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[1]. 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[2]. 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 [2]. 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[3]. 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 theTHP-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 μg/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^{o}C for 2 minutes, 95^{o}C for 10 minutes, and then 40 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 minute. All samples were analyzed in triplicate, and cyclophilin A content was used for normalization as previously described[4].

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^{o}C or internalization, measured after 2 hr at 37 ^{o}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^{o}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[6].

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

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
CD36	CAGCAAGGCCAGATATCACA	TGCAGCTGAGCAGAAAGAGA
LDLR	GAAAAGGCTACTGGCTGTGC	CCAGGACCCGGTCAGTAGTA
ABCA1	TTGTTCCAAAGAGCCATGTG	GCTTGGAATGAGGGCCAATG
CD68	AGGGTGGAAGAAAGGTAAAGC	AGAGCAGGTCAAGGTGAACAG
MSR-A	ATGATCGCTGGGATATACGG	ACCCCAGCATCTTCTGAATG
PSR1	TCTGCTCAATGCACAAGAGG	TGGGCACCTTGTAGTCTTCC
PPAR-γ	ACATAAAGTCCTTCCCGCTGACCA	AAATTCGGATGGCCACCTCTTTGC
CEBP α	AAACAACGCAACGTGGAGA	GCGGTCATTGTCACTGGTC
CEBP β	TGATGCAATCCGGATCAA	CACGTGTGTTGCGTCAGTC
FcγRI	GCAAGTTAGAAGCGATGGCG	TGGGGGTATCTGGACCTGAG
FcγRII	GGAAGGGGAAACCATCACG	CAGAGGGCTGTCTGTACTC
SRB1	CAGTAGTTCTGCCGTTGCTG	TGAATGGCCTCCTTATCCTG
TR4	CAGCCATTGTCAACCACCTA	TTGCTTTATCCGGTCACCA
Cyclophilin	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT

Supplemental Table 2: Plasma Cholesterol levels

Genotype	Weight (gm)		LDL/VLDL (mg/dl)		HDL (mg/dl)	
	CD	HCD	CD	HCD	CD	HCD
ApoE-/-	25-30	30-32	259.4 (±20)	1085 (±146)	52.6 (±6.5)	40.3 (±6)
ApoE-/-Plg-/-	22-27	24-27	346.2 (±78)	832.3 (±113)	20.7* (±6)	27.5 (±3)*

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

(1) Robinet P, Wang Z, Hazen SL, Smith JD. A simple and sensitive enzymatic method for cholesterol quantification in macrophages and foam cells. *J Lipid Res.* 2010;51:3364-3369.

(2) Rahaman SO, Lennon DJ, Febbraio M, Podrez EA, Hazen SL, Silverstein RL. A CD36-dependent signaling cascade is necessary for macrophage foam cell formation. *Cell Metab.* 2006;4:211-221.

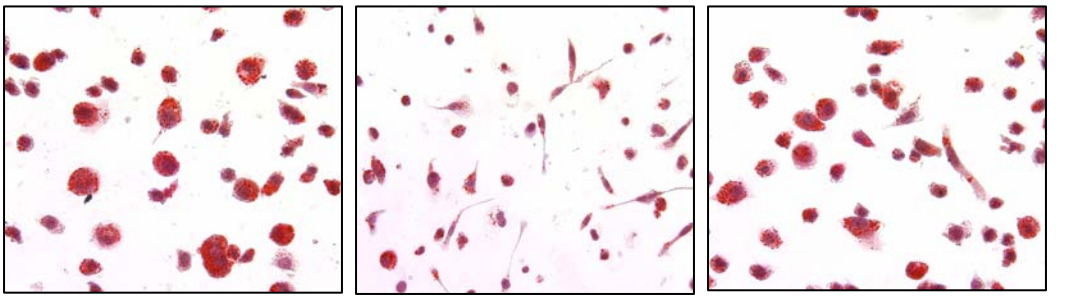
(3) Das R, Burke T, Van Wagoner DR, Plow EF. L-type calcium channel blockers exert an antiinflammatory effect by suppressing expression of plasminogen receptors on macrophages. *Circ Res.* 2009;105:167-175.

(4) Szatmari I, Pap A, Ruhl R et al. PPARgamma controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. *J Exp Med.* 2006;203:2351-2362.

(5) Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, Matsuzawa Y. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation.* 2001;103:1057-1063.

(6) Das R, Burke T, Plow EF. Histone H2B as a functionally important plasminogen receptor on macrophages. *Blood.* 2007;110:3763-3772.

