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

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

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

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

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

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Accepted for publication January 10, 2008.

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

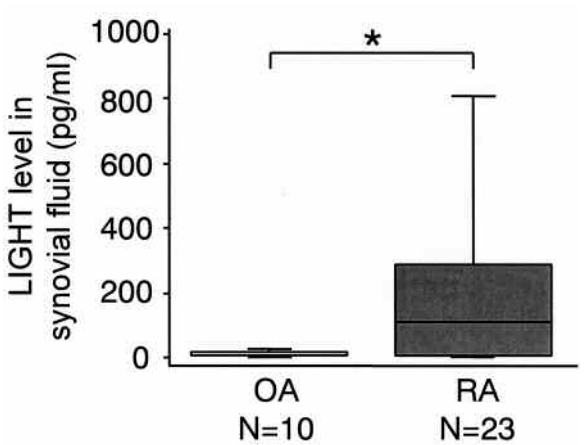

Figure 1. Levels of LIGHT in synovial fluid from RA patients and OA patients, determined by ELISA. Box plots represent 25th to 75th percentiles. Error bars represent 10th to 90th percentiles. Lines inside boxes represent the median. *p < 0.05.

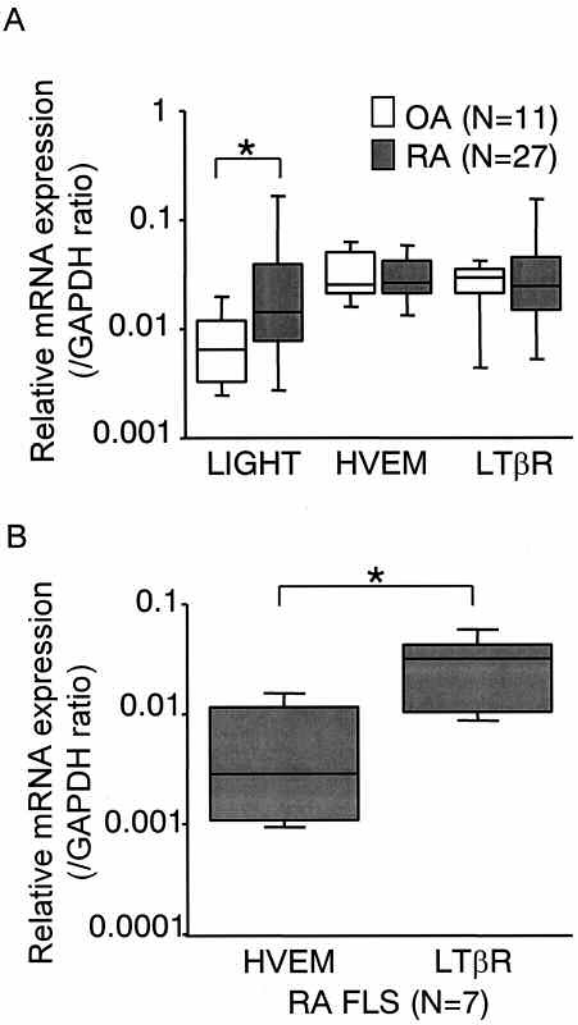

Figure 2. Expression of LIGHT, herpes virus entry mediator (HVEM), and lymphotoxin ß receptor (LTßR) mRNA in synovial tissues and fibroblast-like synoviocytes (FLS): A. In synovial tissues from RA patients and OA patients. B. In RA-FLS. Level was evaluated by real-time quantitative PCR; results are represented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

