A Specific CD36-Dependent Signaling Pathway Is Required for Platelet Activation by Oxidized Low-Density Lipoprotein

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Abstract—Platelet hyperactivity associated with hyperlipidemia may contribute to development of a prothrombotic state. We previously showed that oxidized low-density lipoprotein (oxLDL) formed in the setting of hyperlipidemia and atherosclerosis activated platelets in a CD36-dependent manner. We now show that mitogen-activated protein kinase c-Jun N-terminal kinase (JNK)2 and its upstream activator MKK4 were phosphorylated in platelets exposed to oxLDL. Using *apoE*$^{-/-}$ mice as a model of hyperlipidemia, we showed that JNK was constitutively phosphorylated in platelets in a CD36-dependent manner. Inhibition of src kinase activity reduced JNK phosphorylation by oxLDL. Immunoprecipitations revealed that active phosphorylated forms of src kinases Fyn and Lyn were recruited to CD36 in platelets exposed to oxLDL. Pharmacological inhibition of the mitogen-activated protein kinase JNK or src family kinases abolished platelet activation by oxLDL in vitro. Using a murine carotid artery thrombosis model we demonstrated CD36-dependent phosphorylation of platelet JNK within thrombi. Furthermore, pharmacological inhibition of JNK prolonged thrombosis times in wild-type but not *cd36*-null mice in vivo. These findings suggest that a specific CD36-dependent signaling pathway is required for platelet activation by oxLDL and may provide insights related to development of novel antiplatelet therapies more relevant to atherothrombosis than to normal hemostasis. **(*Circ Res*. 2008;102:1512-1519.)**

Key Words: CD36 ■ JNK ■ thrombosis ■ hyperlipidemia

CD36 is an integral membrane protein expressed on monocytes/macrophages,[1] platelets,[2] microvascular endothelium,[1] fat, and muscle.[3,4] Although initially identified as a receptor for thrombospondin-1 and malaria-infected erythrocytes,[5,6] it is now known to be a class B scavenger receptor that recognizes several unrelated ligands, including thrombospondin-1 and -2,[7,8] oxidized phospholipids expressed on oxidized low-density lipoprotein (oxLDL) and apoptotic cell surfaces,[9,10] long chain fatty acids,[11] amyloidogenic peptides,[12] and specific components of microbial cell walls or cell surfaces.[13]

CD36 is involved in a variety of biological processes including lipid metabolism, inflammation, atherosclerosis, and angiogenesis, depending on the nature of the ligand to which it is exposed and the cell or tissue type on which it is expressed.[14] Although CD36 was first isolated and structurally characterized from platelets,[2] its functional role on platelets remains incompletely characterized. OKM5, a monoclonal antibody directed against CD36, was observed many years ago to induce platelet activation and aggregation.[15] Although the effect was dependent on expression of Fc receptors, it could be blocked by F(ab′)$_2$ fragments of the antibody, suggesting that the CD36 epitope was required and that CD36 may transduce platelet activating signals. Many other CD36 monoclonal antibodies have also been shown to have stimulatory effects on platelets.[16] Our group in collaboration with others recently showed that platelets bind oxLDL via CD36 and this interaction leads to platelet activation, contributing to a prothrombotic state in the setting of hyperlipidemia.[17] However, the mechanisms by which interactions between CD36 and its ligands activate platelets remain unknown.

It is now well established that despite having very short intracytoplasmic domains, CD36 can serve as a signaling molecule. Antibodies to platelet CD36 were shown to coprecipitate the nonreceptor protein tyrosine kinases Fyn, Lyn, and Yes.[18] Studies in other cellular systems have linked the signaling function of CD36 to recruitment/activation of src family kinases and activation of specific mitogen-activated protein (MAP) kinases. For example, on microvascular endothelial cells, thrombospondin-1 induces a CD36-dependent antiangiogenic, proapoptotic signal via activation of Fyn, caspase-3, and p38 MAP kinase.[19] On macrophages, exposure to oxLDL leads to recruitment of Lyn and activation of c-Jun N-terminal kinase (JNK)2 in a CD36-dependent manner. Inhibition of JNK resulted in significant reduction in uptake of oxLDL and foam cell formation.[20] These studies suggest a context-dependent mechanism for CD36 signaling involving

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specific src and MAP kinases. In platelet biology, MAP kinases have not been studied in detail, although it has been demonstrated that the $P2Y_1$ ADP receptor activates p38 MAP kinase, and p38-deficient mice have prolonged thrombotic occlusion time in a ferric chloride ($FeCl_3$)-induced thrombosis model.[21,22] We, thus, hypothesized that CD36-mediated platelet activation might involve specific members of the src and MAP kinase families.

