Macrophage Gene Expression and Foam Cell Formation is Regulated by Plasminogen

Running title: Das et al.; Regulation of foam cell formation by plasminogen

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Abstract:

**Background**—Deciphering the molecular and cellular processes that govern macrophage foam cell formation is critical to understanding basic mechanisms underlying atherosclerosis and other vascular pathologies.

**Methods and Results**—Here, we identify a pivotal role of plasminogen in regulating foam cell formation. Deficiency of plasminogen inhibited macrophage cholesterol accumulation upon exposure to hyperlipidemic conditions *in vitro, ex vivo and in vivo*. Gene expression analysis identified CD36 as a regulated target of plasminogen, and macrophages from Plg-/- mice had decreased CD36 expression and diminished foam cell formation. The plasminogen-dependent CD36 expression and foam cell formation depended on conversion of plasminogen to plasmin, binding to the macrophage surface, and the consequent intracellular signaling that leads to production of leukotrieneB4. Leukotriene B4 rescued the suppression of CD36 expression and foam cell formation arising from plasminogen deficiency.

**Conclusions**—Our findings demonstrate an unanticipated role of plasminogen in regulation of gene expression and cholesterol metabolism by macrophages and identify plasminogen-mediated regulation of leukotrieneB4 as an underlying mechanism.

**Key words:** arteriosclerosis, cholesterol, plasminogen
Introduction

Macrophage-derived foam cell formation, a hallmark in the progression of atherosclerosis, begins with recruitment of monocytes into the subendothelial space of affected blood vessels. In the cytokine-rich subendothelial microenvironment, monocytes differentiate into macrophages with concomitant expression of proteins that mediate uptake of modified lipoproteins and retention of cholesterol in the cells\(^1\). Among the genes upregulated during foam cell formation are scavenger (CD36, MSRA, CD68) and phagocytic (phosphatidylserine receptor, FcgammaR, SRB1, ABCA1) receptors that mediate uptake of oxidized low density lipoprotein (OxLDL); nuclear receptors (PPARs and CEBP) that regulate expression of the receptors involved in LDL uptake; and genes involved in eicosanoid (e.g. HETEs, leukotrienes) biosynthesis that activate the aforementioned nuclear receptors.

Plasminogen (Plg) is synthesized primarily by the liver and circulates in the blood at 1-2 \(\mu M\) \(^3\). Plg is converted to an active serine protease, plasmin (Plm), by plasminogen activators, a transition that is influenced by interaction of Plg with C-terminal lysines of extracellular matrix (ECM) and cell surfaces Plg receptors (Plg-R). Hence, blockade of Plg with lysine analogs, such as tranexamic acid (TXA), or antibodies directed to the C-terminal lysine region of Plg-R, block the interaction of Plg with cells and dampen Plm generation\(^5\).

Beyond the extracellular proteolytic functions of Plg\(^6\), its interaction with cells can trigger intracellular signaling\(^7,8\). Plm activates 5-lipoxygenase (5-LO)\(^7,9\), a key enzyme in leukotriene biosynthesis that generates eicosanoids, including leukotrieneB4 (LTB4), a biologically active lipid mediator associated with cardiovascular pathologies\(^10\). Indeed, the predominant source of blood leukotrienes is inflammatory cells such as activated monocytes and macrophages\(^10\). 5-LO and LTB4 contribute to formation of atherosclerotic lesions in both ApoE-
and Ldr-/- mouse models by enhancing inflammation and foam cell formation\textsuperscript{11,12}.

There are a number of studies in humans that establish a direct association between Plg levels or plasmin activity and the incidence of CAD. As examples, two separate prospective cohort studies, FINRISK,\textsuperscript{9} 92 Hemostasis Study\textsuperscript{13} and the Atherosclerosis Risk in Communities Study (ARIC)\textsuperscript{14} showed that Plg levels were an independent risk factor for CAD. A separate cohort study\textsuperscript{15} reported that the level of plasmin-\(\alpha\)2-antiplasmin, a marker of plasmin generation, was directly associated with abnormal ankle-arm index, a measure of atherosclerosis. Other studies also confirm a direct correlation between blood levels of plasmin-\(\alpha\)2-plasmin complex\textsuperscript{16} or fibrin D-dimer\textsuperscript{14,16,17} and CAD. The role of Plg in atherosclerosis has also been studied in Plg-deficient mice system\textsuperscript{18-20}. In the most recently published study, Kremen et al\textsuperscript{19} demonstrated that Plg knockout mice in an ApoE-/- background displayed a marked reduction in aortic lesion area. However, the mechanism by which Plg influenced lesion development remains unknown.

Here, we present \textit{in vitro} and \textit{in vivo} studies which demonstrate that Plg is critical for transformation of macrophages into lipid-laden foam cells. Surprisingly, this effect depends upon alteration in expression of key genes associated with cholesterol accumulation into macrophages.

\textbf{Material and Methods}

\textbf{Animals}

All animal experiments were performed under institutionally approved protocols. Tenth generation male and female Plg+/- and Plg-/- mice\textsuperscript{4} in C57BL/6J were used, commencing at 8 to 10 wk of age. ApoE-/- mice in C57BL/6J background were from Jackson Laboratories, Bar Harbor, ME and bred with Plg+/+ mice to obtain ApoE-/-Plg+/+ and ApoE-/-Plg-/- mice. Male mice with \textless 20\% variation in weight among ApoE-/- and ApoE-/-Plg-/- genotypes were used.
Four wk old ApoE-/- and Plg-/-ApoE-/- mice were fed either a normal chow (CD) or high cholesterol (HCD) diet (containing 0.15% added cholesterol and 42% milk fat, TD88137, Harlan-Teklad) for 6 wks. Plg mutant mice in which the active site Ser was replaced by Ala were kindly provided by Drs. Ploplis and Castellino (University of Notre Dame, IN, USA). CD36-/- (10x backcrossed to C57Bl/6) mice have been previously described.

Ex vivo and in vivo foam cell formation

Ex vivo foam cell formation was assessed using thioglycollate (TG)-elicited peritoneal macrophages and serum from Plg+/+ or Plg-/- mice. In vivo foam cell formation was performed using ApoE-/- and ApoE-/-Plg-/- mice. Specific protocols for both assays are expanded in supplementary materials. The isolation and utilization of human blood monocyte-derived macrophages (HuPBM), THP-1 and RAW264.7 cell lines are in the supplementary materials.

OxLDL binding and internalization

These assays used OxLDL fluorescently labeled with 1,1'-dioctadecyl-1 to 3,3',3',3'-tetramethylindocarbocyanine perchlorate (Dil, Biomedical Technologies, Inc.) are described in supplementary material.

In vivo transfer of macrophages

This analysis is detailed in supplementary material.

Real time RT-PCR

Isolated RNA from variously treated cells was transcribed into cDNA. Quantitative PCR was performed on cDNA with specific oligonucleotides (Supplemental Table 1).