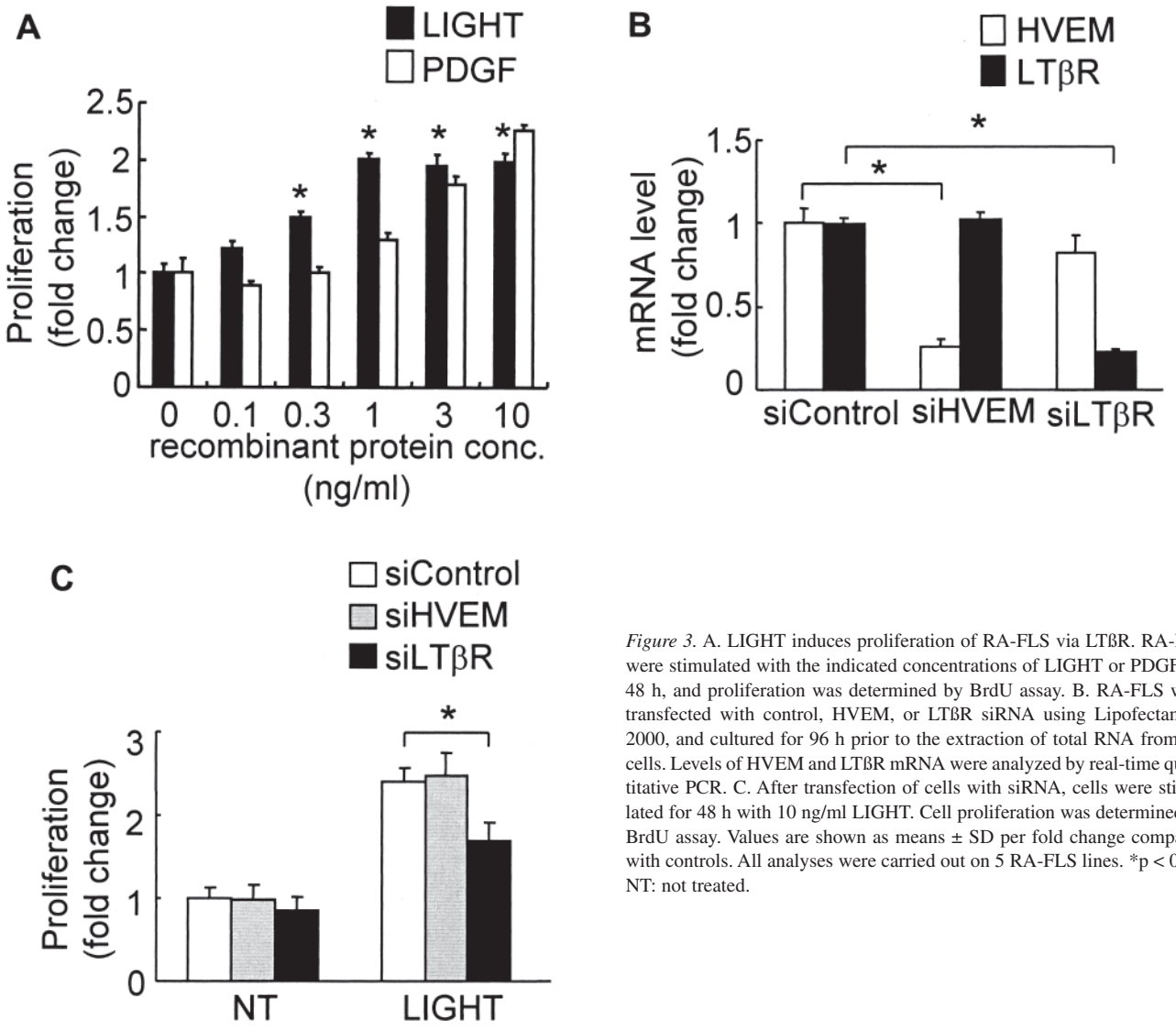

Figure 3. A. LIGHT induces proliferation of RA-FLS via LTßR. RA-FLS were stimulated with the indicated concentrations of LIGHT or PDGF for 48 h, and proliferation was determined by BrdU assay. B. RA-FLS were transfected with control, HVEM, or LTßR siRNA using Lipofectamine 2000, and cultured for 96 h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

