

Inflammatory Interaction Between LIGHT and Proteinase-Activated Receptor-2 in Endothelial Cells

Potential Role in Atherogenesis

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Abstract—The interaction between inflammatory cytokines and endothelial cells is a critical step in atherogenesis leading to endothelial dysfunction and inflammation. We have previously reported that the tumor necrosis factor superfamily member LIGHT could be involved in atherogenesis through its ability to promote vascular inflammation. In the present study we identified proteinase-activated receptor (PAR)-2 as an inflammatory mediator that was markedly enhanced by LIGHT in endothelial cells. We also found that LIGHT acted synergistically with PAR-2 activation to promote enhanced release of the proatherogenic chemokines interleukin-8 and monocyte chemoattractant protein-1, underscoring that the interaction between LIGHT and PAR-2 is biologically active, promoting potent inflammatory effects. We showed that the LIGHT-mediated upregulation of PAR-2 in endothelial cells is mediated through the HVEM receptor, involving Jun N-terminal kinase signaling pathways. A LIGHT-mediated upregulation of PAR-2 mRNA levels was also found in human monocytes when these cells were preactivated by tumor necrosis factor α . We have previously demonstrated increased plasma levels of LIGHT in unstable angina patients, and here we show a similar pattern for PAR-2 expression in peripheral blood monocytes. We also found that LIGHT, LIGHT receptors, and PAR-2 showed enhanced expression, and, to some degree, colocalization in endothelial cells and macrophages, in the atherosclerotic plaques of ApoE^{-/-} mice, suggesting that the inflammatory interaction between LIGHT and PAR-2 also may be operating in vivo within an atherosclerotic lesion. Our findings suggest that LIGHT/PAR-2-driven inflammation could be a pathogenic loop in atherogenesis potentially representing a target for therapy in this disorder. (*Circ Res.* 2009;104:60-68.)

Key Words: endothelial cells ■ atherosclerosis ■ LIGHT/TNF superfamily ligand 14 ■ proteinase-activated receptor 2

The concept that atherosclerosis is an inflammatory disease is no longer controversial. More recent research has focused on understanding what drives this inflammation and how it is regulated. An increasing number of mediators have been suggested to be involved in this process, but there are still components to be clarified, and the precise mechanisms of action of each of these inflammatory mediators, as well as their interactive role in atherogenesis, have not been elucidated.^{1,2}

LIGHT (lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus [HSV] glycoprotein D [gD] for HSV entry mediator [HVEM], a receptor expressed by T lymphocytes; TNFSF14) is a cytokine in the tumor necrosis factor (TNF) superfamily that is involved in innate and adaptive immune responses as well as in regulation of cell survival and proliferation. LIGHT is signaling through 2 distinct members of the TNF receptor superfamily, ie, HVEM and the lymphotoxin β receptor (LT β R), and can

also bind to the soluble decoy receptor 3. Studies in animal models indicate that LIGHT may be important for the development of various autoimmune disorders (eg, inflammatory bowel disease and rheumatoid arthritis) through effects on T cells and T-cell homing into inflamed tissues.³ More recently, this cytokine has been suggested to promote atherogenesis at least partly by inducing matrix metalloproteinase activity in macrophages and inflammation in endothelial cells.^{4,5} Lately, LIGHT was also shown to be involved in regulation of lipid homeostasis.^{6,7}

The interaction between inflammatory cytokines and endothelial cells is a critical step in atherogenesis leading to endothelial cell dysfunction and inflammation within the atherosclerotic lesion, promoting additional recruitment of inflammatory cells in to the vessel wall.⁸ To further elucidate the potential pathogenic role of LIGHT in atherogenesis, we used high-density oligonucleotide microarrays to identify

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genes regulated by LIGHT in endothelial cells. These screening experiments identified proteinase-activated receptor (PAR)-2 as a gene that was markedly enhanced by LIGHT. PAR-2, a heptahelical G protein-coupled receptor, has been identified in various cell types including endothelial cells.⁹ PAR-2 has putative inflammatory roles and was recently proposed to be involved in atherogenesis by contributing to vascular inflammation.¹⁰ In the present study, we further examined the possible pathogenic role of the LIGHT-mediated PAR-2 response in endothelial cells by several approaches, including experimental as well as clinical studies.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at <http://circres.ahajournals.org>.

Isolation of Cells

Peripheral blood mononuclear cells were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep; Nycomed, Oslo, Norway) gradient centrifugation. Further separation of monocytes and CD3⁺ T cells by monodisperse immunomagnetic beads (Dyna, Oslo, Norway) was performed as described elsewhere.^{11,12}

Cell Culture Experiments

Human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cord veins by digestion with 0.1% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany)¹³ and cultured as described previously,⁵ with or without recombinant human (rh) LIGHT (R&D Systems, Minneapolis, Minn). The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, Md) and freshly isolated human monocytes were cultured for 4 days in the presence of rhTNF α (5 ng/mL, R&D Systems) before further incubation with or without rhLIGHT.

Microarray

RNA was isolated from HUVECs using RNeasy (Quiagen, Hilden, Germany). Three micrograms of total RNA were used to generate cRNA, subsequently hybridized to Genechip Human Genome U133A Array (Affymetrix, Santa Clara, Calif) according to standard protocols (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

Real-Time Quantitative PCR

Quantification of mRNA was performed using ABI Prism 7500 Fast Realtime PCR System (Applied Biosystems, Foster City, Calif).¹⁴

Western Blotting

Western blotting was performed as previously described,¹⁵ separating equal amounts of protein from each sample by SDS-PAGE (10%) before transferring it onto polyvinylidene difluoride membranes (NEN Life Science, Boston, Mass).

Preparation and Transfection of Small Interfering RNA

Predesigned small interfering (si)RNA against HVEM (siRNA ID no. 13868) and LT β R (siRNA ID no. 143461) were purchased from Ambion (Austin, Tex), and certificated nonsilencing scramble control siRNA (cat No: 1022076) was obtained from Qiagen (Hilden, Germany).

Multiplex

Protein extracts from HUVECs, stimulated with rhLIGHT (100 ng/mL) or vehicle for 15 and 120 minutes, were subjected to phosphoprotein detection array (multiplexable beads: phosphorylated-Akt; p-activating transcription factor-2 [ATF-2]; p-extracellular signal regulated kinase (ERK) [ERK]1/2; p-inhibitory [I] κ B- α ; p-Jun

N-terminal kinase [JNK]; p-p38 mitogen-activated protein kinase [MAPK]; p-signal transducer and activator of transcription [STAT]3; Bio-Rad, Hercules, Calif) using the Bioplex (Bio-Rad) suspension array technology.

Enzyme Immunoassays

Levels of macrophage chemoattractant protein (MCP)-1, interleukin (IL)-6, IL-8, IL-10, TNF α , and LIGHT were measured by EIAs (R&D Systems).

Mice

Female ApoE^{-/-} and wild-type C57BL/6 mice were obtained from Taconic Europe A/S (Lille Skensved, Denmark) and fed standard chow and water ad libitum as previously described.¹⁶

Immunohistochemistry

Acetone-fixed sections of the ascending aorta of the ApoE^{-/-} and wild-type C57BL/6 mice were further fixed in formalin and stained using affinity-purified polyclonal goat antihuman LIGHT, HVEM, LT β R, or PAR-2 IgG (all Santa Cruz Biotechnology), rat antimouse CD68 IgG (macrophage marker; Serotec Ltd, Oxford, UK), or sheep antirat von Willebrand factor (vWF) IgG (endothelial cell marker; Cedarlane, Ontario, Canada).