CD36 recognizes a variety of pathological ligands including oxLDL,[23] advanced glycation end products,[24,25] apoptotic cells,[26,27] and cell-derived microparticles.[28] We focused on oxLDL because of its essential role in the pathogenesis of atherosclerosis and the known association of oxidative stress, hyperlipidemia, and a prothrombotic phenotype.[17,29,30] In studies outlined here, we identified a CD36-dependent signaling cascade responsible for oxLDL-dependent activation of platelets that includes the src kinases Fyn and Lyn, the upstream MAP kinase kinase (MKK)4, and the MAP kinase JNK2. These data indicate that a CD36-dependent signaling pathway is required for activation of platelets by oxLDL and shed new light on the mechanism of platelet hypersensitivity in the setting of atherosclerosis and/or hyperlipidemia.

Materials and Methods

All human studies were carried out in accordance with Cleveland Clinic IRB and with informed consent from each donor. All animal studies were carried out with prior approval by the Cleveland Clinic IACUC, and mice were cared for in a facility fully accredited by AALAC and in accordance with all federal and local regulations.

Native LDL (nLDL) and oxLDL were prepared as described previously.[20] Whole blood was collected from healthy human volunteers in 0.109 mol/L sodium citrate (1:9 dilution), and platelets were separated by sedimentation, washed, and resuspended in modified Tyrode's buffer. $CaCl_2$ and $MgCl_2$ were added immediately before platelets were stimulated with various agonists and the activated platelets were analyzed by flow cytometry. Human or murine platelets were lysed, and 40 to 60 μg of lysate protein was used for immunoblotting analysis of phosphorylated JNK, total JNK, phospho-MKK4, and MKK4. In some studies, precleared lysates containing 500 μg protein were incubated with protein A–agarose beads conjugated to anti-Lyn or anti-CD36 IgG overnight at 4°C. Beads were washed, boiled in 2× SDS-PAGE loading buffer, and bound material was analyzed by immunoblotting.

Blood from wild-type (WT), *apoE*-null, or *apoE/cd36* double-null mice maintained on chow or high-fat diet for 3 months beginning at 6 weeks of age was obtained by cardiac puncture after animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Platelets in platelet-rich plasma were allowed to adhere to silanized slides (DAKO, Carpinteria, Calif), and then double immunocytofluorescence staining was performed using antibodies to phospho-JNK and CD41. Images were obtained with a laser confocal microscope. In some studies, platelets were lysed and analyzed by immunoblot for JNK activation. Li-Cor Odyssey infrared imaging system was used for signal detection and quantification.

Carotid artery thrombosis was induced in 12-week-old male WT and cd36-null mice by topical application of 12.5% ferric chloride as previously described.[17] After the vessel was allowed to become completely occluded, the carotid arteries were removed, sectioned (4 to 6 μm) across the thrombi, and analyzed by immunohistochemistry using an antibody to phospho-JNK. For quantification, images were scored on the basis of staining intensity by a blinded observer (0 indicated negative; 1, weak; 2, moderate; 3, intense).[31] In some studies, thrombi were dissected away from the vessel wall and pooled (9 per group), and total protein was extracted for analysis by immunoblot.

To study the effect of JNK in thrombosis in vivo, WT or *CD36*-null mice were exposed to 11Gy of external beam irradiation from a cesium 137 source to induce thrombocytopenia with platelet counts <5% of normal after 5 days.[17] Platelets obtained from syngeneic donor mice were labeled with calcein acetoxymethyl ester (final concentration of 0.5 mg/mL) in the presence or absence of the JNK inhibitor SP6000125 (final concentration of 400 nmol/L for 30 minutes). This dose is the minimum active dose and was chosen to avoid potential off-target effects. A total of 2×10^9 donor platelets were injected through the jugular vein of thrombocytopenic mice 10 minutes before carotid injury with 12.5% $FeCl_3$ to allow the transfused platelets to reach equilibrium in the circulation. For further details, see the online data supplement, available at http://circres.ahajournal.org.