Western Blots

Total cell lysates were analyzed by Western blotting (detailed in supplementary material) using anti-CD36 antibody (R&D or Novus Biologicals).
FACS

Cell surface expression of CD36 was analyzed by flow cytometry (detailed in supplementary material) using FITC-labeled rat anti-mouse CD36 (Cayman, Ann Arbor, MI).

Blood cholesterol quantification

Mice were fasted for 16 h prior to blood collection from tail clips. Plasma from the mice was used to measure total LDL/VLDL and HDL content using Quantification Kits from Biovision, Milpitas, CA.

Serum LTB4 measurement

Serum LTB4 levels were quantified using a kit from Cayman Chemical.

Statistical Analysis

A two-tailed $t$ test was used in comparing two groups, and differences between multiple groups were evaluated using either a one-way ANOVA or a two-way ANOVA test followed by Tukey multiple comparison test (detailed in supplementary materials).

Results

Plg regulates macrophage foam cell formation

Plg was shown to support lipid core growth in a murine diet-induced atherosclerosis model\textsuperscript{19, 20}. To dissect the mechanism for this proatherogenic activity, we assessed lipid uptake by TG-elicted peritoneal macrophages derived from Plg+/+ or Plg/-/- mice upon exposure to oxidized LDL (OxLDL), culturing the cells in autologous serum. Lipid uptake, assessed by oil red O (ORO) staining, was dramatically impaired by macrophages derived from Plg/-/- compared to Plg+/+ mice (Fig. 1A). Quantitatively, the reduction in total cholesterol in macrophages derived from Plg/-/- mice was 62.5% ($p<0.001$) less than in WT macrophages (Fig. 1B).
Mixing experiments were performed with macrophages derived from Plg+/+ mice cultured in serum derived from Plg+/+ mice and Plg-/- mice. OxLDL induced no lipid accumulation in the absence of serum (Fig. 1C & D). Macrophages cultured in Plg+/+ serum took up OxLDL and developed a rounded, foam cell appearance (Fig. 1C & D). In contrast, Plg+/+ macrophages cultured in Plg-/- serum displayed reduced ORO staining and cholesterol content (48.4% reduction, p<0.001) compared to the same (WT) macrophages cultured in Plg+/+ serum. The reduced ability of Plg-/- serum to support lipid uptake was restored when Glu-Plg was added at its physiological concentration (1 μM) to Plg-/- serum.

The influence of Plg on cholesterol accumulation also was observed with human monocyte-derived macrophages (HuPBM). In presence of OxLDL, HuPBM accumulated lipids when cultured in autologous serum as indicated by ORO staining (Fig. 1E) and total cholesterol quantification (Fig. 1F). Depletion of Plg from the serum on lysine-Sepharose lowered lipid accumulation by 49.1% (Fig. 1F), and supplementing the depleted serum with Plg reestablished cholesterol accumulation. A similar dependence of lipid uptake on Plg was observed using human monocytoid THP-1 cells (Supplemental Figure S1A) and mouse macrophage RAW264.7 cells (Figure S1B). The RAW264.7 cells cultured in only 1% Nutridoma still showed an increased lipid accumulation in response to OxLDL upon addition of Plg.

Functional requirements for Plg-mediated foam cell formation

The requirement for proteolytic activity of Plm in foam cell formation was evaluated by two approaches. First, aprotinin, a serine protease inhibitor, to macrophages cultured in Plg+/+ serum (Fig 2A & B) inhibited cholesterol accumulation by 48.4% (p<0.001). Second, cholesterol accumulation by macrophages cultured in serum from mice expressing an active site mutant of Plg^{21}, cleavable by Plg activators but does not form an active enzyme (panels [Ac] &
[B]), was suppressed by 67.7% compared to WT serum (p<0.001). Addition of exogenous Plg to the serum from Plg mutant mice enhanced ORO staining and cholesterol uptake (33% recovery, panel [Ad] & [B]), but not as effectively as addition of the same amount of Plg to Plg-/- serum (Fig 1C & D). This partial restoration of lipid uptake may reflect competition of mutant Plg with added WT Plg.

Many cellular functions of Plg depend on its interaction with Plg-R via its kringle-associated lysine binding sites, and TXA, a lysine analog, blocks Plg binding to most Plg-R on macrophages. The recovery of foam cell formation upon addition of Plg to macrophages cultured in Plg-/- serum (Fig. 1C & D) was inhibited by more than 90% (p<0.001) by TXA (panels [Cd] & [D]). Multiple Plg-R have been implicated in the binding of Plg to macrophages, and among these histone H2B plays a particularly prominent in Plg binding23. Fab fragments of monoclonal antibody, G12, raised to the C terminal peptide of H2B (panels [C] & [D]), inhibited the ability of Plg to enhance cholesterol accumulation by macrophages by 53% (p<0.001) compared to non-immune Fab (panels [Cf] & [D]). Hence, the ability of Plg to enhance foam cell formation is dependent on its interaction with the Plg-Rs. H2B does and other Plg-Rs may contribute to this response.

**Plg supports binding and internalization of OxLDL**

To assess the effects of Plg on lipoprotein binding and/or internalization, TG-inflamed peritoneal macrophages were incubated with various concentrations of fluorescently-tagged OxLDL (Dil-OxLDL) for 30 min at 4°C for binding and 2 hr at 37°C for internalization. By flow cytometry, macrophages from Plg-/- mice showed reduced binding (Fig. 3A) and internalization (Fig. 3B) compared to Plg+/+ macrophages. At 10 μg/ml, Plg-/- derived macrophages bound 2.5-fold (p<0.04) less and took up 1.7-fold (p<0.001) less Dil-OxLDL than Plg-/- mice. Binding
and uptake results were confirmed on WT macrophages cultured in either Plg+/+ or Plg-/− mice derive serum for 2 days. Both parameters were lowered (at 10 μg/ml Dil-OxLDL, 2 fold, p<0.001 for binding and 2.2 fold, p≤0.003 for internalization) in macrophages cultured in Plg-/− compared with Plg+/+ serum (Fig 3C & D).

To consider whether plasmin might modify OxLDL and aid in cholesterol accumulation, we cultured the macrophages in Plg+/+ or Plg-/− serum, then washed the cells thoroughly and measured Dil-OxLDL binding in the absence of Plg. Binding to cells cultured in the Plg-/− serum was still 50% less (p<0.001) than macrophages cultured in Plg+/+ serum (Fig. 3E).

Macrophages cultured in Plg-/− serum supplemented with exogenous Plg recovered their capacity to bind Dil-OxLDL, and inclusion of aprotinin with the exogenous Plg during culture inhibited this recovery. Additionally, macrophages cultured in serum from Plg mutant mice bound 62% less Dil-OxLDL (p<0.001) compared to macrophages cultured in Plg+/+ serum. These differences correlated well with cholesterol accumulation and foam cell formation (Fig. 1&2).