Patients and Controls

In a separate experiment, we analyzed PAR-2 expression in monocytes and T cells from 14 patients with unstable angina, 14 patients with stable angina, and 10 healthy controls.

Ethics

The studies were conducted according to the ethical guidelines at our hospital according to the Declaration of Helsinki and were approved by the local ethical committee. All animal experiments were in accordance with national guidelines and approved by the local ethical committee.

Statistical Analysis

Probability values (2-sided) were considered significant at a value of <0.05.

Results

LIGHT Increases PAR-2 Expression in Endothelial Cells

To screen for inflammatory effects of LIGHT in endothelial cells, we used Affymetrix microarrays encoding 22 000 human genes to analyze gene expression profile of HUVECs undergoing 3 hours of rhLIGHT stimulation (100 ng/mL). Data analysis identified PAR-2 as 1 of the genes that were markedly upregulated by LIGHT. This LIGHT-mediated effect on PAR-2 mRNA level was confirmed by real-time RT-PCR that showed a time- and dose-dependent pattern (Figure 1A and 1B). Thus, whereas there was no increase in PAR-2 expression in unstimulated HUVECs, rhLIGHT induced a rapid (maximum effect after 2 hours) and persistent increase in PAR-2 expression showing significantly enhanced mRNA levels of PAR-2 even after 24 hours of stimulation (Figure 1A). The LIGHT-induced expression of PAR-2 was also confirmed at the protein level as assessed by Western blotting, which showed significantly higher protein levels after 20 hours of stimulation (Figure 1C).

In addition to PAR-2, the microarray screening experiment showed that chemokines (ie, MCP-1, growth-related oncogene [GRO] α , and IL-8), the IL-7 receptor, and the TNF superfamily ligand 10 was markedly upregulated (>2-fold increase), and the antithrombotic mediator thrombomodulin

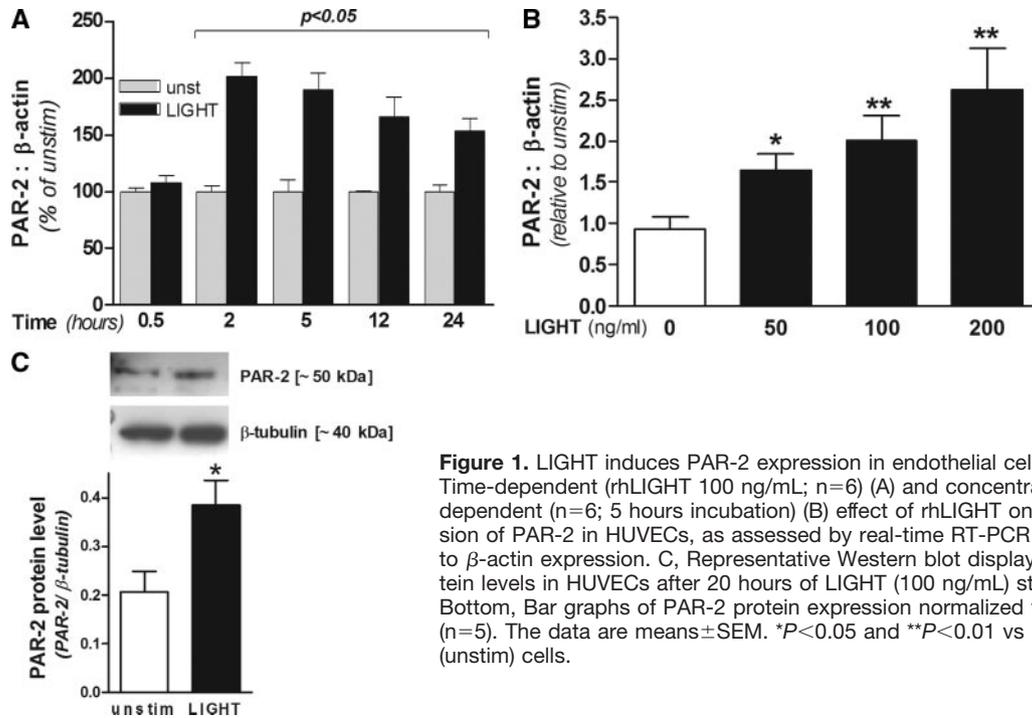


Figure 1. LIGHT induces PAR-2 expression in endothelial cells. A and B, Time-dependent (rhLIGHT 100 ng/mL; n=6) (A) and concentration-dependent (n=6; 5 hours incubation) (B) effect of rhLIGHT on gene expression of PAR-2 in HUVECs, as assessed by real-time RT-PCR and normalized to β -actin expression. C, Representative Western blot displaying PAR-2 protein levels in HUVECs after 20 hours of LIGHT (100 ng/mL) stimulation (top). Bottom, Bar graphs of PAR-2 protein expression normalized to β -tubulin (n=5). The data are means \pm SEM. * P <0.05 and ** P <0.01 vs unstimulated (unstim) cells.

was significantly downregulated (50% reduction) in LIGHT-activated HUVECs. However, LIGHT stimulation of HUVECs had no effect on the expression of TNF α , IL-6, or IL-10, as assessed by real-time RT-PCR and enzyme immunoassay, all being important endothelial-related cytokines, suggesting some degree of selectivity.

PAR-2 Signaling Enhances LIGHT-Mediated Induction of Proatherogenic Cytokines

Both IL-8 and MCP-1 are critically involved in atherosclerosis and plaque destabilization,^{17,18} and we have previously shown that LIGHT is a potent inducer of these chemokines in

endothelial cells.⁵ PAR-2 has also been reported to exert inflammatory effects in these cells, including the induction of IL-6 and IL-8.¹⁰ To examine the biological relevance of the LIGHT-mediated upregulation of PAR-2, we examined the effect of rhLIGHT (100 ng/mL), the PAR-2-activating peptide SLIGKV (10 μ mol/L and 100 μ mol/L), or a combination thereof on the release of IL-8 and MCP-1 in HUVECs after 8 and 20 hours of stimulation. As shown in Figure 2A and 2B, although SLIGKV show no or only modest effects on MCP-1 on its own, it markedly enhanced the LIGHT-stimulated release of this chemokine at both time points in a dose-dependent manner (\approx 2-fold increase when these stimuli were

