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

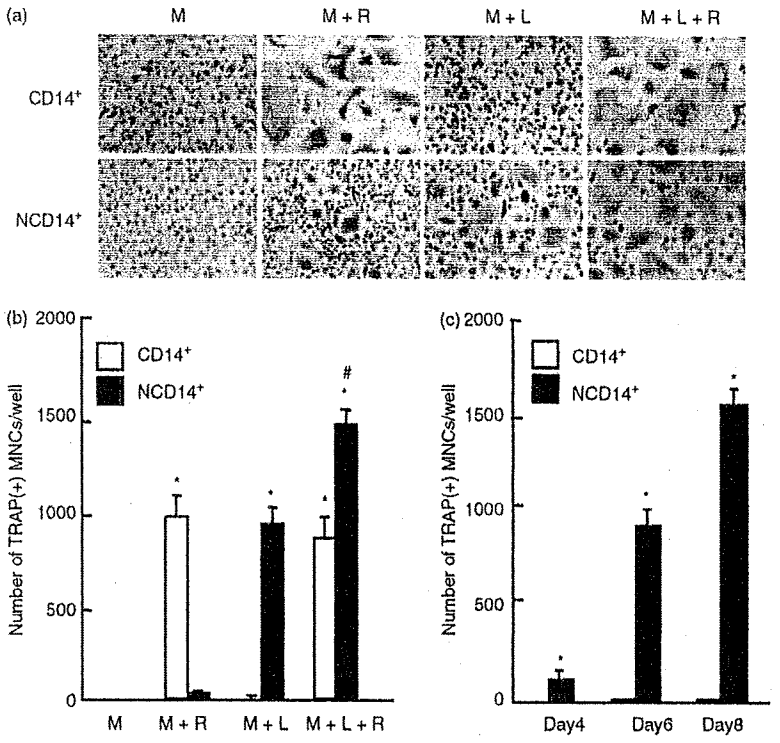

Figure 1. LIGHT induces the differentiation of NCD14$^+$ monocytes into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). (a, b) In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), CD14$^+$ or NCD14$^+$ monocytes were cultured for 6 days with 40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

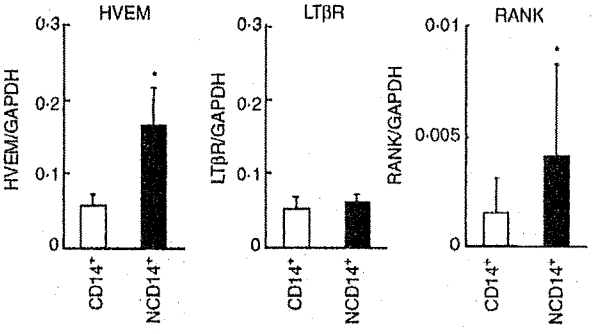

Figure 2. Expression of herpes virus entry mediator (HVEM), lymphotoxin β receptor (LTβR) and receptor activator of nuclear factor-κB (RANK) messenger RNA (mRNA) on CD14$^+$ and NCD14$^+$ monocytes. Total RNA was extracted from CD14$^+$ and NCD14$^+$ monocytes and the mRNA expression levels of HVEM,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

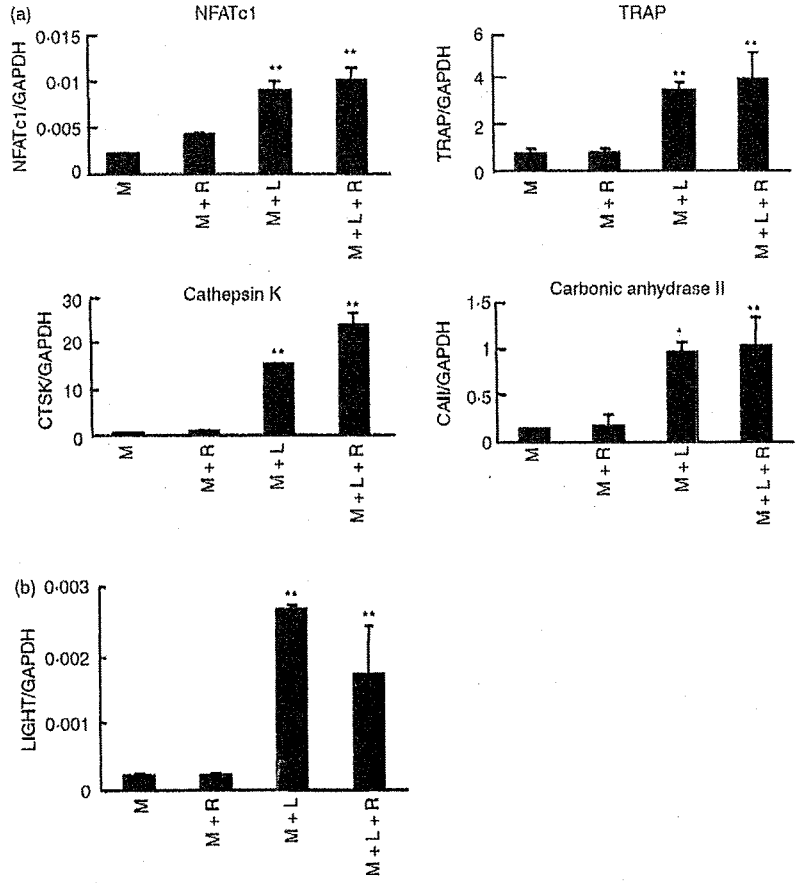

Figure 3. The molecular phenotype of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) derived from NCD14$^+$ monocytes. In the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF; M), NCD14$^+$ monocytes were cultured for 6 days with 40 ng/ml 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