**Foam cell formation in vivo is impaired by the absence of plasminogen**

To translate our observations into an *in vivo* setting, we crossed the Plg-/− and ApoE-/− mice and maintained the resulting ApoE-/−Plg-/− and ApoE-/− mice on a chow diet (CD) or a high cholesterol diet (HCD). After 6 weeks, plasma LDL/VLDL was elevated in mice of both backgrounds fed the HCD compared to the CD and HDL was lower in ApoE-/− mice fed the HCD compared to the CD (supplemental Table 2). Plasma LDL/VLDL levels were similar in the Plg-deficient and Plg-replete ApoE-/− backgrounds, but the HDL level was lower (p≤0.01) in Plg-/−ApoE-/− mice compared with ApoE-/− on both CD and HCD (Supplemental Table 2). TG was used to recruit peritoneal macrophages in these mice; and equal numbers of cells were evaluated...
for lipid and total cholesterol content. Regardless of genotype, macrophages derived from mice fed the HCD showed increased lipid staining and a higher intracellular cholesterol than those fed the CD (Fig 4A & B). However, internal lipid staining and total cholesterol content was dramatically reduced in macrophages derived from ApoE-/Plg-/- mice compared to ApoE-/ mice (panels [A] & [B]). These differences in lipid accumulation in the Plg-/- background were observed in mice fed either CD (60%, p ≤ 0.003) or HCD (68.3%, p ≤ 0.003). Thus, cholesterol accumulation in macrophages in vivo is strongly influenced by Plg.

We considered whether the observed in vivo differences in lipid accumulation might reflect differences in the macrophage populations recruited into the peritoneal cavity of ApoE-/ and Plg-/ApoE-/ mice and performed macrophage transfer experiments. TG-elicited macrophages derived from Plg+/- mice were injected into the peritoneal cavity of recipient ApoE-/ and ApoE-/Plg-/- mice that had been maintained on a CD or HCD for 6 weeks. Cells were recovered 3 days after TG stimulation and analyzed for lipid accumulation. A reduction of ORO staining (Fig 4C) as well as cholesterol content (42.1%, p ≤ 0.001 on CD and 48%, p ≤ 0.001 on HCD, Fig. 4D) was observed in transferred macrophages obtained from ApoE-/Plg-/- recipient mice compared to ApoE-/ recipient mice. Additionally levels of Plg in the peritoneal fluid were found same in ApoE-/ mice on CD and high fat diet mice group (607.1 ng/ml lavage vs. 586.5 ng/ml lavage, p > 0.7). Plg was not detected in the lavage from ApoE-/Plg-/- mice in either diet. Thus, the differences in cholesterol content of macrophages were consequences of both diet and plasminogen deficiency and were not due to differences in the population of recruited macrophages or to diet-associated differences in the peritoneal content of Plg. We also measured the levels of LDL/VLDL in the peritoneal lavage from these mice. In CD fed mice, VLDL/LDL in the lavage were the same in ApoE-/ and ApoE-/Plg-/- mice (38.2 vs. 41.2
mg/ml in 1.5 ml peritoneal wash) but yet these mice still showed a difference in foam cell formation (Fig. 4C & D), supporting the direct role of Plg in lipid uptake. In the HCD mice, a 2-fold difference in VLDL/LDL levels in the ApoE-/-Plg-/- compared with ApoE-/- a mouse (81 vs. 38.1 mg/ml) was noted. Despite this difference in VLDL/LDL levels, the differences in cholesterol content was similar (42.1% and 48% reduction) in Plg-/- and Plg+/+ mice regardless of diet.

**Plg regulates expression of genes involved in foam cell formation**

We next examined how Plg influences expression of selected genes implicated in lipid metabolism. These included receptors involved in OxLDL uptake, CD36, MSRA and CD68, receptors involved in phagocytosis of OxLDL, immune complexes and apoptotic bodies, represented by phosphatidylserine receptor (PSR), Fcγ receptor type 1 (FcγR1), Fcγ receptor type II (FcγRII), SRB1 and ATP-binding cassette transporter (ABCA1); and nuclear receptors known to regulate these receptors, including PPARγ, CEBPα, CEBPβ and TR41,2,5,26. In the absence of OxLDL, no differences were observed in expression levels of genes tested between cells cultured in Plg+/+ and Plg-/- conditions, with the exception of CD36 (Fig. 5A); CD36 expression was inhibited by 30% (p=0.04) in Plg-/- serum vs. Plg+/+ serum. Expression levels of tested genes were consistently higher upon stimulation with OxLDL in macrophages cultured in Plg+/+ serum compared with unstimulated cells (p≤0.01). The exceptions were PSR and CEBPα (panel [B] & [C]), which did not change upon OxLDL stimulation. In absence of Plg, OxLDL-mediated CD36 expression decreased by 57% (p=0.005) vs. Plg+/+ serum (Fig. 5A). Among other genes, OxLDL-mediated CD68 (Fig. 5A), FcγR1 and ABCA1 expression levels were significantly lower in the absence of Plg (Fig 5B). Among the nuclear receptors, OxLDL-induced upregulation of transcripts for PPARγ, CEBPβ and TR4 in macrophages and all were
suppressed in the Plg-/- environment (Fig. 5C).

The expression levels of the most effected genes were further evaluated in macrophages derived from in vivo transfer experiments. Transcript levels of CD36, ABCA1 and PPARγ were significantly suppressed (p≤0.01) in ApoE-/-Plg-/- recipient derived macrophages compared to ApoE-/- recipient derived macrophages (Fig. 5D). Collectively, these results suggest that Plg might enhance macrophage accumulation of cholesterol by affecting expression of various receptors involved in OxLDL uptake.

**Role of CD36 in Plg-mediated cholesterol accumulation**

Among the scavenger receptors tested, CD36 expression was altered by the absence of Plg whether OxLDL was or was not present (Fig. 4A). At the protein level, western blots of whole cell lysates showed lower CD36 in macrophages from Plg-/- mice compared to Plg+/+ mice (Fig 6A). By flow cytometry, CD36 cell surface levels were 2.2-fold (p<0.001) lower in macrophages from Plg-/- vs. Plg+/+ mice (Fig 6B). Expression of CD36 depends upon the differentiation status of macrophages27. Plg+/+ mice derived macrophages cultured for 2 days in Plg+/+ serum showed a 2-fold higher CD36 protein expression than freshly isolated macrophages from Plg+/+ mice both at the whole cell (Fig 6C) and cell surface levels (Fig. 6D). However, CD36 expression did not increase from basal levels when these cells were cultured for 2 days in Plg-/- serum. Upon OxLDL treatment, CD36 expression was enhanced 2-fold in Plg+/+ serum compared with untreated cells but inhibited by 45% when OxLDL treated macrophages were cultured in Plg-/- serum (Fig. 6C & D). These data suggest that CD36 gene and protein expression are regulated by Plg. Regulation of CD36 expression by Plg was confirmed with RAW264.7 cells grown in the absence of serum. When these cells were treated with 1 μM Plg, their CD36 protein expression increased (Supplemental Figure S2A).
CD36 was further implicated in Plg-mediated OxLDL uptake and cholesterol accumulation. When macrophages derived from CD36/-/- mice were cultured in Plg/-/- serum, they showed little accumulation of lipid in response to OxLDL (Fig. 6E), and addition of Plg did not rescue cholesterol accumulation in CD36/-/- macrophages as it did with CD36+/+ cells (Fig. 6E). Together, these data indicate that a major component of Plg-mediated foam cell formation is dependent on its regulation of CD36 expression.

