Lipoprotein-Derived Lysophosphatidic Acid Promotes Atherosclerosis by Releasing CXCL1 from the Endothelium

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SUMMARY

Oxidatively modified low-density lipoprotein (oxLDL) plays a key role in the initiation of atherosclerosis by increasing monocyte adhesion. The mechanism that is responsible for the oxLDL-induced atherogenic monocyte recruitment in vivo, however, still remains unknown. Oxidation of LDL generates lysophosphatidylcholine, which is the main substrate for the lysophosphatidic acid (LPA) generating enzyme autotaxin. We show that oxLDL requires endothelial LPA receptors and autotaxin to elicit CXCL1-dependent arterial monocyte adhesion. Unsaturated LPA releases endothelial CXCL1, which is subsequently immobilized on the cell surface and mediates LPA-induced monocyte adhesion. Local and systemic application of LPA accelerates the progression of atherosclerosis in mice. Blocking the LPA receptors LPA_1 and LPA_3 reduced hyperlipidemia-induced arterial leukocyte arrest and atherosclerosis in the presence of functional CXCL1. Thus, atherogenic monocyte recruitment mediated by hyperlipidemia and modified LDL crucially depends on LPA, which triggers endothelial deposition of CXCL1, revealing LPA signaling as a target for cardiovascular disease treatments.

INTRODUCTION

The formation of atherosclerotic lesions is governed by interactions between circulating leukocytes and activated endothelial cells (Weber et al., 2008). Increased rolling and adhesion of monocytes precedes extravasation into the subendothelial space and constitutes the first morphological sign of inflammation during early atherosclerosis (Mestas and Ley, 2008). Modified lipoprotein particles, such as oxidized low-density lipoproteins (LDLs), which are generated in the vessel wall, upregulate the expression of endothelial cytokines and adhesion molecules that are essential for the monocyte recruitment (Glass and Witztum, 2001). Previous studies in murine models have shown that the CXCR2 ligand *keratinocyte-derived chemokine*/CXCL1, which is the murine ortholog of GRO-α, plays an important role together with other chemokines in atherogenic monocyte recruitment (Weber et al., 2008). CXCL1 in the vessel wall promotes macrophage accumulation and induces monocyte arrest during early atherosclerosis, thus enhancing atherosclerosis (Boisvert et al., 2006; Huo et al., 2001). Minimally modified LDL stimulates CXCL1 deposition on the endothelial cell surface, which is required by chemokines to effectively induce the arrest of leukocytes (Schwartz et al., 1994). However, it is currently unknown how lipoproteins stimulate atherogenic monocyte recruitment in vivo.

Lysophosphatidic acid (LPA) is a phospholipid that is derived from the enzymatic cleavage of lysophospholipids or phosphatidic acid by phospholipases, such as autotaxin. Several LPA subspecies can be found that vary based on the type of fatty acyl chain and the linkage to the glycerol backbone, characteristics that affect biological activity (Aoki et al., 2008). Platelet activation and mild oxidation of LDL leads to LPA biosynthesis (Aoki et al., 2008; Siess et al., 1999), and elevated LPA levels are present in the atherosclerotic lesion core (Rother et al., 2003). LPA effects are primarily mediated by seven G protein-coupled receptors, such as LPA_1, LPA_2, and LPA_3 (Choi et al., 2010). LPA participates in vitro in various biological processes that are associated with atherogenesis (Smyth et al., 2008), e.g., LPA stimulates endothelial-monocyte adhesion in vitro by inducing the expression of adhesion molecules and chemokines (Lin et al., 2007; Rizza et al., 1999). Furthermore, LPA plays an important role in modified LDL-mediated activation of platelets,

monocytes, and endothelial cells (Fueller et al., 2003; Gustin et al., 2008; Siess et al., 1999). Hypercholesterolemia typically is associated with increased serum levels of predominantly unsaturated LPAs (Tokumura et al., 2002). While all these findings indicate that LPA and its receptors play a key role in atherosclerosis, no studies have provided direct evidence for this association.

We studied the role of LPA in lipoprotein-induced monocyte recruitment and atherosclerosis. We found that both modified LDL and unsaturated LPA induced CXCL1-dependent arterial monocyte adhesion, and this effect was dependent upon endothelial LPA receptors and autotaxin. Furthermore, hyperlipidemia-induced atherosclerosis was reduced in $Apoe^{-/-}$ mice by inhibition of LPA receptors, and unsaturated LPA accelerated lesion formation in these mice.

RESULTS

LPA Mediates Modified LDL-Induced Monocyte Recruitment

Ex vivo perfusion of mouse carotid arteries with mildly oxidized (mox)LDL significantly increased rolling and adhesion of human monocytic MonoMac6 cells under flow (Figures 1A and 1B); in contrast, perfusion with native LDL did not have an effect on monocyte rolling and adhesion. Pretreatment of the monocytic cells with an antibody that blocks CXCR2 or perfusion of the artery with a CXCL1 antibody decreased both monocyte adhesion and rolling (Figures 1A and 1B). Blockade of the LPA_1 and LPA_3 receptors with Ki16425 during moxLDL perfusion completely inhibited monocyte rolling and adhesion (Figures 1A and 1B), indicating that these receptors are required for moxLDL-mediated adhesion. Endothelial LPA_1 and LPA_3 protein expression in murine carotid arteries was confirmed by immunostaining (Figures S1A and S1B). Whereas the content of total LPA in moxLDL was not elevated (data not shown), the LPA precursor lysophosphatidylcholine (LPC) was significantly increased (by 21%) as compared to native LDL. Cultured endothelial cells release autotaxin (Figure S1C), which is known to convert LPC into LPA, and blocking autotaxin with S32826 reduced moxLDL-induced monocyte adhesion and rolling (Figures 1A and 1B). Next, the effect of various LPA species on monocyte adhesion in carotid arteries was studied. In contrast to LPA18:0, 10 μM of LPA20:4 significantly increased monocyte rolling and adhesion (Figures 1C and 1D). In the LPA20:4-treated arteries, adherent monocytes directly start to migrate across the endothelium (Figures S1D and S1E). Perfusion with 1-AGP increased monocyte adhesion, but not rolling (Figures 1C and 1D). Inhibition of monocytic CXCR2 or endothelial CXCL1 prevented LPA20:4-induced monocyte rolling and adhesion (Figures 1E and 1F). Treatment with an antibody that inhibits P-selectin reduced monocyte rolling in LPA20:4-treated arteries (data not shown). Treatment of monocytes with LPA20:4 did not increase monocyte adhesion (data not shown). Collectively, these data indicate that autotaxin-dependent generation of unsaturated LPA mediates moxLDL-induced monocyte adhesion through the CXCL1/CXCR2 axis and endothelial LPA receptors.

