LIGHT is Expressed in Foam Cells and Involved in Destabilization of Atherosclerotic Plaques through Induction of Matrix Metalloproteinase-9 and IL-8

Won-Jung Kim and Won-Ha Lee

Department of Genetic Engineering, Kyungpook National University, Daegu, Korea

ABSTRACT

Background: LIGHT (TNFSF14) is a member of tumor necrosis factor superfamily and is the ligand for TR2 (TNFRSF14/HVEM). LIGHT is known to have pro-inflammatory roles in atherosclerosis. **Methods:** To find out the expression pattern of LIGHT in atherosclerotic plaques, immunohistochemical analysis was performed on human carotid atherosclerotic plaque specimens. LIGHT induced atherogenic events using human monocytic cell line THP-1 were also investigated. **Results**: Immunohistochemical analysis revealed expression of LIGHT and TR2 in foam cell rich regions in the atherosclerotic plaques. Double immunohistochemical analysis further confirmed the expression of LIGHT in foam cells. Stimulation of THP-1 cells, which express TR2, with either recombinant LIGHT or immobilized anti-TR2 monoclonal antibody induced interleukin-8 and matrix metalloproteinase(MMP)-9. Electrophoretic mobility shift assay demonstrated that LIGHT induces nuclear localization of transcription factor, nuclear factor (NF)-κB. LIGHT induced activation of MMP-9 is mediated by NF-κB, since treatment of THP-1 cells with the NF-κB inhibitor PDTC (pyrrolidine dithiocarbamate) completely blocked the activation of MMP-9. **Conclusion:** These data indicate that LIGHT is expressed in foam cells in atherosclerotic plaques and is involved in atherogenesis through activation of pro-atherogenic cytokine IL-8 and destabilization of plaque by inducing matrix degrading enzyme. **(Immune Network 2004;4(2):116-122)**

Key Words: Atherosclerosis, inflammation, matrix metalloproteinase, LIGHT, TNFSF

Introduction

Members of tumor necrosis factor superfamily (TNFSF) and TNF receptor superfamily (TNFRSF) play pivotal roles in atherogenesis. TNF-α was found to be expressed in atherosclerotic plaques (1,2) in foam cells, smooth muscle cells (SMCs) (3,4), and mast cells (5). CD40L (TNFSF5) and CD40 (TNFRSF5) were also found to be expressed in vascular endothelial cells, SMCs, and macrophages (6). The interaction between CD40 and CD40L, as well as the interaction between TNF-α and its receptor, elicits diverse biological responses involved in atherosclerosis, such as secretion of pro-inflammatory cytokines and matrix metalloproteinases, and expression of adhesion molecules and tissue factor (7,8). These responses are known to make the plaque unstable.

LIGHT (TNFSF14), a newly identified member of TNFSF, is expressed in spleen cells, activated peripheral blood lymphocytes, $CD8^+$ tumor infiltrating lymphocytes, granulocytes, immature dendritic cells and monocytes (9-13). LIGHT can interact with various cellular receptors such as lymphotoxin β receptor, which is expressed on epithelial and stromal cells, (14) and TR2 (TNFRSF14/HVEM) (10). Recently, LIGHT (TNFSF14) and its receptor TR2 (TNFRSF14/HVEM) have been implicated in atherogenesis (15). By Western blot approach, LIGHT expression was found to be higher in atheromatous plaques than in fibrous plaques. But the cell type(s) expressing LIGHT in atherosclerotic plaque is not known yet.

TR2 is expressed on immature DC (16,17), T and B lymphocytes, NK cells, monocytes, and endothelial

Correspondence to: Won-Ha Lee, PhD, Department of Genetic Engineering, Kyungpook National University, Daegu 702-701 Korea. (Tel) +82-53-950-5388, (FAX) +82-53-943-6925, (E-mail) whl@knu.ac.kr

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cells (11,18,19). TR2 also serves as a mediator for herpes simplex virus type 1 entry into activated T lymphocytes (10,20). In atherosclerotic plaques, TR2 expression was found in foam cells and the expression pattern overlapped with that of matrix matalloproteinases (MMP) such as MMP-1, MMP-9, and MMP-13. Stimulation TR2 in human monocytic cell line THP-1 caused induction of pro-atherogenic cytokines/chemokines and MMPs (15).

