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Supplementary material

The following supplementary material is available for this article online:

Table S1 Primers used for real-time, quantitative RT-PCR analysis

Bridge over troubled water: milk fat globule epidermal growth factor 8 promotes human monocyte-derived macrophage clearance of non-blebbing phosphatidylserine-positive target cells

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Dear Editor,

Externalization of phosphatidylserine (PS) serves as a recognition signal for neighboring phagocytes,[1] and studies published 10 years ago demonstrated that blebs on the surface of apoptotic cells are sites of enhanced procoagulant activity, perhaps owing to the aggregation of PS molecules on these membrane protrusions.[2] However, the putative importance of plasma membrane blebbing for engulfment of dying cells has not been investigated. Phagocytes may not only recognize PS directly, but also secrete so-called opsonins or bridging molecules that bind PS on the dying cell, thereby facilitating corpse clearance. Indeed, deletion in mice of milk fat globule epidermal growth factor 8 (MFG-E8), a PS-binding bridging molecule,[3] results in impaired engulfment of apoptotic cells and autoimmune disease, thus demonstrating the physiological importance of PS-mediated cell clearance.[4] Moreover, the involution of mammary glands is impaired in the absence of MFG-E8, indicating that this bridging molecule also plays a role in the removal of apoptotic cells during organ remodelling.[5] In the present study, we show that plasma membrane blebbing and PS externalization can be dissociated during apoptosis and, moreover, that non-blebbing target cells are less efficiently ingested when compared with their blebbing counterparts. MFG-E8 was found to restore clearance of non-blebbing target cells by human macrophages.

Fas stimulation of Jurkat T cells induced vigorous blebbing of the plasma membrane and activation of caspase-3-like enzymes (Figure 1a and b). These cells also expressed PS on the cell surface, as demonstrated by labelling with fluorescein isothiocyanate (FITC)-conjugated annexin V (Figure 1c). Preincubation of Jurkat cells with cytochalasin B (CB), a fungal metabolite that prevents actin polymerization and promotes the disruption of the microfilament network, completely abrogated blebbing, yet failed to diminish other features of the apoptotic program. Similar results were obtained when Y-27632, a specific inhibitor of ROCK I kinase,[6] was tested (Supplementary information, Figure S1). In contrast, the pan-caspase inhibitor, zVAD-fmk, prevented all of the aforementioned apoptotic events. To address whether plasma membrane blebbing is reversible, CB was added to the medium after 3 h of treatment with agonistic anti-Fas antibodies, at which time marked blebbing was seen. Jurkat blebbing was completely reversed under these conditions, whereas poly(ADP-ribose) polymerase cleavage and PS exposure remained unaffected (Supplementary information, Figure S2). In addition, when Jurkat cells were triggered to undergo apoptosis by staurosporine (STS), an inhibitor of protein kinases, no blebbing was seen despite a considerable degree of PS exposure (Figure 1d), consistent with the notion that apoptotic membrane blebbing is controlled by phosphorylation.[7] Blebbing, thus, appears to be a dynamic event that can be divorced from other features of apoptosis even under conditions when cells are committed to die.

Next, we aimed to test the importance of plasma membrane blebbing for phagocytosis. Fas-stimulated Jurkat cells were readily ingested upon cocultivation with the murine J774A.1 macrophage cell line (Supplementary information, Figure S3); however, the degree of phagocytosis was significantly reduced when Jurkat cells were pretreated with CB. Comparable data were obtained when apoptotic Jurkat cells pretreated or not with CB were added to the human THP.1 macrophage cell line (Supplementary Figure S3). Extensive washing of Jurkat cells was performed before cocultivation in order to exclude a direct effect of residual amounts of CB on macrophages. Pretreatment of Fas-triggered Jurkat cells with Y-27632, an inhibitor of membrane blebbing, also suppressed phagocytosis (Supplementary Figure S1). Moreover, STS-triggered (non-blebbing) Jurkat cells were less efficiently engulfed by THP.1 macrophages when compared to Fas-triggered (blebbing) target cells (Supplementary Figure S3). These results indicate that PS externalization in the absence of blebbing may be insufficient to render dying cells appetizing to phagocytes. One explanation for this conundrum is that the pattern of PS distribution on the surface of apoptotic cells may determine whether PS is recognized by its corresponding phagocytic receptor(s). CED-1, a phagocytosis receptor in *Caenorhabditis elegans*, was previously shown to cluster around dying cells, and it was concluded that this receptor clustering was a direct response to a recognition signal, possibly PS, that distinguishes cell corpses from living cells.[8] Furthermore, studies of galectin-1-treated human T-cell lines have shown that PS localizes to apoptotic blebs protruding from the cell surface.[9] In line with these observations, our confocal microscopic analysis of Fas-triggered Jurkat cells are suggestive of a colocalization of annexin V with cholesterol-rich lipid raft domains in the plasma membrane (Supplementary information, Figure S4). In sum, although we cannot exclude the possibility that CB and Y-27632 inhibit

