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Lysophosphatidic Acid Induces Lymphangiogenesis and IL-8 Production *in Vitro* in Human Lymphatic Endothelial Cells

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The bioactive phospholipid lysophosphatidic acid (LPA) and its receptors LPA_{1-3} are aberrantly expressed in many types of human cancer. LPA has been reported to induce tumor cell proliferation, migration, and cytokine production. However, whether LPA exerts an effect on lymphatic endothelial cells (LECs) or on lymphangiogenesis, a process of new lymphatic vessel formation that is associated with increased metastasis and poor prognosis in cancer patients, has been unknown. Here, we show that LPA induces cell proliferation, survival, migration, and tube formation, and promotes lymphangiogenesis *in vitro* in human dermal LECs. In addition, LPA induces IL-8 expression by enhancing IL-8 promoter activity via activation of the NF-κB pathway in LECs. Using IL-8 siRNA and IL-8 neutralizing antibody, we revealed that IL-8 plays an important role in LPA-induced lymphangiogenesis *in vitro*. Moreover, using siRNA inhibition, we discovered that LPA-induced lymphangiogenesis *in vitro* and IL-8 production are mediated via the LPA_2 receptor in LECs. Finally, using human sentinel afferent lymphatic vessel explants, we demonstrated that LPA up-regulates IL-8 production in the LECs of lymphatic endothelia. These studies provide the first evidence that LPA promotes lymphangiogenesis and induces IL-8 production in LECs; we also reveal a possible new role of LPA in the promotion of tumor progression, as well as metastasis, in different cancer types. ***(Am J Pathol 2012, 180:2170–2181; DOI: 10.1016/j.ajpath.2012.03.003)***

The bioactive phospholipid lysophosphatidic acid (LPA) has been reported to induce tumor cell proliferation, migration, cytokine production, metastasis, and angiogenesis.[1] LPA binds to specific G protein-coupled receptors (LPA_{1-6}) to influence cell behavior.[1] Among these receptors, the endothelial differentiation gene (EDG) G protein-coupled receptor subfamily (EDG2/LPA_1, EDG4/LPA_2, and EDG7/LPA_3) are the most widely expressed and best characterized.[2] The majority of extracellular LPA is produced by autotaxin (ATX) from lysophosphatidylcholine; ATX is a secreted lysophospholipase-D initially identified from melanoma cell lines[3], and lysophosphatidylcholine is the most abundant phospholipid.[4] Although low in normal plasma and tissues, LPA levels have been shown to be elevated in malignant effusions of patients with ovarian cancer.[5] Overall, LPA receptors have been shown to be highly expressed in several human cancers, including ovarian, endometrial, cervical, breast, and gastric cancers and multiple myeloma.[6–8]

Lymphangiogenesis is a complex process of new lymphatic vessel formation that requires coordination of lymphatic endothelial cell (LEC) proliferation, migration, and tube-like network formation. In the adult, the quiescent LECs in lymphatic vasculature undergo lymphangiogenesis during tissue repair or regeneration or in pathological conditions, including tumor growth and metastasis and tumor-associated severe ascites.[9–12] Tumor-in-

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duced lymphangiogenesis facilitates the dissemination of tumor cells to the regional lymph nodes via the afferent lymphatic vessels, thus establishing a preferred route for lymphatic metastases in many solid tumors; indeed, tumor-induced lymphangiogenesis has been associated with increased metastasis and poor prognosis in cancer patients.[10] Although several lymphangiogenic growth factors,[13] including vascular endothelial growth factors VEGF-A, VEGF-C, and VEGF-D and fibroblast growth factor 2 (FGF-2),[14] have been recognized in recent years, the molecular and cellular regulation of lymphangiogenesis is still largely unknown.

Previous studies suggest a possible role of the ATX-LPA axis in lymphangiogenesis. LPA_1 knockdown resulted in lymphatic vessel malformation in zebrafish, suggesting that LPA_1 is necessary for embryonic lymphatic vessel development.[15] In a study using specimens from patients with gastric cancer, LPA_2 expression correlated with increased lymphatic invasion, venous invasion, and lymph node metastasis.[8] In mice, ATX was essential for vascular development through the production of LPA.[16] In an *in vitro* study, LPA induced expression of several lymphatic-specific markers (Prox-1, LYVE-1, and podoplanin) and increased VEGF-C production in blood vascular endothelial cells [human umbilical vein endothelial cells (HUVECs)].[17,18]

LPA induces the expression of IL-8 in several types of cells, including ovarian cancer cells, granulosa-lutein cells, and HUVECs.[19–22] IL-8 has also been shown to induce proangiogenic responses.[23] Several signaling pathways have been implicated in LPA-induced IL-8 production, including a nuclear factor-κB (NF-κB)-dependent pathway in granulosa-lutein cells[20] and Rho kinase signaling through p38 and JNK activation in HUVECs.[22] However, whether LPA has an effect on IL-8 expression in human LECs is currently unknown.

The objective of the present study was to determine the effect of LPA on human LECs and lymphangiogenesis and to elucidate the mechanism underlying LPA effects. The results of our *in vitro* and *ex vivo* investigations reveal a new role of LPA in promoting lymphangiogenesis *in vitro* via up-regulation of IL-8 expression in LECs.

Materials and Methods

Reagents

Lysophosphatidic acid (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate; 18:1) was purchased from Avanti Polar Lipids (Alabaster, AL). LPA stock was prepared fresh before each experiment as 10 mmol/L in PBS and 0.1% (w/v) charcoal-stripped bovine serum albumin (Sigma-Aldrich, Munich, Germany). Mouse anti-human IL-8 neutralizing antibody and mouse IgG_1 control antibody were purchased from R&D Systems (Minneapolis, MN). Calcein AM was purchased from Biotium (Hayward, CA).

Cell Culture and Cell Proliferation

Human adult dermal LECs were purchased from PromoCell (Heidelberg, Germany). Three lots of LECs were used, and all yielded similar results. LECs were cultured in endothelial growth medium MV2 (EGM-MV2), comprised of endothelial basal medium MV2 (EBM-MV2) with supplement pack (PromoCell). LECs were used between passages 2 and 5. LECs were made quiescent in a starvation medium [EBM-MV2 supplemented with 1% fetal bovine serum (FBS), 1 μg/mL ascorbic acid, and 0.2 μg/mL hydrocortisone] for 16 hours before and during LPA treatment. The serum and supplement concentrations in the starvation medium were chosen to maintain cell viability. Starvation medium with 5% FBS was used as positive control for cell proliferation, migration, and tube formation assays. LECs were treated with 0 to 10 μmol/L LPA, and cell proliferation was first evaluated by tetrazolium salt colorimetric assay (MTS), using a CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions. Proliferation was also examined by cell counting using a hemocytometer at 72 hours after treatment with 0 to 10 μmol/L LPA.

Cell Migration, Scratch Wound, and Tube Formation Assays

Cell migration was measured using a modified Boyden chamber assay as described previously.[24] Inserts (3-μm pores; BD Discovery Labware, Bedford, MA) were precoated with 1× attachment factor containing gelatin (Invitrogen Life Technologies, Carlsbad, CA) before use. LECs were incubated with treatment or control medium for 24 hours before seeding into upper chambers and treated medium was added into lower chambers. Migration was assessed after staining with Calcein AM by measuring fluorescence of cells migrated to the lower chamber using a fluorescence SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA) from the bottom at 485/535 nm excitation/emission wavelengths. The migrated cells were identified by the ratio of fluorescence in LPA-treated LECs compared with non-LPA-treated controls. Images of the migrated cells in the lower chambers were captured under a Leica DM IL inverted microscope using a digital microscope camera (Optronics, Goleta, CA).

