The TNF superfamily member LIGHT contributes to survival and activation of synovial fibroblasts in rheumatoid arthritis

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Objectives. The TNF superfamily member LIGHT has a T-cell co-stimulatory role and has previously been associated with inflammation and autoimmunity. To investigate its role in rheumatoid arthritis (RA), a disease where activated T cells contribute in a prominent way, we have analysed the expression of LIGHT and its receptors in RA and analysed its effects on synovial fibroblasts in vitro.

Methods. The expression of LIGHT was measured in synovial tissues and fluids and the receptors of LIGHT were detected on synovial fibroblasts derived from patients with RA and osteoarthritis (OA). The effects of recombinant LIGHT on the production of proinflammatory cytokines and proteases and on the apoptosis of synovial fibroblasts was assessed.

Results. LIGHT mRNA was present in synovial tissues of patients with RA but not with OA. Correspondingly, soluble LIGHT protein could be detected in RA synovial fluid samples at much higher levels than in synovial fluid from patients with OA. Immunohistochemical detection of LIGHT and analysis of synovial fluid cells by flow cytometry revealed CD4 T cells as the major source of LIGHT in the rheumatoid joint. Synovial fibroblasts from RA patients were found to express the LIGHT receptors HVEM and LTβR. Recombinant LIGHT induced RA synovial fibroblasts to upregulate MMP-9 mRNA, CDS4 and IL-6 in an NF-κB-dependent fashion. In vitro, exposure of cultured synovial fibroblasts to LIGHT reduced FAS-mediated apoptosis significantly, without affecting the rate of spontaneous apoptosis.

Conclusions. The results provide evidence for a novel T-cell-dependent activation of synovial fibroblasts by LIGHT in joints of patients with RA, contributing to an inflammatory and destructive phenotype.

Key words: Rheumatoid arthritis, Stromal cells, Apoptosis, Cell activation, Inflammation.

Introduction

Rheumatoid arthritis (RA) is a chronic debilitating joint disease affecting 1% of the population. Although the aetiology is unclear, the accumulation of activated T cells and macrophages in the joints and the presence of rheumatoid factor (RF) autoantibodies suggest an immune dysregulation. Over the last years it has become increasingly evident that proinflammatory cytokines of the TNF superfamily produced mainly by macrophages and T cells play an important role in the development of joint inflammation. Clinical studies in patients with RA, specific blockers of TNF-α have proven to be highly effective anti-arthritic drugs.

Recently, the TNF family member LIGHT (for homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes), a 29 kD type II transmembrane protein produced by activated T cells, as well as monocytes and granulocytes, and immature dendritic cells (DCs) was cloned [1]. LIGHT binds three different receptors, the transmembrane receptors lymphotixin β receptor (LTβR) and HVEM and the soluble decoy receptor 3 (DcR3). Both HVEM and LTβR are associated with TNF receptor-associated factors (TRAFs), which bind to the cytoplasmic domains of the TNF receptor family. LTβR binds TRAF2, TRAF3 and TRAF5, whereas HVEM binds TRAF2 and TRAF5. TRAF2 and TRAF5 activate several transcriptional pathways including NF-κB [2–5]. By signalling through HVEM and LTβR, LIGHT participates in multiple immunological functions. Gene disruption studies in mice indicate that LIGHT plays a role in T cell development. More directly it has been shown that LIGHT serves as a CD28-independent co-stimulatory molecule for T cell activation, inducing enhanced proliferation, Th1-type cytokine production, and NF-κB translocation [6]. Importantly, the enhanced expression of LIGHT as a transgene in mice under the control of a T-cell-specific promoter resulted in T cell expansion and caused severe autoimmunity disease [7, 8]. Furthermore, a role for LIGHT-HVEM co-stimulation in allograft rejection has been proposed based on experiments with HVEM-Ig given after cardiac transplantation in a murine model [9] and even more interestingly it was shown that forced expression of LIGHT in the tumour environment induces massive infiltration of naive T-lymphocytes and leads to the rejection of established tumours at local and distal sites [10].