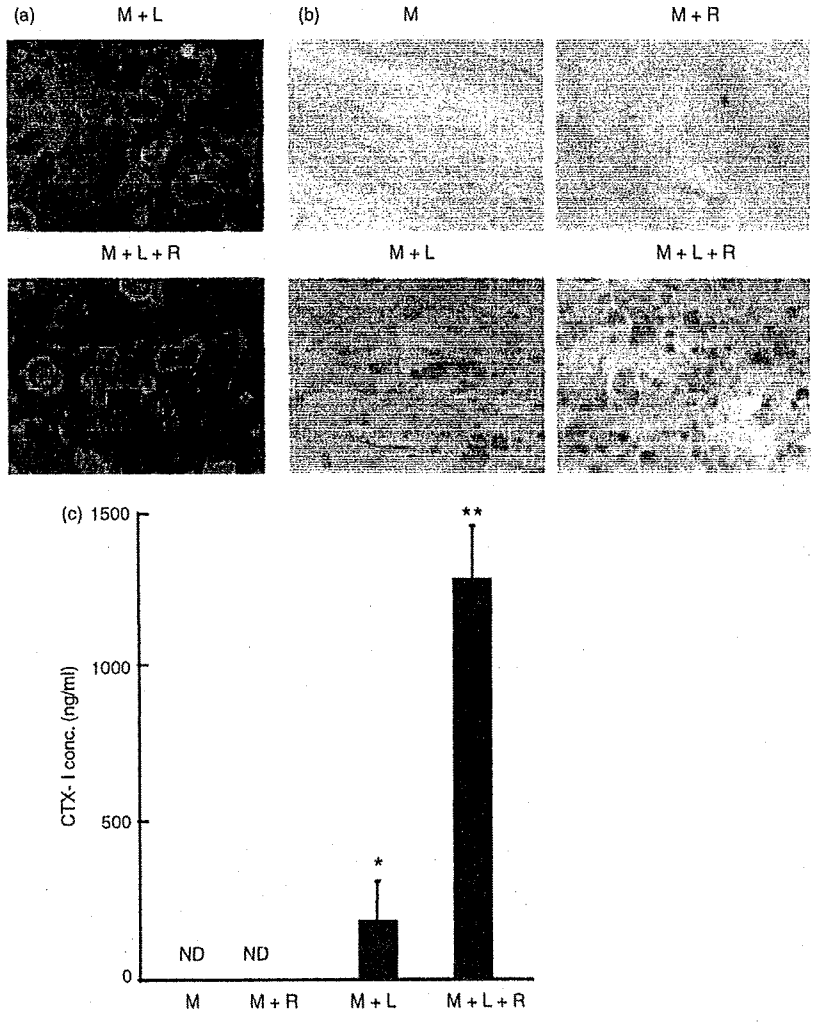

Figure 4. Osteoclastic functions of LIGHT-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) from NCD14^{+} monocytes. (a) For actin ring formation, NCD14^{+} monocytes were stimulated, in the presence of 25 ng/ml macrophage colony-stimulating factor (M-CSF), with 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. $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ 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.

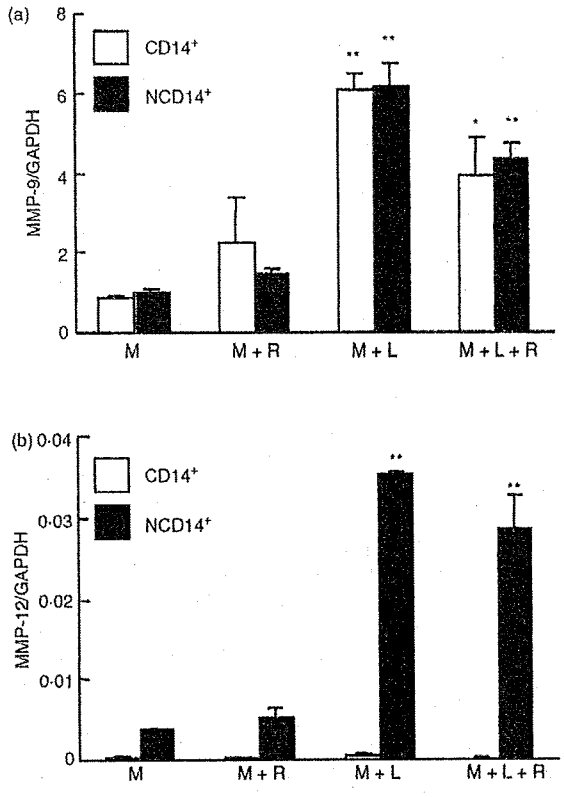

Figure 5. Comparison of matrix metalloproteinase-9 (MMP-9) and MMP-12 messenger RNA (mRNA) expression in $CD14^+$ or $NCD14^+$ monocyte-derived tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs). In the presence of 25 ng/ml macrophage-colony-stimulating factor (M-CSF; M), $CD14^+$ 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. $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ 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.

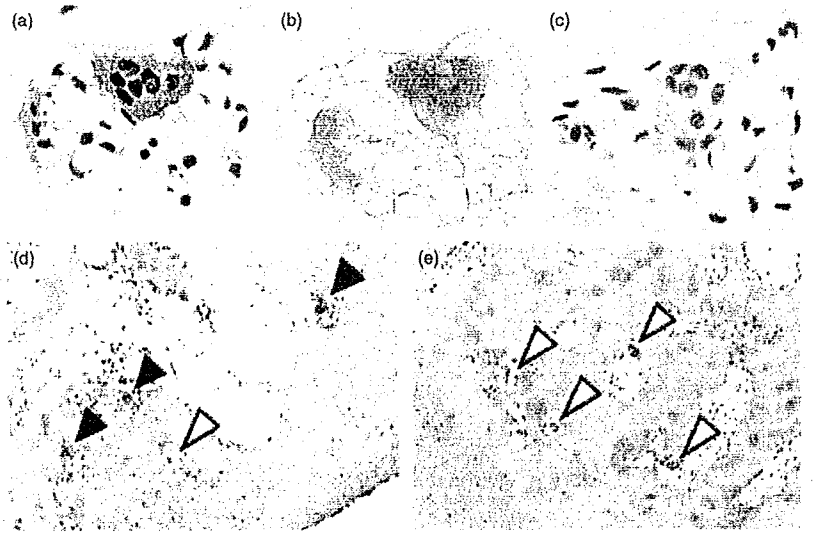

Figure 6. Detection of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) expressing matrix metalloproteinase-12 (MMP-12) in the erosive area of the rheumatoid arthritis (RA) joint. Affected subchondral bone samples were obtained from the resected knee joints of patients 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

SATORU ISHIDA, SHOJI YAMANE, SAORI NAKANO, TOSHIHITO MORI, TAKUO JUJI, NAOSHI FUKUI, TSUNETOSHI ITOH, TAKAHIRO OCHI, and RYUJI SUZUKI

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. (First Release April 15 2008; J Rheumatol 2008;35:960–8)

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,

From the Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Sagamihara, Kanagawa; Discovery Research Laboratories, Shionogi & Co., Ltd., Toyonaka, Osaka; and Department of Immunology and Embryology, Tohoku University School of Medicine, Aoba-ku, Sendai, Japan.

S. Ishida, MSc; S. Yamane, PhD, Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Discovery Research Laboratories, Shionogi & Co., Ltd.; S. Nakano, BSc; T. Mori, MD, PhD; T. Juji, MD, PhD; N. Fukui, MD, PhD, Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital; T. Itoh, MD, PhD, Department of Immunology and Embryology, Tohoku University School of Medicine; T. Ochi, MD, PhD; R. Suzuki, DVM, PhD, Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital.

Address reprint requests to S. Ishida, Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Sakuradai 18-1, Sagamihara, Kanagawa, 228-8522, Japan. E-mail: satoru.ishida@shionogi.co.jp

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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

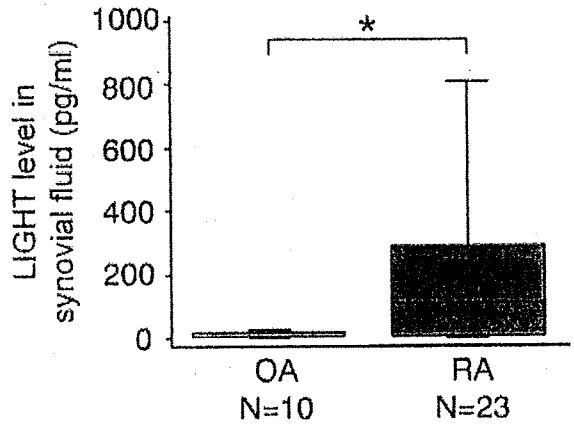

Figure 1. Levels of LIGHT in synovial fluid from RA patients and OA patients, determined by ELISA. Box plots represent 25th to 75th percentiles. Error bars represent 10th to 90th percentiles. Lines inside boxes represent the median. *p < 0.05.