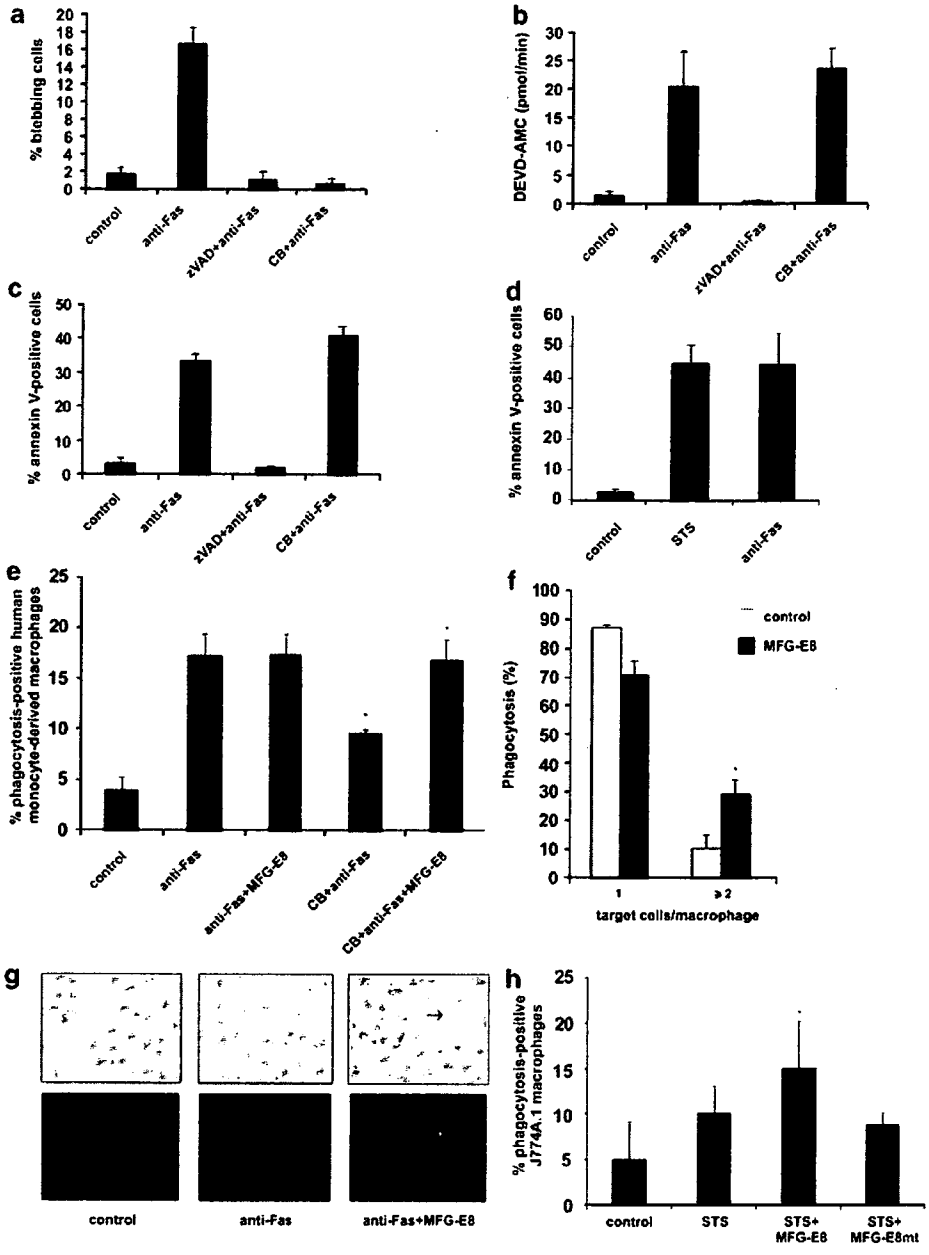

Figure 1 MFG-E8 restores engulfment of non-blebbing target cells. (**a**) Jurkat cells were incubated in the presence or absence of the pan-caspase inhibitor, zVAD-fmk (10 μM, Enzyme Systems Products, Livermore, CA, USA) or the cytoskeleton-disrupting agent, CB (5 μg/ml, Sigma, St. Louis, MO, USA) for 30 min and then treated or not with agonistic anti-Fas antibodies (250 ng/ml, Medical & Biological Laboratories, Nagoya, Japan) for 4 h. Blebbing cells were counted under the light microscope. (**b**) Jurkat cells were stimulated as above and cell lysates were mixed with the fluorogenic substrate, DEVD-AMC (50 μM, Peptide Institute, Osaka, Japan). The rate of AMC release, indicative of caspase-3 activation, was then determined as described.[1] Data shown in (**a** and **b**) are mean values $\pm$ S.D. ($n=3$). (**c**) Flow cytometric detection of PS exposure in Jurkat cells treated as above using FITC-conjugated annexin V (Oncogene Research Products, Cambridge, MA, USA). Cells were analyzed on a FACScan operating with CellQuest software (BD Biosciences, San Jose, CA, USA). The percentages of annexin V-positive and propidium iodide-negative cells are depicted. Data shown are mean values $\pm$ S.D. ($n=4$). (**d**) Quantification of PS exposure in Jurkat cells stimulated with STS (1 μM, Sigma) for 2 h or anti-Fas antibodies (250 ng/ml) for 4 h was performed as described above. Data are depicted as mean values $\pm$ S.D. ($n=5$). The percentage of blebbing cells in the STS-treated samples was negligible (0.4 ± 0.3). (**e**) Jurkat cells pretreated or not with CB (5 μg/ml) were incubated in the presence or absence of Fas-antibodies (250 ng/ml) for 4 h and co-cultured with HMDMs. Recombinant murine MFG-E8 (1 μg/ml) was added to the co-cultures and the percentages of phagocytosis-positive macrophages were determined as described.