[A]

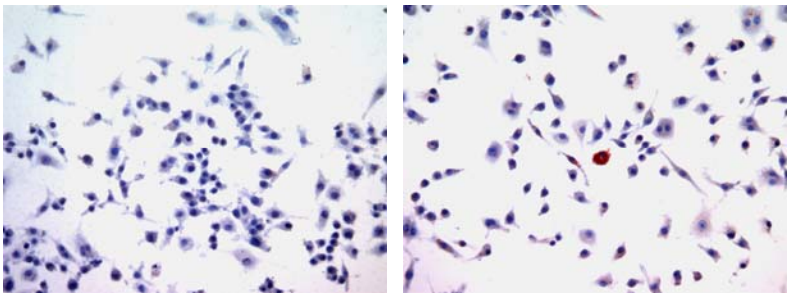


FBS + OxLDL

Plg- depleted FBS + OxLDL

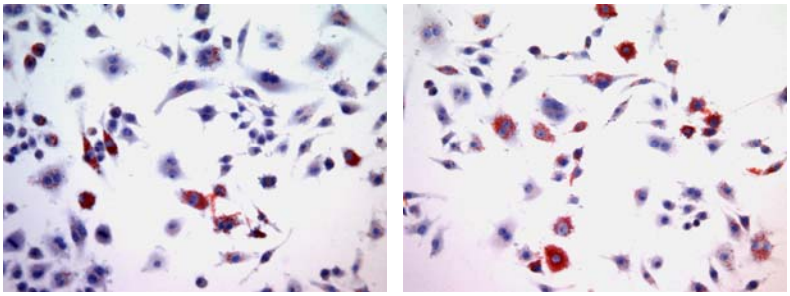
Plg-depleted FBS + Glu-Plg + OxLDL

[B]



No OxLDL

OxLDL



OxLDL + 0.5 μM Plg

OxLDL + 1 μM Plg

Figure S1

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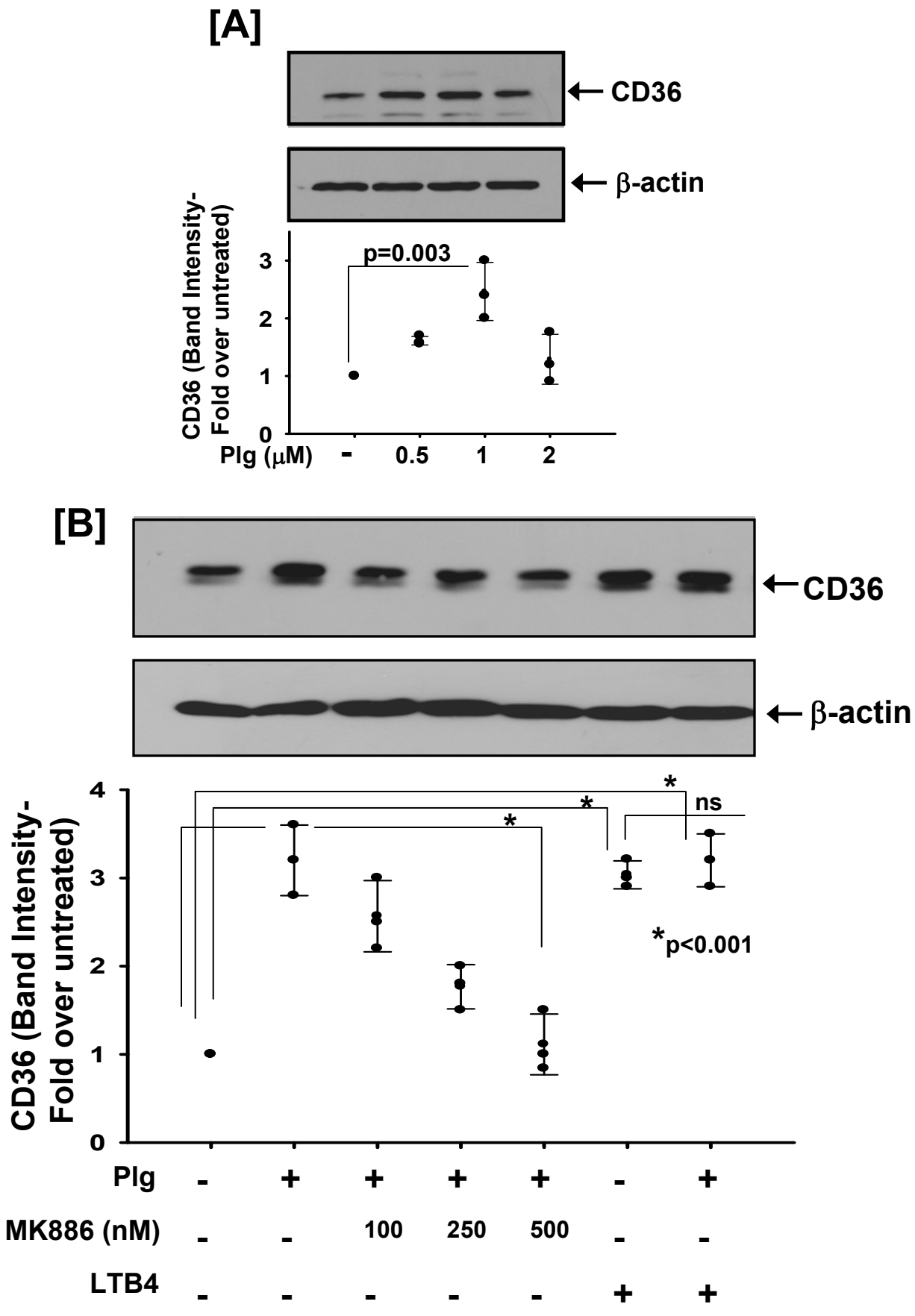