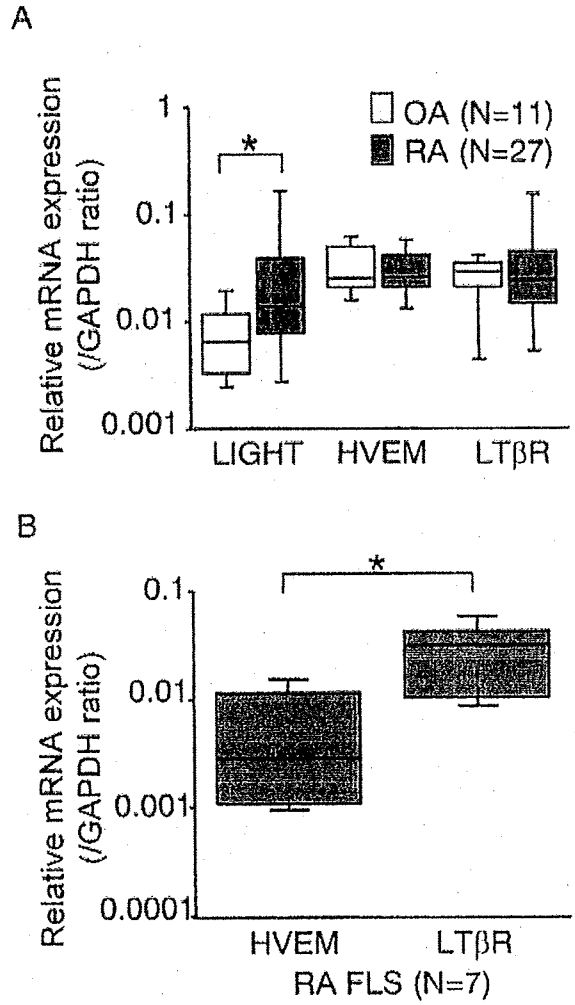

Figure 2. Expression of LIGHT, herpes virus entry mediator (HVEM), and lymphotoxin ß receptor (LTßR) mRNA in synovial tissues and fibroblast-like synoviocytes (FLS): A. In synovial tissues from RA patients and OA patients. B. In RA-FLS. Level was evaluated by real-time quantitative PCR; results are represented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

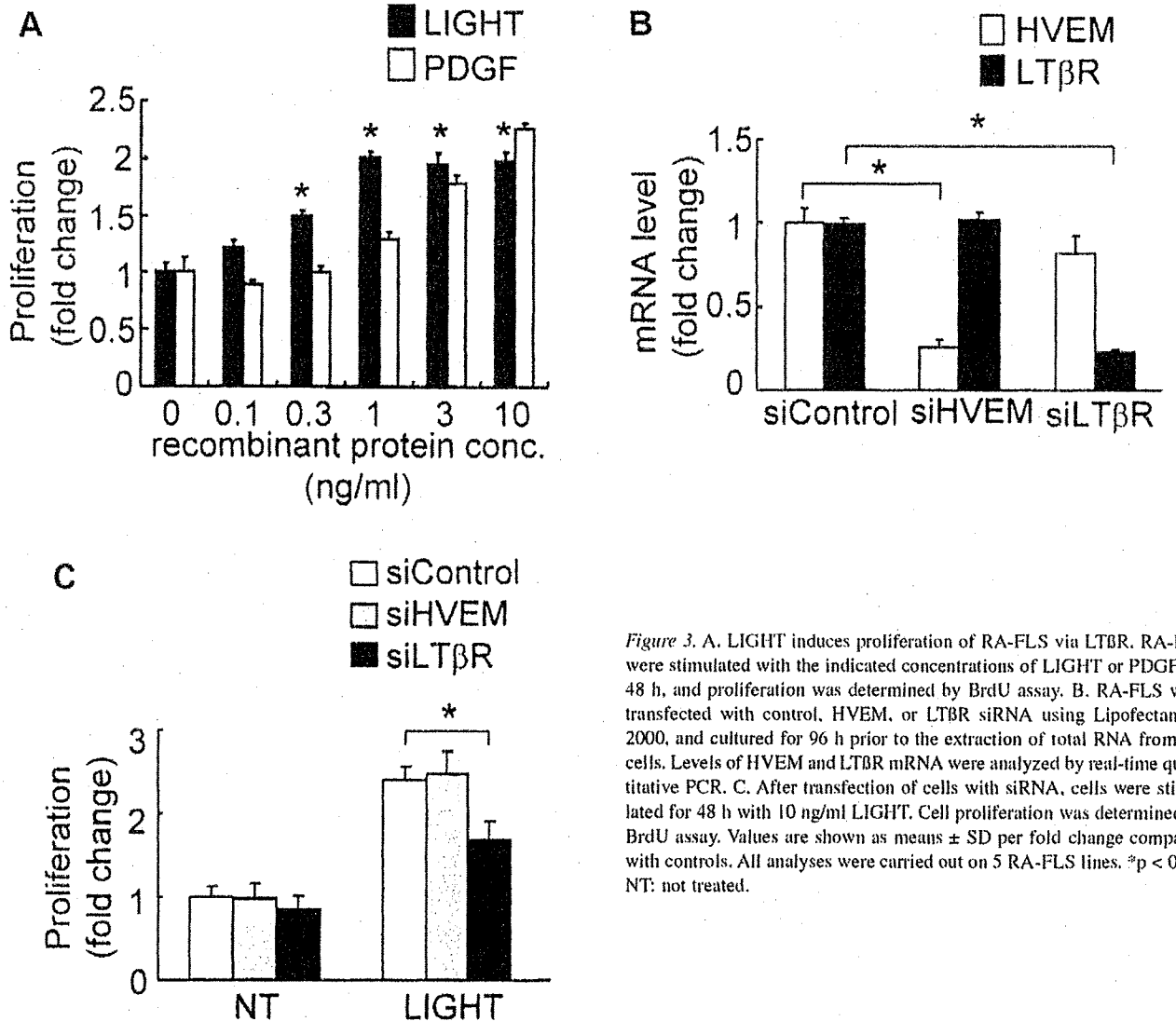

Figure 3. A. LIGHT induces proliferation of RA-FLS via LTßR. RA-FLS were stimulated with the indicated concentrations of LIGHT or PDGF for 48 h, and proliferation was determined by BrdU assay. B. RA-FLS were transfected with control, HVEM, or LTßR siRNA using Lipofectamine 2000, and cultured for 96 h 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.

