, the American Rheumatism Association) criteria[20] for the diagnosis of RA. Patients with RA ranged in age from 41 to 74 years (mean ± SD 66.0 ± 12.0 yrs). Patients with OA ranged in age from 39 to 90 years (mean ± SD 64.1 ± 14.7 yrs). All patients were women. Synovial tissues were obtained from 27 patients with RA and 11 patients with OA at the time of knee prosthetic replacement surgery. RA-FLS were established from the synovia of RA patients as described[21]. RA-FLS were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine. RA-FLS from passages 4–9 were used for each experiment. SF were obtained by arthrocentesis from 23 RA patients and 10 OA patients and, after centrifugation at 20,000 × g for 10 min, the supernatants were collected and frozen at –80°C until used. All specimens were obtained from patients who gave written informed consent, according to the protocol approved by the institutional review board of the National Hospital Organization, Sagamihara National Hospital.

LIGHT in synovial fluids. The amount of LIGHT in SF was measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions. The minimum and maximum detection levels of the ELISA were 7.8 pg/ml and 2000 pg/ml, respectively.

RNA extraction, cDNA synthesis, and real-time quantitative polymerase chain reaction (PCR) analysis. Total RNA was extracted from synovium and FLS using an RNeasy Micro kit (Qiagen). cDNA was generated from RNA using Omniscript Reverse Transcriptase (Qiagen) and used as a template for real-time quantitative PCR on a LightCycler (Roche Diagnostics). PCR was performed using SYBR Premix Ex Taq (Takara). The primers used for real-time PCR were as follows: for IL-6, 5'-AAG CCA GAG CTG TGC AGA TGA GTA-3' and 5'-TGT CCT GCA GCC ACT GGT TC-3'; for IL-8, 5'-ACA CTG CGC CAA CAC AGA AAT TA-3' and 5'-TTT GCT TGA AGT TTC ACT GGC ATC-3'; for granulocyte macrophage-colony stimulating factor (GM-CSF), 5'-CAT GAT GGC CAG CCA CTA CAA-3' and 5'-ACT GGC TCC CAG CAG TCA AAG-3'; for monocyte chemoattractant protein-1 (MCP-1), 5'-GCT CAT AGC AGC CAC CTT CAT TC-3' and 5'-GGA CAC TTG CTG CTG GTG ATT C-3'; for RANTES, 5'-ACC AGT GGC AAG TGC TCC AAC-3' and 5'-CTC CCA AGC TAG GAC AAG AGC AAG-3'; for MIP-1α, 5'-TCC GTC ACC TGC TCA GAA TCA-3' and 5'-AGC ACT GGC TGC TCG TCT CA-3'; for vascular cell adhesion molecule-1 (VCAM-1), 5'-CGT GAT CCT TGG AGC CTC AAA TA-3' and 5'-GAC GGA GTC ACC AAT CTG AGC A-3'; for intercellular adhesion molecule-1 (ICAM-1), 5'-CCT GAT GGG CAG TCA ACA GCT A-3' and 5'-ACA GCT GGC TCC CGT TTC A-3'; for GAPDH, 5'-GCA CCG TCA AGG CTG AGA AC-3' and 5'-ATG GTG GTG AAG ACG CCA GT-3'; for LIGHT, 5'-TCA CGA GGT CAA CCC AGC AG-3' and 5'-CCC AGC TGC ACC TTG GAG TAG-3'; for HVEM, 5'-TTT GCT CCA CAG TTG GCC TAA TC-3' and 5'-CAA TGA CTG TGG CCT CAC CTT C-3'; and for LTßR, 5'-ATG CTG ATG CTG GCC GTT C-3' and 5'-AGG CTC CCA GCT TCC AGC TA-3'.

PCR was performed under the following conditions: initial denaturation at 95°C for 10 s, then 40 cycles of 95°C for 5 s and 60°C for 20 s. When SYBR Green dye was used to monitor PCR, melting curves were routinely recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized to the level of GAPDH.

Proliferation assay. RA-FLS were seeded into a 96-well plate at a density of 5.0×10^3 cells/well. After 24 h of preculture, the cells were stimulated for 48 h by the addition of LIGHT or PDGF used as a positive control. A previous report described stimulation of RA-FLS proliferation by PDGF[22]. Bromodeoxyuridine (BrdU) was added for the last 24 h of culture, then incorporation of BrdU was measured using a cell proliferation ELISA (Roche Diagnostics) according to the manufacturer's instructions.

Measurement of cytokine and chemokine levels in culture supernatants. TNF-α, IL-1ß, IL-6, IL-8, and GM-CSF levels were measured in the supernatants of RA-FLS cultures using a Human Inflammatory Five-Plex Antibody bead kit (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions, on a Luminex 100 instrument (Luminex, Austin, TX, USA). The levels of MIP-1α, MIP-1ß, MCP-1, eotaxin, and RANTES in the supernatant were measured using a Human Chemokine Five-Plex Antibody bead kit (Biosource).

