

LIGHT (TNFSF14), a Novel Mediator of Bone Resorption, Is Elevated in Rheumatoid Arthritis

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Objective. Human osteoclast formation from mononuclear phagocyte precursors involves interactions between tumor necrosis factor (TNF) ligand superfamily members and their receptors. LIGHT is a transmembrane protein expressed and shed from the surface of activated T cells. Since activated T cells have been implicated in osteoclastogenesis in rheumatoid arthritis (RA), this study sought to determine whether LIGHT can regulate RANKL/cytokine-induced osteoclast formation, to identify the mechanism by which LIGHT influences osteoclastogenesis, and to investigate the presence of LIGHT in the serum of RA patients.

Methods. The effect of LIGHT on human and murine osteoclast formation was assessed in the presence and absence of neutralizing reagents to known osteoclastogenic factors. Serum levels of LIGHT in RA patients were measured by enzyme-linked immunosorbent assay.

Results. In the presence and absence of RANKL, LIGHT induced osteoclast formation from both human peripheral blood mononuclear cells and murine macrophage precursors, in a dose-dependent manner, whereas no inhibition was observed by adding osteoprotegerin, RANK:Fc, TNF α , or interleukin-8 or by blocking the LIGHT receptors herpesvirus entry mediator or lymphotoxin β receptor. However, formation of osteoclasts

was significantly decreased by the soluble decoy receptor for LIGHT, DcR3, and by blocking antibodies to the p75 component of the TNF receptor. A significant increase in LIGHT levels in the serum of RA patients compared with normal controls was also noted.

Conclusion. Our results indicate that LIGHT promotes RANKL-mediated osteoclastogenesis and that it can induce osteoclast formation by a mechanism independent of RANKL. The increased concentration of LIGHT in patients with RA raises the possibility that LIGHT may play a role in immunopathogenic conditions that are associated with localized or systemic bone loss.

Osteoclasts are multinucleated cells that specifically function in lacunar bone resorption (1). Osteoclast differentiation from hematopoietic and circulating precursors requires the presence of macrophage colony-stimulating factor (M-CSF) and the receptor activator for nuclear factor κ B ligand, RANKL (2–5). RANKL is a tumor necrosis factor (TNF) superfamily member that is expressed by osteoblasts and T cells and interacts with RANK on osteoclast precursors (5–8). Osteoprotegerin (OPG) acts as a decoy receptor for RANKL and blocks RANKL-mediated osteoclast differentiation and stimulation of osteoclast-resorbing activity (9,10). Although TNF α has also been shown to promote osteoclast formation by a RANKL-independent mechanism, the presence of TNF α results in considerably less lacunar resorption than that in RANKL-induced osteoclast formation (11,12).

LIGHT (homologous to lymphotoxins exhibiting inducible expression and competing with herpes simplex virus glycoprotein D for herpesvirus entry mediator [HVEM], a receptor expressed by T lymphocytes) is a newly identified member of the TNF superfamily (TNFSF14), that is expressed by activated T lymphocytes, monocytes, granulocytes, spleen cells, and imma-

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ture dendritic cells (13,14). LIGHT is a type II transmembrane protein that is known to bind 2 membrane-bound TNFSF signaling receptors: HVEM, which is predominantly expressed by T cells, and lymphotoxin β receptor (LT β R), which is expressed by stromal cells and nonlymphoid hematopoietic cells (13,15,16). LIGHT also binds to a soluble nonsignaling receptor, decoy receptor 3 (DcR3), which can modulate the function of LIGHT in vivo (13,17,18). DcR3 has been implicated in T cell development and homeostasis (19,20), dendritic cell maturation (21), atheroma formation, and tumor development (22–24).

LIGHT can also costimulate T cell responses via HVEM (25), which is constitutively expressed in most lymphocyte subpopulations, including CD4⁺ and CD8⁺ T cells (26,27). In addition, LIGHT has been shown to suppress tumor formation in vivo (16) and to induce tumor cell apoptosis (28) via the up-regulation of intercellular adhesion molecule 1 and an increased lymphocyte adhesion to cancer cells (29). Thus, LIGHT is being actively investigated as a possible basis for cancer treatment.

Whereas TNFSF members such as RANKL and OPG are known to play key roles in both normal and pathologic bone resorption, the effect of LIGHT on osteoclast formation and bone resorption is unknown. T cells express RANKL (30), and activated T cells have been shown to support osteoclast formation directly through increased expression of RANKL (31). Since LIGHT is also produced by activated T cells, and T cell products have been shown to influence osteoclast formation in a number of pathologic conditions associated with bone and joint destruction, such as rheumatoid arthritis (RA) (32–34), we sought, in the present study, to determine whether LIGHT plays a role in osteoclast formation and bone resorption. Our findings show that LIGHT not only promotes RANKL-dependent osteoclast formation, but also can independently induce a significant amount of osteoclast formation and bone resorption. We also show that the serum levels of soluble LIGHT are elevated in RA patients.

PATIENTS AND METHODS

Reagents. For all incubations, α -minimum essential medium (α -MEM) (Invitrogen, Paisley, UK) was supplemented with 100 IU/ml penicillin, 10 μ g/ml streptomycin, 10 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS). Recombinant human M-CSF, LIGHT, TNF α , RANK:Fc, DcR3, LT β R:Fc chimera, anti-HVEM, and neutralizing antibodies to TNF receptors p55 (type I) and p75 (type II)

were purchased from R&D Systems, Europe (Abingdon, UK). Mouse and human soluble RANKL and human and murine OPG were purchased from PeproTech (London, UK). All incubations were carried out at 37°C in 5% CO₂.

Cultures of mouse and human osteoclast formation in the presence of LIGHT. Short-term cultures of murine RAW264.7 cells (kindly provided by Professor Mike Rogers, Aberdeen, UK) were set up by adding 5×10^3 cells to glass coverslips or dentine slices in 24-well plates in the presence of increasing concentrations of human LIGHT (1–150 ng/ml). As a positive control, RAW264.7 cells were also set up in the presence of both mouse and human soluble RANKL. Cultures were maintained for up to 12 days, during which time the entire culture medium containing all factors was replenished every 3 days.