Scratch wounds were produced in a confluent monolayer of LECs. Images of the subsequent LEC migration process were captured at 30 minutes and at 48 hours. Wound area was calculated using ImageJ software version 1.43t (NIH, Bethesda, MD), as described previously.[25]

Tube formation assays were performed and total tube length was calculated as described previously.[24] LECs were treated with or without LPA in starvation medium for 24 hours before they were seeded on Matrigel (BD Biosciences, San Jose, CA) in fresh starvation medium with or without LPA. Images of the tube formation process were captured at 6 hours and at 16 hours; total tube length was calculated as described previously.[24]

Table 1. Probes Used in TaqMan qPCR Assays

Probe	Assay ID	Probe sequence
Human IL-8	Hs00174103_m1	5′-CAGCTCTGTGTGAAGGTGCAGTTTTGCCAA-3′
Human LPA_1	Hs00173500_m1	5′-CACAGCCCCAGTTCACAGCCATGAATGAAC-3′
Human LPA_2	Hs00173704_m1	5′-TGGTCAAGACTGTTGTCATCATCCTGGGGGC-3′
Human LPA_3	Hs00173857_m1	5′-TGACTGTCTTAGGGGCGTTTGTGGTATGCT-3′
Human ACTB	Hs99999903_m1	5′-CCTTTGCCGATCCGCCGCCCGTCCACACCC-3′
Human GAPDH	Hs99999905_m1	5′-TATTGGGCGCCTGGTCACCAGGGCTGCTTT-3′

qPCR and siRNA Transfection

RNA was isolated using a RiboPure RNA isolation kit (Ambion, Austin, TX) and was reverse-transcribed using MMLV reverse transcriptase (Clontech, Mountain View, CA) according to the manufacturer's instructions. Gene expression was quantified using quantitative real-time PCR (qPCR) on an ABI StepOnePlus system using TaqMan gene expression assays with custom TaqMan probes specific for each gene (Applied Biosystems; Life Technologies, Foster City, CA). Relative gene expression was determined and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or human β-actin (ACTB). Probes are listed in Table 1.

Nontargeting control or targeted siRNAs (50 nmol/L; Dharmacon, Lafayette, CO) were transfected with DharmaFECT reagent-1 into LECs according to the manufacturer's instructions. The transfection efficiency of siRNA was measured by GFP generated by concomitant transfection of GFP-reporter plasmid into LECs. The blocking effect of siRNA on LECs was monitored by a positive control GAPDH siRNA in LECs. Gene knockdown was assessed by qPCR at 24 hours or by enzyme-linked immunosorbent assay (ELISA) at 48 hours after siRNA transfection. The effects of LPA on cell growth, survival, migration, tube formation, and IL-8 expression in LECs after siRNA treatment were observed at 24 hours after siRNA transfection.

ELISA

Cell supernatants were collected, and equal amounts of total protein (~3 μg) from each well of a 96-well plate were loaded for measurement of IL-8 level using a human IL-8 ELISA kit II (BD Biosciences) according to the manufacturer's instructions.

Cell supernatants were collected, and equal amounts of total protein (~3 μg) were loaded for measurement of VEGF-A, VEGF-C, VEGF-D, and FGF-2 levels by ELISA (R&D Systems) according to the manufacturer's instructions.

Reporter Constructs and Luciferase Assay

Two firefly luciferase reporter plasmid constructs were used, one driven by human IL-8 promoter (−1481 to +44 bp) (pGL3-IL-8-Luc) versus its control (pGL3-Luc)[26,27] and another driven by wild-type human IL-8 promoter [pGL2-IL-8(wt NF-κB)-Luc] versus human IL-8 promoter with a mutation at the NF-κB binding site [pGL2-IL-8 (mut NF-κB)-Luc]. Procedures were as described previously.[28,29]

LECs were transfected with 200 ng relevant firefly luciferase reporter plasmids and 2 ng pB-actin-RL *Renilla* plasmid (Promega) (transfection efficiency control) using FuGene HD reagent (Roche, Basel, Switzerland) in 24-well plates (80% confluency) according to the manufacturer's instructions. After 24 hours, LECs were incubated with or without 5 and 10 μmol/L LPA in starvation medium for an additional 4 hours. Luciferase/*Renilla* signals were then measured using a dual-luciferase reporter assay kit (Promega).

ChIP Assays

Chromatin immunoprecipitation (ChIP) assays were performed with the ChIP-IT Express kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 7×10^6 LECs were treated with or without 5 μmol/L LPA in 150-mm culture dishes for 60 minutes before cellular proteins and DNA were cross-linked by 1% formaldehyde. Cell lysates were sonicated to generate DNA fragments from 200 to 1500 bp. Protein G magnetic beads with 1 μg of either anti-phospho-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-IgG (negative control) were added to the chromatin-containing supernatants and incubated overnight at 4°C. After reversal of cross-linking of the protein/DNA complexes to free DNA, PCR was performed using primers flanking the NF-κB binding site in the IL-8 promoter: 5′-GGGCCATCAGTTGCAAATC-3′ and 5′-TTCCTTCCGGTGGTTTCTTC-3′.[30]

Apoptosis Assay

LECs grown to 80% to 90% confluency in four-well chamber slides were treated with serum-free EBM-MV2 medium for 16 hours before being treated with or without LPA (5 μmol/L). Apoptosis was examined by TUNEL assay at 36 hours, using the DeadEnd fluorometric TUNEL system (Promega) or caspase-3 immunostaining at 24 hours using caspase-3 (cleaved) antibody (Biocare Medical, Concord, CA).

Reverse-Phase Protein Array

LECs were treated with 5 μmol/L LPA in 30-mm dishes, and cells were harvested at several time points (0, 5, 10, 20, 30, 45, 60, and 120 minutes) and lysed with lysis buffer containing 1% Triton X-100, 50 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1

mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L NaPPi, 10% glycerol, 1 mmol/L Na_3VO_4, and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The protein lysates were normalized to a concentration of 1 mg/mL, and then denatured in 1% SDS for 10 minutes at 95°C. Reverse-phase protein array (RPPA) analysis was performed by staff at the MD Anderson Functional Proteomics RPPA Core as described previously.[31,32] A logarithmic value reflecting the relative amount of each protein in each sample was generated for analyses.

Western Blotting and IHC

Western blotting was performed as described previously.[33] For IHC of LECs, cells were grown on chamber slides (Falcon; BD Discovery Labware); for IHC of human sentinel afferent lymphatic vessels, frozen sections were fixed in cold acetone. Immunostaining was then performed as described previously.[33] Antibodies used were rabbit anti-human phospho-NF-κB p65 (Ser^{536}) (Cell Signaling Technology, Danvers, MA), rabbit polyclonal anti-IL-8 (Invitrogen Biosource, Carlsbad, CA), rabbit anti-human CXCR1/IL8RA (IL-8 receptor α chain) and CXCR2/IL8RB (IL-8 receptor β chain) antibodies (R&D Systems), rabbit anti-human podoplanin (Fitzgerald Industries International, Acton, MA), mouse anti-Prox-1 (Fitzgerald Industries International), rabbit anti-human phospho-ERK1/2 (Cell Signaling Technology), and mouse anti-total ERK1/2 (Cell Signaling Technology). SYTOX Green (Invitrogen-Molecular Probes, Carlsbad, CA) was used to visualize cell nuclei. Confocal images were acquired using an LSM 510 laser-scanning microscope (Carl Zeiss, Thornwood, NY).