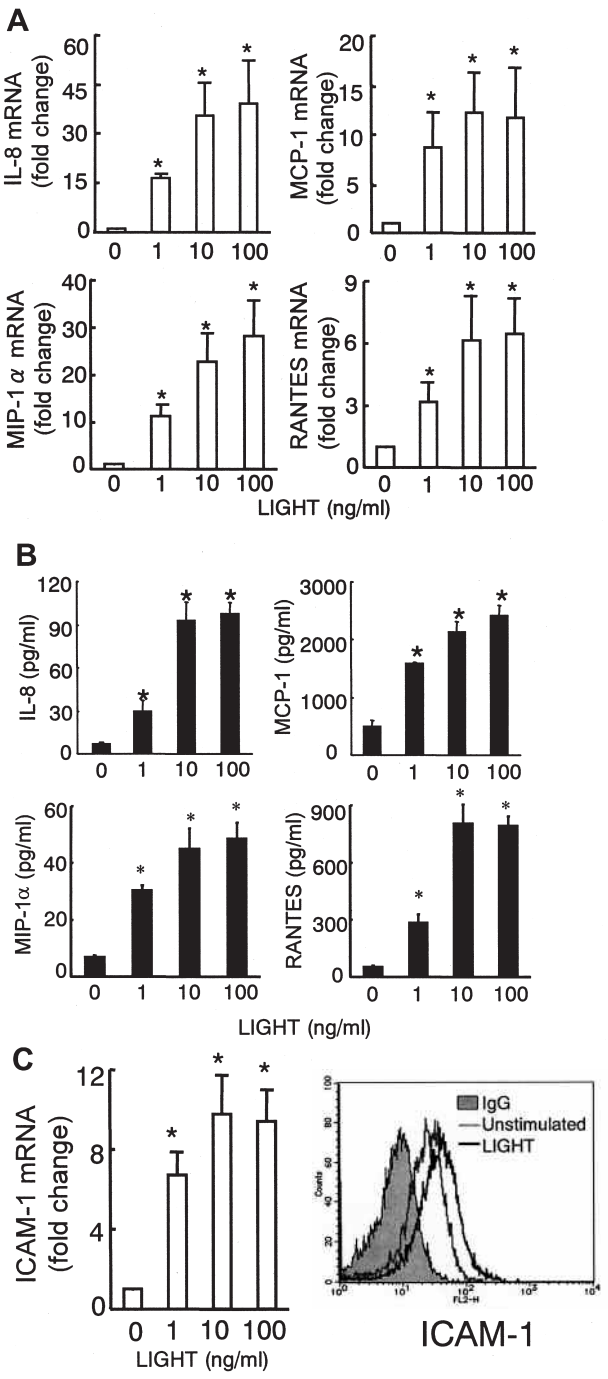

Figure 4. Upregulation of IL-8, MCP-1, MIP-1α, RANTES, and ICAM-1 expression in RA-FLS by LIGHT. A. RA-FLS were stimulated with the indicated concentrations of LIGHT for 3 h, and real-time quantitative PCR was performed to determine levels of IL-8, MCP-1, MIP-1α, and RANTES mRNA expression. Values are 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 LTßR suppressed this series of LIGHT-induced gene expression in RA-FLS. Compared with control siRNA, LTßR siRNA, but not HVEM siRNA, significantly decreased the expression of IL-8, MCP-1, and ICAM-1 mRNA induced by LIGHT (Figure 5). Similarly, LTßR 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 LTßR 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 LTßR siRNA, but not by HVEM siRNA (Figure 6C).

DISCUSSION

We observed that LIGHT, but not HVEM or LTßR, 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 LTßR, which implies that RA-FLS are

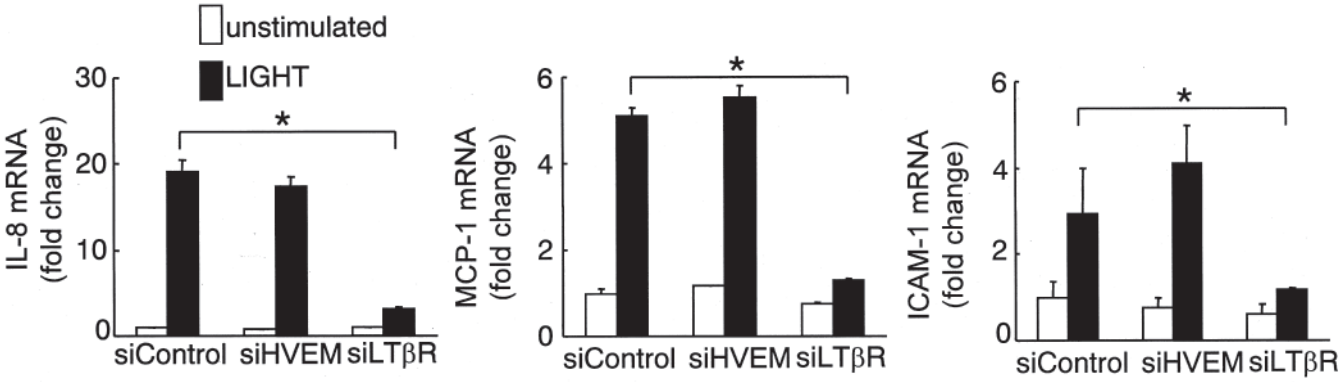

Figure 5. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 in RA-FLS via LTßR. RA-FLS were transfected with control, HVEM, or LTßR siRNA using Lipofectamine 2000. After 96 h incubation, cells were stimulated with 10 ng/ml LIGHT for an additional 3 h. Levels of IL-8, MCP-1, and ICAM-1 mRNA were analyzed 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

