Materials and Methods

CD36 blocking antibody (clone FA6-152) was from Abcam. Isotype-matched control IgG was from Jackson Immunoresearch Laboratory. Anti-Fcγ receptor IIa antibody clone IV.3 was generated from the hybridoma cell line from the American Type Culture Collection (Manassas, VA) as previously described (27). Antibody to β-actin (clone AC-15), PEG-Catalase, Sepharose 2B, and Cyclosporin A were from Sigma-Aldrich. Anti-caspase-3 polyclonal antibody was from Cell Signaling Technology. The broad spectrum Src inhibitor PP2 and control PP3 were from CalBiochem. Small molecule MEK5/ERK5 inhibitors BIX 02188 and XMD8-92 were from ApexBio Technology. Tissue Factor was from Innovin. Fluorophore-labeled Annexin V and BAPTA-AM were from ThermoFisher. Thrombin was from Chrono-Log. Convulxin was from Enzyme Research Laboratories. ABT-737 was from Santa Cruz Biotechnology. Caspase-3 Activity Assay, Z-VAD-FMK, and Z-DEVD-FMK were from R&D Biosolution. FITC-labeled lactadherin was from Haematologic Technologies. GPRP was from GenScript. DyLight488-labeled anti-GPIb antibody was from Emfret. Atherogenic Western High Fat Diet was from Harlan Teklad (catalog no. 88137).

Native LDL was oxidized by copper sulfate as previously described (9,10). For human platelets, 24-hour LDL oxidized in 5 μM copper sulfate with conjugated diene ratios of 2.5:1 (oxLDL:LDL) were used. Since mouse platelets are less sensitive to activation by oxidized lipids (2), LDL oxidized for 24 hour in 10 μM copper sulfate with conjugated diene ratios of 2.7-3.0:1 were used.

Platelet Preparation

Mice were anesthetized with ketamine and xylazine (100 mg/kg and 20 mg/kg, respectively) and whole blood was drawn from the inferior vena cava into 100 μL of 3.8% sodium citrate. Citrated whole blood was then diluted by adding Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5 mM HEPES) containing 0.1% (w/v) glucose at 70% of the volume of whole blood. PRP was obtained by centrifugation at 100g for 5 min at room temperature. Platelets were gel purified by pouring PRP into a Sepharose 2B column and then were re-suspended in buffer to final concentration of 300,000/μL. Human platelets were isolated and washed by centrifugation as previously described (10).

Detecting PSer Surface Exposure

Gel-filtered mouse platelets were stimulated with oxLDL up to 30 min at 37°C then rapidly stained with fluorophore-conjugated Annexin V for 2 minutes followed by fixation with 2% paraformaldehyde. Samples were analyzed immediately by flow cytometry.

Caspase Activity Assay

Platelets isolated from healthy human donors were washed by centrifugation, concentrated to 600,000/μL, and rested up to 1 hour. 1 mM Ca^{2+}/Mg^{2+} were added before incubating with 50 μg/mL LDL or oxLDL, or 50 nM ABT-737 for 1 hour at 37°C; 0.1 U/mL thrombin plus 500 ng/mL convulxin for 7 minutes at 37°C were used to maximally stimulate PSer exposure. ABT-737-stimulated conditions did not include 1 mM Ca^{2+}/Mg^{2+}. In some experiments, washed platelets were pre-incubated with 1 μg/mL of non-immunizing IgG or anti-CD36 blocking antibody (FA6),10 μM BIX02188, or DMSO for 15 minutes prior to stimulating with lipoproteins. Stimulated platelets were lysed with a buffer provided by the Caspase 3 Activity Kit (R&D Biosolution). Protein

levels were normalized between samples. Caspase-3 colorimetric substrate (DEVD-pNa) were added to the samples followed by continuous absorbance reading of 405nm at 37°C up to 2 hours.

Immunoblotting

Platelet samples were lysed with a buffer provided by the caspase 3 activity assay kit. 40 μg of protein was loaded on a 12% SDS PAGE gel followed by standard western blot analysis.

In vivo Arterial Thrombosis Microscopy

For the laser-induced thrombosis model, the mice were anesthetized with ketamine (100 mg/kg) and dexdomitor (1 mg/kg) and the body temperature was maintained at 37°C using a thermoregulated heating pad.

In vivo fibrin accumulation was also studied in a large artery model in which thrombosis was induced by exposing flowing blood to adventitial connective tissue. In this model a segment of epigastric artery is exteriorized and then transplanted into the carotid artery as previously described (10,31).