Human Lymphatic Vessel Assay

Small segments (3 to 5 mm) of anatomically confirmed human sentinel afferent lymphatic vessels were obtained from patients undergoing intraoperative lymphatic mapping and sentinel lymph node biopsy procedures as part of planned surgical treatment for melanoma under an Institutional Review Board-approved protocol, as described previously.[34] Briefly, 1 to 3 mL of 1% isosulfan blue (Lymphazurin) is injected intradermally around the tumor or biopsy site immediately before lymphatic mapping; the protocol includes intradermal injection of 0.5 to 1 mCi of ^{99m}Tc-labeled sulfur colloid 1 to 4 hours before surgery. Intraoperative localization of the sentinel lymph nodes is aided by the use of a handheld gamma counter (Neoprobe 1500 or Neoprobe Neo 2000; Neoprobe, Dublin, OH) and visualization of the blue dye in lymphatic vessels and sentinel lymph nodes. Tiny segments of human afferent lymphatic vessels attached to the sentinel lymph node were harvested. Sentinel afferent lymphatic vessels were placed in cold EGM-MV2 medium with 10% FBS, transported immediately to the laboratory on wet ice, carefully dissected to remove perilymphatic tissues under a dissecting microscope, and then cut lengthwise to expose the luminal surface. To ensure adequate experimental controls, each vessel segment was transected into two subsegments. Each pair of open vessel subsegments were cultured in two separate wells supplemented with EGM-MV2 medium and 20% FBS con-

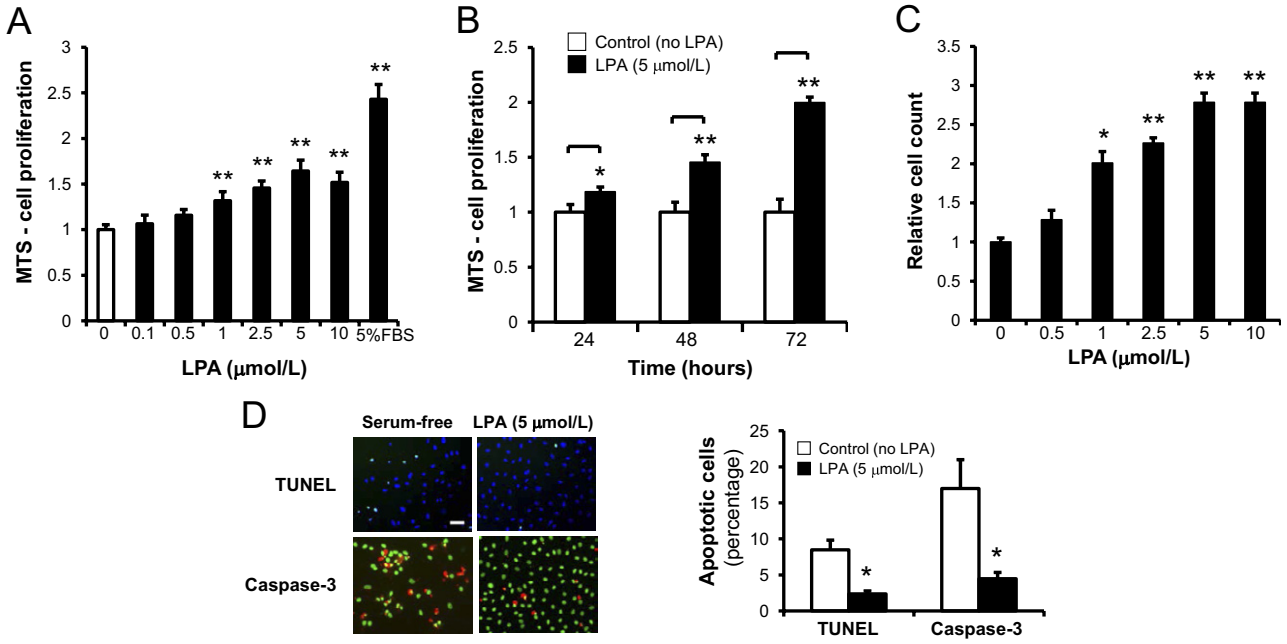

Figure 1. LPA induces LEC proliferation and promotes LEC survival. **A:** LPA increases LEC proliferation in a dose-dependent manner at 48 hours. FBS (5%) was used as a positive control. **B:** LPA (5 μmol/L) induces LEC proliferation in a time-dependent manner. **C:** LPA induces increased LEC cell counts in a dose-dependent manner at 72 hours, relative to no-treatment control. **D:** LPA reduces LEC apoptosis induced by serum-free starvation detected by TUNEL assay (green; nuclei stain blue) at 36 hours and by IHC for cleaved caspase-3 (red; nuclei stain green) at 24 hours. Data are expressed as means ± SEM. Relative values compare LPA-treated LECs with non-LPA-treated controls. $^*P < 0.05$, $^{**}P < 0.01$. Scale bar = 50 μm. Original magnification, ×20.

taining antibiotics for 16 hours before treatment with or without 10 μmol/L LPA with 10% FBS and antibiotics for an additional 24 hours. Each vessel segment was embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Torrance, CA) for frozen section preparation and IHC. For the present study, a total of four vessels from two patients were used.

Statistical Analysis

Student's *t*-test was used to evaluate the data. Data are expressed as means ± SEM. *P* values of <0.05 were considered statistically significant.

Results

LPA Promotes LEC Proliferation, Survival, Migration, and Tube Formation

Lymphatic endothelial cell lineage was confirmed for LECs used in the present study by immunohistochemical analysis. The LECs used (passages 2 to 5) all expressed the lymphatic endothelial cell markers podoplanin and Prox-1 and the panendothelial cell marker CD31, and did not express the blood vascular endothelial cell marker CD34 (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). As expected, HUVECs expressed both CD31 and CD34, but did not express podoplanin or Prox-1. LPA induced a dose- and time-dependent increase in the proliferation of LECs (Figure 1, A and B) and a dose-dependent increase in the number of LECs (Figure 1C). We next tested the effect of LPA on LEC survival. LPA (5 μmol/L) reduced apoptosis of LECs induced by serum starvation as measured by TUNEL assay and cleaved caspase-3 immunohistochemical staining (Figure 1D). Taken together, these data demonstrate that LPA induces LEC proliferation and promotes LEC survival *in vitro*.

LPA treatment significantly increased LEC migration in a dose- and time-dependent manner, as measured by both a modified Boyden chamber assay (Figure 2, A and B) and a scratch wound assay (Figure 2C). We next assessed whether LPA induces formation of tube-like networks by LECs in Matrigel. LPA promoted LECs to form tube-like networks, compared with non-LPA-treated controls (Figure 2D). Together with the observed effects on growth and survival, these data provide evidence that LPA promotes behaviors of LECs that are essential for lymphangiogenesis *in vitro*.