In collagen-induced arthritis (CIA), studies interfering with the LIGHT/lymphotoxin pathway have produced conflicting results. The study by Fava et al. has described LTβR-Ig fusion protein as an effective inhibitor of CIA development, arresting even established disease [11]. In contrast, a recent publication showed that the blockade of LT signalling leads to more severe and prolonged autoimmune arthritis [12]. However, LTβR-Ig blocks LTβ as well as LIGHT and studies evaluating directly the role of LIGHT in this murine model of RA have not been published so far.

Besides its regulatory role for various functions in the immune system, LIGHT has also been reported to influence non-immune cells. While LIGHT induces apoptotic cell death in HT-29 cells and some tumour cells as does LTα [13, 14], it has been shown that LIGHT prevents TNF-α-mediated apoptosis in primary hepatocytes [15].

Considering the fact that LIGHT is produced by activated T cells and that T cell immune responses are important in the pathogenesis of RA, we have analysed the expression and function of LIGHT and its receptors in the synovial tissue of patients with RA.

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The results suggest an important role for LIGHT in the activation of synovial fibroblasts thereby enhancing the sustained inflammatory response in joints of patients with RA.

Materials and methods

Patients and controls

Synovial fluid samples were obtained during arthrocentesis from the knee joints of patients diagnosed as having either RA (n = 42; mean age 63 yrs, range 51–78 yrs) or OA (n = 25; mean age 65 yrs, range 48–74 yrs). Sera from another set of patients with RA (n = 27) and OA (n = 33) were collected, centrifuged and stored frozen at −70°C until analysis. Synovial fluid was collected and centrifuged to remove cells and debris. Aliquots were frozen at −70°C until analysed. Before analysis, samples were pre-treated for 1 h at 37°C with 1 mg/ml of hyaluronidase (Fluka Chemie, Buchs, Switzerland). Synovial fluid cells were obtained from knee joint aspirates of six patients with RA and six patients with other types of inflammatory arthritis (reactive arthritis n = 2, spondyloarthritis n = 4) and were analysed immediately after collection. Synovial tissue was obtained from individual patients undergoing total joint replacement surgery. RA patients were diagnosed according to the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) [16]. All patient samples were collected following Institutional Review Board guidelines after ethical approval and informed patient consent had been obtained, and all patients were seen at the Rheumatology Department of the University Hospital Zürich in Switzerland or the Department of Rheumatology at the University of Leipzig in Germany.

Cell culture

Immediately after joint replacement surgery, the synovial tissues were minced and digested with Dispase at 37°C for 60 min. After washing, the cells were grown in Dulbecco’s MEM (Gibco, Basel, Switzerland) supplemented with 10% foetal calf serum, 50 IU/ul penicillin-streptomycin, 2 mM l-glutamine, 10 mM HEPES and 0.2% fungizide (Gibco). Cell cultures were maintained in a 5% CO2-humified incubator at 37°C. Cultured synovial fibroblasts were used between passages 4 and 7.

Stimulation assays

Total 5 × 10^4 synovial fibroblasts per well were seeded onto 12-well culture plates and subsequently stimulated with various concentrations of recombinant human (rh) LIGHT or TNF-α (10 ng/ml; both R&D Systems, Abingdon, UK). Isolation of mRNA was carried out after 24 h. Additional cultures were stimulated for 48 h and analysed by FACS. Culture supernatants were collected after 24 h and kept at −80°C for ELISA. All reagents were tested routinely for endotoxin using the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD, USA).

Determination of apoptosis and proliferation

Total 5 × 10^4 RA synovial fibroblast (RA-SF) were plated onto 6-well plates. After 24 h, medium was changed and the cells were incubated with medium or recombinant human FasL (Alexis Biochemicals, Montreal, Canada) (100 ng/ml) in the presence or absence of LIGHT at a concentration of 100 ng/ml. Apoptosis was determined using FITC-conjugated annexin V and propidium iodide according to the manufacturer’s instruction (Bender Med Systems, Vienna, Austria). Briefly, 24 h after cytokine addition, the cells were trypsinized, washed with medium, and pelleted by centrifugation. After being resuspended, the cells were stained in a suspension buffer containing annexin-V and propidium iodide. Stained cells were analysed on a FACSScalibur using CELLQuest software (Becton Dickinson, San Josè, CA, USA).