Statistical Analysis

The data were analyzed by either one- or two-way ANOVA with Tukey's or Dunnett's post-hoc analysis. A *P*-value of less than 0.05 is considered statistically significant.

Supplemental Figure Legends

Supplemental Figure 1. Impact of oxLDL on mitochondrial membrane potential, caspase-dependent PSer externalization by CVX and oxLDL, and fluorescence intensity of oxLDL-sensitized PSer externalization by CVX/GPVI. Mitochondrial membrane potential measured by a loss of tetramethylrhodamine methylester (TMRM) was analyzed by flow cytometry to detect mitochondrial polarity after platelets were stimulated with PBS, 50 μg/mL LDL or oxLDL, and 0.1 U/mL thrombin with 500 ng/mL CVX for 15 min (A). In (B), PSer externalization measured by Annexin V binding was analyzed by flow cytometry after platelets were treated with 5 μM CsA or 100 μM Z-VAD-FMK followed by stimulation with 500 ng/mL CVX for 7 min (n of 3 separate donors). In (C), human platelets were pre-treated with 10 μM PP3 or PP2, followed by activation with 500 ng/mL CVX for 5 min (n of 3 separate donors). In (D), human platelets were pretreated with 100 μM Z-DEVD-FMK. Platelets were then sensitized with oxLDL up to 30 minutes followed by 5 minutes of GPVI activation by 500 ng/mL CVX (n of 3 separate donors). In (E), platelets were stimulated as in Figure 3D. Median fluorescence intensity were plotted. Data represented as mean ± SEM.

Supplemental Figure 2. Video microscopy images of the adventitial tissue-mediated thrombosis model. Video microscopy images of fibrin accumulation in Figure 6D-E.

Table S1. Curve fitting quantification of fibrin formation. Human platelets were treated according to Figure 5. The curves of fibrin formation were fitted using a two-site

competition linear regression model. The onset time of fibrin formation was indicated by a change of 5% in relative absorbance of the fitted curve from the baseline. The peak fibrin formation was indicated by the maximal relative absorbance of the fitted curve. In the case where there was no fit with the two-site competition linear regression model, greater than 20 minutes was assigned to that reaction. The averages of each curve for onset time or peak fibrin formation are shown with their respective ± SEM.

Supplemental Videos

Supplementary Videos 1-4: Representative videos of laser-induced cremasteric artery thrombosis from Figure 6A-C. ApoE null or apoE:CD36 double null mice were fed on control standard chow diet or a high fat diet for at least 6 weeks. Platelets were labeled with anti-GPIbβ-DyLight488 antibody and fibrin were labeled with anti-fibrin-AlexaFluor 647 antibody (clone 59D8).

Video 1. High fat diet-fed apoE null mice.