Figure S2 CIRCULATIONAHA/2012/121871 (Supplem.): R14

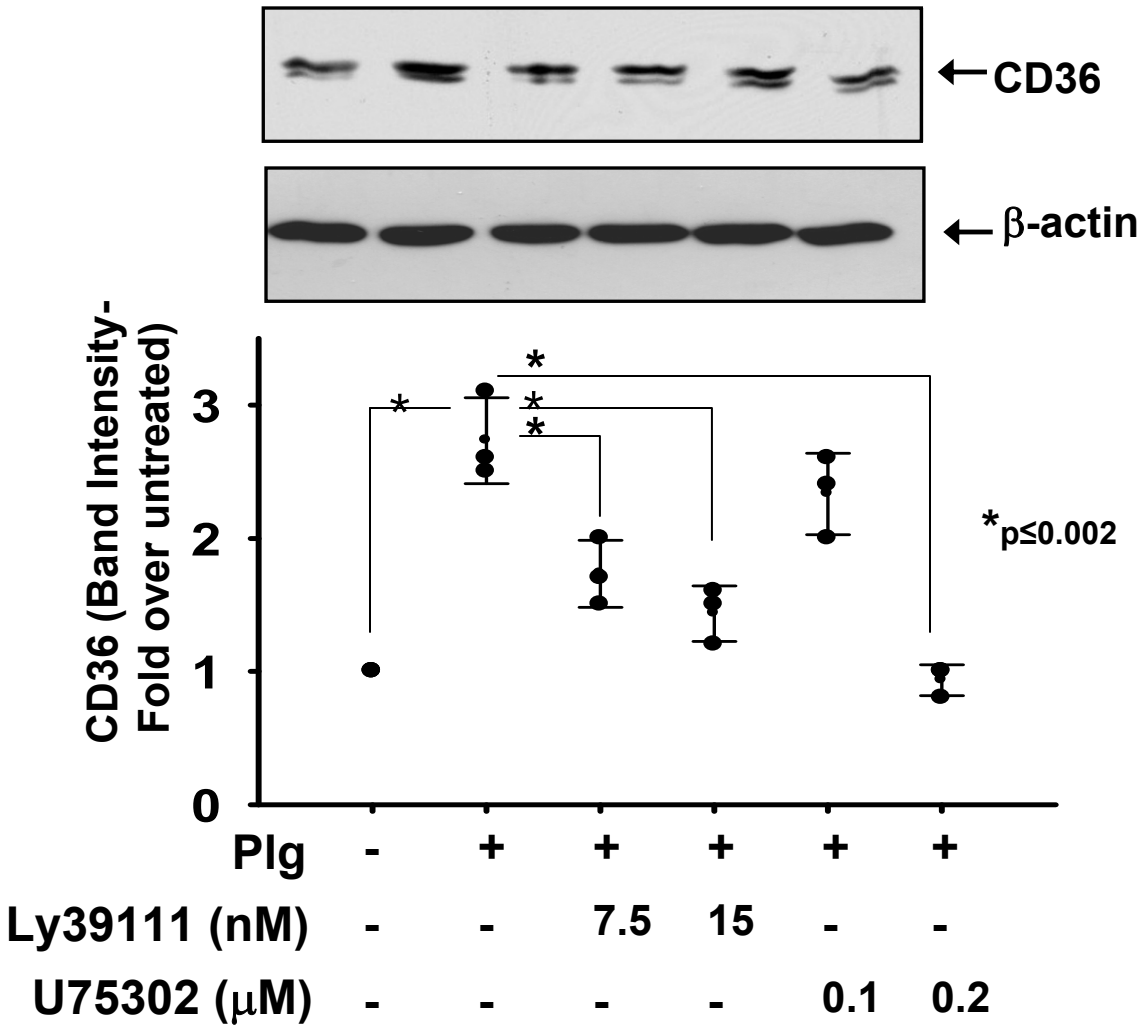