[1] (**f**) Co-cultivation of apoptotic Jurkat cells and HMDMs was performed as in (**e**) and the percentages of macrophages that carried 1 or $\geqslant 2$ target cells in the presence or absence of MFG-E8 (1 μg/ml) were determined. Data shown in (**e** and **f**) are mean values $\pm$ S.D. ($n=3$). $^*P<0.01$. (**g**) Representative images depicting HMDM engulfment of TAMRA-labelled target cells in the presence or absence of MFG-E8 (1 μg/ml). Jurkat cells were preincubated with CB (5 μg/ml) before Fas stimulation. Phase contrast and fluorescence images were captured using a Nikon Eclipse E600 microscope equipped with a DS-5M digital camera operating with NIS elements software. Arrows indicate a 'super-efficient' macrophage that has ingested numerous apoptotic target cells. Original magnification $\times$ 40. (**h**) Jurkat cells treated with STS (1 μM) for 2 h were cocultured with J774A.1 macrophages (known to use the integrin-receptor system for recognition of apoptotic cells)[15] in the presence or absence of MFG-E8 or the D89E mutant and the percentages of phagocytosis-positive macrophages were determined as above. Data are reported as mean values $\pm$ S.D. ($n=3$). $^*P=0.02$

other events besides blebbing that contribute to efficient phagocytosis, our combined data nevertheless suggest that the clustering of PS on membrane protrusions or blebs may play a role in corpse clearance.