Effect of Plg on leukotriene B4 biosynthesis

LTB4 influences many crucial steps in early development of atherosclerosis. Plg/Plasmin induces monocyte production of several leukotrienes, including LTB4. We hypothesized that reduced LTB4 production could be a mechanism underlying Plg-dependent foam cell formation. We first quantified LTB4 levels in serum from Plg-/- and Plg+/+ mice (Fig. 7A). Serum from Plg-/- mice contained 2-fold less LTB4 than from Plg+/+ mice (1.8±0.2 vs. 3.3±0.4 nM, n=5, p<0.001). LTB4 levels were also measured in ApoE-/- mice. LTB4 levels were 2.3-fold higher (p<0.001) in ApoE-/- mice fed the HCD compared to animals fed the CD for 6 wk, consistent with previous reports. Most notably, in ApoE-/-/Plg-/- mice, LTB4 levels in serum were ~40% lower in both CD (1.7±0.4 in ApoE-/-/Plg-/- mice vs. 3.8±0.4 nM in ApoE-/-/Plg+/- mice, n = 5, p=0.03) and HCD fed mice (4.4±1.2 in ApoE-/-/Plg-/- mice vs. 10.2±1.6 nM in ApoE-/-/Plg+/- mice, n=5, p<0.001) (Fig. 7B). Additionally, in ApoE-/-/Plg-/- mice, LTB4 levels in peritoneal fluid were 46% lower (0.06 nM vs. 0.1 nM on CD mice and 0.2 nM vs. 0.4 nM on HCD mice) compared with ApoE-/- mice. Thus, Plg influences LTB4 production in vivo.

LTB4 bypasses the effects of plasminogen deficiency on foam cell formation and gene expression.
TG-elicited peritoneal macrophages were presented with OxLDL in Plg+/+ or Plg-/- serum (Fig. 7C & D), with or without supplemental Plg or LTB4. Addition of Plg or LTB4 overcame the suppressive effects of Plg deficiency (Fig. 7C & D). As noted above, macrophages grown in Plg-/- serum have less CD36 on their surface than macrophages in Plg+/+ serum. Addition of either Plg or LTB4 (500 nM)\(^2\) to the Plg-/- serum enhanced cell-surface expression of CD36 (Fig. 7E and Supplemental Fig. S2B). Adding Plg and LTB4 together to Plg-/- serum did not have an additional effect on ORO staining (Fig. 7C), cholesterol accumulation (Fig. 7D) or CD36 expression (Fig. 7, Supplemental Fig. S2B) compared to LTB4 alone, suggesting that LTB4 is a downstream effector of Plg. When Plg was added to Plg-/- culture media, LTB4 levels were fully recovered (Fig. 7F).

5-LO catalyzes LTB4 synthesis requires an integral membrane protein, 5-LO activating protein (FLAP). MK886 blocks LTB4 secretion by inhibiting FLAP activity. When RAW264.7 cells were pretreated with MK886 and then with Plg, Plg-mediated upregulation of CD36 protein expression was completely suppressed at 500 nM MK886 (Supplemental Figure S2B). LTB4 can signal intracellularly or by binding to G-protein coupled receptors, BLT\(_1\) and BLT\(_2\). Blocking BLT1 (the higher affinity LTB4 receptor) with two unrelated inhibitors, LY293111 and U-75302, reduced Plg-mediated CD36 expression (Supplemental Figure S3, 75% with LY293111 and >90% with U-75302) suggesting that Plg-induced LTB4 acts primarily via its extracellular release and interaction with BLT\(_1\). Besides LTB4, Plg also induces biosynthesis of cysteinyl-LTs. Adding LTE4, the most stable of the cys-LTs, did not enhance CD36 expression in RAW264.7 cells (Supplemental Figure S4), suggesting that among these two leukotrienes, the effect of Plg on CD36 expression depends on LTB4.
Discussion

It is now clear that the role of Plg in vivo extends well beyond its function in fibrinolysis. By virtue of its capacity to degrade various ECM proteins, to activate certain MMPs, and to cleave secreted growth factors, Plg facilitates tissue reorganization and enhances cell migration. In the present study, we report an unexpected effect of Plg on the capacity of macrophages to take up lipids and become foam cells. Surprisingly, Plg exerts these effects by controlling the expression of genes involved in OxLDL uptake by macrophages, most notably CD36, and this regulation is dependent on Plg-dependent activation of the 5-LO biosynthetic pathway. Thus, we have assigned a novel function to Plg in macrophage biology and have begun to define the molecular pathway underlying this function.

The importance of Plg in cholesterol accumulation was demonstrated with primary cells and cell lines of murine (peritoneal macrophages and RAW264.7 cells) and human (HuPBM and THP-1) origin. With all these cells, culture in Plg-/ serum reduced ORO staining and cholesterol content in response to OxLDL compared to Plg+/+ serum, and supplementing the Plg-deficient media with exogenous Plg overcame this suppression. Decreased cholesterol accumulation by macrophages under Plg-deficient conditions was very extensive (e.g., cholesterol accumulation was reduced by 60% in macrophages from Plg-/-ApoE-/- mice compared to those derived from Plg+/+ApoE-/- mice). The pathway giving rise to residual cholesterol uptake in the absence of Plg is unknown. SRA1 expression was unaffected by the absence of Plg but was induced by OxLDL, and is a candidate for mediating Plg-independent cholesterol accumulation. Growth factors such as insulin and many cytokines like IL-10 also support foam cell formation and could contribute to Plg-independent lipid uptake. The molecular and cellular requirements for fat cell formation during adipogenesis often parallel those for
macrophage transformation into foam cells.Hints as to a possible role for Plg in lipid uptake can be derived from two prior studies of adipogenesis. Selvarajan et al\textsuperscript{33} described a role for Plg in lipid absorption during preadipocyte differentiation to adipocytes, and Hoover-Plow et al\textsuperscript{34} demonstrated reduced body weight and less accumulation of fat in Plg-/+ compared to Plg+/- mice. Thus, the influence of Plg on lipid uptake may not be restricted to macrophages.

Correlating with Plg-dependent changes in cholesterol accumulation, binding and internalization of OxLDL was also impaired in the absence of Plg. In one arm of these studies, the Dil-OxLDL tracer was not directly exposed to Plg but yet macrophages cultured in Plg-deficient culture conditions showed reduced binding. These data suggest that the primary role of Plg is to affect the macrophages’ capacity to take up OxLDL rather than to modify the OxLDL ligand. Nevertheless, we do not exclude that plasmin may modify OxLDL over the 24-48h of the foam cell formation assays. Consistent with binding data among the tested scavenger receptors, mRNA and protein levels of CD36 molecule was significantly suppressed in macrophages derived from Plg-/- mice vs. Plg+/- mice or in WT macrophages cultured in Plg-/- serum. CD36 accounts for almost 80% of foam cell formation by OxLDL generated by a myeloperoxidase-hydrogen peroxide-nitrite pathway\textsuperscript{35} and 60% of OxLDL generated by copper oxidation\textsuperscript{22}. Furthermore, studies of hypercholesterolemic CD36-/- mice have shown the importance of this receptor in uptake of OxLDL and foam cell formation\textsuperscript{22}. We verified the role of CD36 in Plg-dependent foam cell formation using macrophages from CD36 KO mice. Addition of exogenous Plg to Plg-deficient medium, which fully rescued cholesterol accumulation in WT macrophages, had little effect on CD36 KO macrophages.