Transfection of RA-FLS with small interfering RNA (siRNA). All siRNA were purchased from Qiagen. The sense strand sequences of the RNA duplexes were as follows: HVEM, 5'-GGC ACU GCC UCA CAG CCA AdTdT-3'; LTßR, 5'-CAU CUA CAA UGG ACC AGU AdTdT-3'; and control siRNA 5'-UUC UCC GAA CGU GUC ACG UdTdT-3'. The day before transfection, RA-FLS were seeded into 6-well culture plates at a density of 4×10^4 cells/well, or 96-well plates at 5×10^3 cells/well, in complete medium without antibiotics. The next day, siRNA (at final concentration of 50 nM) were introduced into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions; 24 h after transfection, media were replaced with regular culture media. The cells were then cultured for 96 h before analysis of the gene-silencing effects. mRNA levels were measured by real-time quantitative PCR analysis.

Immunofluorescence assay for NF-κB localization. To examine the nuclear translocation of NF-κB, RA-FLS were seeded at a density of 5×10^3 cells/well in 8-well Lab-Tek chamber slides (Nalgen Nunc International, Naperville, IL, USA). Cells were stimulated with 10 ng/ml LIGHT for 40 min, washed with cold PBS, and then fixed in PBS with 4% paraformaldehyde for 10 min. The cells were permeabilized with PBS and 0.1% Triton-X100 for 10 min. Nonspecific binding was prevented with blocking buffer

containing 2% goat serum diluted in PBS. The cells were incubated with mouse monoclonal anti-NF-κB p65 antibody or an isotype control for 1 h at room temperature, then with AlexaFluor 488-conjugated goat anti-mouse antibody for 30 min at room temperature. Slides were coverslipped and examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Western blotting analysis. For measurement of IκBa by Western blotting, RA-FLS at a density of 1.5×10^6/well were seeded into 6-well plates in culture medium for 24 h. After incubation with 10 ng/ml LIGHT for 40 min, cells were washed twice in ice-cold PBS and lysed in 100 μl of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris HCl, pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.001% bromophenol blue). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis in 12% polyacrylamide gels, and transferred onto nitrocellulose membranes (Invitrogen). After blocking, membranes were incubated with either anti-ß-actin or anti-IκBa antibody, overnight at 4°C, and then with secondary antibody conjugated to horseradish peroxidase (Dako), at room temperature for 1 h. The signals were visualized using chemiluminescence reagent (ECL; Amersham Biosciences, Little Chalfont, UK).

Statistical analysis. Comparisons of ≥ 3 populations were made using the Kruskal-Wallis test. Comparisons of 2 independent data sets were by Mann-Whitney U-test. P values less than 0.05 were considered statistically significant.

RESULTS

Increased expression of LIGHT in SF of patients with RA. To examine whether LIGHT is involved in the pathogenesis of RA, we analyzed the level of LIGHT in SF from 23 RA patients and 10 OA patients by ELISA. SF from OA patients were used as controls, because they were not available from healthy individuals. The concentration of LIGHT in SF from RA patients was significantly higher than in those from OA patients (Figure 1). The median levels of LIGHT in SF from RA and OA patients were 108.5 pg/ml and 7.8 pg/ml, respectively.

Expression of LIGHT and its receptors in RA synovial tissue and RA-FLS. Because RA patients had more LIGHT in their SF than OA patients, we investigated whether LIGHT and its membrane-bound receptors HVEM and LTßR were expressed in the RA and OA synovial tissues. Although quantitative real-time PCR analysis revealed that mRNA expression of LIGHT in synovial tissue was significantly higher in RA patients than in OA patients (Figure 2A), HVEM and LTßR levels were not different between RA and OA patients.

Further, we investigated the mRNA expression of LIGHT, HVEM, and LTßR in RA-FLS by quantitative real-time PCR. RA-FLS from all 7 patients expressed HVEM and LTßR mRNA, and the level of LTßR mRNA was significantly higher than that of HVEM mRNA, whereas no LIGHT expression was detected (Figure 2B).