MFG-E8 is a PS-binding molecule that is secreted by certain subsets of macrophages and dendritic cells.[3] We reasoned that MFG-E8 should bind to PS molecules on the surface of apoptotic cells irrespective of whether or not these cells are blebbing, and thus serve to promote phagocytosis. Indeed, recombinant FLAG-tagged MFG-E8 was found to bind Fas-stimulated Jurkat cells, but not to untreated cells (data not shown). The degree of MFG-E8 binding to apoptotic Jurkat cells was similar in the presence or absence of CB and correlated with the percentage of PS-positive cells. MFG-E8 was then added to cocultures of Jurkat cells and human monocyte-derived macrophages (HMDMs) that express the $\alpha_V\beta_3$ integrin receptor to which MFG-E8 binds,[10] and the degree of phagocytosis was determined. HMDM-mediated clearance of non-blebbing Jurkat cells was diminished when compared with blebbing Jurkat cells (Figure 1e). MFG-E8 restored phagocytosis of non-blebbing cells, whereas no increase in the percentage of phagocytosis-positive macrophages was seen when MFG-E8 was added to blebbing cells. Previous studies in a murine model system have indicated that MFG-E8 may increase the phagocytic index, that is, the number of apoptotic target cells engulfed per macrophage.[10] Careful enumeration of apoptotic Jurkat cells contained within each macrophage revealed a significant increase in the phagocytic index upon addition of MFG-E8 (Figure 1f). Indeed, numerous instances of highly efficient HMDMs (i.e., macrophages that had ingested $\geqslant 8$ target cells) were observed in MFG-E8-treated cultures (Figure 1g), whereas such cells were never seen in the absence of MFG-E8. Finally, recombinant MFG-E8 also promoted macrophage engulfment of STS-treated Jurkat cells (Figure 1h), thus supporting the notion that uptake of PS-positive, non-blebbing cells is enhanced by this bridging protein. The recombinant D89E mutant form of MFG-E8,[10] however, did not affect macrophage uptake of target cells.

In conclusion, the present study demonstrates that plasma membrane blebbing can be dissociated from other features of the apoptotic program, including the exposure of PS. Furthermore, our data suggest that blebbing may play a role in the recognition and clearance of apoptotic cells by macrophages, insofar as these membrane protrusions may provide a context for the aggregation of PS molecules. Our results are thus congruent with recent studies implicating PS clustering on the cell surface in the selective engulfment of apoptotic versus non-apoptotic cells.[11] The current observation that the PS-binding protein MFG-E8 can restore clearance of non-blebbing target cells by human macrophages could be explained either by the potential ability of this protein to cluster PS molecules on apoptotic cell membranes or by direct effects of MFG-E8 on phagocytes.[12] Finally, although deletion of MFG-E8 in mice results in autoimmune disease,[13] silencing of the expression of several *C. elegans* genes containing motifs similar to those of MFG-E8 fails to produce any significant phenotype (D Xue, personal communication). Hence, although PS-dependent mechanisms of corpse clearance have been conserved through evolution,[14] the involvement of bridging molecules in this process may be specific to mice and men.

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LIGHT induces cell proliferation and inflammatory responses of rheumatoid arthritis synovial fibroblasts via lymphotoxin □À receptor.

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Title page

Title of full-length article: LIGHT induces cell proliferation and inflammatory responses of rheumatoid arthritis synovial fibroblasts via lymphotoxin β receptor.

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A short running footline: synovial activation of LIGHT

Key Indexing Terms: TNFSF14 Rheumatoid Arthritis

Fibroblasts-like synoviocytes Lymphotoxin beta Receptor

Abstract

Objective. To investigate the effects of LIGHT 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 enzyme-linked immunosorbent assay (ELISA), and compared them with those in osteoarthritis (OA) SF. The expression levels of LIGHT and its receptors in RA FLS and synovium were assessed using reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR. RA FLS proliferation was examined by a bromodeoxyuridine (BrdU) assay. The expression of interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) and intercellular adhesion molecule 1 (ICAM-1) was examined by real-time PCR, ELISA, and flow cytometry. The effects of LIGHT on NF-κB activation were investigated using immunofluorescence and Western blotting.

Results. LIGHT was upregulated in both SF and synovium of RA compared with those of OA. Herpes virus entry mediator (HVEM) and lymphotoxin β receptor (LTβR), but not LIGHT, were detected in RA FLS. LIGHT significantly promoted RA FLS proliferation and induced expression of MCP-1, IL-8 and ICAM-1 by RA FLS. Additionally, LTβR small interfering RNA (siRNA), but not HVEM siRNA, inhibited

these effects of LIGHT. LIGHT induced IκBα degradation and NF-κB translocation, and an NF-κB inhibitor suppressed the effects of LIGHT on RA FLS.

Conclusions. Our findings suggest that LIGHT signaling via LTβR plays an important role in the pathogenesis of RA by affecting key process, such as the proliferation and activation of RA FLS. Regulation of LIGHT-LTβR signaling may represent a new therapeutic target for RA treatment.