Results

oxLDL Induces Phosphorylation of JNK2 and MKK4 in Platelets

Previously, we showed that oxidized LDL formed in the setting of hyperlipidemia and oxidant stress binds platelets and promotes platelet activation via CD36 (also see Figure I in the online data supplement)[17]; however, the underlying mechanism by which platelet CD36 transduces signals and contributes to platelet activation is unknown. Because CD36 signaling in a variety of cell types involves MAP kinases, and we recently showed that the specific MAP kinase JNK was a key component of a macrophage CD36-initiated signaling cascade induced by oxLDL,[20] we investigated whether JNK was involved in platelet CD36 signaling induced by oxLDL. We assessed the phosphorylation state of JNK by immunoblot and found that human platelets exposed to oxLDL (50 μg/mL) but not nLDL, had a 3- to 5-fold increase in phosphorylation of JNK2 and its upstream activator MKK4 (Figure 1A). Phosphorylation was time-dependent (Figure 1B), with detectable levels after 1 minute and reaching maximum by 15 minutes. At these time points, neither nLDL nor buffer alone effected JNK phosphorylation (data not shown). JNK phosphorylation was also concentration-dependent (Figure 1C). Concentrations as low as 5 μg/mL were effective at activating JNK, with a maximal effect seen at 25 μg/mL. Treatment of platelets with an RGDS peptide (1 mmol/L) to block fibrinogen binding, or the ADP scavenger apyrase (0.5 U/mL), did not block JNK activation by oxLDL, indicating that phosphorylation was not dependent on outside-in integrin signals or secreted ADP (Figure 1D).

Elevated Levels of Platelet JNK Phosphorylation In Vivo Are Associated With Hyperlipidemia and Are CD36-Dependent

We recently identified specific oxidized phospholipids in oxLDL that serve as high-affinity ligands for CD36.[9] These oxidized lipids are present in the plasma of "western diet"–fed mice rendered hyperlipidemic by genetic deletion of *apoE* and transduce prothrombotic signals in a CD36-dependent manner.[17] To characterize these signals, we isolated platelet-rich plasma from WT, $apoE^{-/-}$, and $apoE^{-/-}$;$cd36^{-/-}$ mice fed normal chow or high-fat western diets and examined the platelets by immunofluorescence microscopy for the presence of phospho-JNK. As shown in Figure 2A and 2B, there was minimal phospho-JNK2 expression in resting platelets from WT mice fed a chow diet. Platelets from $apoE^{-/-}$ mice on