For the detection of viability and proliferation of cells, the ViaLight HS kit (Cambrex, Rutherford, NJ, USA) was used according to the manufacturer’s instructions. Briefly, 7000 RA-SF were plated onto 24-well plates and were incubated with medium or recombinant human FasL (100 ng/ml) in the presence or absence of LIGHT at a concentration of 100 ng/ml. The samples were measured in triplicates on a luminometer (Berthold Detection Systems, Pforzheim, Germany).

Immunohistochemistry/immunofluorescence

For immunohistochemistry, paraformaldehyde fixed and paraffin embedded tissue sections of 3 μm were used. Antibody retrieval procedure was performed in a microwave oven using 10 mM citrate buffer, pH 6.0. Blocking of endogenous peroxidase activity was perfomed with 0.6% hydrogen peroxide in methanol and avidin blocking according to manufacturers instructions (Vector laboratories, Burlingame, CA, USA). LIGHT was detected by incubation with affiinity-purified goat antibodies (sc-7767, Santa Cruz, CA, USA) at 0.2–0.5 μg/ml for 12 h at 4°C, followed by biotinylated rabbit anti-goat IgG (sc-2774, Santa Cruz) for 1 h, and final incubation for 20 min with peroxidase-labelled streptavidin. To identify monocytes/macrophages, slides were additionally incubated with mouse anti-human CD68 (clone PG-M1, IgG3; Dako, Carpenteria, CA, USA) at 0.5 μg/ml for 1 h, followed by alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG for 1 h and mouse AP-conjugated anti-AP (AAPA) (both from Dako) for 30 min. Peroxidase-labelled cells were visualized using AEC and AP-labelled cells using NBT-BCIP (Dako). As negative control, goat anti-LIGHT antibodies were pre-incubated for 1 h with the LIGHT peptide against which they were raised (sc-7767P, Santa Cruz). Isotype-specific mouse IgG was used as a negative control for the anti-CD68 staining. Cells expressing HVEM were detected by anti-human HVEM (sc-SC21718, Santa Cruz) antibody at 0.2–0.5 μg/ml for 12 h at 4°C, followed by biotinylated donkey anti-goat IgG for 1 h and an incubation for 20 min with peroxidase-labelled streptavidin. For the visualization of HRP AEC substrate kit was used.

For concurrent staining of LIGHT and CD4, cryostat sections of OCT (Tissue Tek, Nußloch, Germany) embedded tissue of four RA patients undergoing joint replacement surgery were used. To detect CD4-positive cells, slides were incubated for 2 h with biotinylated anti CD4 antibody (ImmuneTech, Marseille, France), followed by streptavidine HRP and tyramide signal amplification technology with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). LIGHT was detected using the same primary and secondary antibodies in combination with vector red AP substrate kit (Vector Laboratories, UK). Nuclei were counterstained with DAPI (Molecular Probes).

ELISA

IL-6 was detected by ELISA with OptEIA® Kit (BD Pharmingen, Basel, Switzerland), and LIGHT, IL-1α, β, TGF, β, VEGF, CCL21 and CCL22 protein were measured by using Quantikine® ELISA Kits (R&D Systems, Heidelberg, Germany) according to the manufacturer’s instruction.