5-Lipoxygenase and its product LTB4 have been implicated in CD36 expression\textsuperscript{12}. We found a reduced level of LTB4 in serum from TG-treated Plg-/- versus Plg+/- mice. This
influence of Plg in LTB4 biosynthesis was also substantiated in proatherogenic ApoE/- mice, where reduced LTB4 levels were observed in serum of ApoE/-/Plg/- mice compared to ApoE/- mice. Of particular note, we found that addition of LTB4 to Plg-deficient cultures overcame the reduction in cholesterol accumulation by macrophages. The effects of Plg and LTB4 on cholesterol accumulation and CD36 expression suggest that these molecules function in the same pathway since LTB + Plg did not have an additive effect. Additionally, Plg-mediated CD36 expression required BLT1, a high affinity LTB4 receptor. These data support the model depicted in Fig. 8. Accordingly, Plg and/or plasmin bind to receptors, including but not limited to H2B. Plasmin stimulates 5-LO activity, which in turn generates LTB4. LTB4, in part by release from the cell and engaging its high affinity receptor, BLT1, generates nuclear signals that enhance CD36 mRNA levels, protein and cell-surface expression. Our present study does not establish how Plg activates the 5-LO pathway to induce CD36 expression. Activation of 5-LO can be mediated by p38 MAP kinase activation and plasmin is known to be a p38 MAP kinase activator. LTB4 also can activate intracellular nuclear factors PPARα and weakly PPARγ, but these nuclear factors do not support foam cell formation. Plasmin-induced leukotriene secretion and chemotaxis have been shown to be sensitive to pertussis toxin which supports our suggestion that that LTB4 signals via binding to BLT1, a G-protein coupled receptor. While our model suggests that released LTB4 exerts its effects on expression of CD36 on the same cell, released LTB4 may stimulate other macrophages or other cells sensitive to the LTB4. LTB4 but not LTE4 enhanced CD36 expression, but other products of Plg-induced signaling may exert other far-reaching effects.

Kremen et al demonstrated that Plg deficiency markedly reduced atherosclerosis lesion growth, an observation entirely consistent with our data. In contrast, Xiao et al suggested that
Plg deficiency accelerated lesion development. In the Kremen study, mice were fed an atherogenic diet and the Xiao study, a low fat diet. However, our data showed a diet independent effect of Plg in macrophage foam cell formation in ApoE-/- mice. In the Xiao and Kremen studies the lesions were observed at different stages of development (15 wks in the former vs. 18-25 wks in the latter study). Plg exerts numerous effects in vivo and its proatherogenic activities (e.g., enhanced ingress of inflammatory cells or enhanced lipid uptake) may dominate over its antiatherogenic effects (e.g., turnover of ECM or cytokines) at different stages of lesion development. Such counterbalancing effects of Plg may have contributed to the absence of a significant benefit of tPA therapy on the incidence of mortality and nonfatal myocardial infarction in patients with unstable angina or non-ST elevation myocardial infarction; the effects of Plg on foam cell formation and accelerated atherosclerosis may have offset the benefits of thrombolysis.

In sum, we conclude that Plg is a key regulator of foam cell formation. Inactivation of Plg reduces OxLDL driven foam cell formation by: a) reducing the biosynthesis of LTB4; b) reducing expression of CD36; c) suppressing OxLDL driven expression of multiple genes involved in foam cell formation. The combination of these effects identifies a prominent role of Plg in atherogenesis. These findings provide a molecular explanation for the association of Plg with CAD and provide impetus to further explore Plg-mediated lipid metabolism and to consider Plg and Plg-R as targets for therapeutic intervention in cardiovascular disease.

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Conflict of Interest Disclosures: None.

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2009.


**Figure Legends:**

**Figure 1.** Plg regulates lipid uptake by macrophages and foam cell formation. Panel A to D: Thioglycollate-elicited peritoneal macrophages from Plg+/+ or Plg-/- mice were induced to form foam cell with OxLDL in the absence of serum or in the presence of serum derived from either Plg+/+ or Plg-/- mice. Panels E&F: Human monocyte-derived macrophages (HuPBM) were induced to form foam cells by addition of OxLDL and cultured in autologous serum or autologous serum depleted of Plg. In some murine or human cultures, Plg-deficient serum was supplemented with 1 μM Glu-Plg. Panels A,C&D: ORO staining of foam cells at original magnification 40X. Images are representative of 2 independent experiments. Panel B,D&F: Total cholesterol was measured in foam cells. Dots represent an individual data point and the number of replicates is 4. Error bars are ±SD of the mean.

**Figure 2.** Plasminogen receptors and plasmin activity are required for Plg-mediated foam cell formation. Panels A&B: Thioglycollate-elicited macrophages from Plg+/+ mice were untreated
or treated with aprotinin (100 KIU/ml). The cells were then allowed to form foam cells by adding OxLDL in the presence of serum derived from Plg+/+ mice or Plg mutant mice (expressing active site mutant of Plg). Panel B&C: Tranexamic acid (TXA, 200 μM), Fab fragments of anti-H2B mAb (G12, 8 μM), nonimmune mouse IgG Fab (NM, 8 μM) or buffer was added to TG-elicited macrophages and foam cell formation was induced with OxLDL. Panel A&C: ORO staining of foam cells at original 40X magnification. Images are representative of 2 independent experiments. Panel B&D: Total cholesterol was measured in the cultured macrophages. Each dot is one of four replicates and error bars are ±SD of the means.

Figure 3. Role of Plg in OxLDL binding and internalization. A&B: TG-elicited peritoneal macrophages derived from Plg+/+ (filled circle) or Plg-/- mice (open circle) were allowed to bind (30 min at 4°C, [A]) or internalize (2 hr at 37°C, [B]) Dil OxLDL. C&D: Peritoneal macrophages from Plg+/+ mice were cultured in presence of serum derived from Plg+/+ (filled circle) or Plg-/- mice (open circle), and then allowed to bind (panel [C]) or internalize (panel [D]) varying concentration of Dil-OxLDL. Points are ±SD of means from triplicate samples.

Panel E: Macrophages derived from Plg+/+ mice were cultured in either Plg+/+, Plg-/- serum or serum from Plg active site mutant mice. In some panels, Plg (1 μM) or aprotinin (blocks plasmin activity) was added. Cells were washed and allowed to bind Dil-OxLDL in presence or absence of excess unlabeled OxLDL. Bound or internalized Dil-OxLDL was measured by flow cytometry. Specific binding values are displayed and were obtained by subtracting residual non-specific binding in the presence of excess OxLDL. Each dot is an average of triplicates.