In order to confirm the findings in RAW264.7 cells, parallel experiments were also set up using human peripheral blood mononuclear cell (PBMC) cultures. Mononuclear cells were isolated from the leukocyte fraction of the peripheral blood of healthy male volunteers (ages 25–35 years) as previously described (12). Briefly, blood was collected and diluted 1:1 in MEM, layered over Histopaque (Sigma-Aldrich, Poole, UK), and then centrifuged (693g), washed, and resuspended in MEM/FBS. The number of cells in the resultant suspension of PBMCs was counted in a hemocytometer. The cell suspension of PBMCs (5×10^5 cells/well) was added to 96-well tissue culture plates containing dentine slices or coverslips.

After 2 hours' incubation, dentine slices or coverslips were removed from the wells and washed vigorously in MEM/FBS to remove nonadherent cells; they were then placed in a 24-well tissue culture plate containing 1 ml of MEM/FBS supplemented with 25 ng/ml M-CSF. As positive controls, PBMC cultures were maintained in the presence of 30 ng/ml soluble RANKL and 25 ng/ml M-CSF. As negative controls, PBMC cultures were maintained with M-CSF alone. All PBMC cultures were incubated for up to 21 days, during which time the entire culture medium containing all factors was replenished every 2–3 days.

PBMCs were cultured with increasing doses of human LIGHT (1–150 ng/ml) in the presence of M-CSF. Because the results suggested that 50 ng/ml of LIGHT is an optimal concentration for induction of osteoclast formation in PBMC cultures, all subsequent studies were performed using this concentration.

Analysis of LIGHT receptor expression on human PBMCs. To establish the potential for LIGHT signaling in osteoclast precursors, the presence of each of the signaling receptors for LIGHT (HVEM and LT β R) was investigated. Human PBMCs were isolated as described above and labeled with anti-human HVEM (NeoMarkers, Fremont, CA) or anti-human LT β R (Becton Dickinson, Oxford, UK) and anti-human CD14 (IDS, Frankfurt, Germany). The level of fluorescence was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).

Analysis of the effect of LIGHT on human osteoclast formation. Since most commercially available neutralizing antibodies are directed against the relevant human proteins/receptors, only PBMCs were used for assessment of human osteoclast formation. To determine the mechanism by which LIGHT influences human osteoclast formation, PBMC cultures were incubated in the presence of M-CSF alone or with

excess concentrations of the following factors: M-CSF (25 ng/ml), M-CSF with LIGHT (50 ng/ml), M-CSF with LIGHT plus OPG (500 ng/ml), M-CSF with LIGHT plus RANK:Fc chimera (500 ng/ml), M-CSF with LIGHT plus DcR3 (500 ng/ml), M-CSF with LIGHT plus anti-TNF α (100 μ g/ml), M-CSF with LIGHT plus anti-interleukin-8 (IL-8) (100 μ g/ml), M-CSF with LIGHT plus anti-human HVEM (500 ng/ml), M-CSF with LIGHT plus LT β R:Fc chimera (500 ng/ml), and M-CSF with LIGHT plus anti-TNF receptor p55 or p75 (10 μ g/ml each).

The concentrations of OPG, RANK:Fc, anti-TNF α , and anti-TNF receptors used in these experiments have previously been shown, in our laboratory, to completely inhibit RANKL- and TNF α -induced osteoclast formation (12). The biologic activity of the blocking agents directed against LIGHT receptors was confirmed by the fact that the addition of 500 ng/ml anti-human HVEM or LT β R:Fc chimera markedly inhibited LIGHT-induced proliferation of Jurkat T cells (which are known to express HVEM) and LIGHT-induced apoptosis of MDA-MB436 breast cancer cells (which are known to express LT β R), respectively.

Analysis of the effect of DcR3 on LIGHT-induced osteoclast formation. To determine the effect of DcR3, a soluble decoy receptor for LIGHT, on LIGHT-induced osteoclastogenesis, PBMC cultures were incubated with M-CSF (25 ng/ml) in the presence of LIGHT (50 ng/ml) and increasing concentrations of DcR3 (0, 25, 50, 100, 250, and 500 ng/ml).

Analysis of the effect of LIGHT on RANKL- or TNF α -induced osteoclast formation. To assess the effect of LIGHT on RANKL- and TNF α -induced osteoclast formation, PBMC cultures were incubated with M-CSF (25 ng/ml) in the presence of LIGHT (50 ng/ml) alone, soluble RANKL (30 ng/ml) alone, a combination of LIGHT plus soluble RANKL, TNF α (20 ng/ml) plus IL-1 α (10 ng/ml), a combination of LIGHT plus TNF α and IL-1 α , or a combination of LIGHT, soluble RANKL, and DcR3 (500 ng/ml).

Characterization of osteoclasts formed on coverslips and dentine slices. *Tartrate-resistant acid phosphatase (TRAP).* Histochemical staining for TRAP, a marker of osteoclasts (35), was carried out using a commercially available kit (Sigma-Aldrich) after 7 days and 14 days of incubation of RAW264.7 cells and PBMC cultures, respectively. Cell preparations were fixed in citrate/acetone solution and stained with acid phosphatase, using naphthol AS-BI phosphate as a substrate, in the presence of 1.0M tartrate; the product was reacted with fast garnet GBC salt.

Vitronectin receptor (VNR). Human PBMC cultures on coverslips were stained immunohistochemically by an indirect immunoperoxidase technique. This technique utilized the monoclonal antibody 23C6 (Serotec, Oxford, UK), which is directed against the VNR, CD51, an osteoclast-associated antigen (36).

F-actin ring. Multiple rows of podosomes are often localized in the sealing zone of osteoclasts (37). Podosomes consist of an F-actin core surrounded by a number of actin-binding proteins (38). To detect F-actin ring structure (39), dentine slices were fixed with 4% formaldehyde for 5 minutes and then permeabilized for 6 minutes in 0.5% Triton X-100 (in phosphate buffered saline [PBS]) and rinsed with PBS. The cells on dentine slices were then incubated with tetramethyl-

rhodamine isothiocyanate-conjugated phalloidin (Sigma-Aldrich) for 30 minutes and observed using a fluorescence microscope (Nikon, Tokyo, Japan).