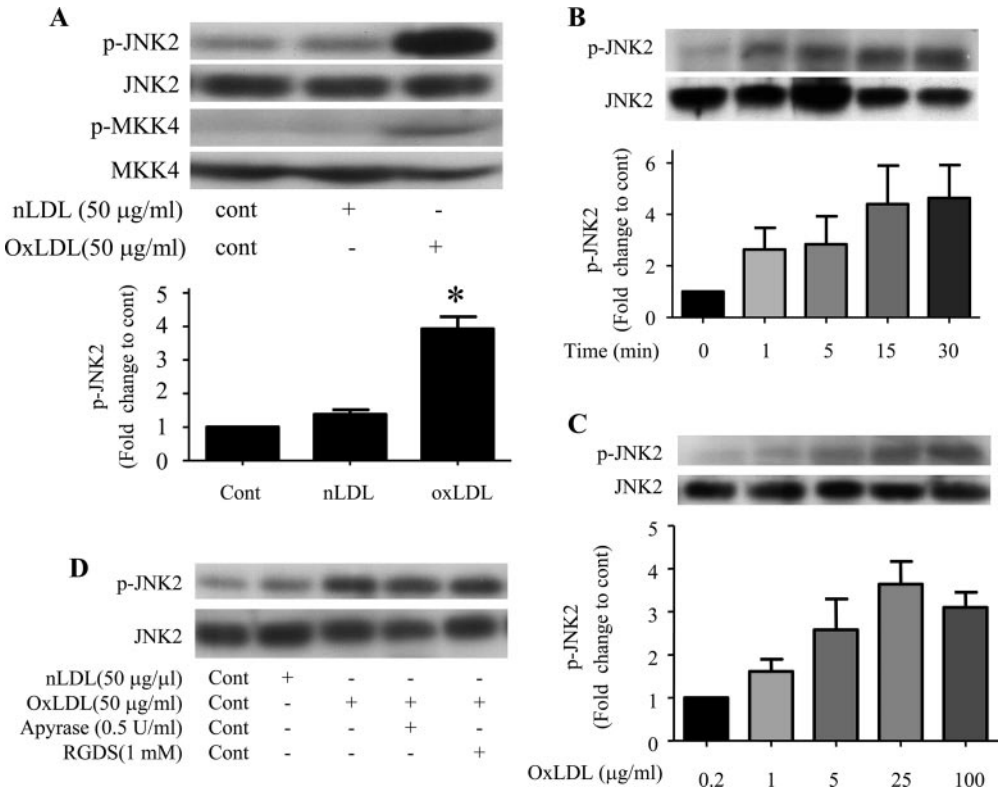

Figure 1. oxLDL induces phosphorylation of JNK2 and MKK4 in platelets. Washed human platelets (2×10^8 /mL) containing 2 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ were incubated with nLDL or various concentrations of oxLDL over various time points and then lysed. The lysates were analyzed by immunoblot with antibodies specific for phospho-JNK (p-JNK2) (A through C) and phospho-MKK4 (p-MKK4) (A). The membranes were then stripped and reprobed with antibodies to the total relevant proteins to normalize the protein loaded. D, Platelets were incubated with 50 μg/mL nLDL (lane 2) or oxLDL with PBS (lane 3), 0.5 U/mL apyrase (lane 4), or 1 mmol/L RGDS (lane 5) for 5 minutes and then lysed. The lysates were analyzed by immunoblot as above for phospho-JNK (p-JNK) and total JNK. Results are representative of at least 3 independent experiments from different donors. The bar graph represents quantification of the phosphorylation of JNK2 (ratio of phosphorylated/total) expressed as relative values when compared with platelets without any treatment (A and B) or platelets treated with 0.2 μg/mL oxLDL (C) (n=5 for A, n=3 for B, and n=4 for C). c indicates control. *$P<0.05$ when compared with control or nLDL treatment.

chow diet showed a modest increase in JNK phosphorylation, whereas platelets from *apoE*$^{-/-}$ mice on the western diet showed a marked increase in phospho-JNK staining ($P<0.001$ compared with those from either of the chow fed strains). Diet-induced JNK phosphorylation was completely eliminated in *apoE*$^{-/-}$ mice that were also deficient in CD36. Flow-cytometric analysis of platelets in suspension confirmed that those from *apoE*$^{-/-}$;*cd36*$^{-/-}$ mice on a western diet had significantly lower levels of phospho-JNK than those from *apoE*$^{-/-}$ mice on a western diet (n=3, $P<0.05$, data not shown). As additional confirmation, we also examined the level of phospho-JNK by immunoblot (Figure 2C) and found an increase in platelets from *apoE*$^{-/-}$ mice on a western diet compared with platelets from *apoE*$^{-/-}$ mice on chow diet. The increase was not seen in platelets from *apoE*$^{-/-}$;*cd36*$^{-/-}$ mice on a western diet. These data demonstrate that the interaction between endogenous oxidized lipid ligands and CD36 triggers a signaling cascade leading to JNK activation in platelets.

oxLDL-Induced JNK2 Phosphorylation Is Mediated By src Family Kinases

The mechanism by which oxLDL induces JNK phosphorylation was assessed using a panel of specific pharmacological inhibitors. Pretreatment of platelets with the broad spectrum src kinase inhibitor AG1879 blocked oxLDL induced JNK2 activation, whereas the phosphatidylinositol 3-kinase inhibitor LY294002 and the broadly active PKC inhibitor GO6983 had no effect (Figure 3A). These data suggest that JNK activation is downstream of src family kinases and independent of PI3K or PKC signaling pathways.