Figure 4. Effect of Plg deficiency on foam cell formation in vivo. Panels A&B: Foam cells were
measured in thioglycollate-elicited peritoneal macrophages derived from ApoE-/- or ApoE-/- Plg-/- mice fed either a chow diet (CD) or high cholesterol diet (HCD). Panels C&D: Foam cell formation was assessed by macrophages transferred to ApoE-/- and ApoE-/-/Plg-/- recipient mice that had been fed a CD or HCD. Panels A &C: ORO staining at an original 40X magnification. Images are representative of cells from 5 mice. Panels B&D: Quantification of total cholesterol. Each dot represents a data point from one of five mice. Error bars are the SD of the means.

**Figure 5.** Plg deficiency causes reduced expression of genes involved in lipid uptake. Panels A,B&C: Real-time PCR quantification of transcripts of genes related to lipid uptake (A), phagocytosis (B) or nuclear receptors (C) on foam cells either treated with Plg+/+ or Plg-/- mice derived serum. Gray bars: Plg+/+ serum, OxLDL untreated; white bars: Plg-/- serum, OxLDL untreated; black bars: Plg+/+ serum, OxLDL treated; and striated bars: Plg-/- serum, OxLDL treated. Bars are means ±SD of triplicates. *P<0.01 vs. Plg+/+ serum. #p=0.04 vs. Plg+/+ serum. †P<0.002 vs. Plg+/+ serum treated with OxLDL. Data are representative of 2 independent experiments. (D) Real time PCR quantification on mRNA of transferred macrophages derived from hyperlepidemic ApoE-/- (black bars) and ApoE-/-Plg-/-(gray bars) recipient mice. Bars are means ±SD of triplicates. *P<0.001 vs. Plg+/+ serum treated with OxLDL. mRNAs are pooled from macrophages derived from 5 mice.

**Figure 6.** Role of CD36 in Plg-mediated foam cell formation. Panels A: Western blot for CD36 (upper panel A) of macrophages derived from either Plg+/+ mice or Plg-/—mice. CD36-/—macrophages were analyzed as a negative control. Panel B: Flow cytometry for cell surface
Relationship between plasminogen, leukotriene B4 and foam cell formation. Panel A: Serum LTB4 levels in thioglycollate-treated Plg+/+ and Plg-/- mice. Panel B: Serum LTB4 levels in ApoE-/- mice (black bars) or Plg-/-ApoE-/- mice (dotted bars) maintained on a high cholesterol diet (HCD) or a chow diet (CD). Error bars are means ±SD from 5 mice. Panels C, D&E: OxLDL induced foam cell formation by TG-elicited macrophages in Plg+/+ serum or Plg-/ - serum with or without added Plg (1 μM) and LTB4 (500 nM). Panel C: ORO staining (original magnification, 40x). Images are representative of 2 independent experiments. Panel D: Total cholesterol quantification. Each dot is the data point from one of four individual wells. Error bars are the SD of means. Panel E: Flow cytometry for surface CD36 expression. Bars are mean fluorescence intensities ±SD of triplicates. Panel F: Plg+/+ mice derived macrophages were cultured in serum derived from Plg+/+ or Plg-/ - mice in the absence or presence of Plg. Culture media were collected and LTB4 levels measured. Each dot is the value for three independent experiments. Error bars are ±SD of means.
Figure 8. The plasminogen-dependent pathway of foam cell formation. Plg binding to Plg-receptors activates 5-lipoxygenase in a plasmin-dependent manner and leads to LTB4 production and secretion by the macrophage (i). Secreted LTB4 binds to BLT1 (ii) and activates transcription of the CD36 gene (iii), CD36 protein synthesis and translocation to the cell membrane (iv). The increased expression of CD36 facilitates uptake of OxLDL and accumulation of cholesterol within the cells (v). The accumulated sterols induce a positive feedback loop for further cholesterol accumulation by enhancing expression many relevant genes, including those for scavenger and phagocytic receptors and for the transcription factors that regulate these genes (vi). The Plg dependence of this pathway for CD36 expression and foam cell formation can be bypassed by exogenous LTB4 (vii). This pathway shows the actions of Plg on a single cell but released LTB4 may affect adjacent cells.
Figure 1

[A] Oil red O staining of Plg+/+ cells and Plg-/- cells in the presence of Plg+/+ serum and Plg-/- serum.

[B] Graph showing the total cholesterol levels (μg/0.1 mg protein) of Plg+/+ and Plg-/- cells in the presence of Plg+/+ serum and Plg-/- serum. The difference is statistically significant (p<0.001).

[C] Oil red O staining of Plg+/+ cells in the presence of No serum, Plg+/+ serum, Plg-/- serum, and Plg-/- serum + Plg.

[D] Graph showing the total cholesterol levels (μg/0.1 mg protein) of Plg+/+ and Plg-/- cells in the presence of No serum, Plg+/+ serum, Plg-/- serum, and Plg-/- serum + Plg. The difference is statistically significant (p<0.001).
Figure 1, cont’d
Figure 2

[A] 
- a. Plg+/+ serum
- b. Plg +/+ serum + Aprotinin
- c. Plg mutant serum
- d. Plg mutatn serum + Exo Plm

[B] 
- Total cholesterol (μg/0.1 mg protein)
- *p<0.001
Figure 2, cont’d
Figure 3
Figure 3, cont’d
Figure 4
Figure 5
Figure 6
Figure 6, cont’d
Figure 7

[A] Serum $LTB_4$ (nM)

[B] Serum $LTB_4$ (nM)

[C] [D] Cholesterol

$\mu$g/0.1 mg protein

Plg+/+    Plg-/-

P=0.03

ApoE--/-- x Plg--/-

Plg+/+ serum + Plg

Plg--/- serum + Plg + $LTB_4$

Plg--/- serum + $LTB_4$

* p≤0.001

ns
Figure 7, cont'd

**Panel E:**

- **FITC-CD36 (relative fluorescence intensity)**

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<td></td>
</tr>
<tr>
<td>Plg-/- serum + Plg</td>
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<tr>
<td>Plg-/- serum + Plg + LTB4</td>
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* *p < 0.002

**Panel F:**

- **Media LTB₄ (nM/10⁶ cells)**

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<td>Plg-/- serum + Plg</td>
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<tr>
<td>Plg-/- serum + Plg + LTB4</td>
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</tbody>
</table>

p = 0.003

p = 0.04

p = 0.003

n.s.
Figure 8
Macrophage Gene Expression and Foam Cell Formation is Regulated by Plasminogen
Riku Das, Swetha Ganapathy, Ganapati H. Mahabeleshwar, Carla Drumm, Maria Febbraio, Mukesh Jain and Edward F. Plow

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Supplementary materials

Supplemental Material and Methods:

Ex vivo foam cell formation

Plg+/+ and Plg-/- mice were injected i.p. with 0.5 ml of 4% TG and blood (via inferior vena cava) and peritoneal cells (90% macrophages) were collected after 3 days. Serum was obtained by incubating the collected blood at 22°C for 15 min followed by centrifugation. Isolated peritoneal macrophages were incubated with 200 μM TXA followed by three washes with PBS. This step was performed to remove surface bound Plg from the macrophages and did not affect cell viability as assessed by trypan blue exclusion. Cells were plated onto 8-well chamber slides (Lab-Tek) or 12-well plates (Corning) in RPMI 1640. After 2 h, non-adherent cells were removed, and fresh medium with 10% serum derived from Plg+/+ or Plg-/- mice was added for 24 hr. Cells were then incubated with 50 μg/ml OxLDL (Biomedical Technologies, Inc, Stoughton, MA) for 24-48 hr in the presence or absence of various inhibitors. Throughout the course of these studies, similar results were obtained with at least 7 different lots of OxLDL. Cells in the 8-well chamber slides were fixed with 4% paraformaldehyde, stained with Oil-Red-O (ORO, Sigma), counterstained with hematoxylin QS (Vector laboratories) and mounted in VectaMount AQ (Vector Laboratories) for microscopic examination. Cells in 12-well plates were used to extract lipids and proteins as described previously. Briefly, total lipids were extracted from cells by adding hexane: isopropanol at a 3:2 ratio. The solvent was collected to measure total cholesterol using the Cholesterol/Cholesteryl Ester Quantification Kit II from Biovision, Milpitas; CA. Proteins were extracted from the
cells using 0.1 M NaOH and quantified by the Bradford method (BioRad). Values of total cholesterol were normalized to the total protein content of extracts.

**In vivo foam cell formation**

ApoE-/- or ApoE-/-Plg-/- mice, fed either CD or HCD, were injected i.p. with TG and peritoneal cells were collected after 3 days and allowed to adhere for 30 min in the 8-well chamber slides or 12-well plates. After removing non-adherent cells, the adherent macrophages were either stained with ORO or extracted to measure total cholesterol.

**In vivo transfer of macrophages**

This experiment was performed as described previously. TG-elicited peritoneal macrophages were isolated from male Plg+/+ donor mice and washed with TXA. A total of 12 x10^6 live cells were then injected i.p. into recipient male ApoE-/- or ApoE-/-Plg-/- mice, which had been maintained on either chow or HC diet for 6 weeks. After 3 days, peritoneal macrophages were collected and assessed for ORO staining and total cholesterol as described above.

**Foam cell formation in THP-1 and in human peripheral blood monocytes**

THP-1 cells were obtained from ATCC and cultured as described before. For foam cell formation assay, THP-1 cells were cultured in fibronectin-coated plastic wells (Calbiochem) and treated with 15 nM PMA on for 24 h in complete media. Cells were then washed and treated with OxLDL (50 μg/ml) for an additional 48 hr in either 10% FBS or 10% FBS depleted of Plg in the THP-1 medium. Cells were analyzed for foam
cell formation by either staining with Oil Red O or quantifying total cholesterol as described above.

To obtain HuPBM, peripheral blood was obtained from healthy donors using an informed consent form approved by Institutional Review Board of Cleveland Clinic. A portion of the blood was used to purify serum while the other portion was used to isolate monocytes. Monocytes were isolated using Ficol Hypaque (Amersham) followed by adherence to fibronectin coated plates (BD Bioscience). The adherent monocytes were cultured for 48 h in RPMI-1640 in either 10% autologous serum or Plg-depleted 10% autologous serum to obtain human peripheral blood derived macrophages. Cells were then treated with 50 \( \mu \text{g/ml} \) OxLDL for 24 h in presence of the autologous serum or autologous serum depleted of Plg. Peripheral blood was collected from three different donors and performed foam cell formation experiment independently.

**RAW264.7 cells culture and treatment.**

RAW264.7 cells were obtained from ATCC and cultured in DMEM containing 10% fetal bovine serum, 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1 mM sodium pyruvate. For experimental studies, the RAW264.7 cells were transferred to 1% Nutridoma-SP (Roche, Germany) in DMEM and cultured for 2 h. Cells were washed, added to fresh 1% Nutridoma medium and treated with either Glu-Plg (Enzyme Research, South Bend, IN) or MK-886, Ly39111, U75302 (Cayman, Ann Arbor, MI) and then treated with Plg for 24 h.
Real time RT-PCR

Total RNA was extracted from macrophages using RNeasy minikits (Qiagen) followed by digestion of genomic DNA using RNase free DNase 1 (Fermentas). A total of 1 μg RNA was transcribed into cDNA using iScript reverse transcriptase (BioRad) and a mixture of oligo (dT) and random primers in a total volume of 25 μl. Reverse transcribed RNA was primed with oligonucleotides specific for each of the genes (supplemental Table 1) to be analyzed. Quantitative PCR was performed in a 20 μl reaction volume containing 2 μl of a 10-fold diluted cDNA, 10 μl 2X SYBER Green PCR master mix (BioRad) and 0.5 pmol sense or antisense primers. The qPCR reactions were performed on optical 96-well strips with optical caps in the BioRad iCycler PCR system (Model: MyiQ2, BioRad). The same thermal profile conditions were used for all primers sets: 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All samples were analyzed in triplicate, and cyclophilin A content was used for normalization as previously described4.

Western blots

Cells were disrupted in lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). Lysates were analyzed by SDS-PAGE on 10% gels and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% BSA in TBS-T (Tris borate saline containing 0.1% Tween-20), probed with rat anti-mouse-CD36 (R&D) or rabbit anti-mouse CD36 (Novus Biologicals) followed by HRP-anti-rat or HRP- anti-rabbit
(Calbiochem) and subsequently developed with ECL detection kits (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The intensities of Western blot bands were measured by using Kodak ID 3.6 software, assigning the intensity of each band of interest in the unstimulated cells a value of 1.0; and the fold change of a particular protein was calculated relative to the corresponding control band.

**Flow Cytometry**

Cells were detached from plastic wells using enzyme-free dissociation buffer (Invitrogen), washed and Fc receptors blocked with seroblocker (AbD Serotec, Raleigh, NC). The extent of cell surface CD36 expression was measured by incubating cells with FITC-labeled rat anti-mouse CD36 antibodies (Cayman, Ann Arbor, MI). Cell fluorescence was measured using instruments from BD Bioscience (Bedford, MA) and Cell Quest software.

**OxLDL binding and internalization**

Macrophage binding and internalization of OxLDL were performed using OxLDL labeled with fluorescent probe, 1,1’-dioctadecyl-1 to 3,3,3’,3’-tetramethylindocarbocyanine perchlorate (Dil, Biomedical Technologies, inc) as described previously^5. After specified culture conditions, cells were extensively washed and incubated with different concentration of Dil-OxLDL in DMEM containing 2% lipoprotein-deficient human serum in absence or presence of 20 fold excess OxLDL. The extent of binding, measured after 30 min at 4°C or internalization, measured after 2 hr at 37°C was determined by flow cytometry. Specific mean fluorescence intensity (MFI)
values were obtained by subtracting the MFI of Dil-OxLDL + Excess OxLDL from MFI of Dil-OxLDL alone. The resulting specific binding values were used to construct ligand binding and internalization curves.