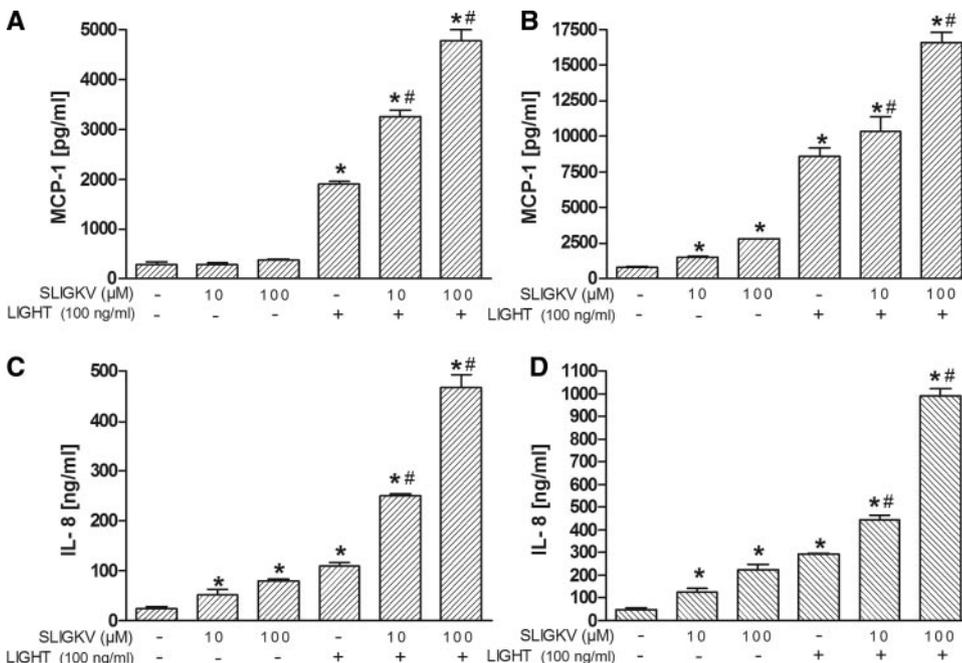


Figure 2. PAR-2 signaling enhances LIGHT-mediated chemokine release from HUVECs. Cells were incubated with the PAR-2-activating peptide SLIGKV (10 μ mol/L and 100 μ mol/L), rhLIGHT (100 ng/mL), or a combination thereof. Levels of MCP-1 (A and B) and IL-8 (C and D) were measured in HUVEC supernatants after 8 (A and C) and 20 hours (B and D) of stimulation by means of enzyme immunoassay. * P <0.05 vs unstimulated cells; # P <0.05 vs LIGHT (100 ng/mL) and SLIGKV (100 μ mol/L) when given alone. The data are means \pm SEM (n=6).

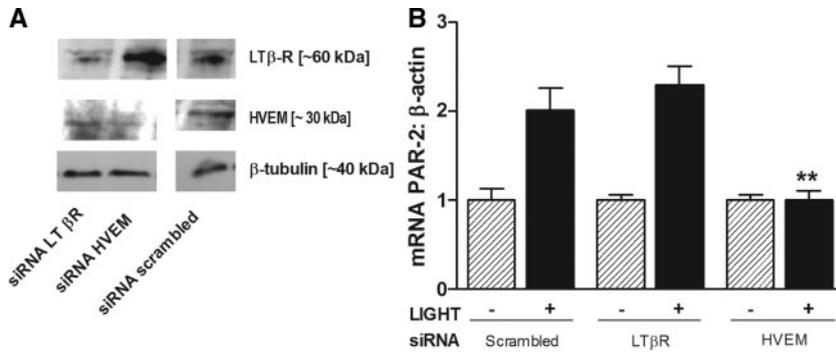


Figure 3. The LIGHT-induced upregulation of PAR-2 in HUVECs is mediated through HVEM. Transfected cells with silencing RNA for both LIGHT receptors, LTβR and HVEM, were incubated with rhLIGHT (100 ng/mL) for 5 hours, and PAR-2 mRNA levels were measured by real-time quantitative RT-PCR and normalized to β-actin expression. A, Gene-silencing efficiency evaluated by Western blot, demonstrating that siRNA transfection reduced both LTβR and HVEM expression by ≈90% as compared to scrambled control siRNA-transfected HUVECs (culture time, 8 hours; 1 representative experiment of 6). B, HVEM silencing

completely abolished the LIGHT-mediated PAR-2 upregulation, whereas LTβR silencing rendered similar PAR-2 levels as compared to scramble control. ** $P < 0.01$ vs scramble control. The data are means \pm SEM ($n = 6$).

combined, 100 μ mol/L SLIGKV). Furthermore, although PAR-2 activation induced a significant release of IL-8 on its own, comparable to the effect of LIGHT, again, the combination of these stimuli dose-dependently enhanced the release of IL-8 at both time points (≈ 3 -fold increase when these stimuli were combined, 100 μ mol/L SLIGKV) (Figure 2C and 2D). It is noteworthy that even if the microarray screening experiment identified MCP-1 and IL-8 as 2 of the most prominent LIGHT responses (see above), this LIGHT-mediated response was markedly enhanced when costimulated with the PAR-2 agonist, underscoring the inflammatory potential of the interaction between LIGHT and PAR-2 in endothelial cells.

Effect of LIGHT/PAR-2 Costimulation on Pro-/Antithrombotic Mediators and Nitric Oxide Synthase

We next examined the ability of LIGHT/PAR-2 activation to modulate other endothelial related mediators with relevance to atherosclerosis. We have previously shown that LIGHT enhances the expression of tissue factor and plasminogen activator inhibitor (PAI)-1 and downregulates the expression of thrombomodulin in endothelial cells.¹⁹ Whereas costimulation with PAR-2 agonist (100 μ mol/L) had no effect on the LIGHT-mediated (100 ng/mL) expression of tissue factor and thrombomodulin (data not shown), it enhanced the LIGHT-mediated upregulation of PAI-1 mRNA levels in HUVECs (supplemental Figure I, A), suggesting that costimulation with LIGHT/PAR-2 agonist may contribute to a prothrombotic phenotype in endothelial cells. Moreover, whereas either LIGHT or PAR-2 activation modulated the expression of inducible nitric oxide synthase (NOS), LIGHT significantly downregulated the mRNA levels of endothelial NOS, but in contrast to the effect of PAI-1, there was no additional effect of costimulation with SLIGKV (supplemental Figure I, B).

LIGHT Mediates PAR-2 Expression Through HVEM

LIGHT is signaling through 2 distinct members of the TNF receptor superfamily: HVEM and LTβR. Both of these receptors, but not the LIGHT-binding decoy receptor 3, have been reported previously to be expressed in HUVECs,²⁰ and we confirmed strong mRNA transcript of HVEM and LTβR in these cells (data not shown). To examine which receptor

that mediates the LIGHT-induced expression of PAR-2, HUVECs were transfected with siRNA probes silencing each of these receptors. We found successful silencing of these genes, as assessed by Western blotting ($\approx 90\%$; Figure 3A) and real-time RT-PCR ($\approx 85\%$; data not shown), 8 and 5 hours posttransfection, respectively. As shown in Figure 3B, silencing HVEM totally abolished the LIGHT-mediated PAR-2 upregulation after 5 hours, whereas LTβR silencing had no effect compared to scramble control.

LIGHT-Mediated PAR-2 Expression in HUVECs Involves JNK/AP1 and NF-κB Activation

LIGHT signaling has been shown to involve activation of the NF-κB and JNK/AP-1 pathways.^{3,21} Such an intracellular pattern was also seen in endothelial cells when analyzing the effect of LIGHT on HUVECs by multiplex suspension array technology with enhanced phosphorylation of IκBα, resulting in dissociation of NF-κB from its inhibitor, and of JNK (Figure 4A and 4B). Blocking JNK with 100 μ mol/L SP600125 and blocking NF-κB with 20 μ mol/L NF-κB activation inhibitor II (JSH-23) completely abolished LIGHT-mediated PAR-2 expression (Figure 4C and 4D).

LIGHT-Mediated JNK Activation Is Mediated Through HVEM

Our findings suggest that HVEM as well as JNK and NF-κB activation are involved in the LIGHT-mediated induction of PAR-2 in HUVECs, and we next examined the relation between HVEM and the activation of JNK and NF-κB signaling pathways. By using siRNA of HVEM and LTβR, we show that silencing of HVEM, but not of LTβR, markedly downregulated the LIGHT-mediated activation of JNK (supplemental Figure II). This finding suggests a direct link between HVEM, JNK activation, and the LIGHT-induced PAR-2 expression. In contrast, either silencing of HVEM or LTβR significantly downregulated NF-κB activation in LIGHT-activated HUVECs (data not shown), suggesting that blocking of both receptors is necessary for totally abolishing the LIGHT-mediated NF-κB activation in these cells.