Induction of RA-FLS proliferation by LIGHT. Previous studies reported that LIGHT induces cell proliferation in T lymphocytes[7,12] and vascular smooth muscle cells[13]. Since the expression of HVEM and LTßR in RA-FLS had been confirmed, we next evaluated the effect of LIGHT on the proliferation of RA-FLS using a BrdU assay. As shown in

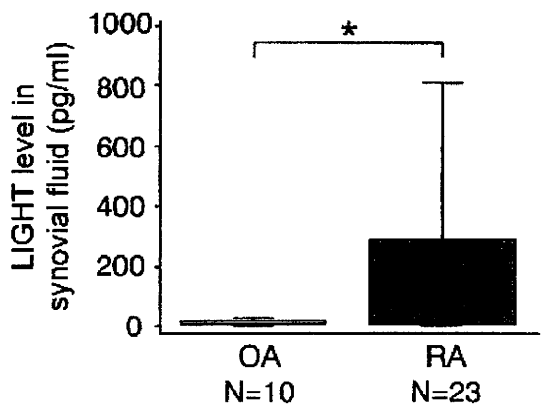

Figure 1. Levels of LIGHT in synovial fluid from RA patients and OA patients, determined by ELISA. Box plots represent 25th to 75th percentiles. Error bars represent 10th to 90th percentiles. Lines inside boxes represent the median. *p < 0.05.

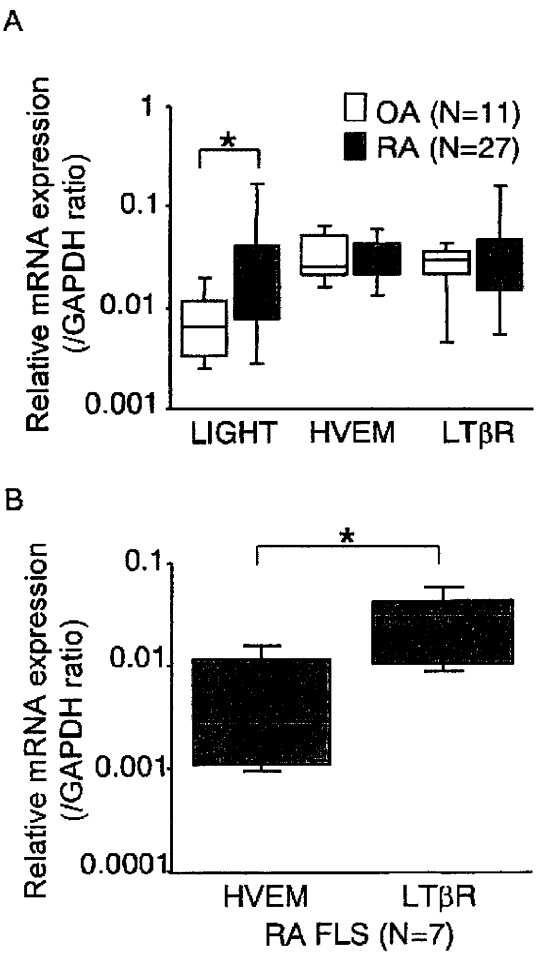

Figure 2. Expression of LIGHT, herpes virus entry mediator (HVEM), and lymphotoxin ß receptor (LTßR) mRNA in synovial tissues and fibroblast-like synoviocytes (FLS): A. In synovial tissues from RA patients and OA patients. B. In RA-FLS. Level was evaluated by real-time quantitative PCR; results are represented as relative ratios to GAPDH levels. *p < 0.05.

Figure 3A, treatment with LIGHT significantly enhanced *de novo* DNA synthesis in RA-FLS in a dose-dependent manner. LIGHT showed a stronger growth-promoting activity than PDGF, at lower concentrations.

To investigate the contributions of HVEM and LTßR to the LIGHT-induced proliferation of RA-FLS, we transfected RA-FLS with HVEM siRNA or LTßR siRNA. Quantitative real-time PCR analysis revealed that the HVEM mRNA level in HVEM siRNA-transfected RA-FLS was reduced by 75% compared with control siRNA-transfected RA-FLS (Figure 3B). Similarly, treatment of RA-FLS with LTßR siRNA led to a 75% reduction in the LTßR mRNA level compared with that in control siRNA-transfected RA-FLS (Figure 3B). Under these conditions, LIGHT-induced growth of RA-FLS was significantly decreased by LTßR siRNA, but not by HVEM siRNA, when compared with RA-FLS transfected with control siRNA (Figure 3C).

LIGHT induces expression of proinflammatory cytokines, chemokines, and adhesion molecules in FLS via LTßR. Previous studies reported that LIGHT induces secretion of various cytokines and augments the expression of adhesion molecules[13,15-17,19]. We examined the effects of LIGHT on inflammatory cytokine and chemokine production by RA-FLS. Treatment with LIGHT enhanced both mRNA and protein expression of IL-8, MCP-1, MIP-1α, and RANTES in RA-FLS, in a dose-dependent manner (Figures 4A, 4B). LIGHT induced IL-1ß, IL-6, and GM-CSF, but not TNF-α, eotaxin, or MIP-1ß (data not shown). Next, to assess whether LIGHT can induce the expression of cell-surface adhesion molecules on RA-FLS, we examined ICAM-1 and VCAM-1 expression on LIGHT-stimulated RA-FLS. LIGHT treatment significantly increased the expression of ICAM-1 mRNA in a dose-dependent manner (Figure 4C). Flow cytometry analysis revealed the augmented expression of ICAM-1 protein on the cell surface of RA-FLS stimulat-