Lacunar resorption. Functional evidence of osteoclast formation was determined by a resorption assay system using cell cultures on dentine slices. After 7–9 days (for RAW264.7 cells) or 21 days (for human PBMC cultures), the dentine slices were removed from the wells, rinsed in PBS, and placed in 0.25M ammonium hydroxide and sonicated for 5–10 minutes. Under these conditions, all cells were completely removed from the dentine slice, permitting examination of the dentine surface for evidence of lacunar resorption. The slices were then washed in distilled water, stained with 0.5% (weight/volume) aqueous toluidine blue, and examined by light microscopy. In some cases the presence of lacunar resorption on dentine slices was confirmed using scanning electron microscopy. Cultures on dentine slices were also assessed by scanning electron microscopy. The cell cultures were fixed in 4% glutaraldehyde and dehydrated by passing through graded alcohols and then through graded (50–100%) hexamethyl-disilazane solution (Sigma-Aldrich), before being air-dried. Dentine slices were then mounted onto aluminium stubs (Agar Scientific Ltd, Stanstead, UK) that were sputtered with gold, and cells were examined using a Philips 505 scanning electron microscope (Mahwah, NJ).

Assessment of normal and RA serum for soluble LIGHT. To determine whether soluble LIGHT may play a role in pathologic bone resorption when associated with a condition in which there is an increase in activated T cells in the joints, synovium, and lymphoid tissues, we measured the serum level of LIGHT in RA patients (n = 11) and compared it with that in healthy age- and sex-matched controls (n = 11). This was carried out using a human LIGHT enzyme-linked immunosorbent assay (ELISA) kit (supplied by R&D Systems). The minimum and maximum detection levels of the ELISA were 31 pg/ml and 2 ng/ml, respectively.

Statistical analysis. The extent of lacunar resorption on dentine slices was determined by measuring the percentage area of resorption on each dentine slice. Each experiment was repeated at least 4–5 times and, for each treatment, the data were expressed as the mean \pm SEM percentage area of lacunar resorption of 3 dentine slices. Statistical significance was determined using 2-way Student's *t*-test or 2-factor analysis of variance with replication. *P* values less than 0.05 were considered significant.

RESULTS

Induction of murine/human osteoclast formation by LIGHT, in a dose-dependent manner. *Murine RAW264.7 cells.* In control (RANKL-treated) RAW264.7 cell cultures, TRAP+ multinucleated cells were found after 7 days in culture, and lacunar resorption pits were noted within 9–12 days. Within 7 days of culturing RAW264.7 cells on glass coverslips with increasing doses of LIGHT, the formation of large,

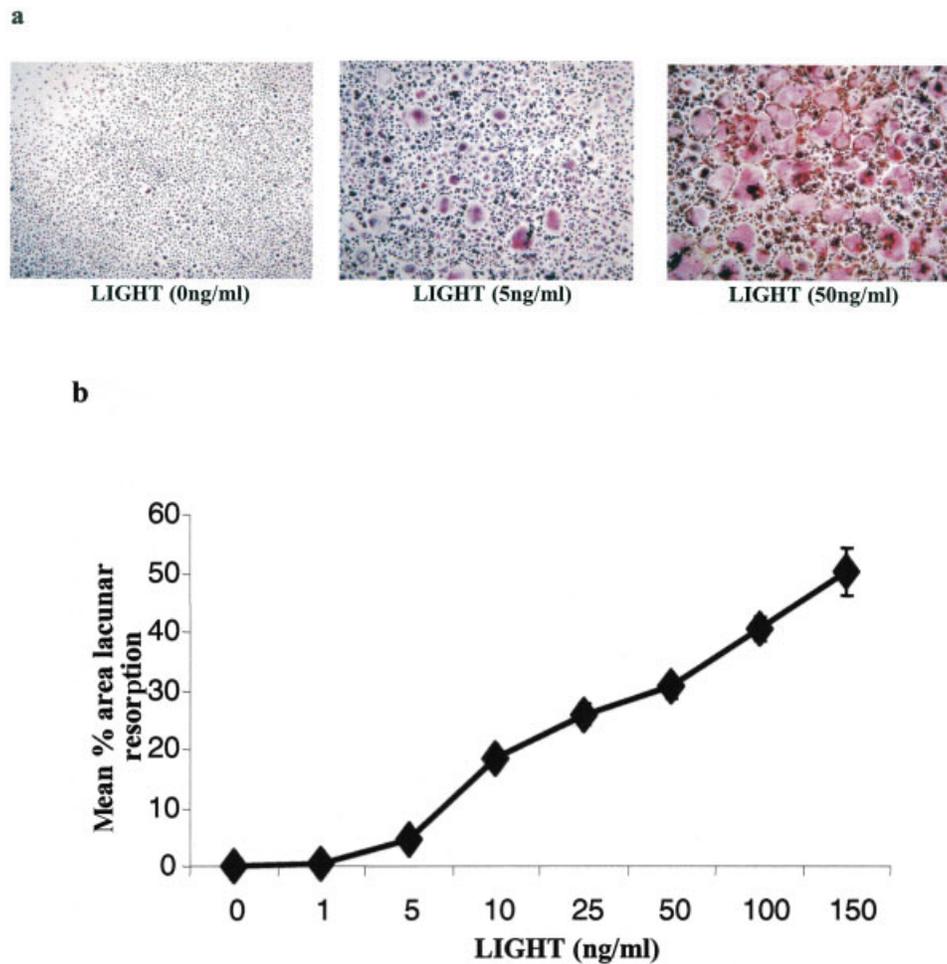


Figure 1. Dose-dependent increases in tartrate-resistant acid phosphatase-positive (TRAP+) multinucleated cell formation and lacunar resorption by LIGHT. **a** and **b**, The effect of varying concentrations of soluble LIGHT on the extent of osteoclast formation and lacunar resorption in RAW264.7 cells is assessed by **a**, the presence of TRAP+ multinucleated cells after 7 days in culture (original magnification $\times 200$) and **b**, the mean \pm SEM percentage area of lacunar resorption after 12 days in culture.

multinucleated TRAP+ cells was observed (Figure 1a). The lowest concentration of LIGHT that induced osteoclast formation was 5 ng/ml. At doses higher than 50 ng/ml, LIGHT-induced TRAP+ multinucleated cells appeared much larger than those formed at lower concentrations of LIGHT.