LPA Receptors Release Endothelial CXCL1

CXCL1 protein expression in endothelial cells of mouse carotid arteries was detected by immunostaining (Figure 1G).

To evaluate whether LPA stimulates endothelial deposition of CXCL1 in vivo, two-photon microscopy was performed on perfused carotid arteries that were stained with a fluorescently labeled CXCL1 antibody (Megens et al., 2007; van Zandvoort et al., 2004). As compared to the control buffer, LPA20:4 treatment visibly increased CXCL1 protein on the endothelial surface (Figure 1H and Movies S1 and S2). In vitro, short-term treatment with LPA20:4 increased CXCL1 secretion and surface deposition on endothelial cells, which was prevented by Ki16425 and by BAY11-7085, an inhibitor of IκB phosphorylation (Figures S1F and S1G). The inhibitor of Rho kinase activation, H-1152, also reduced CXCL1 surface deposition (Figure S1F). In contrast to LPA18:0, LPA20:4 induced CXCL1 mRNA expression via LPA receptors in a NF-κB-dependent manner (Figure S1H). MoxLDL-induced surface immobilization and secretion of CXCL1 was inhibited by Ki16425 (Figures S1I and S1J). These results imply that unsaturated LPA is important in moxLDL-induced endothelial CXCL1 deposition.

LPA_1 and LPA_3 Expression in Atherosclerotic Plaques

In normal mouse arteries, LPA_1 expression is almost twice as high as LPA_2 and LPA_3 expression (Subramanian et al., 2010). Quantitative RT-PCR showed that mice that were fed a HFD for 2 months had a decreased LPA_2 mRNA expression level (approximately 38%) compared to mice that were fed a normal diet (Figure 2A). In contrast, the LPA_3 expression level was slightly increased in the HFD mice, whereas the LPA_1 expression level remained constant (Figure 2A). In $Apoe^{-/-}$ mice, LPA_1 and LPA_3 were present on endothelial cells (Figure 2B), as well as on macrophages within the plaque (Figure S2A). In vitro, macrophages expressed predominantly LPA_2 and LPA_3 (Figure S2B). The endothelia within human plaques also expressed LPA_1 and LPA_3 (Figure S2C). In conclusion, LPA_2 expression is downregulated during early atherosclerosis, and LPA_1 and LPA_3 are expressed in lesional endothelial cells.

Role of LPA in Arterial Leukocyte Recruitment In Vivo

To investigate whether LPA can initiate arterial leukocyte adhesion in vivo, leukocyte adhesion was studied with intravital microscopy (IVM) of the carotid bifurcation after treatment with LPA20:4. As compared to the control, a single injection of LPA20:4 markedly increased the arterial adhesion of leukocytes after 4 days (Figure 2C), although the plasma levels of total LPA after 1 day and 4 days were not elevated (data not shown). Simultaneous administration of an inhibitory CXCL1 antibody completely prevented LPA-induced leukocyte adhesion (Figure 2C). In $CCR2^{-/-}$ mice, leukocyte adhesion was not reduced after treatment with LPA20:4 (Figure S2D). Perivascular treatment of the carotid artery with LPA20:4 also induced CXCL1-dependent leukocyte adhesion (Figure 2D). Ki16425 treatment decreased the hyperlipidemia-induced arterial leukocyte adhesion in $Apoe^{-/-}$ mice by approximately 70% compared to the control (Figure 2E). In $Apoe^{-/-}$ mice that were treated with a CXCL1 antibody, additional Ki16425 treatment did not reduce leukocyte adhesion (Figure S2E). Because Ki16425 inhibits both LPA_1 and LPA_3 (Ohta et al., 2003), LPA receptor-specific siRNA was locally applied to the carotid artery. In contrast to LPA_2-siRNA, treatment with LPA_1- or LPA_3-siRNA
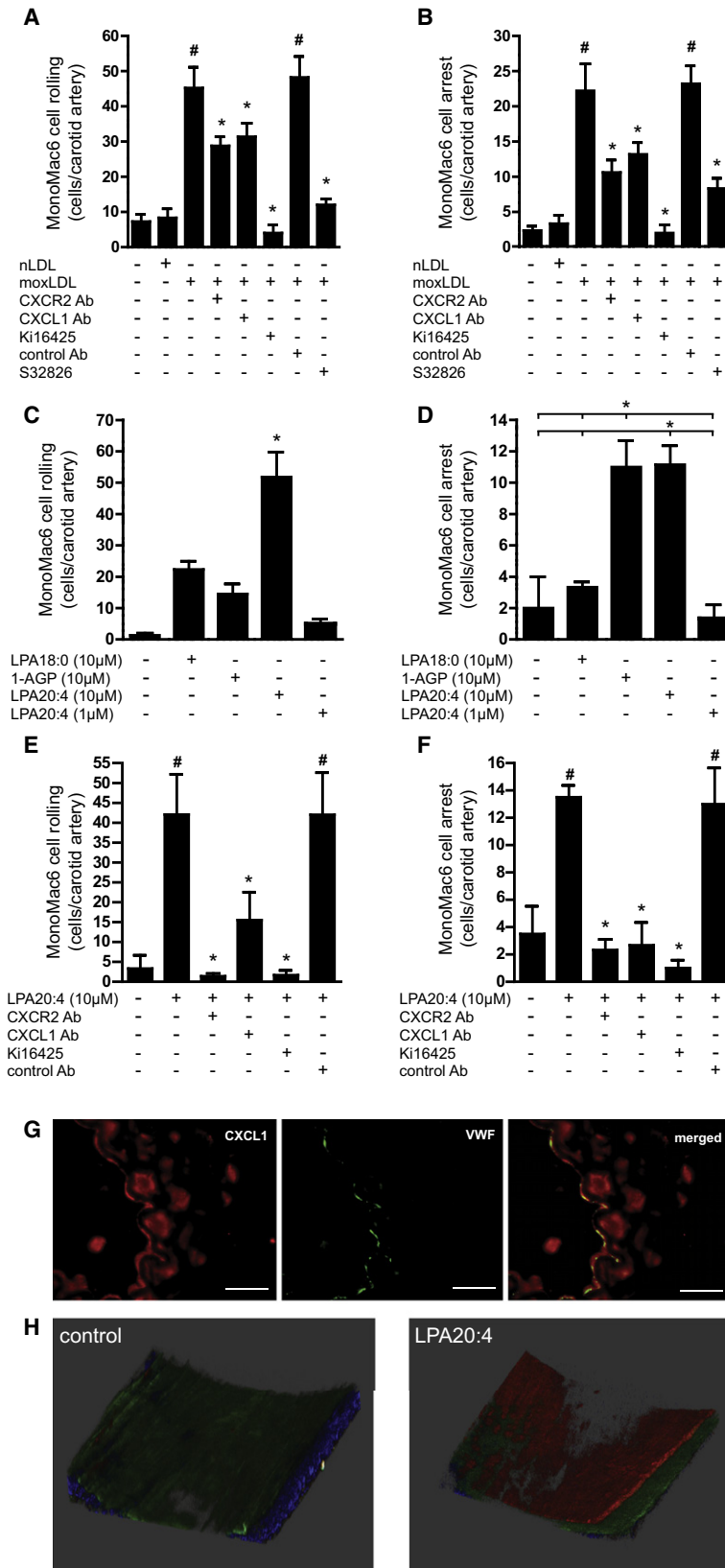
Figure 1. Unsaturated LPA Affects Arterial Monocyte Recruitment