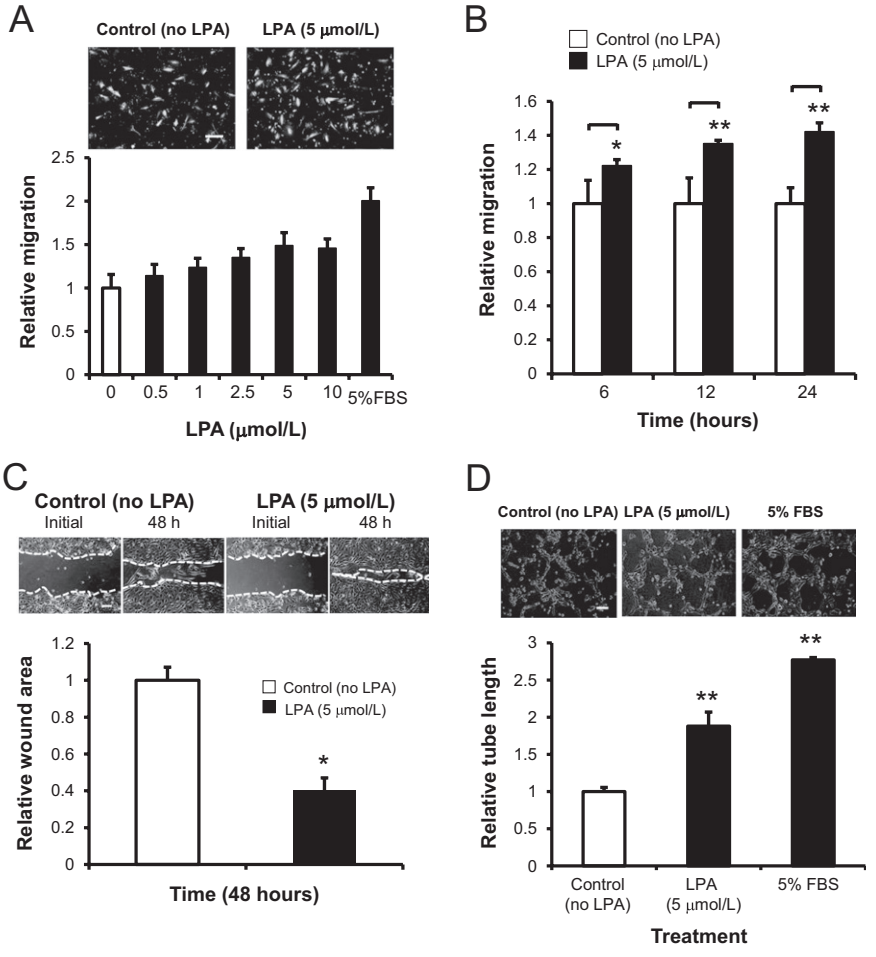

Figure 2. LPA induces LEC migration and tube formation. **A:** LPA induces LEC migration in a dose-dependent manner at 24 hours. Representative photomicrographs of migrated cells in a modified Boyden chamber assay are shown. FBS (5%) was used as a positive control. **B:** LPA (5 μmol/L) induces LEC migration in a time-dependent manner. **C:** LPA promotes LEC migration in a scratch wound assay at 48 hours. **D:** LPA promotes capillary-like LEC tube formation on Matrigel at 6 hours. FBS (5%) was used as a positive control. Data are expressed as means ± SEM. Relative values compare LPA-treated LECs with non-LPA-treated controls. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bar = 50 μm. Original magnification, ×10.

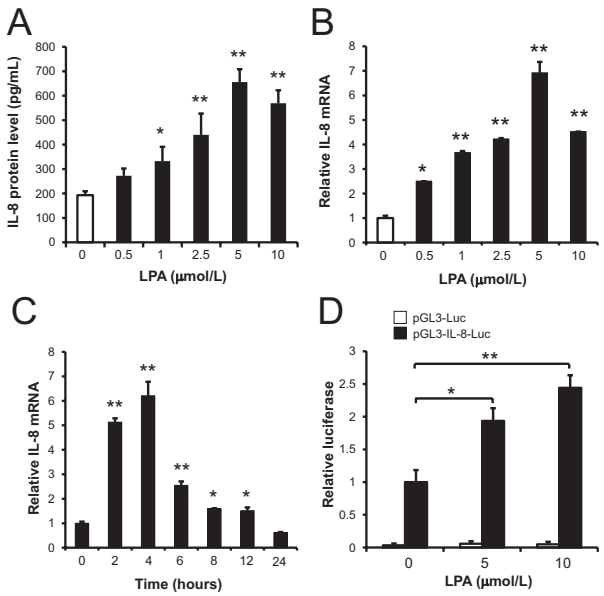

Figure 3. LPA induces IL-8 expression in LECs. **A:** IL-8 protein levels in LEC supernatants as examined by ELISA at 72 hours. **B:** IL-8 mRNA levels at 4 hours, as examined by qPCR. **C:** LPA-induced IL-8 mRNA expression in LECs peaks at 4 hours. **D:** LPA-induced increases in luciferase (Luc) activity are driven by IL-8 promoter. Data are expressed as means ± SEM. Relative values compare LPA-treated LECs with non-LPA-treated controls. $^{*}P < 0.05$, $^{**}P < 0.01$.

LPA Induces IL-8 in LECs in Vitro

LPA is known to induce IL-8 in several cell types. Because IL-8 has been shown to induce proangiogenic responses, we sought to investigate the possible effect of IL-8 in LPA-treated LECs. We found that LPA treatment resulted in a dose-dependent increase in IL-8 secretion by LECs *in vitro*, as determined by ELISA (Figure 3A). qPCR demonstrated that LPA increased IL-8 mRNA expression in LECs in a dose- and time-dependent manner (Figure 3, B and C).

LPA induced IL-8 promoter-driven luciferase activity in LECs in a dose-dependent manner, by 2.5-fold (Figure 3D). These data indicate that LPA up-regulates the transcription and secretion of IL-8 in LECs.

VEGF-A, VEGF-C, VEGF-D, and FGF-2 are known to induce lymphangiogenesis.[13] To examine the potential role of these known lymphangiogenic factors, we examined their expression in LECs in culture after LPA treatment. Importantly, none of these lymphangiogenic factors were induced by LPA treatment as evaluated by ELISA (data not shown), which supports the notion that none of these factors are significantly involved in LPA-induced lymphangiogenesis *in vitro*.

IL-8 Plays an Important Role in LPA-Induced Lymphangiogenesis in Vitro

To explore the significance of LPA-induced up-regulation of IL-8 in LECs, we silenced IL-8 expression in LECs by siRNA. The transfection efficiency of siRNA was 90%, as measured by GFP generated by concomitant transfection of GFP-reporter plasmid into LECs. GAPDH siRNA served as a positive control and reduced up to 81% of GAPDH RNA expression (data not shown). Both LPA-induced and endogenous IL-8 expression were significantly inhibited by IL-8 siRNA in LECs at the protein level as determined by ELISA (see Supplemental Figure S2A at *http://ajp.amjpathol.org*). Suppression of IL-8 expression in LECs inhibited the LPA-induced increase in LEC proliferation (Figure 4A), survival (Figure 4B), migration (Figure 4C), and tube formation (Figure 4D), compared with control cells transfected with nontargeting siRNA. IL-8 neutralizing antibody also blocked LPA-induced cell proliferation, survival, migration, and tube formation (see Supplemental Figure S2, B–E, at *http://ajp.amjpathol.org*). Importantly, both of the IL-8 receptors (CXCR1 and CXCR2) were expressed in cultured LECs (see Supplemental Figure S2F at *http://ajp.amjpathol.org*). Taken together, these data support a significant functional role for IL-8 in LPA-induced lymphangiogenesis in LECs *in vitro*.

NF-κB Is Activated by LPA in LECs and Is Critical to IL-8 Induction

To better understand the mechanism underlying LPA-induced lymphangiogenesis *in vitro*, we examined the expression of total and activation-specific protein markers in kinase signaling pathways in LECs using RPPAs. LPA treatment markedly and rapidly affected the expression of a large number of proteins and phospho-proteins (Figure 5A). Clustering analysis identified coordinated up-regulation of multiple activation-specific markers in the PI3K-AKT pathway, including p-AKT (Ser473), p-GSK-3α/β (Ser21/9), p-PDK1 (Ser241), and p-TSC2 (Thr1462); this analysis also revealed marked increase in components of other kinase cascades, including p-MAPK, p-p38-MAPK, p-JNK, p-S6, and p-SMAD5 (Figure 5A).

