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 LTβR siRNA led to a 75% reduction in the LTβR 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 LTβR siRNA, but not by HVEM siRNA, when compared with control siRNA (Figure 3C). Both LTβR siRNA and HVEM siRNA had little effect on the EGF-induced proliferation of RA FLS (data not shown).

LIGHT induces the expression of proinflammatory cytokines, chemokines and adhesion molecules in FLS via LTβR. Previous studies reported that LIGHT induces the secretion of various cytokines and augments the expression of adhesion molecules (13,15-17,19). We examined the effects of LIGHT on inflammatory cytokine production by RA FLS. Treatment with LIGHT enhanced both mRNA and protein expression of IL-8 and MCP-1 in RA FLS, in a dose-dependent manner (Figure 4A and 4B). LIGHT induced IL-1β, IL-6, GM-CSF, RANTES and MIP-1α, but not TNFα, eotaxin or MIP-1β (data not shown). Next, to assess whether LIGHT can induce 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 4A and 4B). FACS analysis revealed the augmented

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expression of ICAM-1 protein on the cell surface of RA FLS stimulated with LIGHT (Figure 4B). 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 suppresses 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 mRNAs induced by LIGHT (Figure 5). Similarly, LTβR siRNA decreased the LIGHT-induced expression of IL-1β, IL-6, GM-CSF, RANTES and MIP-1α mRNAs 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 (22). Several studies have shown that LIGHT activates the transcription factor NF-κB in different cell types (7,9,13,23-25). To demonstrate 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-1β, IL-6, GM-CSF, RANTES and MIP-1α mRNAs, 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). Furthermore, Western blotting using anti-IκBα mAb showed that IκBα degradation was induced by LIGHT, and that IκBα degradation was inhibited by LTβR siRNA, but not by HVEM siRNA (Figure 6C).

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Discussion

In the present study, we demonstrated that LIGHT, but not HEVM or LTβR, is overexpressed in the synovial tissues of RA patients compared with those of OA patients. The expression of LIGHT was not detected in RA FLS, which is one of the major components of the RA synovium. The RA synovium is histologically characterized by prominent infiltration of macrophages and lymphocytes (26). 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 target cells of LIGHT. Indeed, in the present study, we first showed that LIGHT induced RA FLS proliferation to the same extent as EGF did. The proliferation of RA FLS is one of the most critical pathological changes in RA. Therefore, our findings suggested that increased expression of LIGHT might lead to the synovial hyperplasia of RA. Anti-cytokine therapies targeting TNFα, IL-1 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 (29,30). In addition, recent studies have indicated that LIGHT reduces Fas-mediated apoptosis in FLS (31), that LIGHT may function as a mediator of bone resorption through the induction of osteoclastogenesis (32), and that LTβR-Ig protein blocks the induction of experimental arthritis in mice (18). Thus, a neutralizing antibody against LIGHT could be a useful tool for the inhibition of synovial hyperplasia and bone destruction in RA.

The enhanced effects of LIGHT on RA FLS proliferation were significantly inhibited by LTβR siRNA, but not by HVEM siRNA, suggesting that LTβR, rather than HVEM, is involved in the LIGHT-induced proliferation of RA FLS. The exact

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mechanism by which LIGHT influences RA FLS proliferation through LTβR is unknown. A potential mechanism underlying RA FLS proliferation induced by LIGHT may involve cell cycle regulators, including cyclin-dependent kinase (CDK). The mammalian cell cycle is controlled by holoenzymes composed of a catalytic CDK and regulatory cyclin. The expression of p21 was reduced in RA synovial linings and FLS compared with the level in those of OA patients (33). Overexpression of p21 or p16 by adenovial-mediated delivery suppresses FLS growth in vitro (34,35). Furthermore, LIGHT induces cell proliferation, down-regulates the CDK inhibitors p21, p27 and p53, and inversely up-regulates cyclin D and Rb hyper-phosphorylation in vascular smooth muscle cells (13). Thus, it is possible that LIGHT promotes FLS proliferation by shortening the cell cycle of FLS in RA patients. Wang reported that LTβR null mice show reduced BrdU incorporation in dendritic cells (36). This supports our claim that LTβR signaling is involved in the proliferation of RA FLS.

In the current study, we have demonstrated that LIGHT also induces the production of inflammatory cytokines and chemokines and the expression of adhesion molecules on RA FLS. Inflammatory cytokines such as TNFα and IL-1β are involved in the pathogenesis of RA (1,2). 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 the inflammation in the synovial lining layer, as well as 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 (31,37). Although these reports are consistent with our present results, it has not been clear which of two receptors is involved in the induction of these genes in FLS. Our knockdown analysis using siRNA revealed that LIGHT induced the proliferation and the gene expression by signaling via LTβR, but not HVEM. Braun et al. has shown that LTβR is expressed on RA FLS, and that LTα1β2, which is a ligand for LTβR, induces the expression of inflammatory cytokines, chemokines and ICAM-1 (38). This supports our claim that LTβR 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 (22). In fact, 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 the present 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 by the LIGHT-induced nuclear translocation of NF-κB p65. Moreover, LIGHT induced IκBα degradation in RA FLS, an effect that was inhibited by LTβR siRNA, but not by HVEM siRNA. These findings are consistent with previous studies showing that LTβR ligation can lead to activation of NF-κB (23,42-45). However, it is unknown why LIGHT prefers the LTbR signaling pathway in RA FLS, even though HVEM is also expressed on these cells.