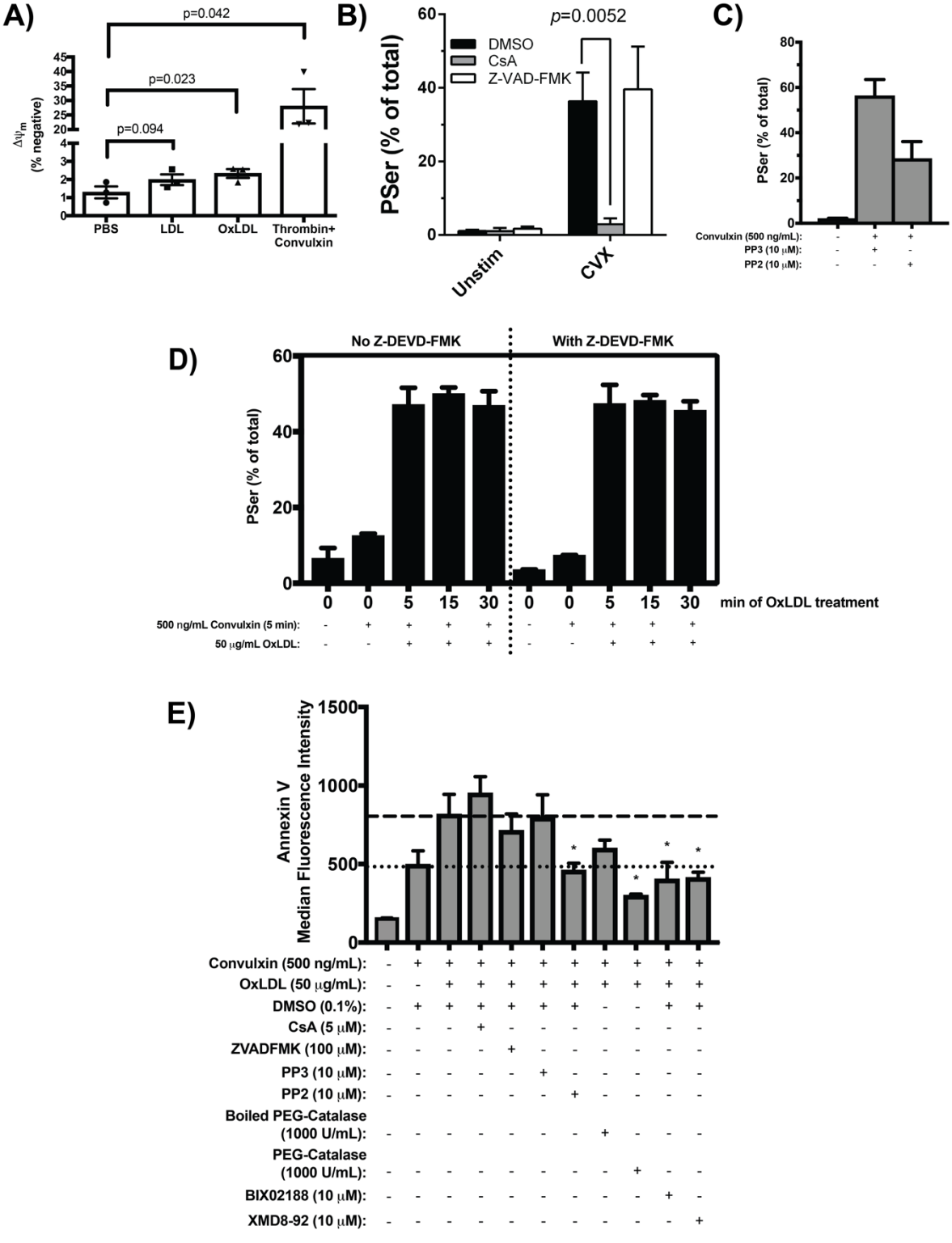
Video 2. High fat diet-fed apoE:CD36 double null mice.

Video 3. Control diet-fed apoE null mice.

Video 4. Control diet-fed apoE:CD36 double null mice.

Table 1. Curve fitting quantification of fibrin formation.					
Figure 4	Reaction	Average Onset Time of Fitted Curve(min)	SEM	Average Peak Fibrin Formation of Fitted Curve (arbitrary absorbance)	SEM
A	PBS	No Fit - Greater than 20 min		No Fit - Greater than 20 min	
	THR/CVX	2.96	0.36	1.52	0.17
	2mM GPRP+THR/CVX	No Fit - Greater than 20 min		No Fit - Greater than 20 min	
	Annexin V+ THR/CVX	No Fit - Greater than 20 min		No Fit - Greater than 20 min	
B	PBS	No Fit - Greater than 20 min		No Fit - Greater than 20 min	
	LDL	No Fit - Greater than 20 min		No Fit - Greater than 20 min	
	OxLDL	No Fit - Greater than 20 min		No Fit - Greater than 20 min	
C	CVX	5.60	0.06	1.10	0.25
	OxLDL+CVX	3.40	0.13	1.30	0.09
D	IgG+CVX	7.45	0.19	1.08	0.04
	IgG+OxLDL+CVX	5.33	0.30	1.11	0.03
	FA6+OxLDL+CVX	6.88	0.09	0.99	0.01
E	DMSO+CVX	6.07	1.06	1.06	0.04
	DMSO+OxLDL+CVX	1.89	0.26	1.26	0.04
	BIX02188+OxLDL+CVX	4.68	0.55	0.96	0.03
	XMD8-92+OxLDL+CVX	4.19	1.34	1.02	0.03
F	CVX	2.96	1.70	0.99	0.06
	OxLDL+CVX	2.23	0.65	1.20	0.04
	THR/CVX	1.92	0.50	1.60	0.04
	ZVADFMK+CVX	No Fit - Greater than 20 min		No Fit - Greater than 20 min	
	ZVADFMK+OxLDL+CVX	5.66	3.2	1	0.045
	ZVADFMK+THR/CVX	3.53	2	1.6	0.071

Supplemental Figure 1



Supplemental Figure 2