(A) Monocytic MonoMac6 cell rolling over perfused mouse carotid arteries ex vivo after perfusion with native (nLDL) or mildly oxidized (moxLDL) LDL for 1 hr. *p < 0.05 versus moxLDL with or without control Ab; #p < 0.05 versus vehicle and nLDL; n = 3–4 mice. Error bars are SEM.

(B) MonoMac6 cell adhesion to perfused mouse carotid arteries ex vivo after perfusion with native or moxLDL for 1 hr. *p < 0.05 versus moxLDL with or without control Ab; #p < 0.05 versus vehicle and nLDL; n = 3–4 mice. Error bars are SEM.

(C) MonoMac6 cell rolling over perfused mouse carotid arteries ex vivo after perfusion with the control buffer, LPA18:0, LPA20:4, or 1-AGP18:1 for 1 hr. *p < 0.05 versus all other groups; n = 4 mice. Error bars are SEM.

(D) MonoMac6 cell adhesion to perfused mouse carotid arteries ex vivo after perfusion with the control buffer, LPA18:0, LPA20:4, or 1-AGP18:1 for 1 hr. *p < 0.05; n = 4 mice. Error bars are SEM.

(E) MonoMac6 cell rolling over perfused mouse carotid arteries ex vivo after perfusion with LPA20:4 for 1 hr. *p < 0.05 compared to LPA20:4 with or without control Ab; #p < 0.05 versus vehicle; n = 5 mice. Error bars are SEM.

(F) MonoMac6 cell adhesion to perfused mouse carotid arteries ex vivo after perfusion with LPA20:4 for 1 hr. *p < 0.05 versus LPA20:4 with or without control Ab; #p < 0.05 versus vehicle; n = 5 mice. Error bars are SEM.

(G) Immunostaining for CXCL1 and von Willebrand factor (vWF) in normal mouse carotid arteries. Scale bar, 20 μm.

(H) Two-photon microscopy of a perfused mouse carotid artery ex vivo after treatment with control buffer or LPA20:4. CXCL1 was detected with a fluorescently labeled antibody (red) in 3D reconstructions of the Z stack images. Green, elastic laminae; blue, collagen.
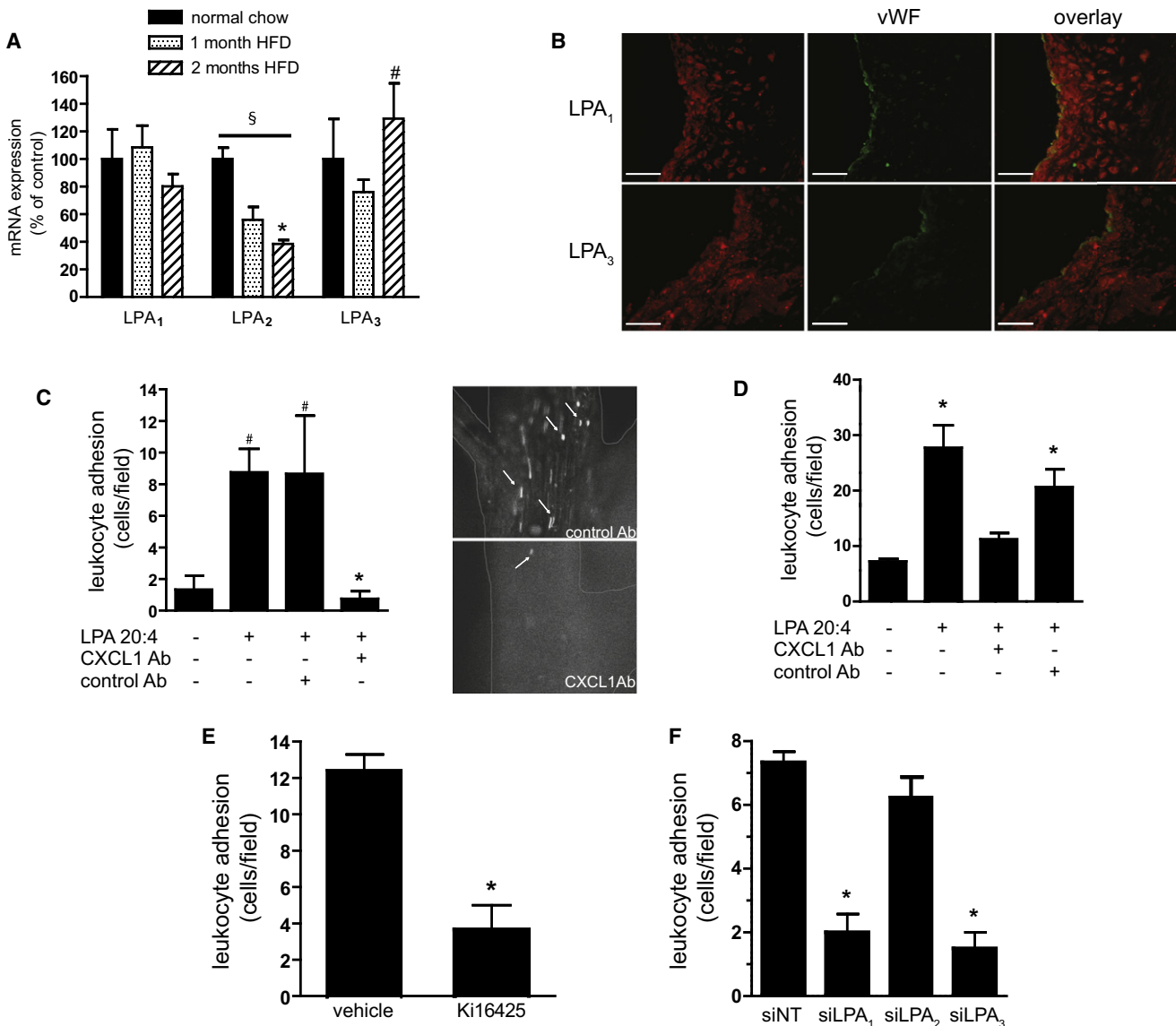
Figure 2. LPA and CXCL1 in Leukocyte Adhesion In Vivo