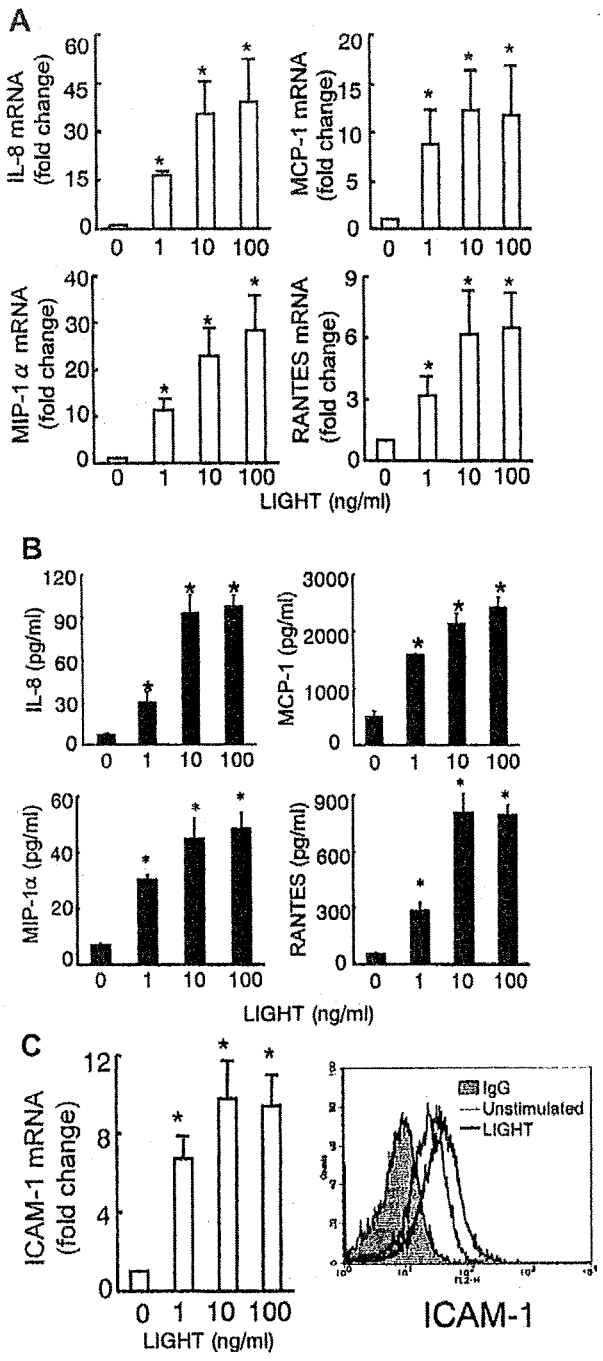

Figure 4. Upregulation of IL-8, MCP-1, MIP-1α, RANTES, and ICAM-1 expression in RA-FLS by LIGHT. A. RA-FLS were stimulated with the indicated concentrations of LIGHT for 3 h, and real-time quantitative PCR was performed to determine levels of IL-8, MCP-1, MIP-1α, and RANTES mRNA expression. Values are shown as means ± SD per fold change compared with controls. B. RA-FLS were stimulated with the indicated concentrations of LIGHT for 72 h. Concentrations of IL-8, MCP-1, MIP-1α, and RANTES in cell culture supernatants were determined by multiplex bead array assays. Values are shown as means ± SD pg/ml. C. RA-FLS were stimulated with the indicated concentrations of LIGHT for 3 h, and real-time quantitative PCR was performed to determine levels of ICAM-1 mRNA expression. ICAM-1 surface expression on RA-FLS was detected by flow cytometry after stimulation with 10 ng/ml LIGHT for 24 h. All analyses were carried out on 4 RA-FLS lines; flow cytometry profiles of one representative result are shown. *$p < 0.05$.

ed with LIGHT (Figure 4C). Similar increases in VCAM-1 mRNA and protein expression were also seen when stimulated with LIGHT (data not shown). Moreover, we investigated whether knockdown of HVEM or LTβR suppressed this series of LIGHT-induced gene expression in RA-FLS. Compared with control siRNA, LTβR siRNA, but not HVEM siRNA, significantly decreased the expression of IL-8, MCP-1, and ICAM-1 mRNA induced by LIGHT (Figure 5). Similarly, LTβR siRNA decreased the LIGHT-induced expression of IL-1ß, IL-6, GM-CSF, RANTES, and MIP-1α mRNA in FLS (data not shown).

Activation of NF-κB in RA-FLS via LTβR by LIGHT. It is known that activation of NF-κB has a key role in inflammatory disease[23]. Several studies have shown that LIGHT activates the transcription factor NF-κB in different cell types[7,9,13,24-26]. To investigate the involvement of NF-κB in LIGHT-induced gene expression, we examined the effect of the NF-κB inhibitor PDTC on the expression of IL-8, MCP-1, and ICAM-1 by real-time quantitative PCR. PDTC completely abolished the LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 (Figure 6A). The LIGHT-induced expression of IL-1ß, IL-6, GM-CSF, RANTES, and MIP-1α mRNA in RA-FLS was also inhibited by PDTC treatment (data not shown). The concentration of PDTC used in these experiments had no cytotoxic effect, as demonstrated by cell viability studies using trypan blue exclusion, which showed that > 95% of cells remained viable over the entire period of the experiment (data not shown).

In the immunocytofluorescence analysis using anti-NF-κB p65 mAb, enhanced nuclear translocation of NF-κB p65 was observed in LIGHT-stimulated RA-FLS (Figure 6B). Further, Western blotting using anti-IκBa mAb showed that IκBa degradation was induced by LIGHT, and that IκBa degradation was inhibited by LTβR siRNA, but not by HVEM siRNA (Figure 6C).

DISCUSSION

We observed that LIGHT, but not HVEM or LTβR, is overexpressed in the synovial tissues of patients with RA compared with those of patients with OA. The expression of LIGHT was not detected in RA-FLS, which comprise one of the major components of the RA synovium. RA synovium is histologically characterized by prominent infiltration of macrophages and lymphocytes[27]. Although LIGHT has been supposed to be produced by activated T lymphocytes *in vitro*[4,5], a recent study reported that LIGHT was overexpressed in CD68-positive macrophages in RA synovial tissue compared with those in OA synovial tissue, and that expression levels of LIGHT were low in areas rich in lymphocytes[19]. Thus, macrophages rather than FLS and lymphocytes could be the major source of LIGHT in the RA synovium.

We further demonstrated that *in vitro*-cultured RA-FLS express HVEM and LTβR, which implies that RA-FLS are

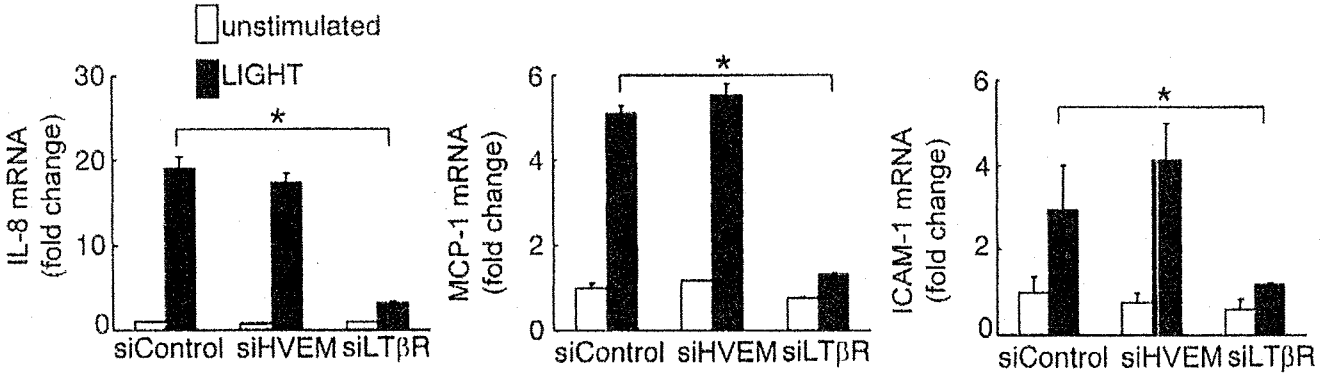

Figure 5. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 in RA-FLS via LTßR. RA-FLS were transfected with control, HVEM, or LTßR siRNA using Lipofectamine 2000. After 96 h incubation, cells were stimulated with 10 ng/ml LIGHT for an additional 3 h. Levels of IL-8, MCP-1, and ICAM-1 mRNA were analyzed by real-time quantitative PCR. Values are shown as means ± SD per fold change compared with controls. All analyses were carried out on 4 RA-FLS lines. *$p < 0.05$.