**Serum LTB4 measurement**

Serum was isolated from blood as described above and stored at -70°C until assay. LTB4 levels in serum were quantified using a kit from Cayman Chemical, Ann Arbor, MI, according to manufacturer’s protocol. The assay is based on a competition between the serum LTB4 and an LTB4-acetylcholineesterase conjugate for a limited amount of LTB4 antiserum coated on plastic wells.

**LTB4, Plg and LDL/VLDL measurement.**

These components were measured in 1.5 ml of 1X PBS used to lavage the peritoneal cavity of TG stimulated ApoE-/- and ApoE-/-Plg-/- mice fed with either CD or HFD. LTB4 levels in peritoneal fluid were measured using LTB4 measurement kits which are based on a competition between the serum LTB4 and an LTB4-acetylcholineesterase conjugate for a limited amount of LTB4 antiserum coated onto plastic wells. LDL/VLDL levels in peritoneal fluid were measured using a quantification kit from Biovision, Milpitas, CA. Plg levels in peritoneal wash was quantified by using an ELISA kit designed for detecting mouse Plg (American Diagnostica, GmbH).
Preparation of plasminogen-depleted fetal bovine serum

FBS was recycled three times through a lysine-Sepharose 4B column (GE Healthcare, Piscataway, NJ) and flow-through from the third pass was collected. A >70% depletion of Plg in flow-through serum was confirmed based on plasmin activity as measured using the S2251 chromogenic substrate S2251 and LMW uPA.

Statistical Analysis

A two-tailed t test was used in comparing two groups, and differences between multiple groups were evaluated using either a one-way ANOVA or a two-way ANOVA test followed by Tukey multiple comparison test. Normality of data was tested using Shapiro-Wilk test. These statistical analyses were performed using either SigmaPlot 12 software or R software (version 2.15.1, Vienna, Austria). Values are expressed as means ± SD, and p values of ≤0.05 were considered significant. Analysis for Figures 1[B], 6[B] and 7[B] were performed using two tailed t-tests. Analysis for Figure 1[D], 1[E], 2[B], 2[D], 3[E], 6[D], 7[D], 7[E], 7[F] and supplemental Figure S2&S3 were performed using a one-way ANOVA followed by Tukey multiple comparison test. Analysis for Figures 3[A]-3[D], 4[B] and 4[D] were performed using a two-way ANOVA followed by Tukey multiple comparison tests. All these analyses were performed using SigmaPlot version 12. Analysis for Figure 6[E] was performed with a combination of one-way and two-way ANOVA followed by Tukey multiple comparison tests. Figure 7[B] and Figure S4 were analyzed by one-way ANOVA followed by Tukey multiple comparison tests. All these analysis were performed using R software version 2.15.1.
Supplemental Tables

Supplemental Table 1. Primers

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Supplemental Table 2: Plasma Cholesterol levels

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<td>(±78)</td>
<td>(±113)</td>
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Values are expressed as mean ± SD, n=5.

*p<0.01 vs. ApoE-/-.
Supplemental Figure legends.

Figure S1. ORO staining to detect formation of foam-like cells by THP-1 (panel A, at original 20X magnification) and RAW 264.7 cells (panel B, at original 20X magnification) (A) Depletion of Plg from fetal bovine serum (FBS) impairs OxLDL mediated foam cell formation by differentiated (PMA, 15 nM) THP-1 cells. Addition of exogenous Plg (1 μM) restores the foam cell phenotype associated with Plg depletion. (B) Plg (0.5 to 1 μM) induces OxLDL mediated foam cells by RAW264.7 cells cultured in 1 % Nutridoma (Roche) in DMEM for 24 hr.

Figure S2. Plasminogen regulates CD36 expression by activating 5-LO pathway.

Panels A&B: Western blot analysis for CD36 protein (upper panels in RAW264.7 cells treated with either Plg or with the 5-LO inhibitor, MK886, and treated with Plg (1 μM) in DMEM supplemented with 1% Nutridoma. Some cells were treated with LTB4 (500 nM) or with Plg + LTB4. Western blot of actin was used as a loading control (lower panels in A&B). Each dot in lower panels of A&B is one of three replicate fold values. Fold values are derived from 3 sets of blots from 3 independent experiments. Error bars are the SD of the means. In panel A CD36 protein was detected by an antibody obtained from R&D. In this panel, bands lower than expected molecular weight of CD36 show the similar pattern of changes as CD36 bands.

CD36 protein in panel B was detected by an antibody derived from Novus Biologicals.
**Figure S3. Plg mediated CD36 expression is regulated via LTB4 receptor, BLT1.**

Western blot analysis for CD36 protein (upper panels) in RAW264.7 cells treated either with Plg or with the BLT1 blockers, Ly39111 or U75302 (Cayman Chemicals) and then treated with Plg (1 μM) in DMEM supplemented with 1% Nutridoma. Western blot of actin was used as a loading control. CD36 was detected by an antibody derived from Novus Biologicals. Each dot in lower panel is one of three replicate fold values. Fold values are derived from 3 sets of blots from 3 independent experiments. Error bars are the SD of the means.

**Figure S4. Plg mediated CD36 expression is mediated by LTB4 but not LTE4.**

Western blot analysis for CD36 protein in RAW264.7 cells treated with Plg (1 μM), LTB4, or LTE4 in DMEM supplemented with 1% Nutridoma. Actin was used as a loading control in the Western blots. Data are representative of 3 independent experiments. CD36 was detected by an antibody derived from Novus Biologicals. Each dot in lower panels of A&B is one of three replicate fold values. Fold values are derived from 3 sets of blots from 3 independent experiments. Error bars are the SD of the means.
Supplemental Reference List


Figure S1

[A]

- FBS + OxLDL
- Plg-depleted FBS + OxLDL
- Plg-depleted FBS + Glu-Plg + OxLDL

[B]

- No OxLDL
- OxLDL
- OxLDL + 0.5 μM Plg
- OxLDL + 1 μM Plg
Figure S2

**Figure S2**

Panel A: Graph showing the change in CD36 expression fold over untreated with different concentrations of Plg. The graph indicates a significant increase in CD36 expression with p-value of 0.003.

Panel B: Graph showing the change in CD36 expression fold over untreated with different treatments of MK886 and LTB4. The graph indicates significant differences with p-values of less than 0.001 for some comparisons.

**Legend:**
- **CD36** and **β-actin** are indicated on the western blot images.
- **p** values are shown on the graphs.
**Figure S3**

- CD36
- β-actin

<table>
<thead>
<tr>
<th>Plg</th>
<th>Ly39111 (nM)</th>
<th>U75302 (μM)</th>
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* *p ≤ 0.002

CD36 (Band Intensity-Fold over untreated)
Figure S4

CD36 (Band Intensity-Fold over untreated)

Pig - + + + + -
LTB4 (nM) - - 250 500 - -
LTE4 (nM) - - - - 250 500

* p<0.001
ns