Flow cytometry

Cultured synovial fibroblasts were harvested by using 2.5 mM EDTA after the indicated incubation time with rhLIGHT, or medium alone. Cells were stained for 15 min with the specific antibodies which were either unlabelled, fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated. The following antibodies were used to determine the expression of surface molecules: anti-HVEM (Labvision, Fremont, CA, USA), anti-LTβR, anti-CD40, anti-CD106, anti-CD44, anti-CD86, anti-CD54 (Beckton Dickinson, Heidelberg, Germany); anti-CD44 (ImmunoTech, Marseille, France).
Synovial fluid cells were isolated by centrifugation from synovial fluid derived from patients with RA and a group of patients with other types of inflammatory arthritis. For extracellular staining, antibodies against LIGHT (sc-7767, Santa Cruz) and HVEM (R&D Systems, Wiesbaden, Germany) as well as anti-CD4, CD8 and CD14 antibodies (all Beckton Dickinson, Heidelberg, Germany) were used. Briefly, cells were incubated with the primary antibodies against LIGHT or HVEM or isotype control antibodies for 30 min and a FITC-labelled rabbit anti-goat IgG or goat anti-mouse IgG (Beckton Dickinson, Heidelberg, Germany) for 20 min followed by directly labelled antibodies against cell surface markers CD4, CD8 or CD14. All analyses were done using FACS Calibur and CellQuest software (BD Biosciences).

**PCR and real-time PCR**

For the qualitative detection of LIGHT, HVEM, LTβR and DcR3 mRNA in tissue or cultured cells, total RNA was isolated using the RNEasy Mini Prep Kit (Qiagen, Basel, Switzerland), reverse transcribed with random hexamers, and amplified by PCR using the primers and conditions indicated in the supplementary online materials. For quantification of matrix metalloproteinase (MMP) mRNA, total RNA was isolated, reverse transcribed and amplified by single-reporter real-time PCR using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Rotkreuz, Switzerland). The specific primers and probes for MMPs are listed in the supplementary online materials (PE Applied Biosystems). The endogenous control 18S cDNA was used for correcting the results, and differences were calculated with the threshold cycle (CT) and the comparative CT method for relative quantification as described by the manufacturer.

**NFκB-driven luciferase reporter assay and EMSA**

RA-SF were plated on 6-well plates at a density of 2 × 10⁶ cells/well and transiently cotransfected with 2 μg of a NFκB-driven luciferase reporter plasmid encoding firefly luciferase as reporter gene and 1.5 μg of pEGFP-C1 plasmid (Clonetech, Palo Alto, CA, USA) encoding an enhanced green fluorescence protein using Lipofectamine 2000 reagent (Life Technologies). After 24 h, equal transfection efficiency was verified by fluorescence imaging on SPECTRmax Gemini II (Molecular Devices, Winnersh, UK) and rhLIGHT or TNF or medium alone were added to different wells and incubated at 37°C. After 5 h, supernatants were removed, the cells lysed in Glo lysis buffer (Promega, Madison, WI, USA), gently shaken for 5 min and then transferred to luminometer tubes. The Bright-Glo Assay reagent was added and incubated for 5 min at room temperature. Luciferase production was assayed in a Turner TD 20/20 luminometer (EG & G Berthold, Bad Wilbad, Germany) by comparison of values obtained in parallel cultures treated with 100 ng/ml of recombinant human LIGHT. Cells were collected by scraping in ice-cold PBS after stimulation at different time points (0, 30, 90 min after stimulation). DNA-binding proteins were extracted from RA-SF according to the method of Andrews and Faller, which utilizes hypotonic lysis followed by high salt extraction of nuclei [17]. The EMSA-binding assay was carried out using a Panomics EMSA ‘gel shift’ kit according to the manufacturer’s instructions (Panomics, Redwood City, CA, USA).

**Statistical analysis**

Values are presented as the mean ± S.E.M. The significance of the results was analysed using Student’s two-tailed t-test, Mann–Whitney U-test or Wilcoxon test where appropriate using SPSS software (V.12.0). P values less than 0.05 were considered significant.

**Results**

**LIGHT and HVEM expression in synovial tissue and synovial fluid cells from patients with RA**

To examine the expression of LIGHT in the synovial compartment, RT-PCR of synovial tissue specimens derived from patients with RA or OA as controls was performed. Using LIGHT-specific primers, mRNA was detected in all tested whole tissue RNA extracts derived from RA patients (n = 5), but in none of the tissue RNA extracts derived from patients with OA (n = 5) (Fig. 1A).