Lacunar resorption was observed in RAW264.7 cells treated with LIGHT for at least 12 days on dentine slices. As shown in Figure 1b, the lowest concentration at which LIGHT could induce resorption was 5 ng/ml, with a mean \pm SEM percentage area of resorption of $4.5 \pm 0.75\%$. At 150 ng/ml, the percentage area of lacunar resorption was $48 \pm 3.6\%$.

Human PBMCs. Human PBMC cultures, in contrast to RAW 264.7 cells, require the exogenous addition of M-CSF throughout the culture period for osteoclastogenesis to occur. As shown in Figure 2, PBMCs incubated with M-CSF and 50 ng/ml LIGHT for 21 days resulted in the formation of TRAP+ multinucleated cells capable of lacunar resorption on dentine slices (Figures 2a, b, and c). Moreover, LIGHT was capable of inducing F-actin ring formation in these cells (Figure 2d).

In parallel cultures in which human PBMCs were incubated with soluble RANKL and M-CSF, extensive lacunar resorption (up to 500 pits; $\sim 50\text{--}60\%$ of

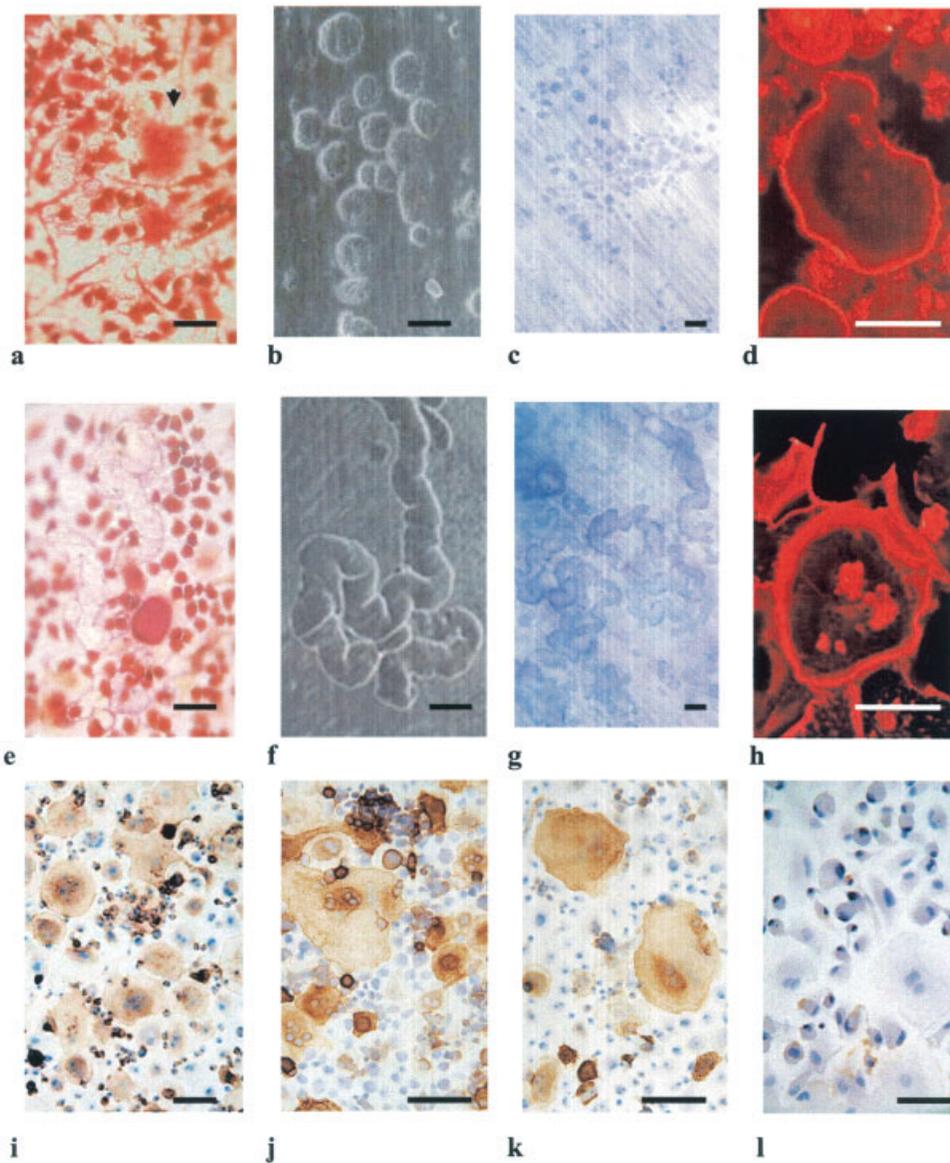


Figure 2. Comparison of osteoclast formation and resorption in LIGHT- and RANKL-treated human peripheral blood mononuclear cell (PBMC) cultures. Multinucleated cells formed in 21-day human PBMC cultures incubated with macrophage colony-stimulating factor and LIGHT (a–d) or RANKL (e–h) showed expression of tartrate-resistant acid phosphatase on dentine slices, the ability to carry out lacunar resorption, and F-actin ring staining. A similar pattern of positive vitronectin receptor (VNR) staining was observed in PBMCs treated with LIGHT (i), RANKL (j), and tumor necrosis factor α (k) compared with the negative isotype control (l). Bars = 50 μ m.

the dentine slice area) was noted (Figures 2e, f, g, and h). In contrast to the large resorption pits and confluent areas of lacunar resorption seen in RANKL-treated PBMC cultures, it was noted that all resorption pits in LIGHT-treated PBMC cultures were small, discrete

(i.e., nonconfluent), and either round or ovoid. Despite this difference, the large, multinucleated cells formed in LIGHT-treated cultures expressed VNR and had similar morphologic features as those formed following RANKL or TNF α stimulation (Figures 2i, j, k, and l).