Introduction

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, aggressively proliferates to form a pannus causing irreversible joint damage in patients with RA. In RA synovial tissue, activated FLS and infiltrating macrophages and lymphocytes produce inflammatory cytokines, including tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β), and IL-6, which 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 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 two members of the TNFR family, herpes virus entry mediator (HVEM,

TNFRSF14) and lymphotoxin β receptor (LTβR, TNFRSF3). HVEM is expressed prominently on monocytes, dendritic cells and lymphocytes (5,8-10), whereas LTβR 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 LTβR-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 (19). These studies have suggested 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.

The aim of this study was to clarify the role of LIGHT in the proliferation and inflammatory response of RA FLS. We demonstrated that the levels of LIGHT in both synovial fluid and synovium were higher in patients with RA than in those with osteoarthritis (OA). In addition, LIGHT signaling via LTβR, but not HVEM, enhanced RA FLS proliferation and induced the expression of inflammatory cytokines, chemokines and adhesion molecules in RA FLS through an NF-κB-dependent signal transduction pathway. We suggest that activation of RA FLS by LIGHT/LTβR signaling may play an important role in the pathogenesis of RA

Patients and Methods

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

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 years). Patients with OA ranged in age from 39 to 90 years (mean ± SD 64.1 ± 14.7 years). 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 according to a previously reported procedure (21). RA FLS were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, streptomycin and L-glutamine. RA FLS from passages 4-9 were used for each

experiment. Synovial fluids were obtained by arthrocentesis from 23 RA patients and 10 OA patients, and after centrifugation at 20,000 × *g* for 10 minutes, 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 synovial fluids 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 2,000 pg/ml, respectively.

RNA extraction, cDNA synthesis and RT-PCR. Total RNA was extracted from synovium and FLS using an RNeasy Micro kit (Qiagen). cDNA was generated from RNA using the Omniscript Reverse Transcriptase (Qiagen). Amplification was performed with 0.5 units of Ex Taq (Takara) in a total reaction volume of 20 μl containing 1× reaction buffer, 200 μM of each dNTP and 10 pmoles of each primer. The sequences of primers used for PCR were as follows: for GAPDH, 5'- GCACCGTCAAGGCTGAGAAC-3' and 5'-ATGGTGGTGAAGACGCCAGT-3'; for LIGHT, 5'-TCACGAGGTCAACCCAGCAG-3' and 5'-CCCAGCTGCACCTTGGAGTAG-3'; for HVEM, 5'- TTTGCTCCACAGTTGGCCTAATC-3' and

5'-CAATGACTGTGGCCTCACCTTC-3'; for LTβR, 5'-ATGCTGATGCTGGCCGTTC-3' and 5'-AGGCTCCCAGCTTCCAGCTA-3'. PCR conditions were as follows: initial denaturation at 95 °C for 1 minute, then 30 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and 72°C for 20 seconds. Ten microliters of the PCR products and 2 μl of loading buffer were run on 2% agarose gels and visualized by staining with ethidium bromide.

Quantitative real-time PCR analysis. cDNA was used 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 follows: for IL-6, 5'-AAGCCAGAGCTGTGCAGATGAGTA-3' and 5'-TGTCCTGCAGCCACTGGTTC-3'; for IL-8, 5'-ACACTGCGCCAACACAGAAATTA-3' and 5'-TTTGCTTGAAGTTTCACTGGCATC-3'; for GM-CSF, 5'-CATGATGGCCAGCCACTACAA-3' and 5'-ACTGGCTCCCAGCAGTCAAAG-3'; for MCP-1, 5'-GCTCATAGCAGCCACCTTCATTC-3' and 5'-GGACACTTGCTGCTGGTGATTC-3'; for RANTES, 5'-ACCAGTGGCAAGTGCTCCAAC-3' and 5'-CTCCCAAGCTAGGACAAGAGCAAG-3'; for MIP-1α, 5'-

TCCGTCACCTGCTCAGAATCA-3' and 5'-AGCACTGGCTGCTCGTCTCA-3'; for VCAM1, 5'-CGTGATCCTTGGAGCCTCAAATA-3' and 5'-GACGGAGTCACCAATCTGAGCA-3'; and for ICAM1, 5'-CCTGATGGGCAGTCAACAGCTA-3' and 5'-ACAGCTGGCTCCCGTTTCA-3'. PCR was performed under the following conditions: initial denaturation at 95°C for 10 seconds, then 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. When SYBR green dye was used to monitor PCRs, melting curves were routinely recorded to verify the singularity of the PCR product. In each sample, the level of cDNA was normalized to the expression 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 hours of preculture, the cells were stimulated for 48 hours by the addition of LIGHT or EGF. Bromodeoxyuridine (BrdU) was added for the last 24 hours of culture, after which the incorporation of BrdU was measured using a cell proliferation enzyme-linked immunosorbent assay (Cell Proliferation ELISA, BrdU; Roche Diagnostic) according to the manufacturer's instructions.