(A) LPA_1, LPA_2, and LPA_3 mRNA expression in the aortas of $Apoe^{-/-}$ mice that were fed either a normal diet, a high-fat diet (HFD) for 1 month, or a HFD for 2 months. *$p < 0.05$ versus normal diet; #$p < 0.05$ versus 1 month HFD; §$p < 0.001$ for linear trend; n = 3–4 mice per group. Error bars are SEM.

(B) Double immunostaining for either LPA_1 or LPA_3 and von Willebrand factor (vWF) in aortic root plaques of $Apoe^{-/-}$ mice. Scale bar, 50 μm.

(C) Intravital microscopy (IVM) of leukocyte adhesion to murine carotid arteries that were treated with vehicle or LPA20:4 (2 nmol) by intraperitoneal injection. #$p < 0.05$ versus untreated; *$p < 0.05$ versus control antibody; n = 3–4 mice per group. Error bars are SEM.

(D) IVM of leukocyte adhesion to murine carotid arteries that were treated with vehicle or LPA20:4 (2 nmol) by perivascular application in pluronic gel. *$p < 0.05$ versus vehicle and LPA20:4 with CXCL1 antibody; n = 3–4 mice per group. Error bars are SEM.

(E) Leukocyte adhesion to the carotid artery of $Apoe^{-/-}$ mice following treatment with Ki16425 or vehicle detected by IVM. *$p < 0.05$; n = 3–4 mice. Error bars are SEM.

(F) IVM of leukocyte adhesion in nontargeting (siNT), LPA_1-, LPA_2-, or LPA_3-specific siRNA-treated carotid arteries of $Apoe^{-/-}$ mice. *$p < 0.05$ versus siNT and LPA_2-siRNA; n = 3–4 mice. Error bars are SEM.

inhibited hyperlipidemia-induced leukocyte adhesion (Figure 2F). Leukocyte adhesion fully recovered 14 days after LPA_1- and LPA_3-siRNA treatment (Figure S2F). These data indicate that LPA elicits sustained CXCL1-dependent arterial leukocyte adhesion in vivo, which may account for the hyperlipidemia-induced leukocyte recruitment via LPA_1 and LPA_3.

Unsaturated LPA Accelerates Atherosclerosis Progression

$Apoe^{-/-}$ mice that were fed a high-fat diet (HFD) for 4 weeks were treated with either LPA20:4, LPA18:0, or vehicle by systemic injection to study the effect on the progression of atherosclerosis. Compared to the control, LPA20:4 treatment
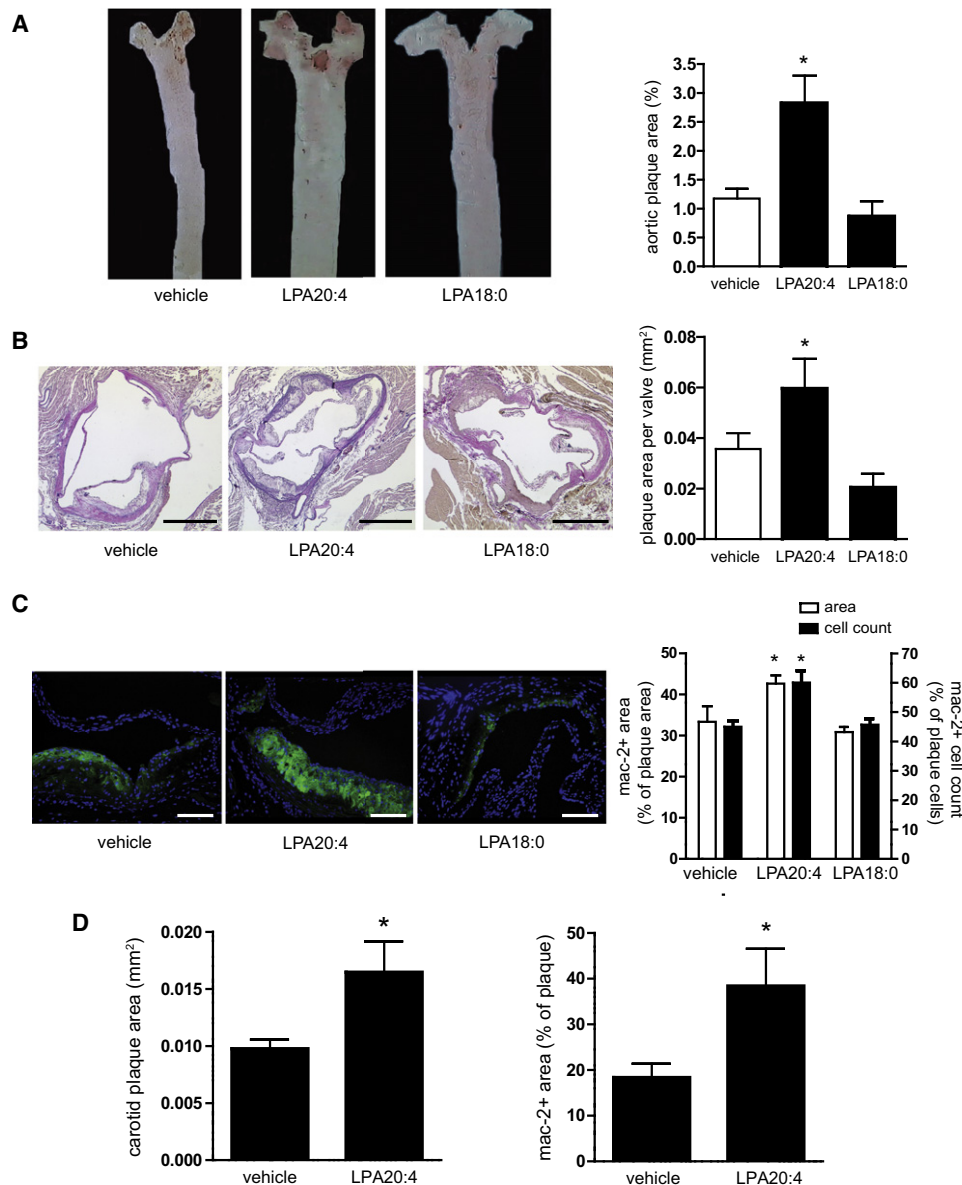
Figure 3. The Role of LPA in Atherosclerosis Progression