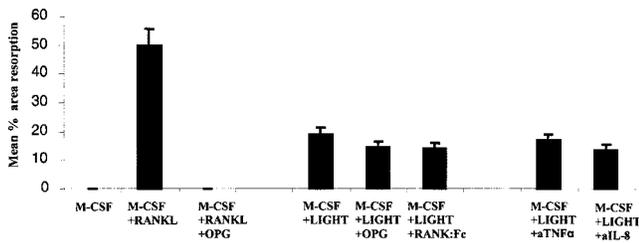


Figure 3. Effect of neutralizing agents for known osteoclastogenic factors on LIGHT-induced lacunar resorption. Excess concentrations of osteoprotegerin (OPG) (500 ng/ml) or RANK:Fc (500 ng/ml), both of which are capable of totally blocking RANKL-induced lacunar resorption, and anti-tumor necrosis factor α (aTNF α) and anti-interleukin-8 (aIL-8) antibodies (both at 100 μ g/ml) had no significant effect on the mean percentage area of lacunar resorption induced by LIGHT (50 ng/ml) in human peripheral blood mononuclear cell cultures. Bars show the mean and SEM. M-CSF = macrophage colony-stimulating factor.

Effect of neutralizing agents on LIGHT-induced osteoclast formation and resorption. To investigate whether LIGHT-induced osteoclast formation occurred via the RANKL/RANK pathway, OPG and RANK:Fc were added to LIGHT-treated human PBMC cultures. Addition of excess molar concentrations of either OPG or RANK:Fc demonstrated no significant decrease in lacunar pit formation in cultures that had been treated with LIGHT alone (Figure 3). The addition of excess antibodies to other known osteoclastogenic factors, i.e., IL-8 and TNF α , also demonstrated no significant decrease in LIGHT-induced lacunar resorption (Figure 3).

Inhibition of LIGHT-induced osteoclast formation and resorption by DcR3. To determine the effect of the soluble decoy receptor of LIGHT, DcR3, on LIGHT-induced osteoclast formation, varying concentrations of DcR3 were added to human PBMC cultures in the presence of LIGHT. It was found that increasing concentrations of DcR3 dose-dependently inhibited LIGHT-induced lacunar resorption, demonstrating up to $88.5 \pm 3.3\%$ inhibition (mean \pm SEM) with 500 ng/ml of DcR3, compared with cultures with M-CSF and LIGHT alone ($P < 0.05$). Moreover, DcR3 alone had no effect on osteoclast formation and resorption (results not shown).

Greater lacunar resorption after cotreatment with LIGHT and RANKL than with LIGHT or RANKL alone. In 21-day cultures of PBMCs incubated in the presence of M-CSF, LIGHT, and RANKL, resorption pit formation was stimulated 2–4-fold as compared with

PBMC cultures treated with RANKL or LIGHT alone (Figures 4a and b). The addition of excess OPG (or RANK:Fc) to PBMC cultures treated with LIGHT and RANKL did not completely inhibit the formation of VNR+ multinucleated cells and reduced lacunar resorption to a level similar to that with LIGHT alone. Furthermore, PBMC cultures maintained in the presence of M-CSF, LIGHT, and soluble RANKL demonstrated $82 \pm 3.8\%$ mean \pm SEM area of lacunar resorption. The addition of DcR3 significantly reduced resorption to $62 \pm 6.8\%$, a level comparable with that seen in control cultures containing M-CSF and soluble RANKL alone ($57 \pm 4.6\%$) (Figure 4b).

Lacunar resorption pits that formed in response to LIGHT alone were generally small and discrete. The morphologic features were similar to those of pits that formed in response to TNF α but larger than those of pits formed in PBMC cultures to which TNF α plus IL-1 α had been added (Figure 4a). The addition of TNF α to LIGHT did not have a significant additive effect on the extent of lacunar resorption (Figures 4a and b).

Presence of LIGHT receptors on human PBMC osteoclast precursors. Flow cytometric analysis utilizing antibodies directed against HVEM and LT β R demonstrated the presence of HVEM on all human PBMCs, whereas LT β R was expressed only on a single human PBMC population. Dual-color flow cytometry using antibodies directed against CD14 and LT β R demonstrated that this single population of PBMCs expressed both CD14 and LT β R (results not shown).

The recent availability of blocking reagents to LIGHT receptors (i.e., LT β R and HVEM) allowed us to examine the possibility that the osteoclastogenic effect of LIGHT is mediated through one or both of these signaling proteins. We found that the addition of excess concentrations of both blocking reagents to LIGHT-treated PBMC cultures failed to prevent the appearance of multinucleated VNR+ cells capable of lacunar resorption (Figures 5a and b).

Since LIGHT is a member of the TNFSF, we also investigated whether LIGHT mediated its osteoclastogenic response through TNF receptors. Antibodies directed against both components of the TNF receptor (i.e., TNFR I [p55 component] and TNFR II [p75 component]) were added to LIGHT-treated PBMC cultures. A marked decrease in the number of TRAP+ multinucleated cells formed was seen in cultures treated with anti-TNFR II (MAB226), which was accompanied by a significant decrease in lacunar formation (Figure 5b). To