Since the cell types that express LIGHT in atherosclerotic plaques are not known, immunohistochemical analysis was performed using human carotid atherosclerotic plaques and, as a result, foam cells were identified as the source of LIGHT. To further investigate the role of LIGHT in atherogenesis, the human monocytic cell line THP-1 was stimulated with recombinant LIGHT and cellular responses were analyzed.

Materials and Methods

Histological analysis. For immunohistochemical analysis, carotid endoarterectomy specimens were obtained from patients, aged from 63 to 81, who underwent the surgery at Samsung Seoul Hospital (generously provided by Dr. Jeong-Euy Park). Atherosclerotic plaque specimens were washed with saline and embedded in OCT to make frozen sections. Standard 5-μm sections were stained using the LSAB kit (DAKO, Copenhagen, Denmark) according to the manual provided by the manufacturer. For double staining of CD68 and LIGHT, specimen was sequentially treated with anti-CD68 monoclonal antibody; biotin-linked secondary antibody; streptavidin-alkaline phosphatase; fuchsin for visualization of CD68 staining (red color); anti-LIGHT monoclonal antibody which was pre-conjugated with horseradish peroxidase using an Animal Research Kit (DAKO, Copenhagen, Denmark) according to the manual provided by the manufacturer; diaminobenzidene (DAB) for visualization of LIGHT (brown color); and finally with hematoxylin (blue color) for visualization of nuclei.

Cell culture and Monoclonal antibodies and reagents. Human monocytic leukemia THP-1 cells (21) were obtained from the American Type Culture Collection (Rockville, MD). Soluble recombinant human LIGHT (rhsRIGHT) was purchased from Alexis (San Diego, CA, USA). Anti-LIGHT monoclonal antibody was purchased from R&D (Minneapolis, MN, USA); monoclonal antibody to TR2 from Immunomics (Ulsan, Korea); monoclonal antibodies to CD68 (KP1) from DAKO (Glostrup, Denmark); and anti-IκB rabbit polyclonal antibody from Chemicon International (Temecula, CA, USA).

Flow cytometry analysis. Flow cytometry analysis of THP-1 cells was performed on FACS-calibur (Becton-Dickinson, Mountain View, CA). Cells (1×10^6) were sequentially incubated with 1μg of monoclonal antibody specific for LIGHT (Alexis, CA, USA) or TR2 (Immunomics, Ulsan, Korea), washed twice, 1 μg of FITC labeled rat anti-mouse IgG (Caltag Laboratories, Burlingame, CA, USA), and washed twice. The fluorescence profiles of 1×10^4 cells were collected and analyzed.

Activation of cells and enzyme-linked immunosorbent assay (ELISA). THP-1 cells (1×10^5) in 100μl of serum-free RPMI1640 medium were added to 96 well plates. Recombinant human soluble LIGHT (rhsLIGHT, Alexis, San Diego, CA) was added in 10 to 100 ng/ml concentrations to the wells. For the activation using anti-TR2 monoclonal antibody, 50μl/well of PBS containing 2 to 20 ng/ml of antibody were incubated overnight in 96 well plates. Wells were washed twice with PBS and THP-1 cells (1×10^5) in 100μl of serum-free RPMI1640 medium were added. Supernatants were collected 48 hours after activation. Cytokines were measured by sandwich ELISA (Endogen Inc., Woburn, MA, USA); detection limits were $<$ 10 pg/ml.