Analysis of mononuclear cells isolated from joint aspirates of patients with RA by flow cytometry revealed the expression of LIGHT on CD4 positive T cells (Fig. 1B and D). However, CD4-positive T cells derived from a group of patients with other types of inflammatory arthritis (n = 6, two reactive arthritis, four spondyloarthropathy) were also expressing LIGHT, but in a significantly lower percentage (14.3% vs 50.5%, P < 0.001) than RA patients (Fig. 1B and F). Neither CD14 expressing monocytes nor granulocytes or fibroblasts were found to express surface LIGHT (data not shown). HVEM, which is carried by quiescent CD4 T cells was expressed by a mean of 76.5% CD4+ T cells isolated from synovial fluid of patients with RA, while a mean of 84.3% of non-RA CD4 T cells was expressing HVEM, a difference not reaching statistical significance (Fig. 1C, E and G).

The immunohistochemical analysis of synovial tissue specimens from patients with RA revealed varying numbers of cells producing LIGHT in all analysed samples (n = 6), but none in the tissue samples derived from patients having OA. LIGHT-positive cells were mainly found in cell-rich sublining regions, predominantly in areas with abundant lymphocytic infiltrations (Fig. 2A). To further characterize the LIGHT-expressing cells, double staining with anti-CD68 antibodies specific for the macrophage/monocyte lineage—restricted form of the CD68 molecule [18]—was performed. LIGHT-expressing cells were not detected by anti-CD68 antibodies, indicating that in the RA synovium LIGHT is not expressed by macrophages (Fig. 2A inset). However, CD68 positive cells and LIGHT positive cells were found in close proximity to each other. Since T cells are known to produce LIGHT [1, 19] and the synovial fluid CD4+ T cells were found to express LIGHT, we performed immunofluorescence double staining with an anti CD4 antibody together with the LIGHT detection. Using this staining protocol, many of the cells in the synovial membrane expressing LIGHT could be identified as CD4+ T cells (Fig. 2E).

In contrast to LIGHT, HVEM was expressed more ubiquitously in the lining and sublining regions of the inflamed synovium presumably including fibroblasts, endothelial cells and macrophages. This expression pattern was confirmed in all investigated tissues of patients with RA, suggesting a constitutive expression of this surface receptor for LIGHT (Fig. 2C).

**LIGHT is up-regulated in synovial fluids of patients with RA, but not in patients with OA**

Cell surface LIGHT can be cleaved by MMPs to yield a soluble form. We have analysed sera and synovial fluids of patients with RA and OA for the presence of soluble LIGHT protein by ELISA. In sera of both, patients with RA (n = 27) and OA (n = 33), soluble LIGHT could be detected at comparable levels (RA mean 118 pg/ml; OA mean 169 pg/ml; P = 0.21). In contrast to the serum concentrations, LIGHT was detectable.
in the synovial fluids of patients with RA in higher concentrations (mean 720 pg/ml) than in sera but was below detection level in synovial fluids of patients with OA (Fig. 3), confirming the RT-PCR results in whole tissue RNA extracts. Within the group of RA patients, no correlation of the concentrations of LIGHT in the synovial fluid and the C-reactive protein as a measure of systemic disease activity was found ($r = 0.21$, $P = 0.34$; data not shown).

**RA-SF express HVEM and LTβR, the receptors for LIGHT**

Since LIGHT protein is present in synovial fluid and synovial tissue of RA patients, cells expressing one or more of the identified receptors HVEM, LTβR are possible targets of this cytokine.

**Fig. 1.** Detection of LIGHT in joints of patients with RA or OA. (A) LIGHT mRNA was amplified by RT-PCR from synovial tissue RNA extracts from patients with RA or OA. As loading control, GAPDH is shown. (B) Surface expression of LIGHT and HVEM (C) on CD4+ T lymphocytes from synovial fluid of patients with RA ($n = 6$) and a group of patients with joint inflammation other than RA (IA, four spondylarthropathy, two reactive arthritis) as determined by flow cytometry using anti LIGHT and anti HVEM antibodies and the respective isotype control antibodies. Each bar represents the mean (±S.E.M.) percentage of LIGHT- or HVEM-expressing cells, *$P < 0.001$. Representative FACS plots are shown for LIGHT positive CD4+ and HVEM positive CD4+ T lymphocytes in RA and inflammatory arthritides (IA) in (D–G).