(A) Lipid quantification of oil red O-stained thoracic aortas of $Apoe^{-/-}$ mice that were treated with vehicle, LPA20:4 (2 nmol/mouse, twice weekly), or LPA18:0 (2 nmol/mouse, twice weekly). *p < 0.05 versus all other groups; n = 5–6 mice per group. Error bars are SEM.

(B) The lesion area in the aortic root of $Apoe^{-/-}$ mice. Scale bars, 500 μm. *p < 0.05 versus all other groups; n = 4–6 mice per group. Error bars are SEM.

(C) Macrophage accumulation in lesions as determined by the Mac-2-immunostained area and the Mac-2+ cell count. Scale bars, 100 μm. n = 5–7 mice per group. *p < 0.05 versus all other groups. Error bars are SEM.

(D) The lesion area (left) and Mac-2 immunostained area (right) in partially ligated carotid arteries of $Apoe^{-/-}$ mice treated perivascularly with LPA20:4 (2 nmol/mouse) or vehicle. *p < 0.05; n = 3–4 mice. Error bars are SEM.

increased aortic lipid deposition (Figure 3A), the plaque area in the aortic root (Figure 3B), and the accumulation of macrophages (Figure 3C). In contrast, LPA18:0 treatment did not affect plaque progression or plaque macrophage content (Figures 3A–3C). There was no statistically significant difference in the lipid levels for the three groups (Table S1). Perivascular treatment of the carotid artery of $Apoe^{-/-}$ mice with LPA20:4 after partial ligation increased the lesion size and the macrophage accumulation (Figure 3D), indicating that LPA can affect lesion growth also

from within the vessel wall. Thus, unsaturated LPA promotes plaque progression and macrophage accumulation.

Inhibition of LPA₁ and LPA₃ Suppresses Diet-Induced Atherosclerosis

To study the role of endogenous LPA in atherogenesis, $Apoe^{-/-}$ mice fed a HFD were injected with Ki16425 daily for 3 months. Treatment with Ki16425 did not affect the serum lipid levels or the renal and liver function (Table S2). Compared to the control
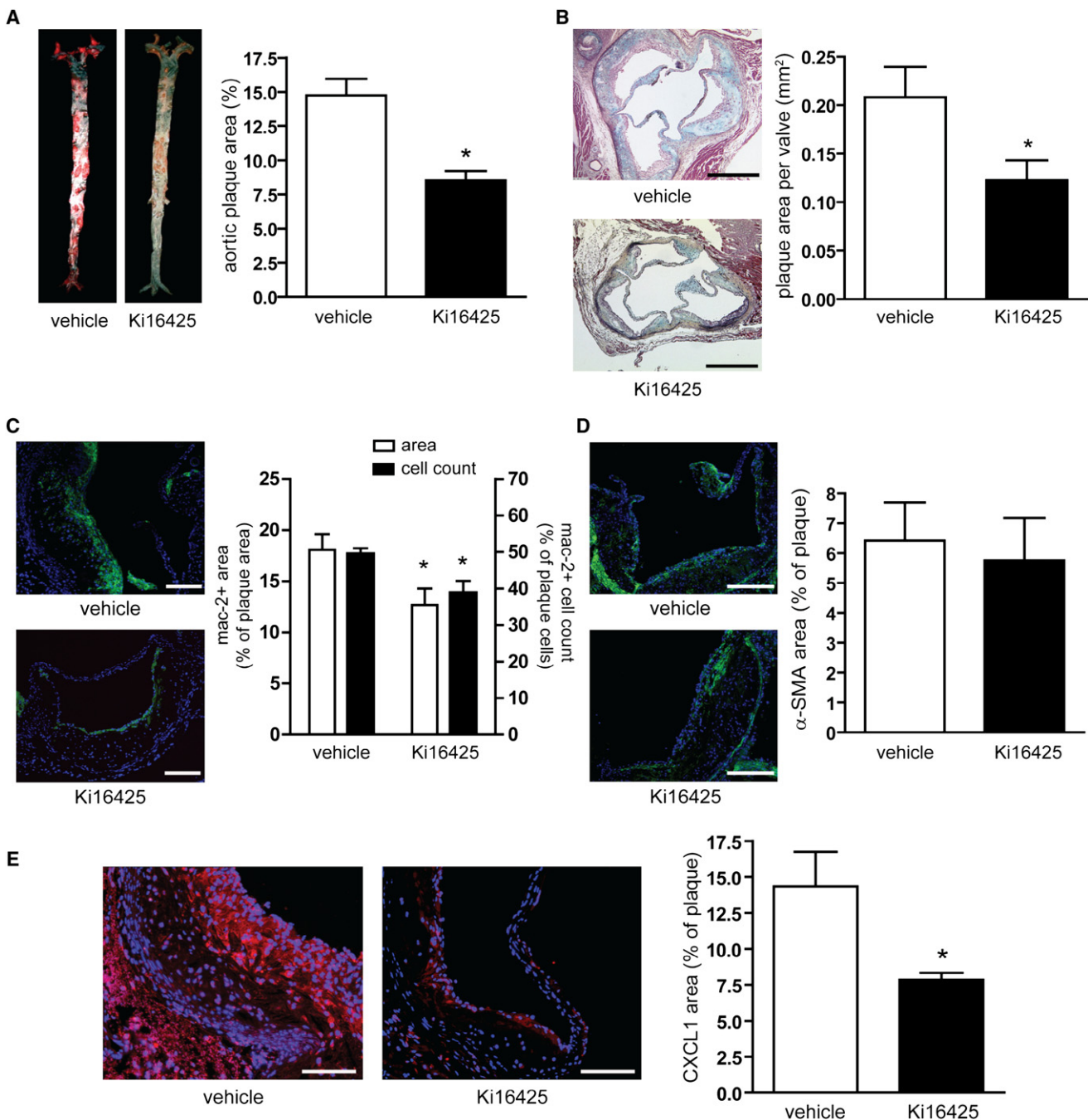
Figure 4. The Effect of the LPA Receptors on Diet-Induced Plaque Formation

(A) Oil red O staining of en face-prepared aortas of $Apoe^{-/-}$ mice that were treated with vehicle or Ki16425 for 3 months. *p < 0.001; n = 9 mice. Error bars are SEM.