Electrophoretic mobility shift assay (EMSA). THP-1 cells were stimulated with 10 and 100 ng/ml of recombinant LIGHT and nuclear extracts were collected after 2 hours. Nuclear extracts (10μg) were mixed with poly (dI-dC) · poly (dI-dC) (1μg) in 10~30μl of binding buffer (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 10 mM DTT, and 30 mM PMSF). End labeled NF-kB probes (Promega, Madison, WI, USA) (0.002~0.008 pmol/5000 cpm) were added 5 min later, and the cells were incubated for 20 min at room temperature. One μl of dye was added to each tube, the mixture were applied to a 4% polyacrylamide gel (29:1) in 1×TEB buffer (0.09 M Tris, 0.09 M boric acid, 0.002 M EDTA) and electrophoresed for an appropriate period at 10 V/cm. The gel was then dried and autoradiographed overnight at -70°C using an intensifying screen. For supershift analysis, 1μg of anti-p65 antibody (SantaCruz Biotechnology, sc-8008) was added during the 20 min incubation period before electrophoresis.

Gelatin zymography. The MMP activity in the culture supernatant was determined by substrate gel electrophoresis, as described previously (22). Briefly, culture supernatants were mixed with running buffer (4% SDS, 20% glycerol, 0.01% bromophenolblue, 0.125 M Tris-Cl, pH 6.8) and separated on SDS-PAGE containing 0.1% gelatin. The gels were then sequentially treated with 2.5% Triton X-100 for 20 minutes each for renaturation of proteins; two changes of distilled water for 20 minutes each for washing; reaction

buffer solution (10 mM Tris, pH7.5, 10 mM $CaCl_2$, 50 mM NaCl , 2μM $ZnCl_2$, 0.25% TX-100, 0.002% NaN_3) for 24 hours at 37°C for the digestion of gelatin; and the gels were finally Coomassie stained. For the pyrolidine dithiocarbamate (PDTC, Calbiochem, CA, USA) inhibition assay, PDTC was added one hour before the addition of LIGHT. Culture supernatants were collected after 48 hours for the assay.

Results

Immunohistochemical analysis of atherosclerotic plaques obtained from carotid endoarterectomy revealed the expression of LIGHT in areas rich in CD68 positive foam cells (Fig. 1A, B). Foam cells were mainly found in neointima while a-actin positive