target cells of LIGHT. Indeed, we first showed that LIGHT had a stronger RA-FLS growth-promoting activity than PDGF, in lower concentrations. The proliferation of RA-FLS is one of the most critical pathological changes in RA. Thus, our findings suggest that increased expression of LIGHT might lead to the synovial hyperplasia of RA. Anticytokine therapies targeting TNF-α, IL-1ß, and IL-6 have been used to treat patients with RA, and it has been demonstrated that such treatments may suppress the accompanying bone destruction as well as the synovitis[28,29]. In addition, recent studies have indicated that LIGHT reduces Fas-mediated apoptosis in FLS[30], that LIGHT may function as a mediator of bone resorption through the induction of osteoclastogenesis[31], and that LTßR-Ig protein blocks the induction of experimental arthritis in mice[18]. Thus, a neutralizing antibody against LIGHT could be a useful tool for inhibition of synovial hyperplasia and bone destruction in RA.

The enhanced effects of LIGHT on RA-FLS proliferation were significantly inhibited by LTßR siRNA, but not by HVEM siRNA, suggesting that LTßR, rather than HVEM, is involved in the LIGHT-induced proliferation of RA-FLS. The exact mechanism by which LIGHT influences RA-FLS proliferation through LTßR is unknown. A potential mechanism underlying RA-FLS proliferation induced by LIGHT may involve cell-cycle regulators, including cyclin-dependent kinases (CDK). The mammal cell cycle is controlled by holoenzymes composed of a catalytic CDK and regulatory cyclin. The expression level of p21 was reduced in RA synovial linings and FLS compared with the level in patients with OA[32]. Overexpression of p21 or p16 by adenoviral-mediated delivery suppresses FLS growth *in vitro*[33,34]. Further, LIGHT induces cell proliferation, downregulates the CDK inhibitors p21, p27 and p53, and inversely upregulates cyclin D and Rb hyperphosphorylation in vascular smooth muscle cells[13]. Thus, it is possible that LIGHT promotes FLS proliferation by shortening the cell cycle of FLS in RA. Wang, *et al* reported that LTßR-null mice show reduced BrdU incorporation in dendritic cells[35]. This supports our claim that LTßR signaling is involved in the proliferation of RA-FLS.

We observed that LIGHT also induces the production of inflammatory cytokines and chemokines and expression of adhesion molecules on RA-FLS. Inflammatory cytokines and chemokines induce the migration of cells and release of mediators of inflammation and angiogenesis, and could be involved in the pathogenesis of RA[1,2,36]. The increased expression of ICAM-1 and VCAM-1 adhesion molecules on activated endothelial cells enhances the recruitment of monocytes, lymphocytes, and neutrophils, leading to inflammation. These findings indicate that LIGHT might play an important role in inflammation in the synovial lining layer, as well as in its hyperplasia. A recent study revealed that LIGHT upregulates the expression of ICAM-1, VCAM-1, and IL-6 in RA-FLS via NF-κB activation[30,37]. Although these reports are consistent with our present results, it has not been clear which of 2 receptors is involved in the induction of these genes in FLS. Our knockdown analysis using siRNA revealed that LIGHT induces proliferation and gene expression by signaling via LTßR, but not HVEM. Braun, *et al* have shown that LTßR is expressed on RA-FLS, and that LTa1ß2, a ligand for LTßR, induces expression of inflammatory cytokines, chemokines, and ICAM-1[38]. This supports our claim that LTßR signaling is involved in the activation of RA-FLS. The NF-κB transcription factor is certainly involved in cytokine- and chemokine-driven responses and is a point of convergence for several upstream proinflammatory pathways[23]. Indeed, NF-κB activation appears to be an important factor in RA, as the expression of NF-κB is enhanced in lining cells[39,40] and in the cartilage-pannus junction in the RA synovium[41]. In our study, treatment with PDTC blocked LIGHT-induced IL-8, MCP-1, and ICAM-1 expression, suggesting that the effects of LIGHT are mediated through NF-κB. The involvement of NF-κB in LIGHT-induced proinflammatory responses was further confirmed