Figure S3

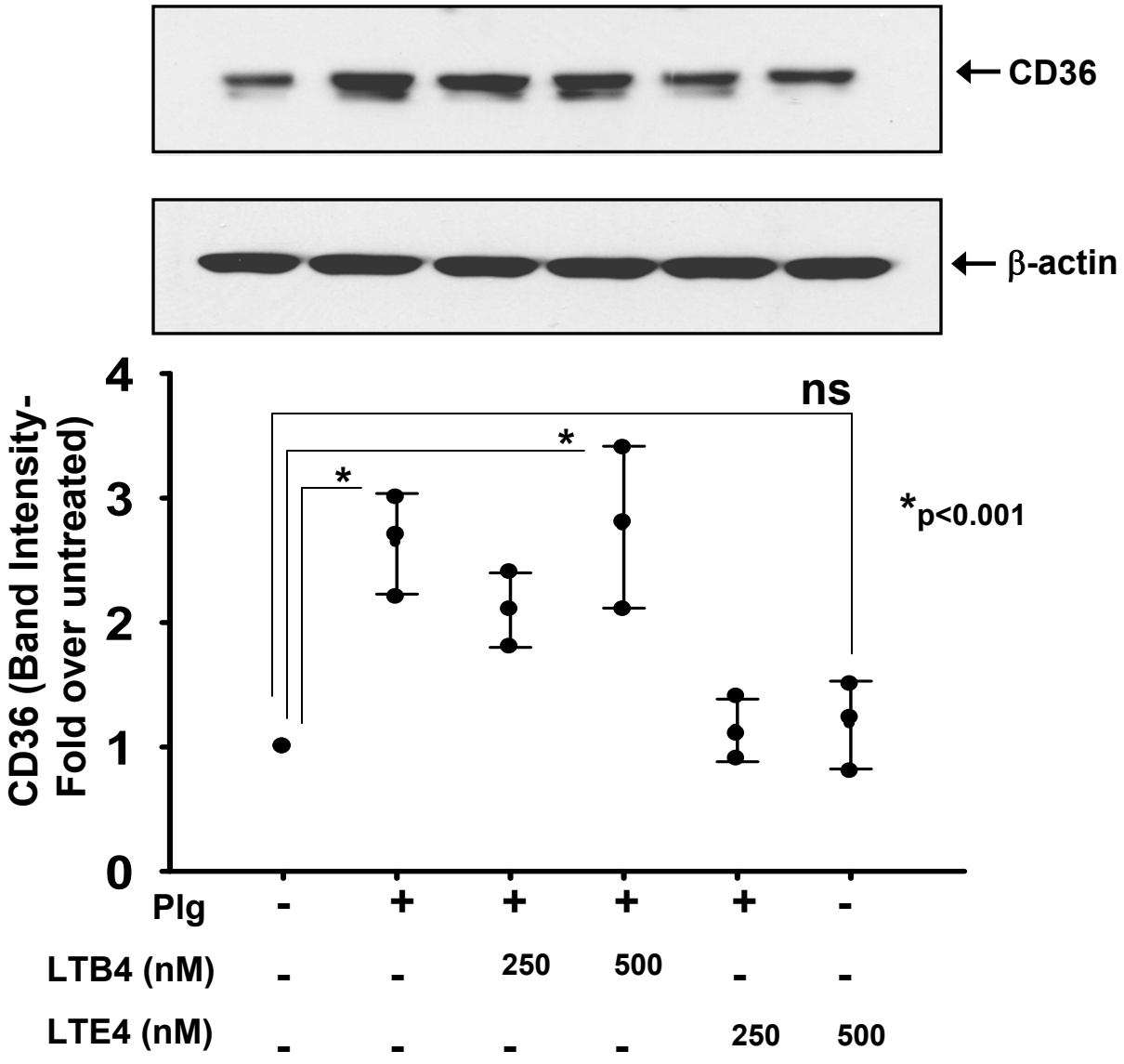

Figure S4