In conclusion, we demonstrated that LIGHT is overexpressed in RA synovial tissues and synovial fluids. 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 LTβR plays an important role in the pathogenesis of RA by affecting key process, such as the proliferation and activation of RA FLS. Therefore, regulation of LIGHT-LTβR signaling may represent a new therapeutic target for the treatment of RA.

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Figure 1. Levels of LIGHT in synovial fluid from RA patients compared with those from OA patients, as determined by enzyme-linked immunosorbent assay. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Error bars represent the 10th to 90th percentiles. Lines inside the boxes represent the median. * = $P < 0.05$.

Figure 2. Expression of LIGHT, HVEM, and LTβR mRNAs in synovial tissues and fibroblast-like synoviocytes. A, Expression of LIGHT, HVEM and LTβR mRNAs in synovial tissues from RA patients OA patients. The expression level of LIGHT mRNA was evaluated by real-time PCR; results are represented as relative ratios to GAPDH levels, and are shown as box plots. B, Expression of LIGHT, HVEM, LTβR, and

GAPDH in RA FLS. RT-PCR was performed using total RNA extracted from FLS from 7 different RA patients. * = $P < 0.05$.

Figure 3. LIGHT induces the proliferation of RA FLS via LTβR. A, LIGHT induces RA FLS proliferation. RA FLS were stimulated with the indicated concentrations of LIGHT or EGF for 48 hours, and proliferation was determined by a BrdU assay. B, RA FLS were transfected with control, HVEM or LTβR siRNAs using Lipofectamine 2000, and cultured for 96 hours prior to the extraction of total RNA from the cells. Expression of HVEM and LTβR mRNA was analyzed by real-time quantitative PCR. C, After the transfection of cells with siRNAs, the cells were stimulated for 48 hours with 10 ng/ml LIGHT. Cell proliferation was determined by a BrdU assay. Values are shown as means ± SD fold change compared with controls. All analyses were carried out on 5 RA FLS lines. * = $P < 0.05$.

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Figure 4. Up-regulation of IL-8, MCP-1 and ICAM-1 expression in RA FLS by LIGHT. A, RA FLS were stimulated with the indicated concentrations of LIGHT for 3 hours, and real-time quantitative PCR was performed to determine the levels of IL-8, MCP-1, and ICAM-1 mRNA expression. Values are shown as means ± SD fold change compared with controls. B, RA FLS were stimulated with the indicated concentrations

of LIGHT for 72 hours. The concentrations of IL-8 and MCP-1 in the cell culture supernatants were determined by multiplex bead array assays. Values are shown as means ± SD pg/ml. ICAM-1 surface expression on RA FLS was detected by FACS after stimulation with 10 ng/ml LIGHT for 24 hours. All analyses were carried out on 4 RA FLS lines; the FACS profiles of one representative result are shown. * = $P < 0.05$.

Figure 5. LIGHT-induced expression of IL-8, MCP-1, and ICAM-1 in RA FLS via LTβR. RA FLS were transfected with control, HVEM or LTβR siRNAs using Lipofectamine 2000. Following 96 hours of incubation, the cells were stimulated with 10 ng/ml LIGHT for an additional 3 hours. The expression levels of IL-8, MCP-1 and ICAM-1 mRNAs were analyzed by real-time quantitative PCR. Values are shown as means ± SD fold change compared with controls. All analyses were carried out on 4 RA FLS lines. * = $P < 0.05$.

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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 hours with or without preincubation for 30 minutes with 30 μM PDTC. The expression levels of IL-8, MCP-1 and ICAM-1 mRNAs were analyzed by real-time quantitative PCR. Values

are shown as means ± SD 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); unstimulated RA FLS (middle) and RA FLS stimulated with 10 ng/ml LIGHT for 30 minutes (right). Results are representative of 2 experiments using 2 FLS lines. C, Ninety-six hours after siRNA transfection, the cells were stimulated with 10 ng/ml LIGHT for 40 mins. IκBα degradation was analyzed by immunoblotting. Results are representative of 2 experiments using 2 RA FLS lines.

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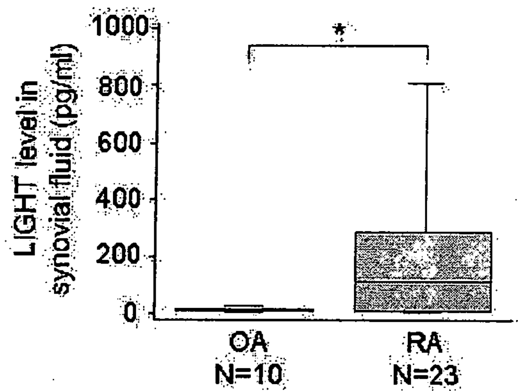

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