LIGHT Induces PAR-2 Expression in Monocytes

In addition to endothelial cells, monocytes/macrophages play an important role in atherogenesis by promoting lipid accumulation and inflammation. To assess whether LIGHT exerts

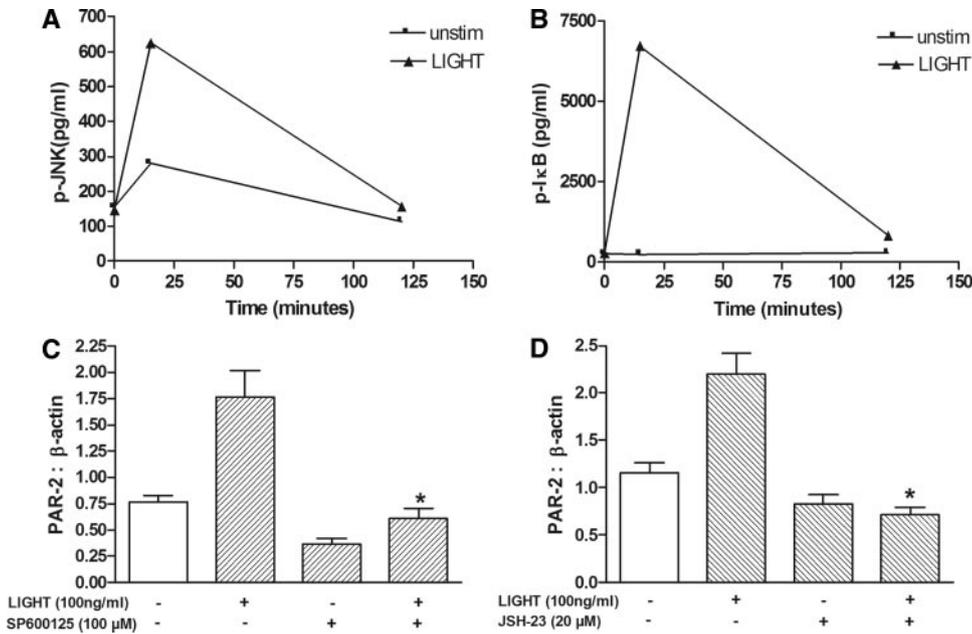


Figure 4. LIGHT-mediated PAR-2 expression in HUVECs involves activation of JNK and NF- κ B intracellular pathways. In A and B, HUVECs were incubated for 0, 15, and 120 minutes with rhLIGHT (100 ng/mL) before cell extracts were applied to phosphoprotein multiplex suspension array technology (see Materials and Methods). Unstimulated (unstim) cells were incubated with vehicle. LIGHT-mediated phosphorylation of JNK (p-JNK) (A) and I κ B α (p-I κ B α) (B) is shown. C and D show that HUVEC incubation with the JNK-blocker SP600125 (100 μ mol/L) (C) and the NF- κ B activation inhibitor II (JSH-23, 20 μ mol/L) (D) for 30 minutes before 5 hours of rhLIGHT (100 ng/mL) stimulation totally abolished the LIGHT-mediated induction of PAR-2, as assessed by real-time quanti-

tative RT-PCR and normalized to β -actin expression. A similar pattern was seen when using the NF- κ B activation inhibitor IV (10 μ mol/L). * P <0.05 vs LIGHT stimulation alone. The data are means \pm SEM (n=8).

similar effects on PAR-2 expression in these cells, THP-1 monocytes and freshly isolated monocytes from 6 healthy individuals (all with CRP levels <1.5 mg/L) were cultured for 4 days before stimulation with rhLIGHT (100 ng/mL) for additional 5 hours. Whereas rhLIGHT had only modest and nonsignificant effects on PAR-2 expression when these cells were cultured in medium alone (data not shown), LIGHT induced a significant increase in PAR-2 mRNA levels in cells that had been preincubated with rhTNF α (5 ng/mL) for 4 days before LIGHT activation, with the same pattern in THP-1 cells and primary monocytes (Figure 5A and 5B). The functional consequences of the LIGHT-mediated upregulation of PAR-2 seem to differ between monocytes and HUVECs. Hence, whereas PAR-2 activation markedly enhanced the LIGHT-mediated release of MCP-1 and IL-8 in HUVECs (Figure 3), no such effects were seen in THP-1 monocytes (data not shown). However, when examining the effects of LIGHT/PAR-2 activation on other chemokines with relevance to atherogenesis by multiplex suspension array technology, we found that SLIGKV (100 μ mol/L)

significantly enhanced the LIGHT-mediated (100 ng/mL) release of RANTES in THP-1 monocytes (Figure 5C), whereas no costimulatory effects was seen in HUVECs (data not shown). Either LIGHT, SLIGKV, or a combination thereof had any effects on IP-10 and MIG in either THP-1 monocytes or HUVECs (data not shown).

Costimulation With LIGHT and PAR-2 Agonist Enhances PAR-2 Expression in HUVECs and Monocytes

Although some differences, our findings suggest enhanced inflammatory responses in both monocytes and HUVECs when these cells are coactivated with LIGHT and PAR-2 agonist. To examine whether this enhancing effect of combined activation could involve upregulation of PAR-2 itself, we examined the costimulatory effect of LIGHT/SLIGKV on PAR-2 expression. Although SLIGKV (100 μ mol/L) had no effect on PAR-2 when given alone, it markedly increased the LIGHT-mediated (100 ng/mL) upregulation of PAR-2 mRNA levels, with the same pattern in THP-1 monocytes and

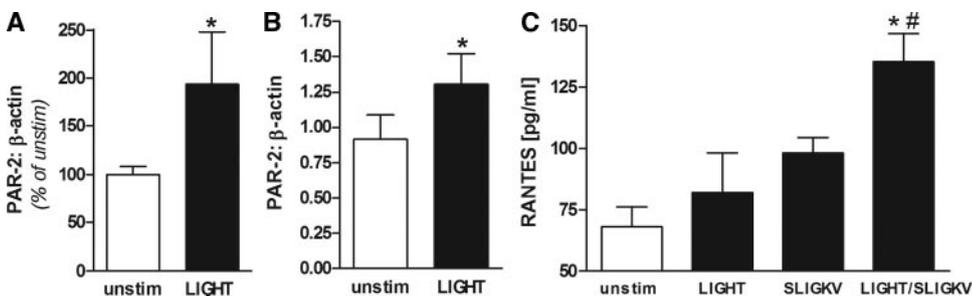


Figure 5. LIGHT induces PAR-2 expression in human monocytes. THP-1 monocytes (n=8) (A) and freshly isolated monocytes from 6 healthy individuals (B) were preincubated with rhTNF α (5 ng/mL) for 4 days before stimulation with rhLIGHT (100 ng/mL) or vehicle (unstim) for additional 5 hours before analyzing mRNA levels of PAR-2 by means of real-time RT-PCR and normalized

to β -actin. * P <0.05 vs vehicle (unstim). C, Effect of LIGHT (100 ng/mL), the PAR-2 agonist SLIGKV (100 μ mol/L), and a combination thereof on RANTES levels in supernatants of THP-1 monocytes (culture time 20 hours) by means of multiplex suspension array technology (n=8). * P <0.05 vs unstimulated cells; # P <0.05 vs LIGHT (100 ng/mL) and SLIGKV (100 μ mol/L) when given alone. The cells in C were preactivated by TNF α as described for the cells in panel A and B. The data are means \pm SEM.