(B) The lesion area of aortic root sections of $Apoe^{-/-}$ mice. Scale bars, 500 μm; *p < 0.05; n = 5–8 mice. Error bars are SEM.

(C) Lesional macrophage accumulation in the aortic root of $Apoe^{-/-}$ mice determined by the Mac-2-immunostained area and the Mac-2+ cell count. Scale bars, 200 μm; *p < 0.05; n = 4–9 mice. Error bars are SEM.

(D) α-SMA immunostaining for lesional smooth muscle cell accumulation in the aortic root of $Apoe^{-/-}$ mice. Scale bars, 200 μm; n = 7–9 mice. Error bars are SEM.

(E) Immunostaining for lesional CXCL1 expression in the aortic root of $Apoe^{-/-}$ mice. Scale bars, 100 μm; *p < 0.05; n = 7–9 mice. Error bars are SEM.

mice, Ki16425 decreased the lipid deposition by 38% (Figure 4A) and the plaque area in the aortic root by 41% (Figure 4B) and reduced the macrophage accumulation (Figure 4C). Whereas the macrophage accumulation was reduced in the Ki16425-treated mice, no difference of the lesional SMC content was detectable (Figure 4D). The CXCL1-immunostained area in the plaque of Ki16425-treated mice was decreased (Figure 4E). The expression of CCL2, CCL5, and CX₃CL1 in the lesions was

not reduced by Ki16425 (Figure S3A). In *Apoe*$^{-/-}$ mice that were treated with a blocking CXCL1 antibody, plaque size and the lesional macrophage content were not decreased by Ki16425 (Figure S3B). Hence, blocking the LPA$_1$ and LPA$_3$ receptors reduces lesional macrophage accumulation mainly through CXCL1, which ultimately impairs atherogenesis.

DISCUSSION

The oxidative modification hypothesis states that modified LDL affects many aspects of atherogenesis (Stocker and Keaney, 2004). Though several experimental studies have supported this hypothesis, the specific LDL components that affect atherosclerosis in vivo are unknown (Steinberg, 2009). We have shown that LPA accelerates the progression of atherosclerosis and recruits leukocytes to the vessel wall during early atherogenesis via LPA$_1$ and LPA$_3$ receptor-mediated release of endothelial CXCL1. Inhibition of the LPA receptors impaired hyperlipidemia-induced arterial leukocyte adhesion and reduced diet-induced atherogenesis, indicating that lipoprotein-derived LPA plays a crucial role in atherosclerosis.

The oxidation of LDL is associated with cleavage of oxidized phospholipids via lipoprotein-associated phospholipase A$_2$, which generates LPC (Steinbrecher et al., 1984). LPC is a physiological substrate of autotaxin, which is a major LPA-producing enzyme (Nakanaga et al., 2010). We demonstrated that moxLDL-induced monocyte adhesion to the arterial wall via CXCL1 is dependent upon the endothelial LPA receptors LPA$_1$ and LPA$_3$ and autotaxin activity. Furthermore, LPA20:4 closely mimics the effect of moxLDL on CXCL1 secretion and monocyte recruitment. Thus, our data are consistent with the concept that oxidation of LDL gives rise to LPC, which is a substrate for LPA generation by autotaxin-related mechanisms. Interestingly, autotaxin that is secreted from endothelial cells recruits lymphocytes to the lymph nodes via LPA production (Kanda et al., 2008; Nakasaki et al., 2008) and is upregulated after vascular injury (Panchatcharam et al., 2008). Although our results do not suggest that the CCL2/CCR2 axis mediates LPA-driven leukocyte adhesion, LPA upregulates the endothelial expression of numerous chemokines and adhesion molecules, which may participate in the CXCL1-dependent recruitment of monocytes in vivo (Lin et al., 2007; Rizza et al., 1999). In addition to the modification of LDL, platelet activation also leads to the generation of LPA, which could affect atherogenesis (Aoki et al., 2008; Davì and Patrono, 2007).

Saturated and unsaturated LPA species as well as the alkyl-LPA analog 1-AGP had differential effects on monocyte adhesion and rolling. Monocyte rolling ex vivo in perfused mouse carotid arteries largely depends on interactions with the endothelial adhesion molecule P-selectin (Ramos et al., 1999), and P-selectin-dependent leukocyte rolling is enhanced by CXCL1 (Zhang et al., 2001). P-selectin is stored within Weibel-Palade bodies (WPB) and is transferred to the cell surface upon stimulation, whereas CXCL1 is stored in non-WPB secretory vesicles in endothelial cells (Øynebråten et al., 2004). Because P-selectin is involved in LPA20:4-induced monocyte rolling, we hypothesize that both WPB and non-WPB endothelial vesicles are released by LPA20:4. In contrast, 1-AGP18:1 may only induce CXCL1 secretion, thus increasing monocyte adhesion, but not rolling.

Treatment with LPA20:4 enhanced the progression of atherosclerosis, and conversely, inhibition of LPA$_1$ and LPA$_3$ decreased atherosclerosis in hyperlipidemic mice. Our findings indicate that the effect of LPA on atherogenesis is due to increased macrophage accumulation following the LPA-induced arterial adhesion of monocytes. Other LPA-dependent mechanisms, such as the inhibition of macrophage emigration from plaques, however, may additionally play a role (Llodrá et al., 2004). The subendothelial space is generally regarded as the primary site for LDL modification, and thus LDL-derived LPA may accumulate in the arterial tissue. LPA locally administered to the carotid wall increased leukocyte adhesion and atherosclerosis like the systemic application of LPA. Therefore, it is unlikely that an unspecific systemic inflammatory response to the LPA injection is responsible for the atherogenic effect. Furthermore, inhibition of hyperlipidemia-induced leukocyte adhesion by targeting the LPA receptors provides evidence that endogenous LPA exerts functions like the administered LPA. This functional similarity suggests that the dose of LPA used in this study represents the amount of endogenously generated LPA, although the LPA levels have not been directly compared.