Quantitative analysis of the RPPA data revealed that the protein that was increased from baseline more than any other marker at both 60 minutes (11.3-fold versus baseline) and 120 minutes (9.2-fold) was p-NF-κB (p65 subunit) (see Supplemental Table S1 at *http://ajp.amjpathol.org*). Western blot analysis confirmed the observed increase in p-NF-κB p65 levels, which was noted as soon as 20 minutes and peaked at 60 minutes after LPA treatment (Figure 5B). Activation of NF-κB was also confirmed by IHC; p-NF-κB p65 was highly expressed in the nuclei of LPA-treated LECs 60 minutes after LPA treatment, but not in untreated controls (see Supplemental Figure S3 at *http://ajp.amjpathol.org*).

Previous studies have demonstrated that NF-κB regulates LPA-induced increases in IL-8 in some, but not all, cell types.[20,22] On the basis of our observation that LPA induced NF-κB activation in LECs, we tested the hypothesis that NF-κB directly mediates the observed LPA-induced increase in IL-8 transcription. To examine whether LPA-activated p-NF-κB p65 binds directly to the NF-κB binding site in the IL-8 promoter in LECs, we performed a ChIP assay using p-NF-κB p65 antibody. Indeed, ChIP assay confirmed that p-NF-κB p65 bound to the NF-κB binding site in the IL-8 promoter in LPA-treated LECs, but not in untreated controls (Figure 5C). To further test whether NF-κB binding to the IL-8 promoter is required

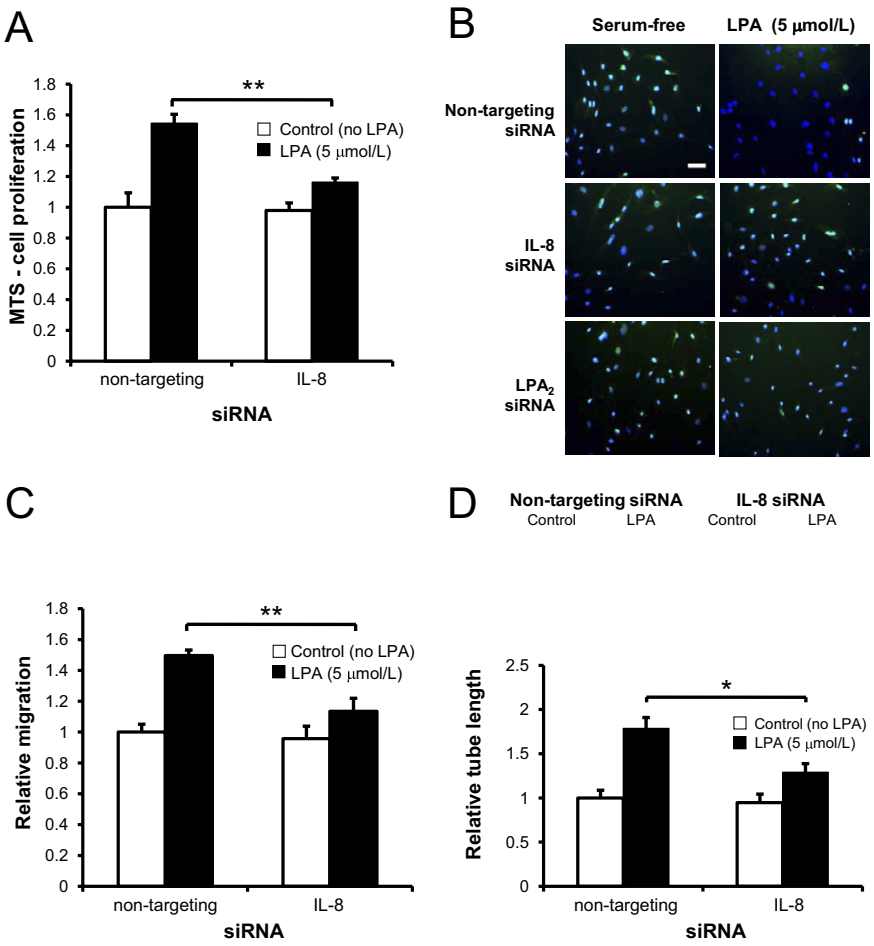

Figure 4. IL-8 plays an essential role in LPA-induced lymphangiogenesis *in vitro*. **A:** IL-8 siRNA partially blocks LPA-induced LEC proliferation, as examined by MTS assay at 24 hours. **B:** IL-8 siRNA and LPA_2 siRNA partially blocks LPA-induced LEC survival by cleaved caspase-3, as examined by IHC at 24 hours (green; nuclei stain blue). **C:** IL-8 siRNA partially blocks LPA-induced LEC migration at 24 hours. **D:** IL-8 siRNA partially blocks LPA-induced capillary-like tube formation of LECs on Matrigel at 16 hours. Scale bar = 50 μm. Original magnification: ×20 (**B**); ×10 (**D**). Relative values compare LPA-treated LECs with nontargeting siRNA-treated, non-LPA-treated controls. Data are expressed as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

for IL-8 induction, we transfected LECs with either a firefly luciferase reporter construct containing the wild-type IL-8 promoter or the same promoter containing a mutation at the NF-κB binding site.[28,29] Transfection of cells with the promoter containing the mutation abolished LPA-induced IL-8 promoter-driven luciferase activity (Figure 5D). Together, these data support the hypothesis that LPA up-regulates IL-8 production by activating the NF-κB pathway.

LPA_2 Is a Critical Mediator of LPA Effects on LECs

The biological activity of LPA is mediated by interaction of LPA with its receptors, the best characterized of which are LPA_1, LPA_2, and LPA_3. Because specific antibodies against LPA_{1-3} receptors are lacking, the expression of these receptors was examined by qPCR. We demonstrated that these receptors are all expressed in LECs (see Supplemental Figure S4A at *http://ajp.amjpathol.org*). To determine which of these LPA receptors are involved in the effect of LPA on LECs, we used siRNA to individually silence each LPA receptor. Each LPA receptor subtype siRNA treatment significantly decreased LPA receptor expression of that subtype; no cross-receptor inhibition was noted (see Supplemental Figure S4B at *http://ajp.amjpathol.org*).

Having demonstrated knockdown efficiency and LPA receptor specificity for the different siRNAs, we then tested their respective influence on the effects of LPA on LECs. LPA_2 siRNA significantly blocked LPA-induced IL-8 up-regulation in LECs, compared with nontargeting siRNA. In contrast, neither LPA_1 nor LPA_3 siRNA abrogated LPA-induced IL-8 activity (Figure 6A). LPA-activated p-NF-κB p65 was blocked by LPA_2 siRNA in LECs at 60 minutes, compared with nontargeting siRNA, but not by LPA_1 or LPA_3 siRNA (Figure 6B). Further experiments demonstrated that LPA_2 siRNA also inhibited LPA-induced increases in cell proliferation (Figure 6C) and migration (Figure 6D), cell survival (Figure 4B), and tube formation (Figure 6E). These data support the hypothesis that LPA_2 is critical to mediating the effects of LPA on LECs.