**Fig. 2.** Localization of LIGHT and HVEM in synovial tissue from patients with RA. (A) Immunohistochemical detection of LIGHT expressing cells (stained with red). In the inset in (A), cells expressing CD68 are stained in dark-blue and cells expressing LIGHT appear in red. (C) Detection of cells expressing HVEM (stained in red). At the inset in (C) a sublining region with lymphocytic infiltration is shown. (E) Immunofluorescence staining of CD4 expressing cells staining in green and LIGHT expressing cells staining in red. Cells expressing both molecules appear in yellow. No specific staining was seen with the respective control reagents as shown in (B) for LIGHT and (D) for HVEM. Nuclei were stained with haematoxylin in A (not inset) and with DAPI (blue) in B, C, D and E. Original magnification ×100 (A and B) and ×200 (C, D, E).

**Fig. 3.** Detection of soluble human LIGHT in sera (white bars) of patients with RA ($n = 27$) and OA ($n = 33$) and synovial fluid samples (grey bars) from RA patients (RA, $n = 42$) or osteoarthritis patients (OA, $n = 25$) by ELISA. The dashed line indicates the minimum detectable dose of the ELISA, *$P < 0.001$. 

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We analysed HVEM, LTβR and DcR3 mRNA expression of cultured RA-SF by conventional RT-PCR. Transcripts of all three receptors could be amplified from synovial fibroblast cultures from both RA and OA patients (Fig. 4A). FACS analysis with anti-HVEM antibodies revealed positive staining in 74% of RA-SF (Fig. 4B), confirming the expression of HVEM in RA-SF observed in immunohistochemical analyses of synovial tissue. Moreover, LTβR was found to be expressed by a mean of 91% of the analysed RA-SFs (Fig. 4C). Expression of HVEM and LTβR is not an exclusive feature of synovial fibroblasts since both markers could also be detected in dermal fibroblasts (data not shown).

**Soluble LIGHT up-regulates the expression of MMP-9, ICAM-1 and IL-6 in RA-SF**

In order to identify the functional effects of LIGHT on RA-SF we incubated cultured synovial fibroblasts derived from RA patients in the presence of different concentrations of recombinant human LIGHT. Subsequently, the RNA was extracted and quantitative RT-PCR was performed for MMP 1, 3, 9, 13 and 14 was performed. Addition of LIGHT resulted specifically in an increased expression of MMP-9 but not of the other MMPs analysed (Fig. 5A). When rhLIGHT stimulated RA-SF were analysed for the surface expression of integrins and activation markers, only CD54 (ICAM-1) was increased significantly (Fig. 5B), while CD40, CD44, CD106, HLA-DR/DP/DQ or CD86 remained unchanged (data not shown). Similar results for LIGHT stimulation were obtained with OA-SF cultures (data not shown).

Additionally, the supernatants of cultures stimulated with rhLIGHT for 24 h were tested for the presence of IL-6, IL-1, IL-1RA, TGFβ, CCL21, VEGF and FGFβ1 by ELISA. However, only the expression of IL-6 in LIGHT stimulated cultures increased significantly and in a dose-dependent manner (Fig. 5C).

**Soluble LIGHT reduces Fas-mediated apoptosis in synovial fibroblasts**

Decreased apoptotic cell death of RA-SF has been widely discussed as a major contribution to the destructive process specific for rheumatoid arthritis and members of the TNF superfamily are well known for their influence on that process. Therefore we analysed the effect of LIGHT on the rate of programmed cell death of RA-SF. However, no differences in the spontaneous rate of apoptosis was observed as assessed by Annexin V and PI staining and cytofluorometry (3.3% vs 2.8%, Fig. 6A) when LIGHT was added to cultured RA-SF. In contrast, LIGHT reduced FasL-induced apoptotic cell death in cultured RA-SF significantly by more than 40% (P < 0.03, Fig. 6A and B).