Ligand-Induced Recruitment of Fyn and Lyn Kinases by Platelet CD36 Is Essential for oxLDL-Induced Signaling Events

Previous studies demonstrated that the specific src family kinases Fyn, Lyn, and Yes were coprecipitated from platelet membrane lysates with anti-CD36 antibodies.[18] In addition, Fyn and/or Lyn has been shown to be involved in CD36-initiated signaling, leading to MAP kinase activation in macrophages, microglia, and endothelial cells.[19,20,32] These studies suggest that the association between CD36 and Fyn and/or Lyn may have a functional role in CD36-mediated signaling in platelets. To test this hypothesis, we performed immunoprecipitation with anti-CD36 monoclonal antibody FA6 and examined the precipitates for the presence of src kinases and their activation state. The amounts of Fyn and Lyn in CD36 immunoprecipitates were markedly increased on oxLDL treatment (Figure 3B). Src family kinases have 2 tyrosine phosphorylation sites; phosphorylation in the activation loop increases kinase activity, whereas phosphorylation in the C terminus renders the kinases inactive. We used an antibody specific to the phosphotyrosine in the activation loop and found that Fyn and to a lesser extent Lyn recruited to CD36 after oxLDL exposure were in the "active" state (Figure 3B). In contrast, oxLDL did not increase the total amount of active tyrosine phosphorylated Fyn or Lyn in the