LPA Induces IL-8 Expression in Luminal LECs of Human Sentinel Afferent Lymphatic Vessel Explants

To corroborate these findings and determine the effect of LPA on LECs in human lymphatic vessels *in vivo*, we

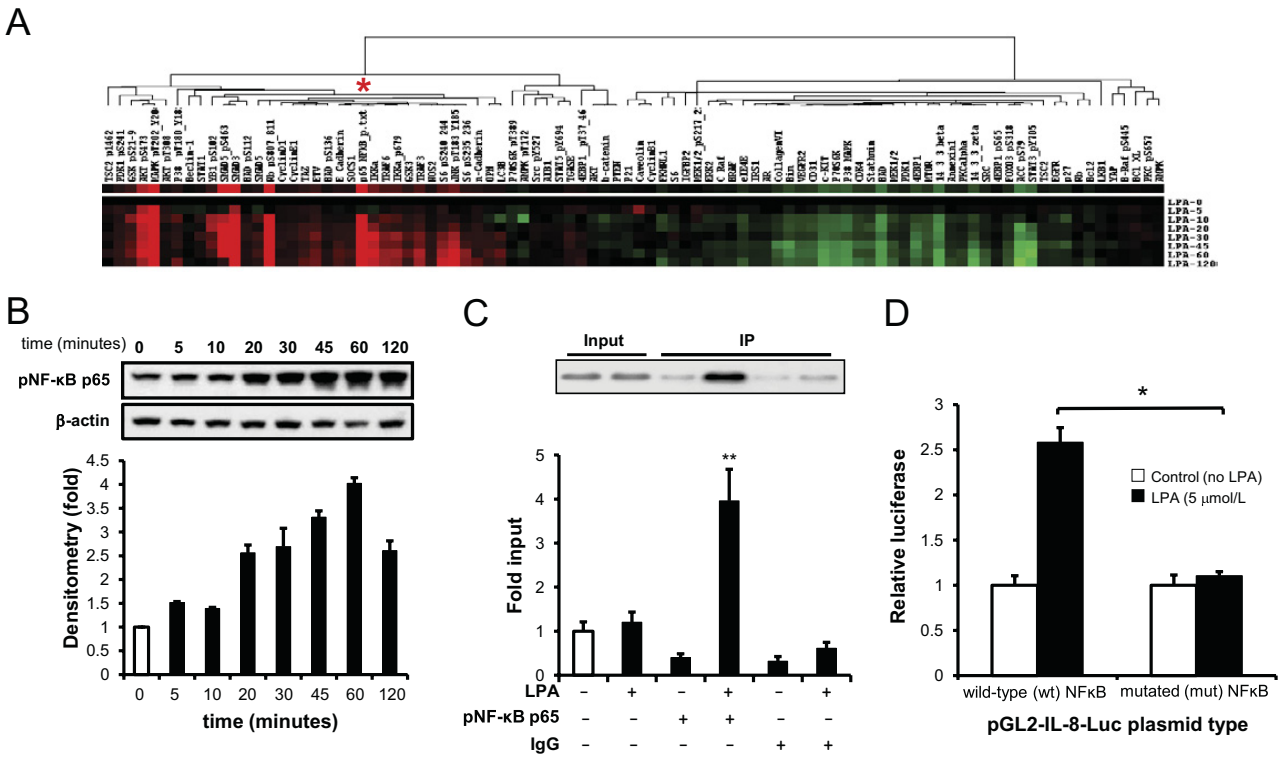

Figure 5. NF-κB is activated by LPA in LECs and is critical to IL-8 induction. **A:** Clustered heat map of results of RPPA analysis. Note activation of NF-κB (p-NF-κB p65) after LPA treatment (**asterisk**). Red indicates high phosphoprotein levels relative to black or green. **B:** Activation of NF-κB (p-NF-κB p65) pathway in LECs by 5 μmol/L LPA as examined by Western blot. **C:** LPA-activated p-NF-κB p65 binds to endogenous IL-8 promoter in LECs as examined by ChIP assay. Fold change is relative to control input. **D:** Mutation of the NF-κB binding site in the IL-8 promoter blocks LPA-induced IL-8 promoter activity. Data are expressed as means ± SEM. Relative values compare LPA-treated LECs with non-LPA-treated controls. $^{*}P < 0.05$, $^{**}P < 0.01$.

isolated human sentinel afferent lymphatic vessels in patients undergoing planned lymphatic mapping surgery (Figure 7A) and sectioned them to expose the luminal surface to either LPA or no treatment *ex vivo* (Figure 7B). To document their lymphatic lineage, we confirmed that these human afferent lymphatic vessels expressed the lymphatic-specific markers podoplanin and Prox-1 (see Supplemental Figure S5 at *http://ajp.amjpathol.org*). LPA increased IL-8 expression in the LECs of lymphatic vessel endothelia in LPA-treated subsegments, compared with matched non-LPA-treated control subsegments from the same lymphatic vessel specimens (Figure 7C). LPA-induced IL-8 expression was indeed observed in each of four lymphatic vessel harvested from two patients. Additionally, the IL-8 receptors CXCR1 and CXCR2 were both expressed in lymphatic vessel endothelia (see Supplemental Figure S6 at *http://ajp.amjpathol.org*); LPA treatment did not alter their expression (data not shown). Together, these data provide further evidence that LPA induces IL-8 expression in LECs in human lymphatic vessel endothelia explants, suggesting a potential role of LPA in IL-8 production and lymphangiogenesis *in vivo*.

Discussion

In the present study, we demonstrated that LPA, a bioactive phospholipid produced by many different tumor cells, promotes lymphangiogenesis *in vitro* and up-regulates IL-8 expression in LECs via the LPA receptor LPA_2. Indeed, LPA induced dose- and time-dependent increases in LEC proliferation, migration, and IL-8 production, each of which was abrogated by LPA_2 siRNA. We also demonstrated that LPA regulates IL-8 expression via the NF-κB pathway. Finally, we corroborated our findings in human lymphatic vessels *in situ* by demonstrating that LPA up-regulates IL-8 production in the luminal LECs of intact human sentinel afferent lymphatic vessel explants.

Tumor-associated lymphatic vessels act in part as a conduit by which tumor cells can disseminate to regional lymph nodes and potentially to distant sites.[10] Tumors interact with the lymphatic vasculature in many ways, including vessel co-option, chemotactic migration and invasion into lymphatic vessels, and induction of lymphangiogenesis. Tumor-induced lymphangiogenesis has been correlatively and functionally associated with metastasis formation and poor prognosis in multiple types of cancer. In preclinical models, experimental blockade of tumor-induced lymphangiogenesis inhibited metastasis formation in lymph nodes and often in other organs, which suggests that lymphatic blockade might suppress metastasis formation.[35]

LPA is a bioactive phospholipid that is produced by activated platelets, leukocytes, epithelial cells, adipocytes, peritoneal mesothelial cells, fibroblasts, and tumor cells.[36] LPA circulates in normal human peripheral blood at plasma concentrations of <100 nmol/L.[6,37,38]