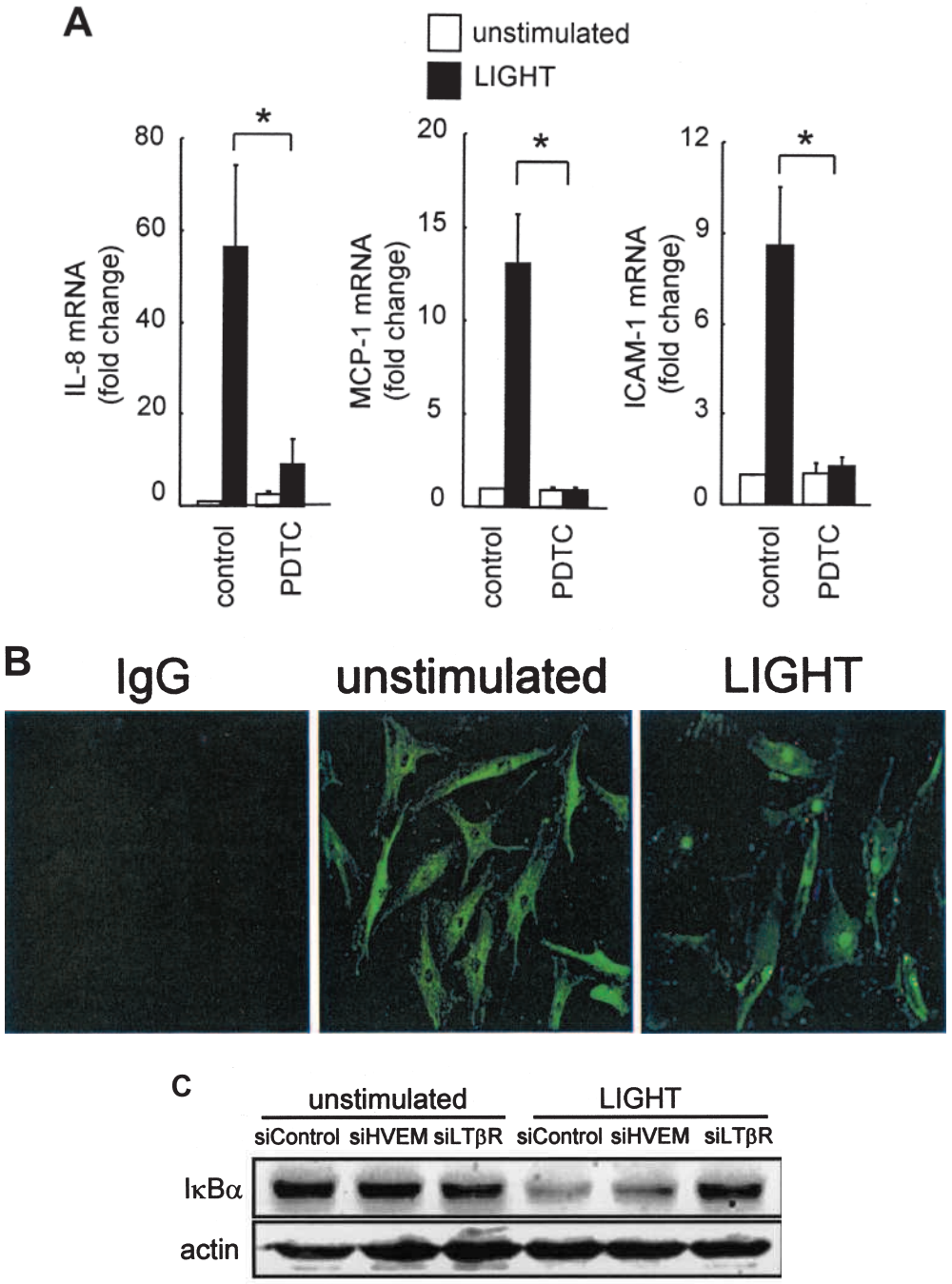

Figure 6. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 through NF-κB-mediated pathways. A. FLS were stimulated with 10 ng/ml LIGHT for 3 h with or without preincubation for 30 min with 30 μM PDTC. Levels of IL-8, MCP-1, and ICAM-1 mRNA were analyzed by real-time quantitative PCR. Values are shown 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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