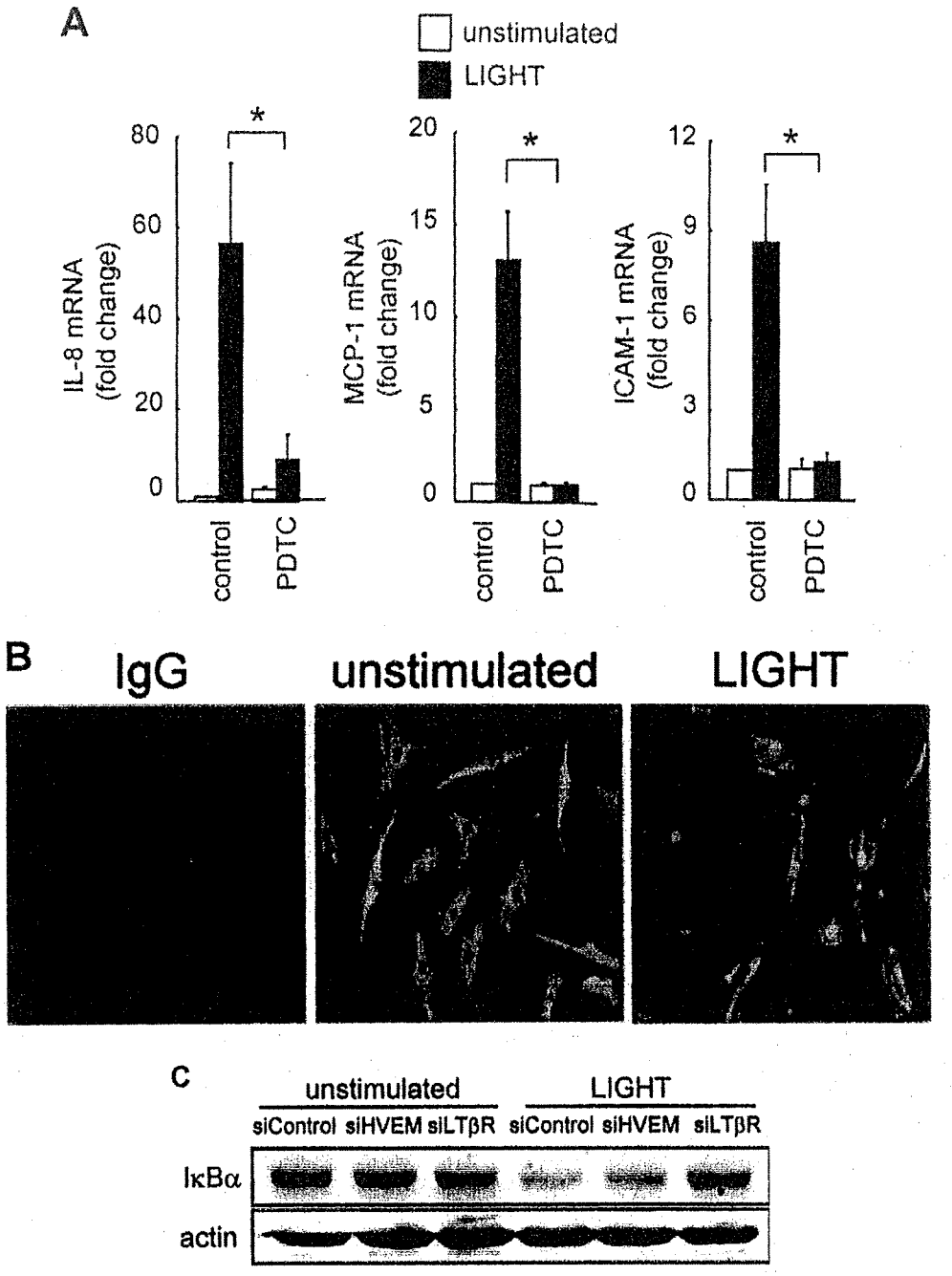

Figure 6. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 through NF-κB-mediated pathways. A. FLS were stimulated with 10 ng/ml LIGHT for 3 h with or without preincubation for 30 min with 30 μM PDTC. Levels of IL-8, MCP-1, and ICAM-1 mRNA were analyzed by real-time quantitative PCR. Values are shown as means ± SD per fold change compared with control. All analyses were carried out on 4 RA-FLS lines. *$p < 0.05$. B. Immunofluorescence staining for NF-κB p65 in RA-FLS. Control in which primary antibodies were replaced with control IgG (left panel); unstimulated RA-FLS (middle); and RA-FLS stimulated with 10 ng/ml LIGHT for 30 min (right). Results are representative of 2 experiments using 2 FLS lines. C. 96 h after siRNA transfection, cells were stimulated with 10 ng/ml LIGHT for 40 min. IκBa degradation was analyzed by immunoblotting. Results are representative of 2 experiments using 2 RA-FLS lines.

by the LIGHT-induced nuclear translocation of NF-κB p65. Moreover, LIGHT induced IκBa degradation in RA-FLS, an effect that was inhibited by LTβR siRNA, but not by HVEM siRNA. These findings are consistent with studies showing that LTβR ligation can lead to activation of NF-κB[24,42-45]. However, it is unknown why LIGHT prefers the LTβR signaling pathway in RA-FLS, even though HVEM is also expressed on these cells.

We have demonstrated that LIGHT is overexpressed in RA synovial tissues and SF. LIGHT induced increased production of inflammatory cytokines, chemokines, and adhesion molecules through NF-κB activation, as well as proliferation of RA-FLS. These findings indicate 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. Therefore, regulation of LIGHT-LTßR signaling may represent a new therapeutic target for the treatment of RA.

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Inhibition of Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase Substrate-1 Reduces the Severity of Collagen-Induced Arthritis

KONAGI TANAKA, TATSUYA HORIKAWA, SATSUKI SUZUKI, KAZUTAKA KITAURA, JUNKO WATANABE, AKITO GOTOH, NORIYUKI SHIOBARA, TSUNETOSHI ITOH, SHOJI YAMANE, RYUJI SUZUKI, NAOSHI FUKUI, and TAKAHIRO OCHI

ABSTRACT. ***Objective.*** To investigate whether the blockade of Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1) has any therapeutic effects on rheumatoid arthritis.

Methods. A functional blocking monoclonal antibody for SHPS-1 (anti-SHPS-1 mAb) was administered at various doses to collagen-induced arthritis (CIA) mice, and severity of the arthritis was evaluated by clinical and histological scores of the limbs. To clarify the mechanisms of action of the antibody, the serum concentration of anti-type II collagen antibody was measured in those mice, and *in vitro* experiments were conducted to determine the effects of the antibody on the induction of osteoclasts and the release of cytokines from mouse spleen cells.

Results. Compared with mice given control IgG, the administration of anti-SHPS-1 mAb significantly reduced the severity of inflammation and destruction of bone and cartilage in CIA mice. This therapeutic effect was observed even when the antibody treatment was started after the onset of arthritis. The appearance of anti-type II collagen antibody in CIA mice was not altered by the antibody treatment. In *in vitro* experiments, the anti-SHPS-1 mAb significantly inhibited osteoclastogenesis of bone marrow cells, and significantly reduced the release of interleukin 1ß (IL-1ß), IL-2, IL-12, interferon-γ, and tumor necrosis factor-α, but not that of IL-4 or IL-10, from the spleen cells after stimulation with concanavalin A.

Conclusion. Administration of a monoclonal antibody for SHPS-1 reduced the severity of arthritis in CIA mice. Regulation of biological functions of SHPS-1 may be a novel and potent strategy to treat patients with rheumatoid arthritis. (First Release Nov 1 2008; J Rheumatol 2008;35:2316–24; doi:10.3899/jrheum.080369)

Key Indexing Terms:
SHPS-1 RHEUMATOID ARTHRITIS THERAPEUTICS

From the Clinical Research Center, National Hospital Organization, Sagamihara Hospital, Sagamihara, Kanagawa; Section of Biological Science, Research Center for Odontology, Nippon Dental University, Chiyoda-ku, Tokyo; Department of Immunology and Embryology, Tohoku University School of Medicine; and Division of Dermatology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan.

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K. Tanaka, MD; K. Kitaura, DVM; J. Watanabe, MD, PhD; A. Gotoh, DDM; N. Shiobara, BS; S. Yamane, PhD; R. Suzuki, DVM, PhD; N. Fukui, MD, PhD; T. Ochi, MD, PhD, Clinical Research Center, National Hospital Organization, Sagamihara Hospital; T. Horikawa, MD, PhD, Division of Dermatology, Department of Clinical Molecular Medicine, Kobe University Graduate School of Medicine; S. Suzuki, DVM, PhD, Section of Biological Science, Research Center for Odontology, Nippon Dental University; T. Itoh, MD, PhD, Department of Immunology and Embryology, Tohoku University School of Medicine.

Address reprint requests to Dr. N. Fukui, Clinical Research Center, National Hospital Organization, Sagamihara Hospital, Sakuradai 18-1, Sagamihara, Kanagawa 228-8522, Japan. E-mail: n-fukui@sagamihara-hosp.gr.jp

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Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects synovial joints systemically[1]. In spite of numerous investigations, there is still no fundamental therapy to treat RA. The hallmarks of this disease are synovial inflammation and destruction of articular cartilage and subchondral bone. Synovial tissue in rheumatoid joints is characterized by a marked intimal-lining hyperplasia due to increased numbers of macrophages and fibroblast-like synoviocytes. Accumulation of T cells, plasma cells, and other types of inflammatory cells in the synovial lining is also obvious[1,2]. Those cells produce cytokines such as interleukin 1ß (IL-1ß), tumor necrosis factor-α (TNF-α), and IL-6, which promote the expression of proteinases that cause tissue degradation in the joints. As well, those cytokines are responsible for the destruction of bone in the disease through the induction of osteoclasts. In joints involved in RA, osteoclasts are derived from precursor cells of the monocyte-macrophage lineage in the presence of several cytokines such as macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-κB ligand (RANKL), IL-1ß, and

TNF-α[3,4]. Therefore, the synovial hyperplasia and bone absorption around the joints in RA is closely correlated through the activity of those cytokines.