Measurement of cytokines and chemokines in culture supernatants. TNFα, IL-1β, IL-6, IL-8, and GM-CSF levels were measured in the supernatants of RA FLS cultures, using a Human Inflammatory Five-Plex Antibody Beads Kit (Biosource, CA) according

to the manufacturer's instructions, on a Luminex 100 instrument (Luminex, Austin, TX). The levels of MIP-1α, MIP-1β, MCP-1, eotaxin and RANTES in the supernatant were measured using a Human Chemokine Five-Plex Antibody Beads Kit (Biosource)

Transfection of RA FLS with siRNAs. All siRNAs were purchased from Qiagen. The sense strand sequences of the RNA duplexes were as follows: HVEM, 5'-GGCACUGCCUCACAGCCAAdTdT-3'; LTβR, 5'-CAUCUACAAUGGACCAGUAdTdT-3'; and control siRNA 5'-UUCUCCGAACGUGUCACGUdTdT-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 the 5×10^3 cells/well, in complete medium without antibiotics. The next day, siRNAs (at final concentration of 50 nM) were introduced into cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Twenty-four hours after transfection, media were replaced with regular culture media. The cells were then cultured for 96 hours before analyzing the gene-silencing effects. The mRNA levels were measured by quantitative real-time PCR analysis.

Immnofluorescence 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). The cells were

stimulated with 10 ng/ml LIGHT for 40 minutes, washed with cold PBS, and then fixed in PBS with 4% paraformaldehyde for 10 minutes. The cells were permeabilized with PBS and 0.1% Triton-X100 for 10 minutes. 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 hour at room temperature, then with AlexaFluor 488-conjugated goat anti-mouse antibody for 30 minutes at room temperature. Slides were coverslipped and examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Western blotting analysis. For measurement of IκBα 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 hours. Following incubation with 10 ng/ml LIGHT for 40 minutes, 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, 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, CA). After blocking, membranes were incubated with either anti-β-actin or anti-IκBα antibody, overnight at 4°C, and then with secondary antibody conjugated to horseradish peroxidase (DAKO), at room temperature for 1 hour. 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 made using the Mann-Whitney U-test. P values less than 0.05 were considered statistically significant.

Results

Increased expression of LIGHT in the synovial fluid of RA patients. To examine whether or not LIGHT is involved in the pathogenesis of RA, we analyzed the level of LIGHT in synovial fluids from 23 RA patients and 10 OA patients by ELISA. The synovial fluids from OA patients were used as controls, because synovial fluids were not available from healthy individuals. The concentration of LIGHT in synovial fluids from RA patients was significantly higher than in those from OA patients (Figure 1). Median levels of LIGHT in synovial fluids 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 synovial fluid than OA patients, we determined whether LIGHT and its membrane-bound receptors, HVEM and LTβR, were

expressed in the synovial tissues from RA patients and OA patients. Although quantitative real-time PCR analysis revealed that the mRNA expression of LIGHT in synovial tissue was significantly higher in RA patients than in OA patients (Figure 2A), HVEM and LTβR levels were not different between RA and OA patients.

Furthermore, we investigated the mRNA expression of LIGHT, HVEM and LTβR in RA FLS by RT-PCR. RA FLS from all seven patients expressed HVEM and LTβR mRNAs, but no LIGHT expression was detected (Figure 2B).

Induction of RA FLS proliferation by LIGHT. Previous studies reported that LIGHT induced cell proliferation in T lymphocytes (7,12) and vascular smooth muscle cells (13). Since the expression of HVEM and LTβR 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 3A, treatment with LIGHT significantly enhanced de novo DNA synthesis in RA FLS in a dose-dependent manner. LIGHT showed growth-promoting activity equivalent to that of epidermal growth factor (EGF), which was used as a positive control.

Next, to investigate the contributions of HVEM and LTβR to the LIGHT-induced proliferation of RA FLS, we transfected RA FLS with HVEM siRNA or LTβR siRNA. Quantitative real-time PCR analysis revealed that the HVEM mRNA level