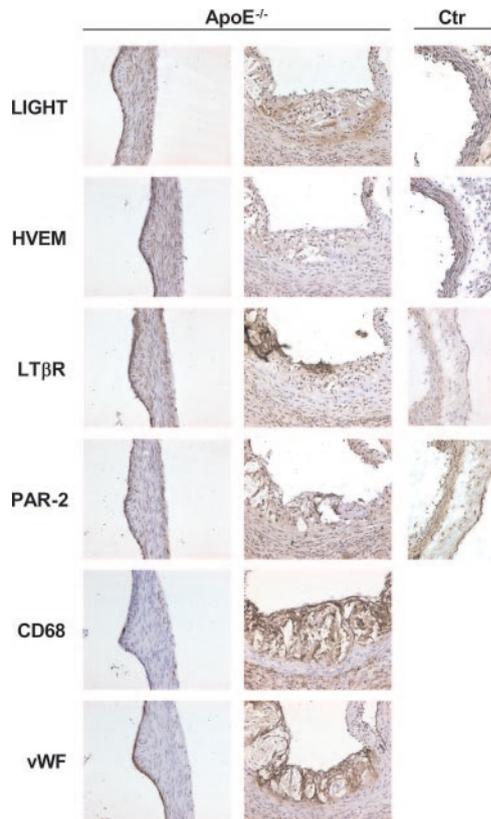


Figure 6. Representative photomicrographs demonstrating LIGHT, HVEM, LT β R, and PAR-2 immunostaining within (middle images) and outside (left images) the atherosclerotic lesions of aorta in a 18-week-old atherosclerotic ApoE^{-/-} mouse and in the aorta of a C57BL/6 nonatherosclerotic control mouse (right images). Weak LIGHT and HVEM immunoreactivities were seen in vascular smooth muscle cells and more intense in vWF-positive endothelial cells (left images) in the control mice and particularly in the ApoE^{-/-} mouse. Weak LT β R and PAR-2 immunoreactivity was also seen in vascular smooth muscle cells in both the ApoE^{-/-} mouse and more intense in the control mouse, whereas immunostaining of LT β R and PAR-2 in vWF-positive endothelial cells was observed only in the ApoE^{-/-} mouse. Within the plaques of ApoE^{-/-} mice, LIGHT, LT β R, and PAR-2 and to a lesser degree HVEM immunoreactivities were seen in regions with CD68-positive macrophages (middle images). Original magnification, $\times 200$.

HUVECs (supplemental Figure III), potentially contributing to the inflammatory response in these cells when coactivated by LIGHT and SLIGKV.

Expression of LIGHT and PAR-2 in Experimental Atherosclerosis

Our data so far demonstrate a LIGHT-mediated upregulation of PAR-2 in endothelial cells and monocytes. To examine the *in vivo* relevance of these findings to atherosclerotic disease, we first examined the protein expression of LIGHT and its receptors, as well as PAR-2 by immunohistochemistry within the atherosclerotic lesions of aorta in a murine model of atherosclerosis, the ApoE^{-/-} mouse (n=4), and in wild-type control mice (n=4) (Figure 6). In general, ApoE^{-/-} mice showed increased immunostaining of LIGHT, HVEM, and LT β R as compared with the aorta of wild-type mice, at least partly reflecting the lack of macrophages within the vessel wall in control mice. However, significant immunostaining of

these mediators was also seen within endothelial cells in ApoE^{-/-} mice, in particular for LT β R. In ApoE^{-/-} mice, PAR-2 immunostaining showed a similar distribution and intensity, colocalized to LIGHT and its receptors in macrophages and in particular in endothelial cells. In contrast to macrophages and endothelial cells, immunostaining of these mediators in vascular smooth muscle cells displayed the same (LIGHT and HVEM) or stronger (LT β R and PAR-2) intensity in control mice as compared with ApoE^{-/-} mice.

To obtain more quantitative data, we also analyzed the mRNA levels of these mediators in aortas from control (n=8) and ApoE^{-/-} (n=8) mice by real-time quantitative RT-PCR, showing a marked and significant upregulation of PAR-2, LIGHT, HVEM, and LT β R in ApoE^{-/-} mice (>2-fold increase for all parameters) (Figure 7).

Expression of PAR-2 in CAD Patients

We have previously reported increased plasma levels of LIGHT in angina patients with the highest levels in those with unstable disease.⁶ Here, we examined PAR-2 mRNA levels in monocytes and T cells, and observed enhanced expression in monocytes from those with unstable angina, but not in T cells (data not shown), when examining cells from 14 patients with stable angina, 14 patients with unstable angina, and 10 healthy controls (Figure 8), with no correlation to plasma levels of LIGHT ($P=0.18$).

Discussion

Increased LIGHT expression has previously been reported in atherosclerotic disorders potentially promoting matrix degradation, lipid accumulation, thrombus formation, and inflammation.⁴⁻⁶ In the present study, we extend these findings by showing that LIGHT is a potent inducer of PAR-2 in endothelial cells, acting synergistically with PAR-2 activation to promote enhanced release of the proatherogenic chemokines IL-8 and MCP-1. A LIGHT-mediated upregulation of PAR-2 was also seen in monocytes that had been preactivated by TNF α , and in both HUVECs and monocytes, coactivation with LIGHT and PAR-2 agonist promoted increased expression of PAR-2 in itself, potentially contributing to the inflammatory interaction between these 2 mediators. Finally, whereas previous studies have shown increased LIGHT expression in peripheral blood in unstable angina patients, as well as within atherosclerotic plaques,^{4,6} our findings in the present study show a similar pattern for PAR-2, underscoring the *in vivo* relevance of our *in vitro* findings, suggesting a role for LIGHT/PAR-2 interaction in atherogenesis.

Atherosclerosis depends critically on altered behavior of the intrinsic cells of the arterial wall including endothelial cells, and atherogenesis is characterized by development of an inflammatory phenotype in these cells. A key event in this process is the localized recruitment of various leukocyte subsets into an inflamed endothelium.⁸ Although most previous studies have focused on the effect of LIGHT on T-cell activation and lymphoid tissue, there are also some reports of LIGHT-mediated effects on endothelial cells promoting increased expression of chemokines (eg, IL-8 and MCP-1) and adhesion molecules (eg, E-selectin and vascular cell adhesion molecule 1).²⁰ Our findings in the present study suggest that

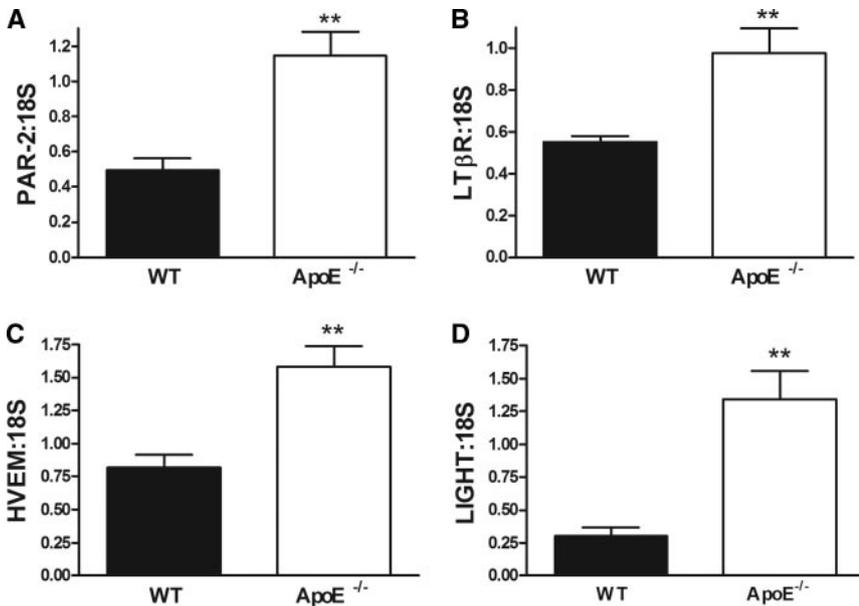


Figure 7. Enhanced expression of PAR-2, LIGHT, LTβR, and HVEM in the aorta of ApoE^{-/-} mice. The graphs show mRNA levels of PAR-2 (A), LTβR (B), HVEM (C), and LIGHT (D) in aorta of wild-type (WT) (n=8) and ApoE^{-/-} (n=8) mice, as assessed by real-time RT-PCR and normalized to 18S expression. ***P*<0.01 vs wild-type mice. The data are means±SEM.