Although diverse functions for LPA$_1$ and LPA$_3$ have been described (Choi et al., 2010), several reports indicate that these receptors have similar effects in vascular cells (Lin et al., 2007; Subramanian et al., 2010), which may be due to the formation of heterodimers of the two different LPA receptors (Zaslavsky et al., 2006). Our findings suggest that the inhibition of either LPA$_1$ or LPA$_3$ may be sufficient to prevent atherogenic monocyte recruitment. Additional in vivo studies, however, are required to define the general role of LPA$_1$ or LPA$_3$ in the recruitment of immune cells in order to assess the therapeutic potential of LPA receptor inhibition in atherosclerosis.

In conclusion, we have identified a mechanism for modified lipoproteins in atherosclerotic progression. Modified lipoprotein-derived LPA promotes CXCL1-induced monocyte adhesion by activating LPA$_1$ or LPA$_3$ receptors and by releasing endothelial CXCL1. Thus, the LPA receptors may be targets for cardiovascular disease treatment.

EXPERIMENTAL PROCEDURES

Animals
Apoe$^{-/-}$ mice were fed a HFD (21% fat, 0.15% cholesterol) and treated with the LPA$_{1/3}$ receptor antagonist Ki16425 (5 mg/kg/day) or vehicle by intraperitoneal (i.p.) injection for 12 weeks. In addition, *Apoe*$^{-/-}$ mice fed a HFD were treated with a CXCL1 antibody (50 μg/mouse, twice weekly, i.p.) and either Ki16425 or vehicle for 4 weeks. Furthermore, *Apoe*$^{-/-}$ mice fed a HFD for 4 weeks were injected with LPA20:4, LPA18:0 (2 nmol, twice weekly for 4 weeks) or PBS. Partially ligated carotid arteries of *Apoe*$^{-/-}$ mice on a HFD were treated perivascularly with LPA20:4 (2 nmol, once weekly) or PBS dissolved in pluronic gel and harvested after 4 weeks. The animal experiments were approved by the local authorities (LANUV NRW) in accordance with the German animal protection laws.

Histology
The en face prepared aortas were stained with oil red O. Serial sections of the aortic root or the carotid artery were stained with Movat's pentachrome or Elastica van Gieson stain.

Immunostaining
Mac-2 (clone M3/38), α-smooth muscle actin (α-SMA, clone 1A4), von Willebrand factor (vWF, polyclonal), CXCL1 (polyclonal), LPA$_1$, and LPA$_3$ (both

rabbit IgG) were detected by immunofluorescence staining using directly conjugated secondary antibodies.

Ex Vivo Perfusion of Carotid Arteries
Isolated, perfused carotid arteries from C57BL/6 mice were transferred to an epifluorescence microscope stage and pretreated as indicated. Calcein-AM-labeled MonoMac6 cells that were either untreated or treated with an antibody against CXCR2 or control IgG were subsequently perfused through the arteries. Monocyte adhesion and rolling were recorded during strobo-scopic illumination.

Intravital Microscopy
Leukocyte-endothelial interactions in the carotid arteries were analyzed by IVM (BX51, Olympus) after labeling of the circulating leukocytes with Rhodamine6G. Following injection or perivascular administration of LPA20:4 (2 nmol), IVM was performed in C57BL/6 mice that were treated with a CXCL1 antibody or control IgG. Additionally, $Apoe^{-/-}$ mice fed a HFD for 4 weeks were studied after treatment with Ki16425 or vehicle for 1 week. Alternatively, carotid arteries were treated perivascularly with control siRNA, LPA_1-, LPA_2-, or LPA_3-specific siRNA (Dharmacon) dissolved in pluronic gel 4 or 14 days before analysis.

Two-Photon Laser Scanning Microscopy
Carotid arteries were perfused ex vivo with LPA20:4 (10 μM) or a control buffer for 1 hr. The CXCL1 deposition after staining with a Cy3-labeled CXCL1 antibody or the transendothelial migration of Calcein-labeled MonoMac6 cells was imaged with an Olympus FV1000MPE multiphoton system.

Statistical Analysis
The data represent means ± SEMs and were compared with either a Student's t test or a one- or two-way ANOVA, followed by a Newman-Keuls or linear trend posttest when appropriate (Prism, GraphPad). ANOVA analysis for the randomized block design was performed with the SAS 8.0 software (SAS Institute Inc., Cary, NC). A p value < 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, three figures, two tables, and two movies and can be found with this article online at doi:10.1016/j.cmet.2011.02.016.

REFERENCES

Aoki, J., Inoue, A., and Okudaira, S. (2008). Two pathways for lysophosphatidic acid production. Biochim. Biophys. Acta 1781, 513–518.

Boisvert, W.A., Rose, D.M., Johnson, K.A., Fuentes, M.E., Lira, S.A., Curtiss, L.K., and Terkeltaub, R.A. (2006). Up-regulated expression of the CXCR2 ligand KC/GRO-alpha in atherosclerotic lesions plays a central role in macrophage accumulation and lesion progression. Am. J. Pathol. 168, 1385–1395.

Choi, J.W., Herr, D.R., Noguchi, K., Yung, Y.C., Lee, C.W., Mutoh, T., Lin, M.E., Teo, S.T., Park, K.E., Mosley, A.N., and Chun, J. (2010). LPA receptors: subtypes and biological actions. Annu. Rev. Pharmacol. Toxicol. 50, 157–186.

Davì, G., and Patrono, C. (2007). Platelet activation and atherothrombosis. N. Engl. J. Med. 357, 2482–2494.

Fueller, M., Wang, D.A., Tigyi, G., and Siess, W. (2003). Activation of human monocytic cells by lysophosphatidic acid and sphingosine-1-phosphate. Cell. Signal. 15, 367–375.

Glass, C.K., and Witztum, J.L. (2001). Atherosclerosis. the road ahead. Cell 104, 503–516.

Gustin, C., Delaive, E., Dieu, M., Calay, D., and Raes, M. (2008). Upregulation of pentraxin-3 in human endothelial cells after lysophosphatidic acid exposure. Arterioscler. Thromb. Vasc. Biol. 28, 491–497.

Huo, Y., Weber, C., Forlow, S.B., Sperandio, M., Thatte, J., Mack, M., Jung, S., Littman, D.R., and Ley, K. (2001). The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. J. Clin. Invest. 108, 1307–1314.

Kanda, H., Newton, R., Klein, R., Morita, Y., Gunn, M.D., and Rosen, S.D. (2008). Autotaxin, an ectoenzyme that produces lysophosphatidic acid, promotes the entry of lymphocytes into secondary lymphoid organs. Nat. Immunol. 9, 415–423.