To detect influences of LIGHT on proliferation and survival of RA synovial fibroblasts, bioluminescent measurement of adenosine triphosphate (ATP) was performed under the same experimental conditions. When no apoptotic stimulus was present, LIGHT did not influence the proliferation of cells (Fig. 6C). However, when FasL was introduced to the cultures, LIGHT led to a significantly higher survival of cells (Fig. 6C), supporting the data of the apoptosis measurement. These results indicate that LIGHT present in the synovium and synovial fluid may contribute to the apoptosis resistance of synovial fibroblasts.

**Stimulation of synovial fibroblasts by soluble LIGHT induces NF-κB**

To analyse whether the stimulatory effects of LIGHT on synovial fibroblasts are mediated via NF-κB we transiently transfected RA-SF with a NF-κB promoter sensitive luciferase gene construct and measured the induced luciferase in a luciferase assay. To exclude variances in the transfection efficacy, an EGFP expression plasmid was cotransfected. Only cultures with similar transfection rates as identified by green fluorescent protein expression using a fluorescence microplate reader were used for the stimulation experiments. After optimization a transfection efficiency between 10% and 15% was achieved, comparable to previous reports [20]. NF-κB activation was subsequently measured in the highly sensitive luciferase assay.

A strong (15–20-fold) increase in luciferase gene expression in RA-SF cultures was detected in response to TNF (10 ng/ml).
Somewhat less potent, the addition of LIGHT to the transfected RA-SF cultures increased the luciferase expression in a dose dependent fashion, which saturated at 250 ng/ml (Fig. 7A). In an electromobility shift assay, LIGHT (100 ng/ml) led to an increase of NF-κB nuclear translocation after 90 min (Fig. 7B). Thus, LIGHT induces NF-κB-driven transcription in RA-SF.

**Discussion**

We have analysed the presence of LIGHT and its receptors in joints of patients with RA and the effects of LIGHT on cultured synovial fibroblasts in vitro, to assess whether it contributes to the activated phenotype of RA-SF found in RA [21].

The results demonstrate the presence of LIGHT in synovial tissue of patients with RA but not with OA. CD4 T cells are a major cell type expressing LIGHT, which can be abundantly found in the inflamed synovium, but also in the synovial fluid. However, the membrane-associated receptors for LIGHT, HVEM and LTβR show no disease specific expression, since both were found in all tested RA and OA samples.

The data on the expression of LIGHT in the synovial tissue of patients with RA differ from the study by Kim et al. [22], who have recently demonstrated expression of LIGHT and HVEM by synovial macrophages of patients with RA but not with OA. In our study LIGHT expressing cells were found in lymphocyte-rich areas and co-stained with CD4 but not with CD68 antibodies, consistent with predominant expression of LIGHT in CD4 T cells. A predominant LIGHT production by CD4 T cells could also offer an explanation as to why LIGHT could not be found in OA synovial tissues, where activated T cells are virtually absent.

LIGHT is expressed mainly as a membrane bound molecule, but evidence has been presented for the existence of other molecular forms lacking the transmembrane domain [23]. In addition proteinases such as MMPs, which are abundantly expressed in the rheumatoid synovium [24] may cleave membrane bound LIGHT resulting in elevated concentrations of soluble LIGHT molecules. Correspondingly, concentrations of LIGHT were strongly elevated in synovial fluid specimens of RA patients compared to patients with OA where no LIGHT could be detected.