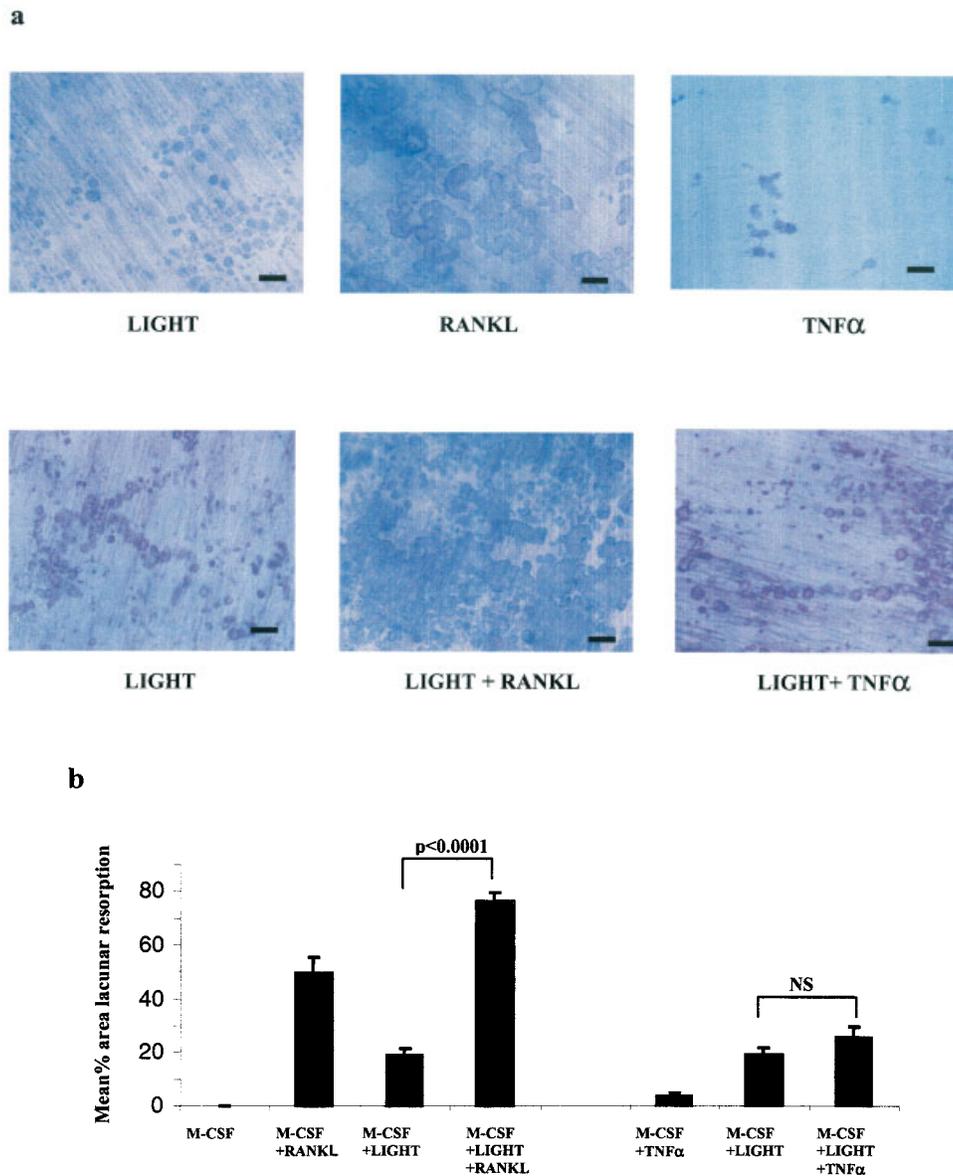


Figure 4. Effect of cotreatment with LIGHT plus RANKL or tumor necrosis factor α (TNF α). **a**, In 21-day peripheral blood mononuclear cell cultures, small resorption pits were formed following LIGHT treatment compared with large resorption lacunae in RANKL-treated cultures or a small number of discrete pits following TNF α stimulation (stained with toluidine blue; bars = 50 μ m). **b**, LIGHT in the presence of RANKL increased lacunar resorption 2–4-fold compared with that in LIGHT-treated cultures alone, whereas addition of TNF α to LIGHT-treated cultures caused no significant (NS) increase. Bars show the mean and SEM.

confirm these findings, we used an alternative neutralizing monoclonal antibody to human TNFR_{II}, MAB726, in an identical, independent study; our results demonstrated a mean \pm SEM 75 \pm 1.5% inhibition in LIGHT-induced lacunar resorption compared with that in cultures with LIGHT plus an isotype control ($P < 0.01$).

Increased expression of LIGHT in the serum of patients with RA. Serum samples from 11 patients with RA who were not currently receiving treatment and 11 age- and sex-matched normal control subjects were collected and assessed for the presence of soluble LIGHT. A significant increase in soluble LIGHT was