enhanced PAR-2 expression should be added to the list of inflammatory responses in LIGHT-activated endothelial cells. More importantly, although LIGHT has been found to be a potent inducer of IL-8 and MCP-1 in endothelial cells,⁵ costimulation with the PAR-2-activating peptide SLIGKV strongly boosted the LIGHT-mediated chemokine response in these cells. Based on the important role of IL-8 and MCP-1 in atherosclerosis, including their ability to promote leukocyte recruitment into the atherosclerotic lesion,¹⁸ this LIGHT/PAR-2 interaction in endothelial cells could contribute to crucial steps in atherogenesis. In fact, we found that LIGHT, LIGHT receptors (ie, HVEM and LTβR), and PAR-2 all showed enhanced expression, and, to some degree, colocalization in endothelial cells and macrophages, in the atherosclerotic plaques of ApoE^{-/-} mice, suggesting that the inflammatory interaction between LIGHT and PAR-2 also may be operating in vivo within an atherosclerotic lesion.

LIGHT interaction with LTβR triggers the production of inflammatory mediators and upregulates adhesion molecule expression in T cells and macrophages, and has also been

shown to be crucial for the inflammatory effects of LIGHT in a mouse model of inflammatory bowel disease.^{22,23} On the other hand, by signaling through HVEM, LIGHT costimulates CD28-independent T-cell activation, preferentially inducing inflammatory T-helper cell type 1 responses.^{21,24} By silencing the LIGHT receptors, we show that the LIGHT-mediated upregulation of PAR-2 in HUVECs is dependent on signaling through HVEM but not through LTβR. These findings may suggest a role for HVEM not only in LIGHT-driven T cell inflammation, but also in the LIGHT-mediated inflammation in endothelial cells, as also recently suggested by others.²⁰ Furthermore, our blocking experiments suggest that the LIGHT-mediated upregulation of PAR-2 involves activation of NF-κB and JNK signaling pathways. Additionally, silencing of HVEM, but not of LTβR, markedly downregulated the LIGHT-mediated activation of JNK, suggesting that HVEM/JNK activation is crucial for the LIGHT-mediated effect on PAR-2. In contrast, either silencing of HVEM or LTβR significantly downregulated NF-κB activation in LIGHT-activated HUVECs, indicating that blocking of both receptors is necessary for totally abolishing the LIGHT-mediated NF-κB activation in these cells. Although LIGHT-LTβR signaling recently has been shown to involve JNK activation in fibroblasts,²⁵ overexpression of HVEM seems to be of particular importance for the LIGHT-mediated activation of JNK.²⁶ Based on our findings in the present study, it is tempting to hypothesize that the LIGHT/HVEM/JNK interaction could represent an important cellular pathway in the promotion of endothelial cell-related inflammation.

Previous in vivo studies in PAR-2-deficient mice highlight a role of PAR-2 in progression of skin and joint inflammation, as well as sepsis,^{27,28} and very recently, Tennant et al reported reduced cellular adhesion to injured vessels with a consequent reduction in neointima formation in mice lacking PAR-2.²⁹ Moreover, Seitz et al have recently shown that PAR-2 activation increases IL-6 and IL-8 expression in endothelial cells.¹⁰ However, although we found that SLIGKV had a slight effect on IL-8 and MCP-1 release in

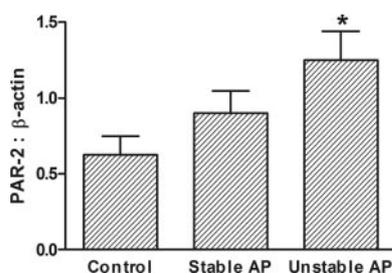


Figure 8. Increased PAR-2 expression in monocytes from unstable angina patients. The figure shows mRNA levels of PAR-2 in freshly isolated monocytes from patients with stable (n=14) and unstable (n=14) angina pectoris (AP) and sex- and age-matched healthy controls (n=10), as assessed by real-time RT-PCR and normalized to β-actin expression. **P*≤0.05 vs controls. The angina patients had raised plasma levels of LIGHT as compared to controls, with particularly high levels in unstable diseases but with no correlation with PAR-2 expression (*P*=0.12). The data are means±SEM.

HUVECs, confirming inflammatory effects of PAR-2 agonists in these cells, our major finding was that PAR-2 activation strongly and dose-dependently boosted the LIGHT-mediated release of these proatherogenic chemokines in endothelial cells. An inflammatory effect of PAR-2 activation was also seen in LIGHT-stimulated monocytes, but the inflammatory interaction between LIGHT and PAR-2 seems to be somewhat less prominent in these cells, depending on preactivation with TNF α . Additionally, the inflammatory profile in LIGHT/PAR-2-activated monocytes was different from that in HUVECs, with enhanced release of RANTES as the significant finding in the former cells. Increased PAR-2 expression has previously been reported in human coronary atherosclerotic lesions,³⁰ and in the present study, we show enhanced expression of PAR-2 in monocytes from angina patients, with particularly high levels in unstable disease. We also found increased mRNA levels of PAR-2 in atherosclerotic lesions of ApoE^{-/-} mice, with strong immunostaining in macrophages and endothelial cells, at least partly colocalized to LIGHT and its receptors. Our findings suggest that the LIGHT/PAR-2 interaction in endothelial cells and, to some degree, also in monocytes may represent a pathogenic loop that could be operating within an atherosclerotic lesion, contributing to local and systemic inflammation, which in turn could induce further inflammatory responses in other endothelial interacting cells such as various leukocyte subpopulations.

It may be argued that the LIGHT concentrations used in the in vitro experiments in the present study were too high. However, it is not inconceivable that within an inflamed atherosclerotic plaque, consisting of activated platelets, macrophages, and endothelium (all important cellular sources of LIGHT), LIGHT levels could be comparable to those used in the in vitro experiments in the present study. Also, we have previously shown that oxidized LDL is a potent inducer of LIGHT in macrophages,⁶ further underscoring the relevance of LIGHT-mediated activation of endothelial cells in atherogenesis. Moreover, the ability of other inflammatory mediators, operating within an atherosclerotic lesion, to enhance LIGHT-mediated effects was further supported by our data showing that preactivation of monocytes with TNF α augments the LIGHT-mediated PAR-2 response in these cells.

In the present study, we show a potent inflammatory interaction between LIGHT and PAR-2 in endothelial cells and, to some degree, also in monocytes. The demonstration of enhanced expression of PAR-2, LIGHT, and its receptors within atherosclerotic lesions underscore the potential in vivo relevance of these finding in relation to atherosclerosis. Our findings suggest that LIGHT/PAR-2-driven inflammation, which engages HVEM/JNK signaling, could represent a pathogenic loop in atherogenesis and plaque progression, potentially representing a target for therapy in this disorder.

Acknowledgments

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Disclosures

None.

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SUPPLEMENT MATERIAL

Inflammatory interaction between LIGHT and Proteinase-activated receptor-2 in endothelial cells – potential role in atherogenesis

Sandberg WJ et al.