Src homology 2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1), also known as signal regulatory protein α1 (SIRPα1)[5], a brain Ig-like molecule with tyrosine-based activation motifs[6], macrophage fusion receptor[7], and p84 neural adhesion molecule[8], is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. The extracellular domain of SHPS-1 consists of 3 parts, the amino-terminal Ig variable (IgV) region and 2 Ig constant (IgC) regions, although the latter may be removed by alternative splicing. The intracellular domain of SHPS-1 contains 2 immunoreceptor tyrosine-based inhibitory motifs, suggesting that it transmits signals for inactivation[9]. SHPS-1 is expressed by macrophages, dendritic cells (DC), neutrophils, and neurons[10]. CD47, another transmembrane glycoprotein belonging to the immunoglobulin superfamily, is a known ligand for SHPS-1[11-14]. CD47 is present on virtually all kinds of hemopoietic cells, including T cells, B cells, and neutrophils, as well as endothelial cells. SHPS-1 binds to CD47 via the IgV domain[11,15-18], which causes its various biological activities[19]. Interactions between SHPS-1 and CD47 are important for cellular fusion or multinucleation, processes necessary for osteoclast formation[7,11,20]. At this time, it is controversial whether CD47-SHPS-1 interaction plays a key role in the activation of T cells and the acquisition of cell-mediated immunity[21,22], or downregulates the activation of T cells by DC[16,17,23,24]. Considering that T cell activation and osteoclast formation are critical events in the pathology of RA, we speculated that inhibiting the interaction between SHIP-1 and CD47 might be beneficial in the treatment of RA.

We previously reported that an antibody against SHPS-1 effectively inhibits the migration of epidermal DC and Langerhans cells, resulting in decreased development of the delayed-type hypersensitivity response[23,24]. Using this SHPS-1 antibody, we conducted a series of *in vivo* and *in vitro* experiments to clarify the role of SHPS-1 in the pathology of RA. The results not only suggested the significance of SHPS-1 in RA, but also indicated a possibility that the administration of the anti-SHPS-1 antibody could be an effective strategy to treat patients with RA.

MATERIALS AND METHODS

Collagen-induced arthritis (CIA). Our study was performed under the approval of the Institutional Review Board of the National Hospital Organization, Sagamihara Hospital. The induction of arthritis in mice was based on a described method[25,26]. Briefly, bovine type II collagen (CII; Collagen Research Center, Tokyo, Japan) was dissolved at 2 mg/ml in 10 mM acetic acid, and was emulsified by mixing with an equal volume of Freund's complete adjuvant (Nippon BD, Tokyo, Japan). Five-week-old male DBA/1JN mice (Charles River Japan, Yokohama, Japan) were immunized by intradermal injection of the emulsion (100 μl) at the base of their tails. Twenty-one days later, the same volume of emulsion was injected again in the same manner as a booster. With this protocol, arthritis developed in 100% of mice at around 4 weeks after the initial immunization.

Treatment with anti-SHPS-1 monoclonal antibody. Hybridoma cells producing anti-mouse SHPS-1 (P84) monoclonal antibody (anti-SHPS-1 mAb) was a generous gift from Dr. C.F. Lagenaur (Pittsburgh University, Pittsburgh, PA, USA)[27]. Ascites fluid was collected from BALB/c nu/nu mice that had been injected intraperitoneally with hybridoma cells, and the p84 antibody was purified from the ascites using a protein A column[23,24]. Rat IgG1 (Sigma Diagnostics, St. Louis, MO, USA) was used as a control immunoglobulin (control IgG). The experiments were performed according to either of the following 2 protocols. In Protocol A, CIA was induced as described, and anti-SHPS-1 mAb, control IgG, or methotrexate (MTX; Wyeth, Tokyo, Japan) were given to the mice every other day from Day 21 (the day of second immunization) until Day 31, 6 times in total. The antibody or control IgG was dissolved in 200 μl phosphate buffered saline (PBS) and injected intraperitoneally. MTX was administered orally. In Protocol B, the administration of the SHPS-1 antibody, control IgG, or MTX was started on Day 29 (8 days after the second immunization) and was repeated 6 times until Day 39, on every other day.

Evaluation of arthritis. The development of arthritis was determined by the presence of redness or swelling in any of the 4 limbs. If these signs were observed in at least 1 limb, the mouse was determined to be positive for arthritis. The incidence of arthritis was defined in each treatment group by the ratio of the number of positive mice to the total number of mice in the group. The occurrence and severity of arthritis were evaluated macroscopically on each hind limb in each mouse by scores from 0 (normal) to 3 (joint deformity or rigidity). The sum of scores for both hind limbs (0 to 6) was used as the clinical score for that animal. The severity of arthritis was also evaluated by the average thickness of footpads of the right and left hind limbs, which was measured using a caliper. The body weight was recorded daily throughout the experimental period.

Histological evaluation. For histology, the mice were sacrificed and their hind limbs were amputated, fixed with 10% formaldehyde, decalcified with EDTA, and embedded in paraffin. Four-micron-thick sections of the ankle and toe joints were prepared in a sagittal plane and were stained with hematoxylin and eosin (H&E). Using a light microscope, the severity of inflammation and joint destruction was assessed semiquantitatively based on a described procedure[28]. The severity of inflammatory change was assessed as a score from 0 to 4, considering the extent of inflammatory cell infiltration, synovial lining-cell hyperplasia, and pannus formation. Further, the severity of bone destruction was evaluated by scores from 0 to 5, according to the following criteria: 0 = normal; 1 = minimal loss of cortical bone at a few sites; 2 = mild loss of cortical and trabecular bone at a few sites; 3 = moderate bone loss at multiple sites; 4 = marked bone loss at multiple sites; and 5 = marked bone loss with distortion of the profile of the remaining cortical surface.

Measurement of anti-type II collagen antibodies. The concentration of anti-CII antibodies in the sera of mice was determined by ELISA[29]. For this, 96-well flat-bottom plates (Iwaki, Tokyo, Japan) were coated with 50 μl CII (2 μg/ml in PBS) overnight at 4°C. Prior to use, the wells were blocked with PBS containing 1% (w/v) bovine serum albumin at 37°C for 1 h. Sera were then diluted appropriately in PBS containing 0.05% (v/v) Tween-20, and were added to the wells. After incubation at 37°C for 2 h, levels of CII-specific IgG2a were measured using biotin-labeled rat anti-mouse IgG2a (R&D Systems, Minneapolis, MN, USA). The amount of biotin-labeled antibody bound was determined by color reaction using streptavidin-peroxidase coupled with peroxidase substrate (Substrate Reagent Pack, Stop Solution; R&D Systems). All measurements were performed in triplicate and averages were calculated.