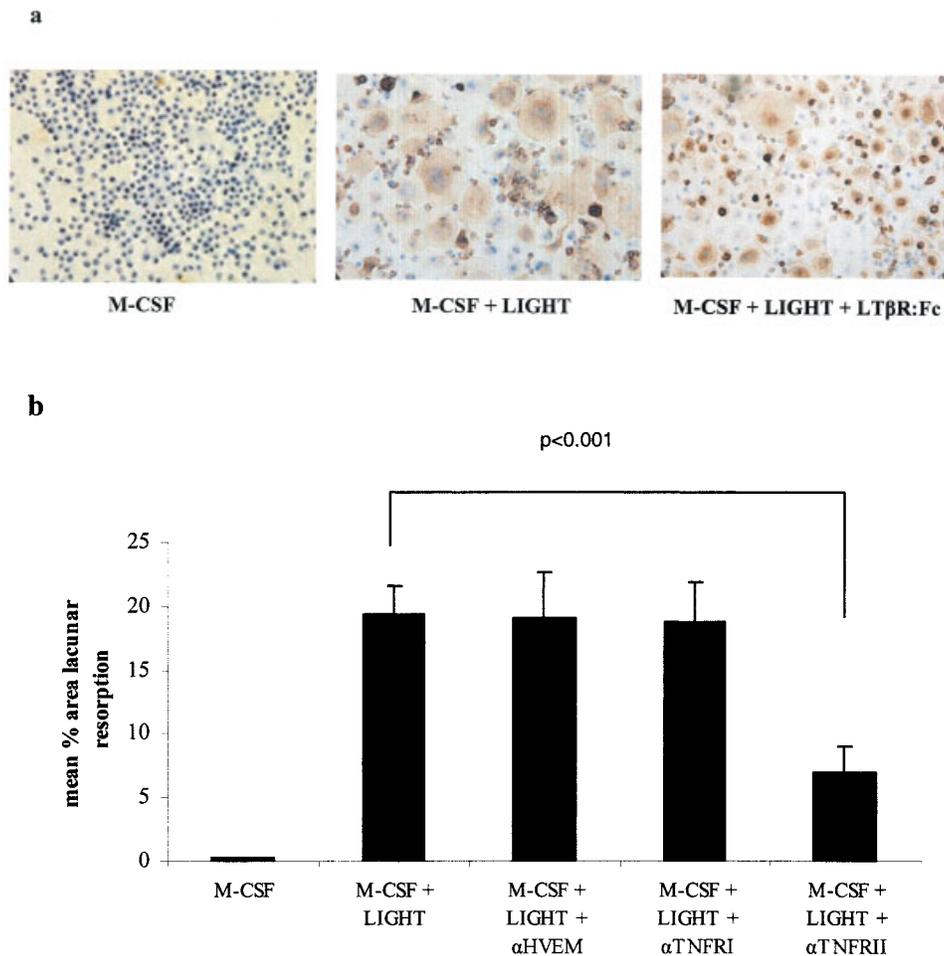


Figure 5. LIGHT-induced osteoclast formation and lacunar resorption mediated through tumor necrosis factor receptor (TNFR) p75. **a**, Addition of the fusion protein for lymphotoxin β receptor (LT β R:Fc) to LIGHT-treated peripheral blood mononuclear cell (PBMC) cultures had no effect on formation of vitronectin receptor-positive multinucleated cells (stained with immunoperoxidase; original magnification $\times 200$). **b**, Addition of a neutralizing antibody to herpesvirus entry mediator (α HVEM) had no significant effect on the percentage area of lacunar resorption (mean and SEM) in LIGHT-treated PBMC cultures. A significant decrease in the mean percentage area of lacunar resorption was noted when anti-TNFR II (directed against TNFR p75) was added to PBMC cultures incubated with LIGHT ($P < 0.001$ compared with LIGHT alone). M-CSF = macrophage colony-stimulating factor.

found in RA serum samples ($P < 0.05$) when compared with control samples (Figure 6).

DISCUSSION

Osteoclasts are formed by fusion of mononuclear phagocyte precursors that circulate in the CD14+ (monocyte) fraction of whole blood (40,41). Osteoclast formation is dependent on the interaction of RANK (expressed on osteoclast precursors) with RANKL, which is mainly expressed on bone stromal cells and T cells (3–5). Activated T cells have been shown to directly

induce osteoclastogenesis and bone resorption (31,32), and T cell products, such as IL-17, TWEAK, granulocyte-M-CSF, and interferon- γ , have previously been shown to regulate osteoclast formation (42–44). In the present study, we have shown that LIGHT, a product of activated T cells, not only promotes RANKL-induced osteoclast formation, but also, in a dose-dependent manner, directly induces osteoclast formation through a mechanism other than the RANKL/RANK pathway. We also have demonstrated that the serum concentration of LIGHT is significantly increased in patients with RA,

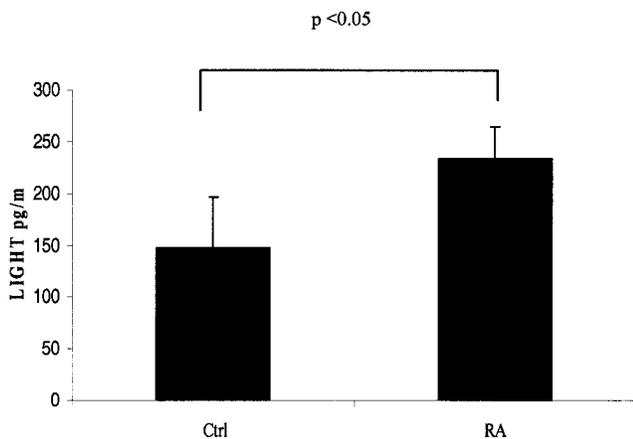


Figure 6. Serum levels of LIGHT in patients with rheumatoid arthritis (RA) ($n = 11$) compared with age- and sex-matched normal control (Ctrl) subjects ($n = 11$). Bars show the mean and SEM.

suggesting that LIGHT may play a role in the joint-based or systemic osteolysis associated with this autoimmune disorder.

LIGHT-induced osteoclastogenesis in cultures of murine osteoclast precursors (RAW264.7 cells) and human PBMCs occurred in the absence of RANKL. This osteoclast formation was not abolished when OPG or RANK:Fc was added, demonstrating that LIGHT-induced osteoclastogenesis was independent of the RANKL/RANK signaling pathway. We and other investigators have previously shown that RANKL-independent osteoclast formation can occur when marrow or circulating mononuclear phagocytes are stimulated with cytokines, such as $\text{TNF}\alpha$ and IL-8 (12,45). In contrast to the large resorption pits and confluent areas of lacunar excavation produced in PBMC cultures treated with RANKL, treatment with LIGHT alone resulted in the formation of numerous small, discrete, round or ovoid resorption pits; this pattern of resorption is similar to that seen following treatment with $\text{TNF}\alpha$ (12). The differences in the size and morphologic features of the resorption pits formed under these conditions are most likely attributable to the fact that smaller TRAP⁺ and VNR⁺ osteoclastic cells are formed from monocytes in cytokine-treated cultures, thus giving rise to smaller and shallower lacunar resorption pits. RANKL is shown to promote the fusion of osteoclast precursors (5,46), and this would account for an increase in the size and multinuclearity of osteoclasts and larger resorption pits formed in RANKL-treated cultures.