Materials and Methods

Isolation of cells

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep; Nycomed, Oslo, Norway) gradient centrifugation. Further separation of monocytes (anti-human CD14-labeled magnetic beads; MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and CD3⁺ T cells (negative selection by monodisperse immunomagnetic beads; Dynal, Oslo, Norway) was performed as described elsewhere.^{1,2} Selected T cells consisted of >90% CD3⁺ cells and the isolated monocytes of >95% CD14⁺ cells (flow cytometry). The cells were stored in liquid nitrogen as pellets for RNA isolation or used for further *in vitro* experiments (monocytes).

Cell culture experiments

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cord veins by digestion with 0.1% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany)³ and cultured as previously described.⁴ HUVEC were passaged by treatment with 0.05% trypsin-EDTA (Gibco, Grand Island, NY) and grown in 12-well plates (Costar, Cambridge, MA) to confluence for 3 to 5 days. The medium was then discarded, and HUVEC were stimulated with different concentrations of recombinant human

(rh)LIGHT (R&D Systems, Minneapolis, MN), the PAR-2 agonist peptide sequence SLIGKV (a kind gift from Ola R Blingsmo at the Biotechnology Centre of Oslo, Oslo, Norway), or a combination thereof. In some experiments, 10-100 μ M SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) (Sigma, St Louis, MO), 10 μ M nuclear factor- κ B (NF- κ B) activation inhibitor IV (Calbiochem, San Diego, CA), or 20 μ M NF- κ B activation inhibitor II, JSH-23 (Calbiochem) was added to HUVEC culture 30 minutes before rhLIGHT stimulation. The cells used in the HUVEC experiments were in passage 3-5. The *human monocytic cell line THP-1* (American Type Culture Collection, Rockville, MD) and freshly isolated *human monocytes* were cultured for 4 days in RPMI 1640 (PAA laboratories, Pasching, Austria) supplemented with 2.5% fetal bovine serum (Gibco) in the presence of rhTNF α (5 ng/ml, R&D Systems), before further incubation with or without rhLIGHT (100 ng/ml). At different time points, cell-free supernatants and cell pellets of HUVEC and monocytes were harvested and stored at -80°C. The endotoxin levels of all stimulants and culture media were <10 pg/mL (Limulus Amebocyte Assay; BioWhittaker, Walkersville, MD). In all experiments with controls and un-stimulated cells, the vehicle of the stimulus was always added.

Microarray

RNA was isolated from HUVEC using RNeasy (Quiagen, Hilden, Germany). Three μ g of total RNA was used to generate cRNA, subsequently hybridized to Genechip Human Genome U133A Array (Affymetrix, Santa Clara, CA) according to standard protocols (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Arrays were scanned using a GeneArray scanner (Agilent Technologies, Palo Alto, CA) with

probe intensities extracted, and subjected to global scaling and statistical evaluations using the free software R.

Real time quantitative RT-PCR

Total RNA was extracted from HUVEC, monocytes, and T cells using RNeasy columns (Qiagen), subjected to DNase I treatment, and stored in RNA storage solution (Ambion, Austin, TX) at -80°C . In the mice experiments, tissue samples were cryohomogenized with a Mikro Dismembrator S (B Braun Biotech International GmbH, Melsungen, Germany), and total RNA was isolated with the RNeasy extraction kit (Qiagen), and reverse transcribed to cDNA using random hexamers and Superscript II reverse transcriptase (Life Technologies, Rockville, MD). Quantification of mRNA was performed using ABI Prism 7500 Fast Realtime PCR System (Applied Biosystems, Foster City, CA).⁵ Primers were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA). Primer sequences could be provided by request. PCR was performed using qPCR Master Mix for SyBr Green I (Eurogentec, Seraing, Belgium) and 300 nM primers. The mRNA levels of the housekeeping genes β -actin and 18S were used for normalization.

Western blotting

Western blotting was performed as previously described,⁶ separating equal amounts of protein from each sample by SDS-PAGE (10%) before transferring it onto polyvinyl difluoride membranes (NEN; Life Science, Boston, MA). The membranes were incubated with rabbit antibody against PAR-2 (Phoenix Pharmaceuticals, Belmont, CA), stripped, and reprobated with human anti- β -tubulin (Sigma) to ensure equal loading, followed by incubation with species-specific horseradish peroxidase-coupled secondary antibodies

(Cell Signaling, Beverly, MA). In a subset of experiments, the siRNA-mediated gene silencing efficiency (see below) was examined using primary antibody against HVEM (goat; Santa Cruz Biotechnology, Santa Cruz, CA) and LT β R (mouse; R&D Systems). The immune complex was visualized by using the Supersignal West Pico Western blot detection system (Pierce, Rockford, IL), exposure to Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK), and detected with the use of the Kodak 440 CF imaging station (Boston, MA). The software Total Laboratory v.1 10 (Phoretix, Newcastle, UK) was used for quantification.

Preparation and transfection of siRNA

Pre-designed siRNA against HVEM (siRNA ID: 13868) and LT β R (siRNA ID: 143461) were purchased from Ambion, and certificated non-silencing scramble control siRNA (cat No: 1022076) was obtained from Qiagen. HUVEC were cultured in 12-wells trays (Costar) and at 80% confluence, the cells were transfected using HiPerFect reagent (Qiagen) and 50 nM siRNA. The transfection reagents and the siRNA duplexes were mixed by vortexing in OptiMEM (Invitrogen), and incubated for 10 minutes at room temperature before the complexes were added drop-wise onto the cells. Thereafter, the cells were incubated for 18 hours with transfection complexes in normal growth medium. After an additional 18 hours, the cells were washed once and cultured for up to 8 hours with or without rhLIGHT (100 ng/ml) before harvesting and subsequent real time RT-PCR analyses. A parallel tray of siRNA-treated cells were assessed for HVEM and LT β R by Western blot to ensure successful gene silencing. The toxicity after siRNA transfections was examined for lactate dehydrogenase leakage using a cytotoxicity detection kit (Roche Applied Science, Penzberg, Germany).

Multiplex

Protein extracts (15 µg) from HUVEC, stimulated with rhLIGHT (100 ng/ml) or vehicle for 15 and 120 minutes, were subjected to phosphoprotein detection array (multiplexable beads: phosphorylated [p]-Akt; p-activating transcription factor-2 [ATF-2]; p-extracellular signal-regulated kinase [ERK]1/2; p-inhibitory [I]κB-α; p-Jun N-terminal kinase [JNK]; p-p38 mitogen-activated protein kinase [MAPK]; p-signal transducer and activator of transcription [STAT]3; BioRad, Hercules, CA) using the Bioplex (BioRad) suspension array technology. Multiplex suspension technology was also used for examining chemokine levels in supernatants of THP-1 cells and HUVEC (Biosource Human Chemokine Plex, Biosource, Camarillo, CA) analyzing concentrations of MCP-1, *interferon (IFN)-γ-induced protein (IP)-10*, *monokine induced by IFN-γ (MIG)*, and *regulated on activation normally T-cell expressed and secreted (RANTES)*. In all experiments, the samples were analyzed on a Multiplex Analyser (BioRad), and quantification was accomplished by using the BioPlex Manager Software (BioRad).

Enzyme immunoassays (EIAs)

Levels of macrophage chemoattractant protein (MCP)-1, interleukin (IL)-6, IL-8, IL-10, TNFα, and LIGHT were measured by EIAs (R&D Systems).