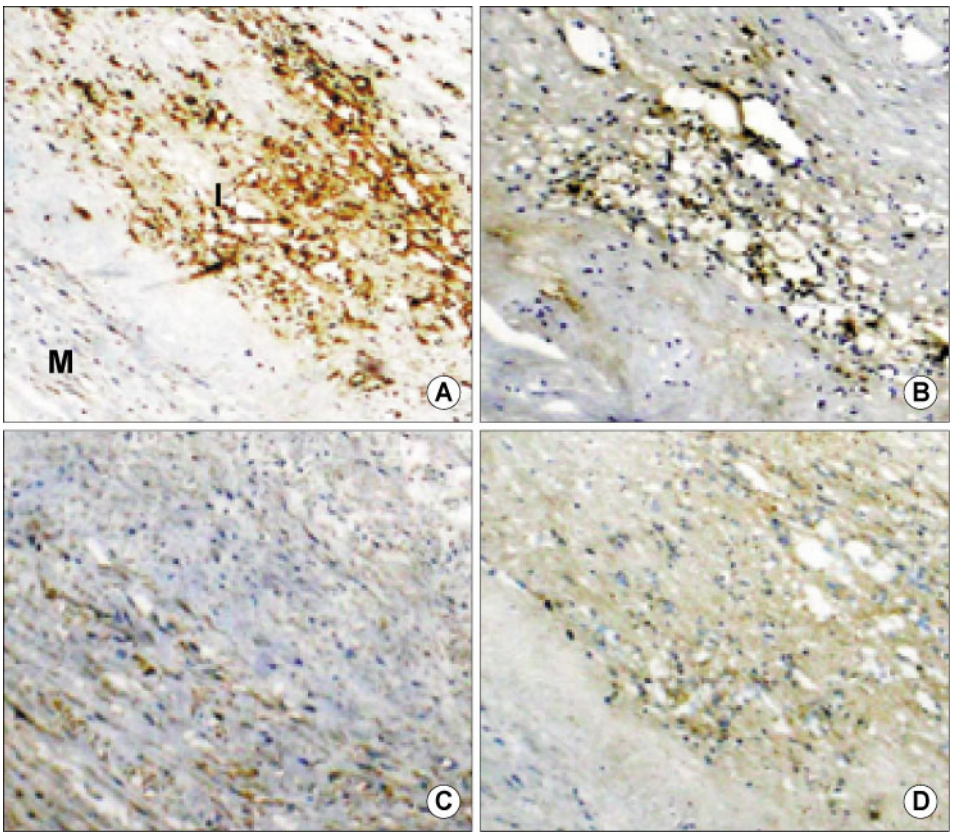

Figure 1. Immunohistochemical analysis of human atherosclerotic plaques reveals the expression of LIGHT in foam cells in atherosclerotic plaques. Consecutive sections were stained with monoclonal antibody to CD68 (foam cell marker, A), LIGHT (B), α-actin (smooth muscle cell marker, C), and TR2 (D). In each section, nuclei were visualized by autohematoxylin staining (blue color). The experiment was repeated using three separate sets of samples with essentially the same results. Magnification, 1:100; M, media; I, intima.

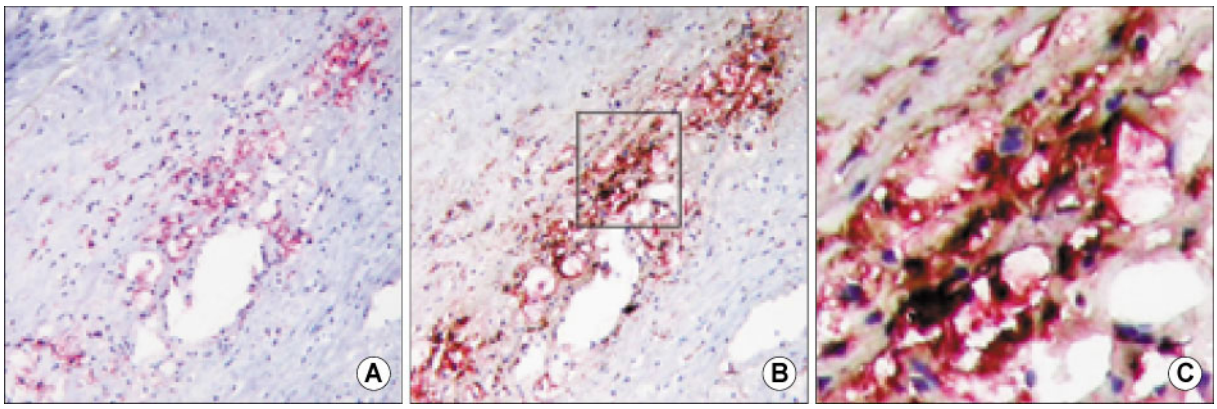

Figure 2. Double immunohistochemical (CD68/LIGHT) analysis of an atherosclerotic plaque reveals that foam cells express LIGHT. Consecutive sections were stained with monoclonal antibody to CD68 (red color, A), CD68 (red) and LIGHT (brown) (B and C) and nuclei were visualized by autohematoxylin staining (blue). Low magnification (1:100) pictures are shown in A and B and high magnification (1:400) images of the foam cell rich regions are shown in C. Box indicate the regions illustrated by the high magnification picture. The experiment was repeated using three separate sets of samples with essentially the same results.

SMCs were concentrated on media (Fig 1A, C). In agreement with a previous report (15), expression of TR2 was also observed in foam cell rich regions (Fig. 1D). To confirm that the cells expressing the LIGHT were foam cells, atherosclerotic plaque specimens were double stained for LIGHT and CD68. As shown in Fig. 2, CD68 staining overlapped with that of LIGHT, indicating that foam cells are responsible for the expression of LIGHT in atherosclerotic plaques (Fig. 2). These data in combination with our previous data (15) indicate that foam cells in the atherosclerotic plaques express both the ligand, LIGHT, and the receptor, TR2. To confirm the coexpression of LIGHT and TR2 at the same cells, flow cytometric analysis in human monocytic cell line, THP-1, was performed (Fig. 3). THP-1 cells expressed both LIGHT and TR2 but the expression levels of LIGHT were relatively lower than that of TR2.