LIGHT is known to interact with 2 membrane-

bound TNFSF signaling receptors, HVEM and $\text{LT}\beta\text{R}$ (47). HVEM expression is mainly restricted to lymphocytes, while $\text{LT}\beta\text{R}$ is expressed primarily on cells of stromal origin (48). Our studies demonstrate, for the first time, the presence of $\text{LT}\beta\text{R}$ on the CD14⁺ subpopulation of cells. The response induced by the binding of LIGHT to its receptors is cell specific (13,16,49,50). HVEM interacts with TRAF2 and TRAF5, leading to activation of the NF- κB or the JNK/activator protein 1 pathway, ultimately resulting in transcription of genes that promote cell survival, cytokine production, or cell proliferation (51). In contrast, LIGHT- $\text{LT}\beta\text{R}$ signaling can lead to cell death by the recruitment of TRAF3 and subsequent caspase activation. The presence of HVEM on cells resistant to the apoptotic effect of $\text{LT}\beta\text{R}$ suggests that HVEM exerts a regulatory role on LIGHT-induced apoptosis, and it is possible that $\text{LT}\beta\text{R}$ -expressing CD14⁺ cells are protected from LIGHT-induced cell death by the expression of HVEM.

Neutralizing reagents to HVEM or $\text{LT}\beta\text{R}$ had no effect on LIGHT-induced osteoclast formation, suggesting that this response was not mediated through HVEM or $\text{LT}\beta\text{R}$. However, the addition of 2 independent neutralizing antibodies along with anti-TNFRII (p75), which is expressed by monocytes (52), resulted in a significant decrease in osteoclast formation and lacunar resorption in separate LIGHT-treated PBMC cultures. Although LIGHT has not previously been shown to bind this receptor, a LIGHT homolog, lymphotoxin α ($\text{LT}\alpha$), is known to bind TNFRI (p55) and TNFRII (p75) (49,53). Interestingly, LIGHT shares a high degree of homology with $\text{LT}\alpha$; shared receptor binding of LIGHT and $\text{LT}\alpha$ with HVEM (13) has also been noted, but stimulation of osteoclast formation by $\text{LT}\alpha$ has not been reported.

In addition to these receptors, LIGHT is also known to bind to DcR3, a soluble decoy receptor. DcR3 lacks a transmembrane domain and modulates the effects of LIGHT signaling on cell function, through competitive inhibition (16,17). We found that the addition of increasing concentrations of DcR3 resulted in a significant inhibition of LIGHT-induced osteoclast formation and bone resorption in human PBMC cultures. Yang et al (54) recently reported that DcR3 was capable of inducing osteoclastogenesis in human PBMC cultures in the absence of RANKL, M-CSF, and other osteoclastogenic factors. They stated that a complex reverse signaling mechanism initiated by DcR3 results in a bidirectional signaling cascade via ERK and p38 MAPK, and that this leads to the stimulation of $\text{TNF}\alpha$ release and consequent osteoclast formation. The requirement

that M-CSF must be present for PBMC attachment and survival in culture is well documented, and most in vitro systems of osteoclast formation require the addition of M-CSF. We found that cultures of human PBMCs treated with similar doses of DcR3, in both the presence and absence of M-CSF, did not induce the formation of VNR+ multinucleated cells capable of resorption. Our findings contradicted those of the aforementioned study, which may be explained, in part, by the differences in the biologic activity and sources of the DcR3 used in these experiments.

We and other investigators have previously shown that cotreatment of RANKL with calciotropic hormones and inflammatory cytokines, such as parathyroid hormone, 1,25-dihydroxyvitamin D₃, glucocorticoids, IL-1, and TNF α , promotes RANKL-induced osteoclastogenesis (55–57). This is predominantly mediated through up-regulation of RANKL and down-regulation of OPG expression on bone stromal cells. We found that in RANKL-treated human PBMC or murine RAW264.7 cultures, the addition of LIGHT resulted in a marked increase in the number of TRAP+ multinucleated cells capable of lacunar resorption. Because stromal cells were not present in our cultures, the mechanism through which the additive effect of LIGHT on RANKL-induced osteoclastogenesis is mediated remains unclear. This mechanism appears to be distinct from RANKL-induced osteoclastogenesis, since the addition of excess concentrations of OPG to RANKL/LIGHT-treated cultures led to a decrease in bone resorption, to a level comparable with that using LIGHT alone. Further evidence that this mechanism is not directly attributable to RANKL is shown by the fact that the addition of excess DcR3 to PBMC cultures, maintained in the presence of LIGHT and soluble RANKL, resulted in a significant reduction in lacunar resorption to a level comparable with that seen in PBMCs cultured with RANKL alone.

LIGHT is thought to play a major role in T cell homeostasis. Overexpression of LIGHT results in T cell-derived autoimmune diseases that affect a variety of tissues. Transgenic mice that constitutively express LIGHT have an enlarged T cell compartment and develop severe autoimmune diseases affecting several tissues, including intestine, skin, and kidney, with increased autoantibody production. Activated T cells in these animals also exhibit increased cytokine and growth factor production that is associated with expansion of the macrophage and granulocyte population, thereby resulting in splenomegaly and lymphadenopathy (58). In atherosclerosis, LIGHT expression by activated CD3+ T cells provides macrophages with a strong inflamma-

tory signal via HVEM during development and progression of atheromatous lesions (59).

Our finding of a significant increase in the serum levels of LIGHT in RA patients mirrors the increase in the number and activity of T cells in this condition (60,61). T cells are abundant in RA joints and are thought to play a crucial role in the activation and maintenance of the inflammatory changes that characterize this condition. A role of LIGHT in the pathogenesis of RA was first suggested by Fava et al (62), who showed that blocking LIGHT reduced the severity of joint damage in murine collagen-induced arthritis. More recently, Kim et al (63) found that both LIGHT and HVEM are expressed by RA synovial macrophages. They also showed that treatment of macrophage-enriched synovial fluid with LIGHT induces the expression of TNF α , IL-6, and IL-8, which are cytokines that have been shown to promote osteoclastic bone resorption.

Our results indicate that LIGHT can promote RANKL-mediated osteoclastogenesis and induce osteoclast formation by a mechanism independent of RANKL. Despite the fact that serum LIGHT levels were lower than those shown to induce monocyte-osteoclast differentiation in vitro, the significant increase in serum LIGHT noted in RA patients suggests that this factor may play a role in the localized and systemic bone loss that occurs in this condition.

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