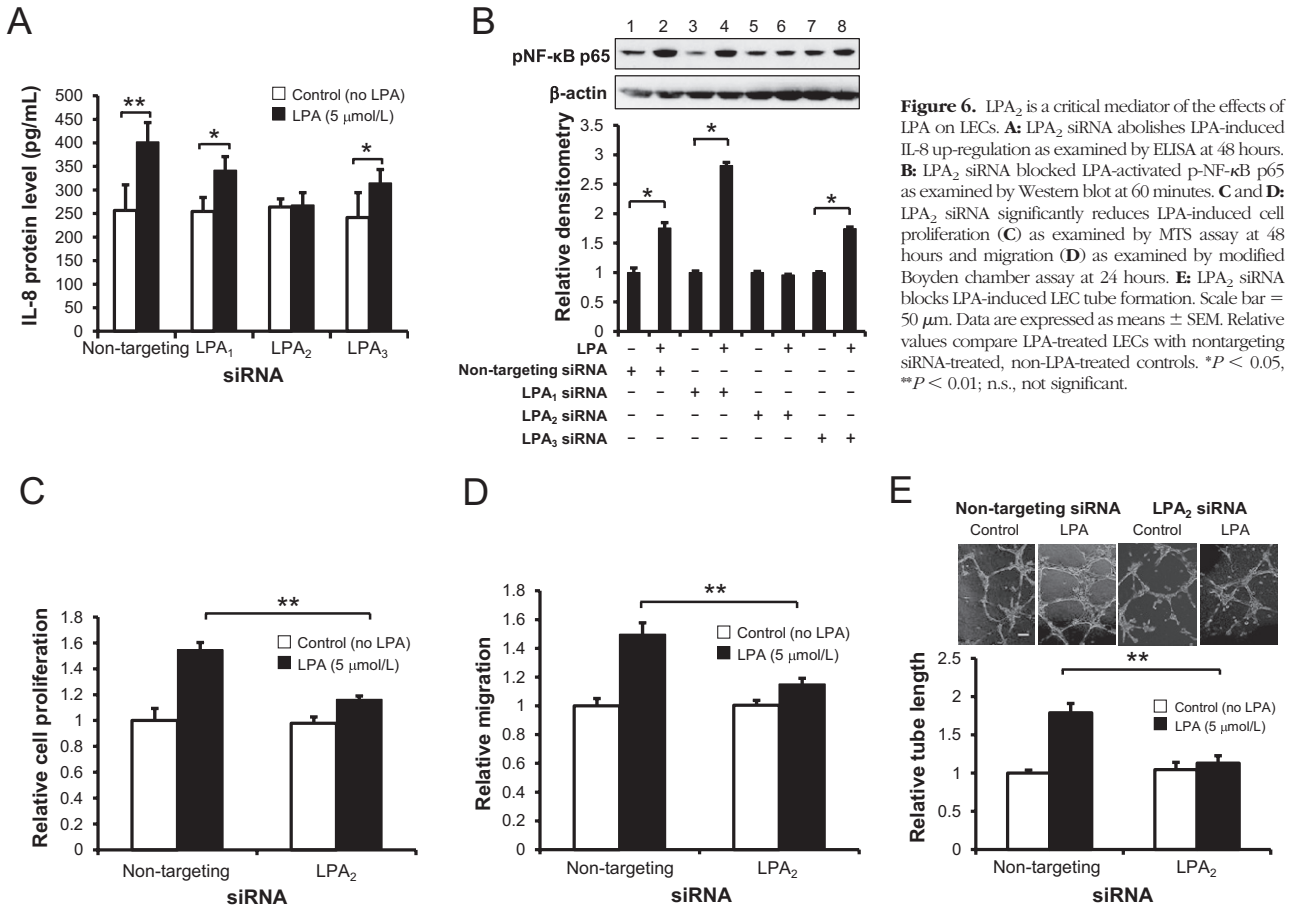

Figure 6. LPA_2 is a critical mediator of the effects of LPA on LECs. **A:** LPA_2 siRNA abolishes LPA-induced IL-8 up-regulation as examined by ELISA at 48 hours. **B:** LPA_2 siRNA blocked LPA-activated p-NF-κB p65 as examined by Western blot at 60 minutes. **C** and **D:** LPA_2 siRNA significantly reduces LPA-induced cell proliferation (**C**) as examined by MTS assay at 48 hours and migration (**D**) as examined by modified Boyden chamber assay at 24 hours. **E:** LPA_2 siRNA blocks LPA-induced LEC tube formation. Scale bar = 50 μm. Data are expressed as means ± SEM. Relative values compare LPA-treated LECs with nontargeting siRNA-treated, non-LPA-treated controls. $^*P < 0.05$, $^{**}P < 0.01$; n.s., not significant.

However, LPA levels have been reported to be increased in 90% of ovarian cancer patients[6] and in at least some patients with myeloma, endometrial cancer, and cervical cancer.[6,7] Macrophage-induced lymphangiogenesis has been shown to directly induce ascites in a murine model of ovarian cancer.[12] Because massive ascites formation is a major cause of morbidity and mortality in patients with ovarian cancer,[12] and LPA is known to be secreted by ovarian cancer cells, our finding that LPA induces lymphangiogenesis *in vitro* sheds light on the mechanisms associated with ascites formation in ovarian cancer patients and suggests that LPA may be a therapeutic target in cancer patients. *In vitro* studies have shown that LPA induces proliferation and migration in both human blood vascular endothelial cells and endometrial carcinoma cells at LPA concentrations of 1 to 10 μmol/L.[17,39] In the present study, LPA induced lymphangiogenesis in LECs *in vitro* at similar LPA concentrations (2.5 to 10 μmol/L; maximal effect at 5 μmol/L). Recent data support the notion that extracellular ATX, responsible for production of LPA, can associate directly with the cell surface and thus could potentially generate the high local concentrations required for cellular effects without necessarily increasing the circulating LPA concentration.[40] Overall, a better understanding of molecular pathways that regulate lymphangiogenesis may reveal ways to interfere with metastasis.

Blood and lymphatic vessels represent two important constituents of the tumor microenvironment and the metastatic cascade.[41] Although LPA has previously been reported to induce angiogenesis[20] and contribute to metastasis,[42] to our knowledge the present study is the first to directly explore the potential role of LPA in lymphangiogenesis *in vitro*. In the present study, LPA functionally contributed to lymphangiogenesis *in vitro*, as evidenced by dose- and time-dependent increases in LEC proliferation, migration, and tube formation (Figures 1 and 2).

Serum IL-8 levels are increased in many cancer patients, and such increases have been shown to correlate with angiogenesis, tumorigenicity, and metastatic potential in several murine models of human cancer.[43–45] Although LPA is reported to induce IL-8 expression in several cell types, including blood vascular endothelial cells,[21] the effects of LPA and IL-8 on LECs have not previously been explored. We show here that LPA induces IL-8 expression in LECs. Indeed, we found that LPA up-regulated IL-8 expression in LECs at both transcriptional and protein levels (Figure 3, A–C) and further confirmed this finding by demonstrating that LPA increased IL-8 expression in the luminal LECs of human sentinel afferent lymphatic vessels *ex vivo* (Figure 7C). The present study shows that IL-8 siRNA partially abrogates LPA-induced lymphangiogenesis *in vitro* (Figure 4), which implies that LPA-up-regulated IL-8 contributes, at