Effect of antibodies on osteoclast formation from murine bone marrow cells. Bone marrow cells were obtained from 6-week male Balb/c mice, and were plated in wells of 24-well plates at 1×10^6 cells per well. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Invitrogen), glut-

amine, streptomycin, penicillin, macrophage colony-stimulating factor (M-CSF, 50 ng/ml; R&D Systems), and RANKL (30 ng/ml; R&D Systems). In this experiment, the effect of anti-SHPS-1 mAb was compared with that of control IgG. Immediately after plating, anti-SHPS-1 mAb or control IgG was added to the media at graded concentrations, and the cells were cultured for 5 days. The medium was then removed and the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using a commercial kit (Sigma Diagnostics Acid Phosphatase Kit; Sigma Diagnostics). TRAP-positive multinuclear cells that had more than 3 nuclei were counted as osteoclasts. The experiment was then repeated with an anti-CD47 monoclonal antibody, and the results were compared. The antibody against CD47 (miap301; anti-CD47 mAb) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Effect of antibodies on cytokine production by murine spleen cells. Spleen cells were obtained from 6-week male Balb/c mice, and were plated at a density of 5×10^5 cells per well in 96-well plates. Cells were maintained in RPMI-1640 medium (Invitrogen) containing 10% FBS, glutamine, streptomycin, and penicillin. One hour after plating, anti-SHPS-1 mAb, anti-CD47 mAb, or control IgG were added to the media at the indicated concentrations. One hour after the addition of antibody or control IgG, the cells were stimulated by 5 μg/ml concanavalin A (ConA; Wako, Osaka, Japan). After 24 h, the supernatants were collected and the concentrations of IL-1ß, IL-2, IL-12, interferon-γ (IFN-γ), TNF-α, IL-4, and IL-10 in the media were determined by ELISA (R&D Systems).

Statistical analysis. For parametric data, statistical significance was determined by 2-way analysis of variance and contrast as a post hoc test. Nonparametric data were analyzed using the Kruskal-Wallis test, and the Dunn procedure was used as a post-hoc test when necessary. Log-rank test was used to determine the difference in the incidence of arthritis. The level of significance was set at $p < 0.05$.

RESULTS

Anti-SHPS-1 antibody reduces incidence and severity of CIA. Six groups of mice, each consisting of 10 animals, were prepared, and CIA was induced in 5 of those groups. The other group was maintained without any treatment and served as a non-CIA control. Each of the 5 CIA-induced groups received 6 consecutive administrations of either anti-SHPS-1 mAb (1, 10, or 100 μg), control IgG (100 μg), or MTX (3 mg), following Protocol A, in which the treatments were started on the day of the second immunization. The incidence of arthritis was significantly reduced by the SHPS-1 antibody treatment (Figure 1A). While arthritis developed in all mice treated with the control IgG, the administration of 10 μg or 100 μg anti-SHPS-1 mAb reduced the incidence by 20% and 30%, respectively, although no significant reduction was observed with 1 μg anti-SHPS-1 mAb. The decline in the incidence was significant in the 10 and 100 μg antibody-treated groups (both at $p < 0.05$). The incidence of arthritis was dramatically reduced by MTX, indicating that the immune response was profoundly involved in the development of arthritis.

In untreated mice, the body weight increased by 1.8 g on average between Day 21 and Day 41 (Figure 1B). Among the 5 experimental groups, the mice treated with control IgG lost 1.0 g in weight during that period, likely due to the general exhaustion associated with the arthritis. This decline in

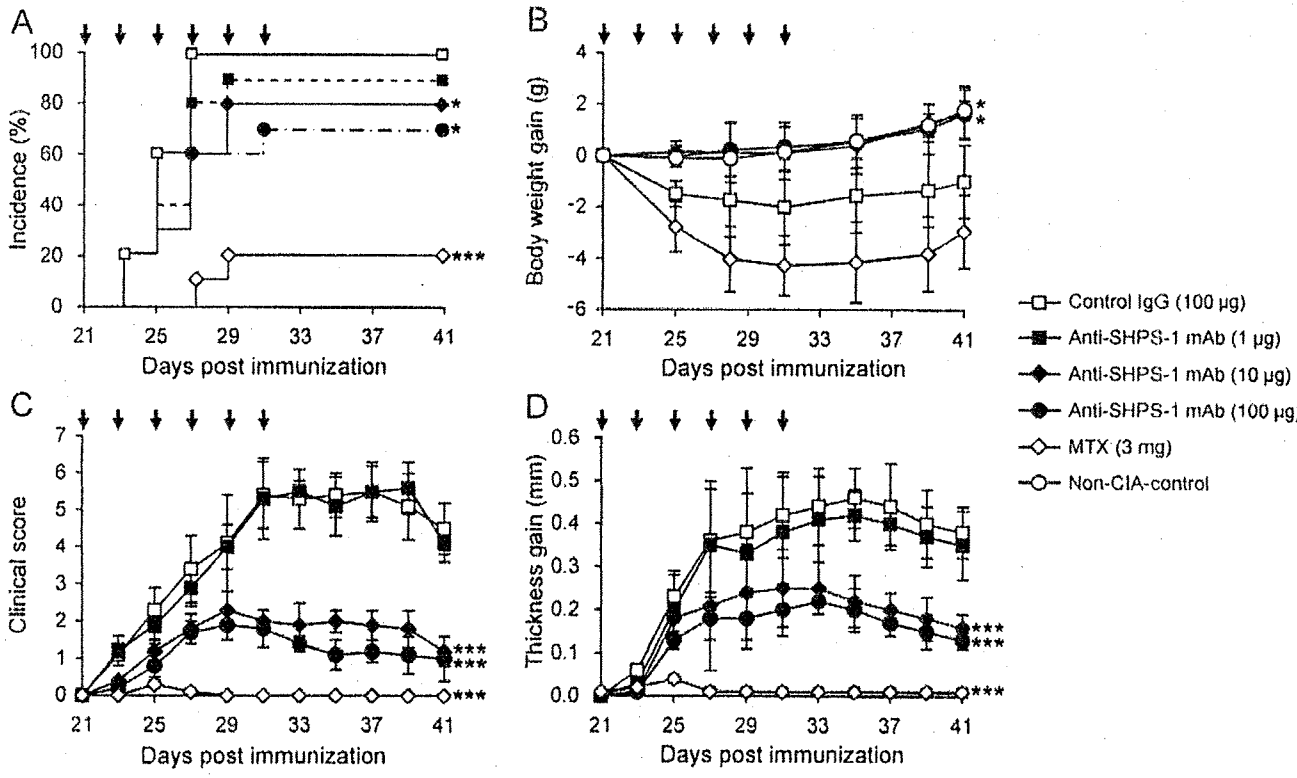

Figure 1. Incidence and severity of arthritis in CIA mice treated with anti-SHPS-1 mAb. Mice were given graded doses of antibody, and the incidence and severity of arthritis were compared with those in control IgG- or MTX-treated mice. Incidence (A), gain of body weight (B), clinical score (C), and increase of footpad thickness (D) from Day 21 to Day 41 are shown. Black arrows indicate the timing of antibody administration. Data are mean ± SD. *$p < 0.05$ and ***$p < 0.001$ compared with control IgG.