The human monocytic cell line THP-1 has been commonly used as a cell line representing the precursor of foam cells (15,23,24). To find out the cellular responses caused by LIGHT mediated activation of TR2, we used THP-1 cells. Treatment of THP-1 cells with rhsLIGHT induced IL-8 in a dose dependent manner (Fig. 4). Similarly, stimulation of TR2 with immobilized anti-TR2 monoclonal antibody also induced lower level activation of IL-8 which was augmented after concomitant treatment with interferon (IFN)-γ (Fig. 4). Treatment of THP-1 cells with either rhsLIGHT or immobilized anti-TR2 monoclonal antibody also induced activation of MMP-9 (Fig. 6A). Activation of NF-κB has been reported to play an important role in the activation of pro-inflammatory cytokines and MMPs. Therefore, the LIGHT induced NF-κB activation in THP-1 cells was investigated by EMSA. As shown in Fig. 5, the nuclear localization of NF-κB was induced by LIGHT in a dose dependent manner. The identity of NF-κB

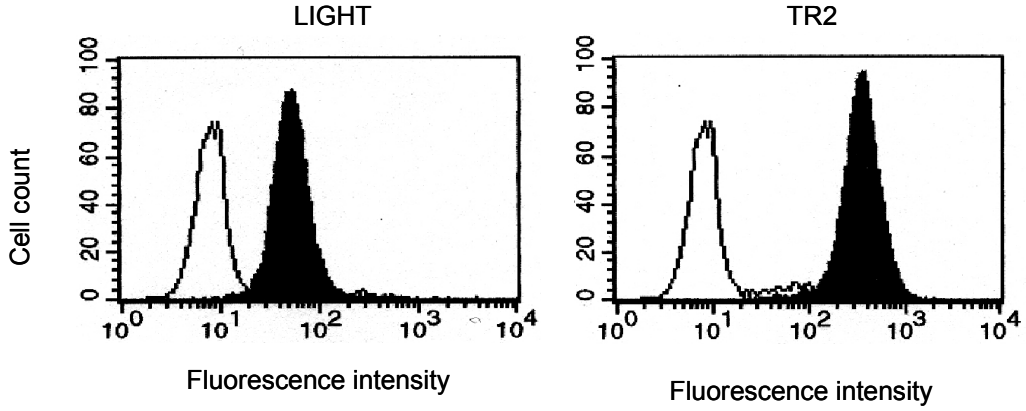

Figure 3. Flow cytometry analysis of THP-1 cells. Expression levels of LIGHT (A) and TR2 (B) on the surface THP-1 cells were measured as described in Materials and Methods. Filled areas represent LIGHT or TR2 specific fluorescence profiles and the open areas represent the background level of fluorescence given by the secondary antibody.

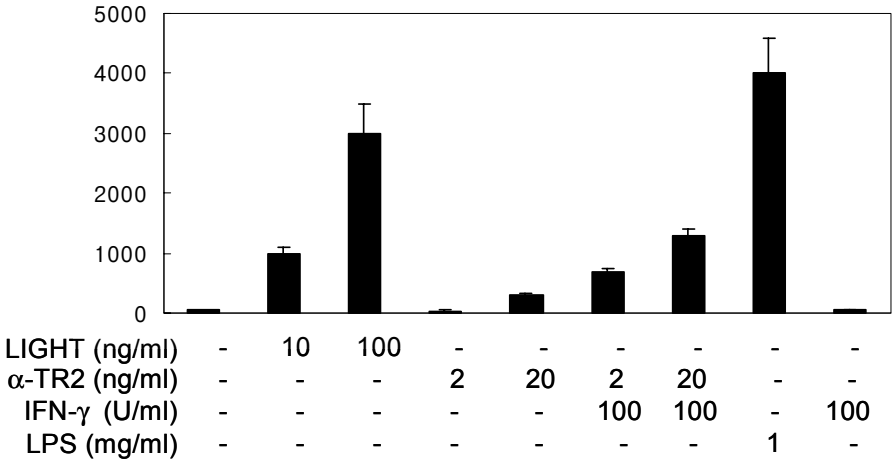

Figure 4. Induction of IL-8 in THP-1 cells. THP-1 cells were stimulated with either rhsLIGHT or immobilized anti-TR2 monoclonal antibody as described in the material and methods. Supernatants were collected 48 hours after activation and the expression levels of IL-8 were measured by ELISA.