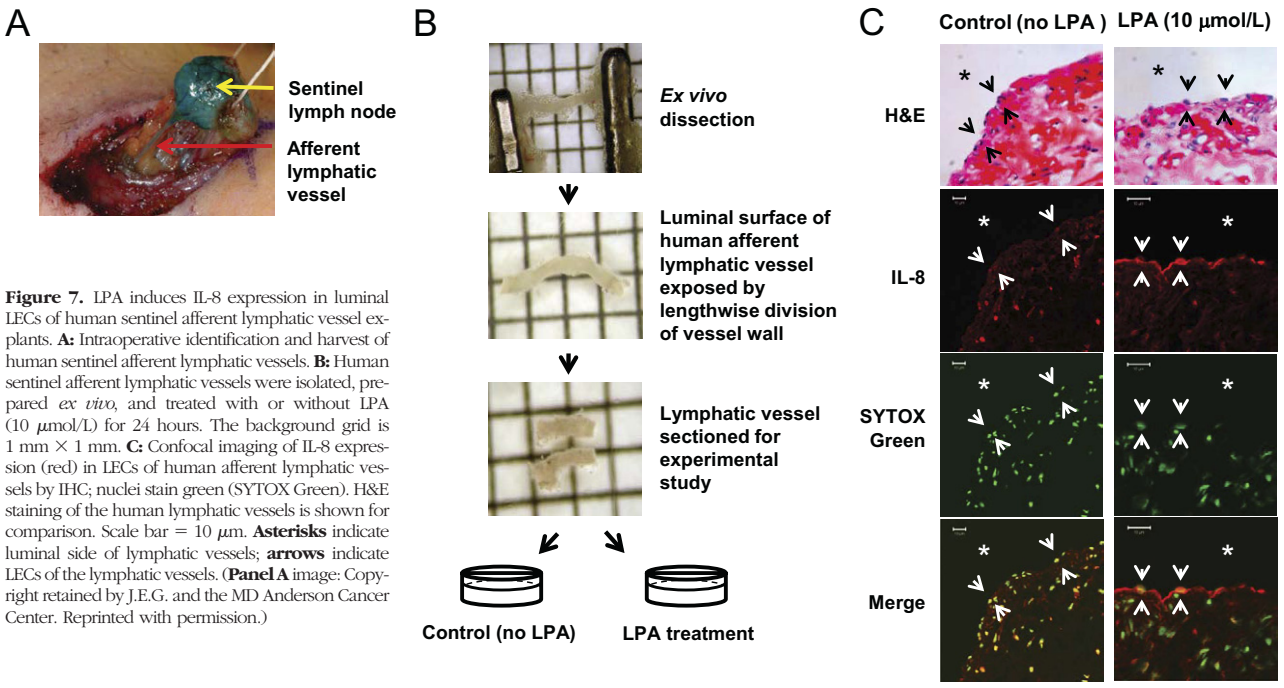

Figure 7. LPA induces IL-8 expression in luminal LECs of human sentinel afferent lymphatic vessel explants. **A:** Intraoperative identification and harvest of human sentinel afferent lymphatic vessels. **B:** Human sentinel afferent lymphatic vessels were isolated, prepared *ex vivo*, and treated with or without LPA (10 μmol/L) for 24 hours. The background grid is 1 mm × 1 mm. **C:** Confocal imaging of IL-8 expression (red) in LECs of human afferent lymphatic vessels by IHC; nuclei stain green (SYTOX Green). H&E staining of the human lymphatic vessels is shown for comparison. Scale bar = 10 μm. **Asterisks** indicate luminal side of lymphatic vessels; **arrows** indicate LECs of the lymphatic vessels. (**Panel A** image: Copyright retained by J.E.G. and the MD Anderson Cancer Center. Reprinted with permission.)

least in part, to LPA-induced lymphangiogenesis *in vitro*. Both CXCR1 and CXCR2 were expressed in LECs *in vitro* (see Supplemental Figure S2F at *http://ajp.amjpathol.org*), and is consistent with previous reports.[46] Overall, the presence of IL-8 receptors on human lymphatic vessel LECs (see Supplemental Figure S6 at *http://ajp.amjpathol.org*) supports a possible direct effect of IL-8 on lymphangiogenesis *in vitro*. Additional studies are needed to further elucidate how IL-8 induction contributes to the effect of LPA on lymphangiogenesis *in vitro*.

Several signaling pathways have been reported to mediate LPA-induced IL-8 production. The NF-κB pathway has been implicated in various cell types, including bronchial epithelial cells[47] and granulosa-lutein cells.[20] In contrast, rho kinase signaling has been shown to mediate LPA-induced IL-8 production in HUVECs through p38 and JNK activation, independent of NF-κB.[22] In the present study, RPPA analysis demonstrated marked induction of activation-specific markers in these and other pathways (Figure 5A). We chose to focus on activation of NF-κB, because its phosphorylation was increased more than that of any other protein in the present study. The induction of NF-κB activation was confirmed by both Western blotting and IHC. ChIP assay further revealed that LPA-activated p-NF-κB p65 bound to an NF-κB binding region in the IL-8 promoter in LPA-treated LECs, an observation supported by the time sequence of activation of p-NF-κB p65 (peak at 60 minutes) and increase in IL-8 transcription (peak at 4 hours) after LPA treatment in LECs. Furthermore, inhibition of NF-κB binding by a mutation of the NF-κB binding region in the IL-8 promoter abolished LPA-induced IL-8 promoter activity, further supporting the contention that LPA regulates IL-8 transcription via the NF-κB pathway. These data strongly support a critical functional role for NF-κB in the regulation of IL-8 production. It is clear from the RPPA experiment that LPA induced the activation of multiple kinase signaling pathways (ie, PI3K-AKT, MAPK). Importantly, the lack of induction in LECs of other known prolymphangiogenic factors (VEGF-A, VEGF-C, VEGF-D, and FGF-2) by LPA in our studies further supports an important role for LPA in the LPA/IL-8/NF-κB axis and a minimal role for these other growth factors. Planned studies in our research group to interrogate the functional role of these signaling pathways, and their possible interactions with NF-κB signaling, should further refine understanding of the signaling pathways involved in the effect of LPA on LECs and on the regulation of IL-8.

Most biological functions of LPA have thus far been shown to be mediated through interaction with the LPA receptors LPA_1, LPA_2, and LPA_3.[2] LPA_2 has been reported to play important roles in tumorigenesis and metastasis in both clinical and experimental studies; in at least one study in patients with gastric cancer, LPA_2 expression correlated with invasion and metastasis.[8] Moreover, in a murine model of mammary carcinoma, overexpression of LPA_2 in mice was associated with a higher incidence and earlier development of mammary carcinoma than that of either LPA_1 or LPA_3.[48] In contrast, the absence of LPA_2 attenuated tumor formation in an experimental murine model of colitis-associated colon adenocarcinoma.[49] LPA_2 has also been shown to mediate LPA-induced activation of growth-regulated oncogene α (GRO-α) in ovarian cancer cells and contribute to both angiogenesis and tumorigenesis.[50] In the present study, we found that LPA_1, LPA_2, and LPA_3 were all expressed in LECs; however, only LPA_2 siRNA blocked LPA-induced IL-8 production (Figure 6A) and LPA activation of p-NF-κB p65 (Figure 6B). LPA_2 siRNA also blocked LPA-induced LEC proliferation and migration (Figure 6, C and D), cell survival (Figure 4B), and tube formation (Figure 6E). These data support the contention

that LPA induces lymphangiogenesis *in vitro* and IL-8 production via interaction with LPA_2 in LECs and are consistent with the observation by others that LPA_2 is involved in tumor progression and metastasis.[8,49] Lymphangiogenesis is also essential for lymphatic vessel system development. However, studies on LPA_{1-3} knockout mice did not reveal cardiovascular developmental defects.[51–54] Further evaluation of the LPA_{1-3}-null mice for lymphatic vascular defects will help to elucidate the role of LPA_{1-3} in lymphangiogenesis.[54]

Our present study demonstrates that LPA induces lymphangiogenesis in LECs *in vitro*. We recognize that, although not all steps of lymphatic vessel formation *in vivo* are recapitulated using LECs in culture, numerous relevant processes can be explored, including cell proliferation, apoptosis, migration (determined by wound scratch assay or Boyden chamber assay), and morphogenesis (ie, tubulogenesis); these lymphatic endothelial cellular functions collectively contribute to lymphatic vessel formation,[55,56] and have been widely used to analyze lymphangiogenesis *in vitro*.[55–62] We extended these observations using human afferent lymphatic vessel explants (a truly unique resource) and also demonstrated LPA-induced IL-8 production in the LECs of these human lymphatic vessels (Figure 7, A–C). Additional studies on LPA and its receptors in lymphatic endothelium *in vivo* will likely further refine our understanding of lymphangiogenesis *in vivo*.

In the present study, we demonstrated for what we believe is the first time that LPA increases human LEC proliferation, survival, migration, and tube formation, promotes lymphangiogenesis *in vitro* via interaction with LPA_2, and up-regulates IL-8 production in LECs via an NF-κB-mediated pathway. We also corroborated our findings by demonstrating that LPA up-regulates IL-8 production in the luminal LECs of human sentinel afferent lymphatic vessels *ex vivo*. Overall, these data shed considerable light on the links among LPA, IL-8, and lymphangiogenesis, advance our understanding of the role of LPA in tumor progression and metastasis, and strongly support accumulating evidence that LPA may serve as a therapeutic target in cancer patients.

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