Mice

Female ApoE^{-/-} and wild-type C57BL/6 mice were obtained from Taconic Europe A/S (Lille Skensved, Denmark) and fed standard chow and water ad libitum as previously described.⁷ Eight ApoE^{-/-} and 8 C57BL/6 mice were euthanized at 18 weeks of age, and the aortas were freed from connective tissue under microscope and snap-frozen.

Immunohistochemistry

Acetone-fixed sections of the ascending aorta of the ApoE^{-/-} and wild-type C57BL/6 mice were further fixed in formalin and stained using affinity-purified polyclonal goat anti-human LIGHT, HVEM, LTβR, or PAR-2 IgG (all Santa Cruz Biotechnology), rat anti-mouse CD68 IgG (macrophage marker; Serotec Ltd., Oxford, UK), or sheep anti-rat von Willebrand factor (vWF) IgG (endothelial cell marker; Cedarlane, Ontario, Canada). The primary antibodies were followed by biotinylated anti-goat, anti-rat or anti-sheep IgG (Vector Laboratories, Burlingame, CA). The immunoreactivities were further amplified using avidin-biotin-peroxidase complexes (Vectastain Elite kit, Vector Laboratories). Diaminobenzidine was used as the chromogen in a commercial metal enhanced system (Pierce). The sections were counterstained with hematoxylin. Omission of the primary antibody served as a negative control.

Patients and controls

In a separate experiment, we analyzed PAR-2 expression in monocytes and T cells from 14 patients with unstable angina (60±12 years, 6 women and 8 men), 14 patients with stable angina (66±8 years, 2 women and 12 men), and 10 healthy controls (59±6 years, 2 women and 8 men). Serum levels of C-reactive protein (CRP) were significantly higher in angina patients ($p < 0.01$) as compared with controls (1.0±0.2 mg/l) with particularly high levels in those with unstable disease (2.2±0.5 mg/l versus 3.5±0.7 mg/l, stable and unstable angina, respectively; $p < 0.05$). All patients with unstable angina had experienced ischemic chest pain at rest within the preceding 48 hours (ie, Braunwald's class IIIB), but with no evidence of myocardial necrosis by enzymatic criteria. Transient ST-T segment depression and/or T-wave inversion were present in all cases. All patients with stable

angina had stable effort angina of >6 months duration and a positive exercise test. The diagnosis of coronary artery disease (CAD) was confirmed in all patients by coronary angiography showing at least 1 vessel disease (>50% narrowing of luminal diameter). Exclusion criteria were myocardial infarction or thrombolytic therapy in the previous month, ECG abnormalities invalidating ST-segment analyses, concomitant inflammatory diseases such as infections and autoimmune disorders, and liver or kidney disease.

Ethics

In the studies involving humans, informed consent for participation was obtained from all individuals. The studies were conducted according to the ethical guidelines at our hospital according to the Declaration of Helsinki and were approved by the local ethical committee. All animal experiments were in accordance with national guidelines and approved by the local ethical committee.

Statistical analysis

For statistical analysis of the *in vitro* experiments in HUVEC and monocytes, the Student paired *t* test was used. For comparisons of three groups of individuals, the nonparametric Kruskal-Wallis test was used. If a significant difference was found, Mann-Whitney *U* test was used to calculate the difference between each pair of groups. For comparisons within the same individuals, Wilcoxon's matched-pair test was used. Probability values (2-sided) were considered significant at a value of <0.05.

References for Supplemental Material

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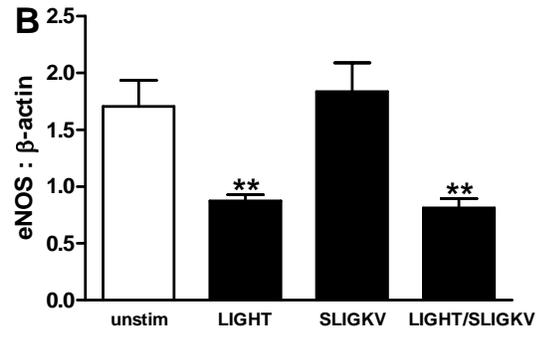
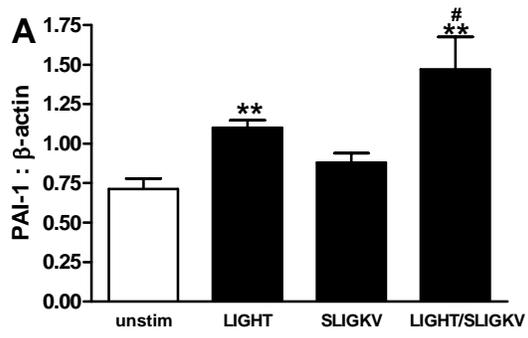
Supplemental Figure Legends

Supplemental Figure I. The effect of PAR-2/LIGHT co-stimulation on PAI-1 and eNOS expression in HUVEC. Cells were incubated with the PAR-2 activating peptide SLIGKV (100 μ M), rhLIGHT (100 ng/ml), or a combination thereof for 5 hours before analyzing mRNA levels of PAI-1 (**A**) and eNOS (**B**) by means of real-time RT-PCR and normalized to β -actin expression. ** $p < 0.01$ versus un-stimulated (unstim) cells; # $p < 0.05$ versus SLIGKV (100 μ M) when given alone. LIGHT/SLIGKV versus LIGHT (100 ng/ml) alone, $p = 0.08$. The data are mean \pm SEM (n=6).

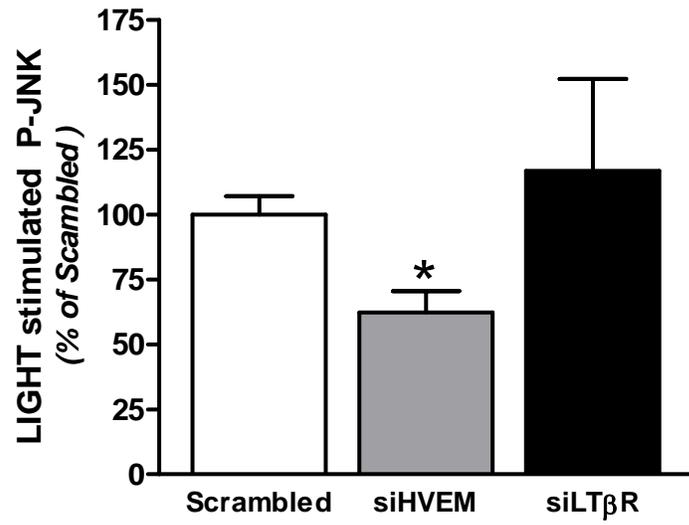
Supplemental Figure II. LIGHT-mediated JNK activation is mediated through HVEM. Silencing of HVEM, but not of LT β R, down-regulated the LIGHT-mediated (100 ng/ml) activation of JNK as assessed by attenuated phosphorylation of JNK (p-JNK) 15 minutes after LIGHT activation (phospho-protein multiplex suspension array technology). * $p < 0.05$ versus scramble control. The data are mean \pm SEM (n=4).

Supplemental Figure III. Co-stimulation with LIGHT and PAR-2 agonist enhances PAR-2 expression. THP-1 monocytes (**A**) and HUVEC (**B**) were incubated with the PAR-2 activating peptide SLIGKV (100 μ M), rhLIGHT (100 ng/ml), or a combination thereof for 5 hours before analyzing mRNA levels of PAR-2 by means of real-time RT-PCR and normalized to β -actin expression. ** $p < 0.01$ versus un-stimulated (unstim) cells; # $p < 0.05$ versus SLIGKV (100 μ M) and LIGHT (100 ng/ml) when given alone. The data are mean \pm SEM (n=8).

Supplemental Figure I



Supplemental Figure II



Supplemental Figure III