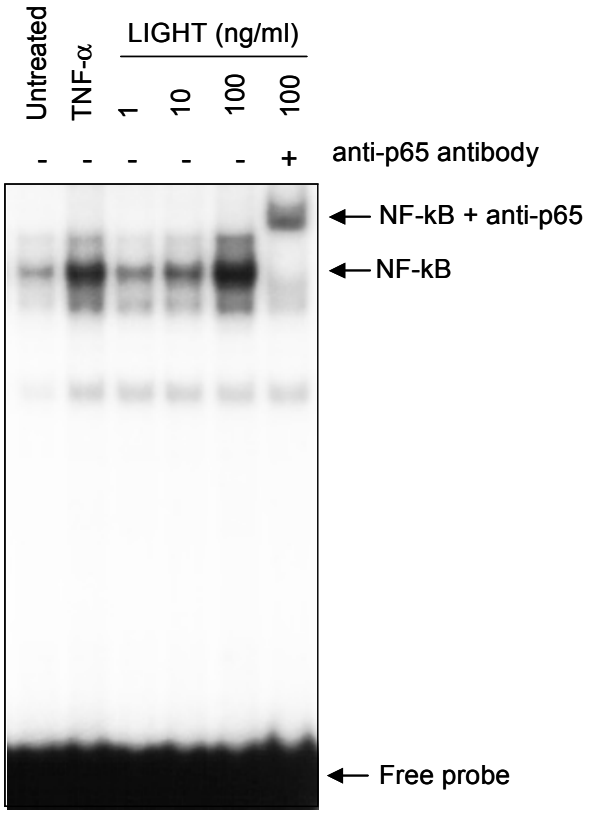

Figure 5. LIGHT induces NF-κB activation in THP-1 cells. EMSA was performed using NF-κB probes and the nuclear extracts isolated from THP-1 cells stimulated with various concentrations of rhsLIGHT for 2 hrs. For supershift of NF-κB band, anti-p65 monoclonal antibody was added as indicated. Nuclear extracts isolated from THP-1 cells treated with 10 ng/ml TNF-α were used as a positive control.

was conformed by supershift induced by anti-p65monoclonal antibody.

To confirm that the activation of NF-κB by LIGHT is required for the induction of MMP-9, THP-1 cells were pre-incubated with the NF-κB inhibitor, PDTC, before LIGHT treatment. PDTC inhibited the activation of MMP-9 in a dose dependent manner and pretreatment with 10μM of PDTC completely blocked the activation of MMP-9 (Fig. 6B). These data indicate that LIGHT mediated induction of MMP-9 requires activation of NF-κB.

Discussion

Immunohistochemistry data indicate that the source of LIGHT in atherosclerotic plaques is the foam cells (Fig. 1, 2). Monocytes/foam cells present within the plaque are under stimulation by various agents including oxidized-LDL, infection, pro-atherogenic cytokines, and cell-to-cell contact with activated T-lymphocytes (25). Since these agents can activate foam cells, it is expected that these agents will also induce expression of LIGHT. It is the subject of further research to find out the atherogenic stimuli which are responsible for the induction of LIGHT in foam cells.

Flow cytometry data (Fig. 3) indicate that LIGHT can be expressed on the surface of monocytic cells. It is expected that LIGHT, like TNF-α (1,26), will be secreted upon activation. Previous analysis already demonstrated that TR2 is also expressed in foam cells and the expression level of TR2 in foam cells is increased by TNF-α and bacterial lipopolysaccharide (LPS) (15). Thus, LIGHT produced by foam cells in atherosclerotic plaques will autoinduce foam cells which are expressing its cognate receptor TR2.