Lin, C.I., Chen, C.N., Lin, P.W., Chang, K.J., Hsieh, F.J., and Lee, H. (2007). Lysophosphatidic acid regulates inflammation-related genes in human endothelial cells through LPA1 and LPA3. Biochem. Biophys. Res. Commun. 363, 1001–1008.

Llodrá, J., Angeli, V., Liu, J., Trogan, E., Fisher, E.A., and Randolph, G.J. (2004). Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques. Proc. Natl. Acad. Sci. USA 101, 11779–11784.

Megens, R.T., Reitsma, S., Schiffers, P.H., Hilgers, R.H., De Mey, J.G., Slaaf, D.W., oude Egbrink, M.G., and van Zandvoort, M.A. (2007). Two-photon microscopy of vital murine elastic and muscular arteries. Combined structural and functional imaging with subcellular resolution. J. Vasc. Res. 44, 87–98.

Mestas, J., and Ley, K. (2008). Monocyte-endothelial cell interactions in the development of atherosclerosis. Trends Cardiovasc. Med. 18, 228–232.

Nakanaga, K., Hama, K., and Aoki, J. (2010). Autotaxin—an LPA producing enzyme with diverse functions. J. Biochem. 148, 13–24.

Nakasaki, T., Tanaka, T., Okudaira, S., Hirosawa, M., Umemoto, E., Otani, K., Jin, S., Bai, Z., Hayasaka, H., Fukui, Y., et al. (2008). Involvement of the lysophosphatidic acid-generating enzyme autotaxin in lymphocyte-endothelial cell interactions. Am. J. Pathol. 173, 1566–1576.

Ohta, H., Sato, K., Murata, N., Damirin, A., Malchinkhuu, E., Kon, J., Kimura, T., Tobo, M., Yamazaki, Y., Watanabe, T., et al. (2003). Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. Mol. Pharmacol. 64, 994–1005.

Øynebråten, I., Bakke, O., Brandtzaeg, P., Johansen, F.E., and Haraldsen, G. (2004). Rapid chemokine secretion from endothelial cells originates from 2 distinct compartments. Blood 104, 314–320.

Panchatcharam, M., Miriyala, S., Yang, F., Rojas, M., End, C., Vallant, C., Dong, A., Lynch, K., Chun, J., Morris, A.J., and Smyth, S.S. (2008). Lysophosphatidic acid receptors 1 and 2 play roles in regulation of vascular injury responses but not blood pressure. Circ. Res. 103, 662–670.

Ramos, C.L., Huo, Y., Jung, U., Ghosh, S., Manka, D.R., Sarembock, I.J., and Ley, K. (1999). Direct demonstration of P-selectin- and VCAM-1-dependent mononuclear cell rolling in early atherosclerotic lesions of apolipoprotein E-deficient mice. Circ. Res. 84, 1237–1244.

Rizza, C., Leitinger, N., Yue, J., Fischer, D.J., Wang, D.A., Shih, P.T., Lee, H., Tigyi, G., and Berliner, J.A. (1999). Lysophosphatidic acid as a regulator of endothelial/leukocyte interaction. Lab. Invest. 79, 1227–1235.

Rother, E., Brandl, R., Baker, D.L., Goyal, P., Gebhard, H., Tigyi, G., and Siess, W. (2003). Subtype-selective antagonists of lysophosphatidic Acid receptors inhibit platelet activation triggered by the lipid core of atherosclerotic plaques. Circulation 108, 741–747.

Schwartz, D., Andalibi, A., Chaverri-Almada, L., Berliner, J.A., Kirchgessner, T., Fang, Z.T., Tekamp-Olson, P., Lusis, A.J., Gallegos, C., Fogelman, A.M., et al. (1994). Role of the GRO family of chemokines in monocyte adhesion to MM-LDL-stimulated endothelium. J. Clin. Invest. 94, 1968–1973.

Siess, W., Zangl, K.J., Essler, M., Bauer, M., Brandl, R., Corrinth, C., Bittman, R., Tigyi, G., and Aepfelbacher, M. (1999). Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc. Natl. Acad. Sci. USA 96, 6931–6936.

Smyth, S.S., Cheng, H.Y., Miriyala, S., Panchatcharam, M., and Morris, A.J. (2008). Roles of lysophosphatidic acid in cardiovascular physiology and disease. Biochim. Biophys. Acta 1781, 563–570.

Steinberg, D. (2009). The LDL modification hypothesis of atherogenesis: an update. J. Lipid Res. Suppl. 50, S376–S381.

Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L., and Steinberg, D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc. Natl. Acad. Sci. USA 81, 3883–3887.

Stocker, R., and Keaney, J.F., Jr. (2004). Role of oxidative modifications in atherosclerosis. Physiol. Rev. 84, 1381–1478.

Subramanian, P., Karshovska, E., Reinhard, P., Megens, R.T., Zhou, Z., Akhtar, S., Schumann, U., Li, X., van Zandvoort, M., Ludin, C., et al. (2010). Lysophosphatidic acid receptors LPA1 and LPA3 promote CXCL12-mediated smooth muscle progenitor cell recruitment in neointima formation. Circ. Res. 107, 96–105.

Tokumura, A., Kanaya, Y., Kitahara, M., Miyake, M., Yoshioka, Y., and Fukuzawa, K. (2002). Increased formation of lysophosphatidic acids by lysophospholipase D in serum of hypercholesterolemic rabbits. J. Lipid Res. 43, 307–315.

van Zandvoort, M., Engels, W., Douma, K., Beckers, L., Oude Egbrink, M., Daemen, M., and Slaaf, D.W. (2004). Two-photon microscopy for imaging of the (atherosclerotic) vascular wall: a proof of concept study. J. Vasc. Res. 41, 54–63.

Weber, C., Zernecke, A., and Libby, P. (2008). The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat. Rev. Immunol. 8, 802–815.

Zaslavsky, A., Singh, L.S., Tan, H., Ding, H., Liang, Z., and Xu, Y. (2006). Homo- and hetero-dimerization of LPA/S1P receptors, OGR1 and GPR4. Biochim. Biophys. Acta 1761, 1200–1212.

Zhang, X.W., Liu, Q., Wang, Y., and Thorlacius, H. (2001). CXC chemokines, MIP-2 and KC, induce P-selectin-dependent neutrophil rolling and extravascular migration in vivo. Br. J. Pharmacol. 133, 413–421.