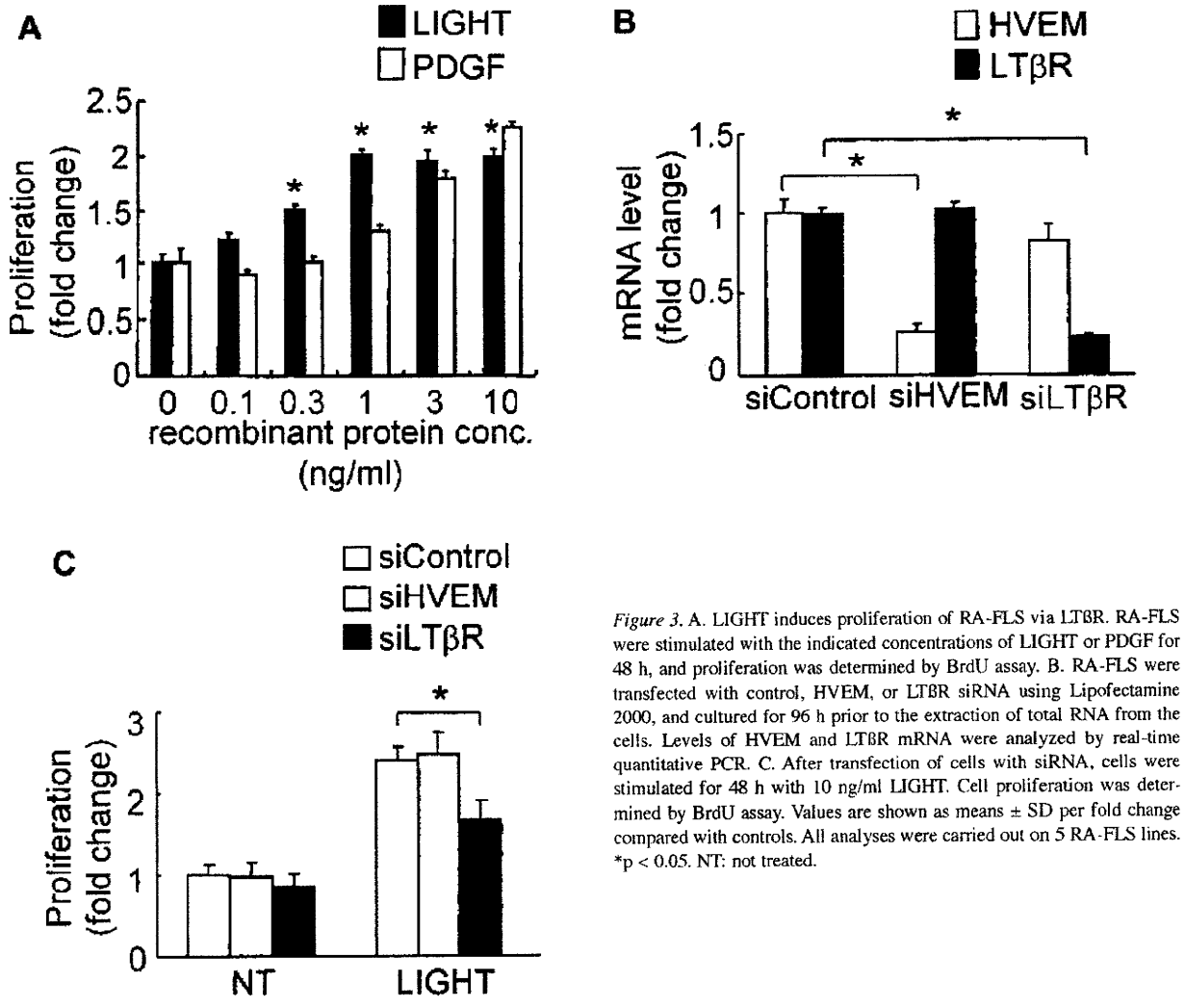

Figure 3. A. LIGHT induces proliferation of RA-FLS via LTßR. RA-FLS were stimulated with the indicated concentrations of LIGHT or PDGF for 48 h, and proliferation was determined by BrdU assay. B. RA-FLS were transfected with control, HVEM, or LTßR siRNA using Lipofectamine 2000, and cultured for 96 h prior to the extraction of total RNA from the cells. Levels of HVEM and LTßR mRNA were analyzed by real-time quantitative PCR. C. After transfection of cells with siRNA, cells were stimulated for 48 h with 10 ng/ml LIGHT. Cell proliferation was determined by BrdU assay. Values are shown as means ± SD per fold change compared with controls. All analyses were carried out on 5 RA-FLS lines. *$p < 0.05$. NT: not treated.