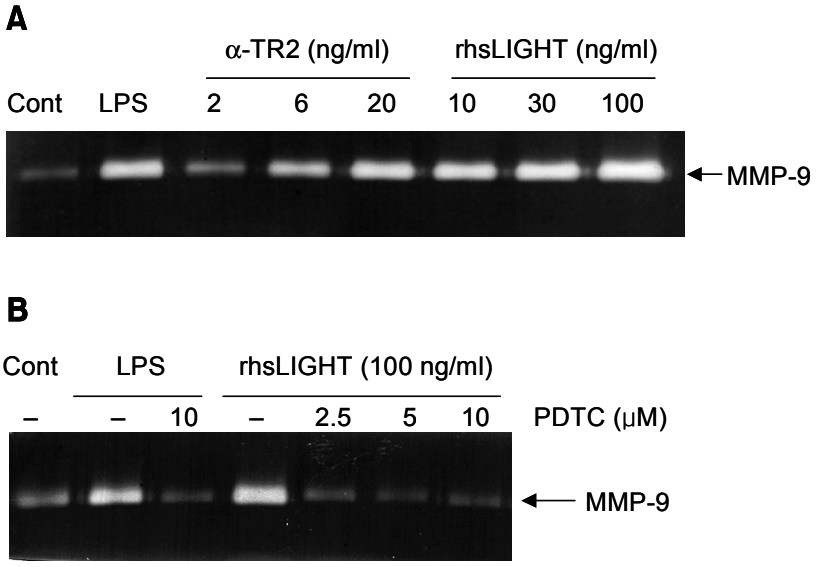

Figure 6. Induction of MMP-9 is mediated by NF-κB. (A) THP-1 cells (1×10^5/well in 96 well plate) were stimulated with 1 mg/ml of LPS, rhsLIGHT, or immobilized anti-TR2 monoclonal antibody at indicated concentration. Supernatants were collected 48 hours after stimulation and the levels of MMP-9 were tested by gelatin zymography. (B) THP-1 cells were stimulated with 1μg/ml of LPS (as a positive control) or 100 ng/ml of recombinant LIGHT in the presence or absence of PDTC. Cell culture supernatants were collected after 48 hours and subjected to gelatin zymography.

LIGHT in concert with other atherogenic stimuli will synergistically induce pro-atherogenic cytokines and matrix metalloproteinases in foam cells.

IFN-γ has been reported to be expressed in atherosclerotic plaques and to regulate the expression of macrophage scavenger receptors (27). IFN-γ was also found to be involved in atherogenesis, by acting on SMCs to potentiate growth-factor-induced mitogenesis (28). Our data showing that IFN-γ can synergize with LIGHT for the induction of MMP-9 and IL-8 indicate that both IFN-γ and LIGHT can work in concert to induce these mediators during the development of atherosclerotic plaques.

The rupture of atherosclerotic plaques depends greatly on the integrity of the fibrous cap, which in turn depends on its content of extracellular matrix protein. Dysregulation of the expression of matrix degrading enzymes (MMPs) is believed to be responsible for the rupture of atheroslcerotic plaques (29). IL-8, a member of CXC (α type) chemokines, enhances inflammatory responses by working as a potent chemokine and an activator of matrix degrading enzymes (30). Our observation that both MMP-9 and IL-8 are induced by LIGHT emphasizes the importance of LIGHT in the pathogenesis of atherosclerosis.

Current data in combination with previous reports suggest that LIGHT and TR2 are involved in atherosclerosis via the induction of pro-atherogenic cytokines and decrease in plaque stability by inducing extracellular matrix degrading enzymes. Since these functions have also been linked to other cytokines, such as TNF-α and CD40 and CD40L,(1,3,5-7,31), it can be expected that LIGHT and TR2 are contributing factors in atherosclerosis. Further investigations are required to find out whether blocking the interaction between LIGHT and TR2 could suppress the atherogenic process.

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