Since RA-SF carry both membrane receptors for LIGHT and play an important role in the disease process, we investigated the effects of LIGHT on these cells in vitro. Stimulation of cultured synovial fibroblasts with recombinant human LIGHT resulted in an up-regulation of MMP expression (MMP-9), increased surface expression of the adhesion molecule CD54 and in an increased release of the proinflammatory cytokine IL-6. However, RA-SF did not produce CCL-21 in response to LIGHT [25], the expression of surface HVEM on RA-SF remained stable upon incubation with LIGHT (data not shown). For some patients with RA, concentrations of LIGHT measured in the synovial fluid were higher than the smallest concentrations of recombinant soluble LIGHT necessary to induce significant stimulatory activity in vitro, while most patients had concentrations significantly below the in vitro used concentrations. However, LIGHT was shown to act as cell membrane anchored protein and synovial fluid concentrations...
may not reflect properly the local effective concentrations reached in the intercellular spaces of the inflamed synovial tissue. The receptor system, comprising LTβR, HVEM, and DcR3, did not find to differ significantly between synovial fibroblasts derived from RA or OA patients. Elevated expression levels of DcR3 might theoretically antagonize stimulatory effects of LIGHT on cells expressing HVEM or LTβR. However, in our experiments there was no indication of an overexpression of DcR3 in RA-SF vs OA-SF.

Cytometric analyses of synovial fluid cells from RA patients revealed LIGHT staining of more than 50% of CD4+ T cells, supporting an activated phenotype of these cells [25, 26]. Therefore, in addition to soluble LIGHT, activated T cells carrying the membrane associated form of LIGHT may play a role in a cell-contact dependent activation of HVEM- or LTβR-expressing cells.

It has to be noted that there were no differences in the response of RA-SF vs OA-SF to LIGHT stimulation in vitro. These results correspond to a similar expression level of the LIGHT receptors in RASF and OA-SF in vitro. However, the concentration of LIGHT in the synovial fluid was significantly higher in RA as compared with OA, possibly explained by the virtual absence of activated T cells in the synovium of patients with OA. Therefore, the availability of LIGHT protein rather than increased expression of LIGHT receptors in synovial fibroblasts may be responsible for increased LIGHT signaling in RA synovial tissue.

LIGHT induced activation of NF-κB has been shown previously for a monocytic cell line [27] and for primary hepatocytes [15]. However, the functions of the signalling pathways associated with receptors of the TNF superfamily are often cell-type specific. We demonstrate that in RA-SF, at least parts of the activities of LIGHT are induced via NF-κB-driven transcription.

Alterations in the apoptosis of RA-SF have been described and associated with the pathogenesis of RA [28–30]. These changes contribute to both chronic inflammation and hyperplasia. The resistance of RA-SF to apoptosis has been linked closely to the progressive destruction of articular cartilage [31] and a reduced sensitivity to Fas-induced apoptosis has been described [32]. In in vitro experiments, the presence of LIGHT led to a significant decrease of Fasl-induced cell death of RA-SF, whereas the spontaneous apoptosis rate was unaffected. Proliferation of RA-SF, which might have contributed to the observed decrease in apoptotic cell death measurements, was not affected by LIGHT. Contrasting to the observed effect in synovial fibroblasts, LIGHT has been shown to increase apoptotic cell death [13, 33]. However, in another study in hepatocytes LIGHT also had an antiapoptotic effect, but this was only observed for TNFα and not for Fas-induced apoptosis [33]. The antiapoptotic effect of LIGHT observed on RA-SF may argue for a prevailing effect mediated via HVEM, since LTβR-mediated signalling was shown to induce cell death in HT-29 cells [13].

Taken together, LIGHT is up-regulated in the rheumatoid but not osteoarthritic joint and may contribute to the inflammatory process by several mechanisms. As reported in the transgenic mouse models increased LIGHT concentrations may lead to T cell co-stimulation and enhanced lymphocytic infiltration. Moreover, LIGHT may activate non-lymphoid cells expressing HVEM or LTβR, such as synovial fibroblasts or macrophages. Additionally, by decreasing susceptibility to apoptotic stimuli LIGHT may contribute to synovial hyperplasia. Finally, as reported recently, LIGHT can induce osteoclast formation and may thereby contribute to localized bone loss [34]. Based on these results, LIGHT signalling pathways may represent promising therapeutic targets for the treatment of RA.

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