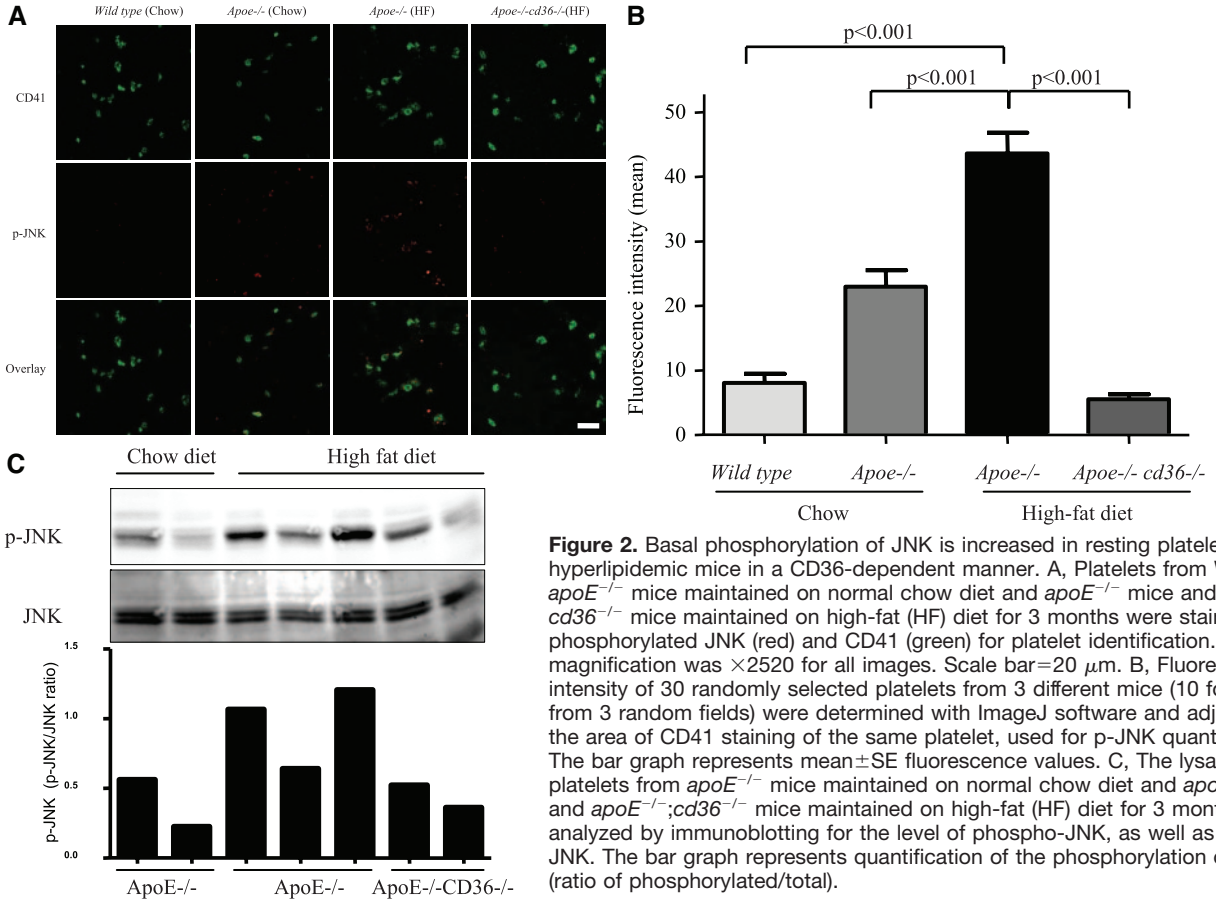

Figure 2. Basal phosphorylation of JNK is increased in resting platelets from hyperlipidemic mice in a CD36-dependent manner. A, Platelets from WT and *apoE*$^{-/-}$ mice maintained on normal chow diet and *apoE*$^{-/-}$ mice and *apoE*$^{-/-}$;*cd36*$^{-/-}$ mice maintained on high-fat (HF) diet for 3 months were stained for phosphorylated JNK (red) and CD41 (green) for platelet identification. Original magnification was ×2520 for all images. Scale bar=20 μm. B, Fluorescence intensity of 30 randomly selected platelets from 3 different mice (10 for each from 3 random fields) were determined with ImageJ software and adjusted to the area of CD41 staining of the same platelet, used for p-JNK quantification. The bar graph represents mean±SE fluorescence values. C, The lysates of platelets from *apoE*$^{-/-}$ mice maintained on normal chow diet and *apoE*$^{-/-}$ mice and *apoE*$^{-/-}$;*cd36*$^{-/-}$ mice maintained on high-fat (HF) diet for 3 months were analyzed by immunoblotting for the level of phospho-JNK, as well as total JNK. The bar graph represents quantification of the phosphorylation of JNK (ratio of phosphorylated/total).

non–CD36-associated fraction (data not shown). We also performed immunoprecipitation with anti-Lyn antibody and probed the tyrosine phosphorylation state with a phosphotyrosine specific antibody 4G10 and found that there was no significant increase in total Lyn tyrosine phosphorylation after exposure to oxLDL (Figure 3C). In sum, these data suggest that recruitment of activated Fyn and Lyn to CD36 in response to oxLDL is a key step in CD36-mediated signaling leading to JNK2 phosphorylation.

oxLDL-Induced Activation of Platelets In Vitro Is Mediated by JNK and src Family Kinases

We next used pharmacological inhibitors to study the functional role of JNK and src kinases in oxLDL-induced platelet activation, using a flow cytometric–based assay for surface exposure of P-selectin as a marker for platelet activation. We found that specific pharmacological inhibition of JNK by SP600125 markedly reduced platelet activation in response to oxLDL (≈40% inhibition) (Figure 4A). The inhibitor had minimal effect on platelet activation by other agonists as exemplified by ADP or TRAP (SFLLRN) (Figure 4B and 4C). We also found that inhibition of src family kinases by AG1879 blocked oxLDL-induced platelet activation (≈55% inhibition) (Figure 4D). These results show that JNK and src family kinases are required for oxLDL-induced platelet activation and suggest that JNK is specific to oxLDL-initiated platelet signaling.

CD36-Dependent Activation of JNK Promotes In Vivo Thrombus Formation

To determine whether JNK signaling occurs during thrombosis in vivo, we performed immunohistochemical analysis of carotid artery thrombi induced in mice by $FeCl_3$ injury using a specific antibody for phospho-JNK. As shown in Figure 5A, phospho-JNK was detected in thrombi from both WT and *cd36*$^{-/-}$ mice. The staining intensity, however, was significantly lower (P=0.003) in thrombi from *cd36*$^{-/-}$ mice, suggesting that CD36-mediated JNK signaling occurred during thrombus formation in vivo (Figure 5B). As an alternative approach to quantify phospho-JNK levels, we dissected carotid artery thrombi from WT and c*d36*$^{-/-}$ mice and examined pooled lysates from 9 thrombi in each group by immunoblot. Thrombi from *cd36*$^{-/-}$ mice had ≈16% less phospho-JNK than those from WT mice (supplemental Figure II). These data suggest that CD36 contributed to JNK phosphorylation during thrombus formation in vivo.

Platelet JNK Inhibition Prolonged Time to Thrombosis in a CD36-Dependent Manner

To define the functional effect of JNK signaling in thrombosis, we transfused platelets pretreated with the JNK inhibitor SP600125 into mice rendered severely thrombocytopenic by irradiation and then monitored carotid artery thrombus formation in vivo in response to injury with 12.5% $FeCl_3$. As shown in Figure 6, inhibition of platelet JNK significantly prolonged the time to thrombosis in mice transfused with WT

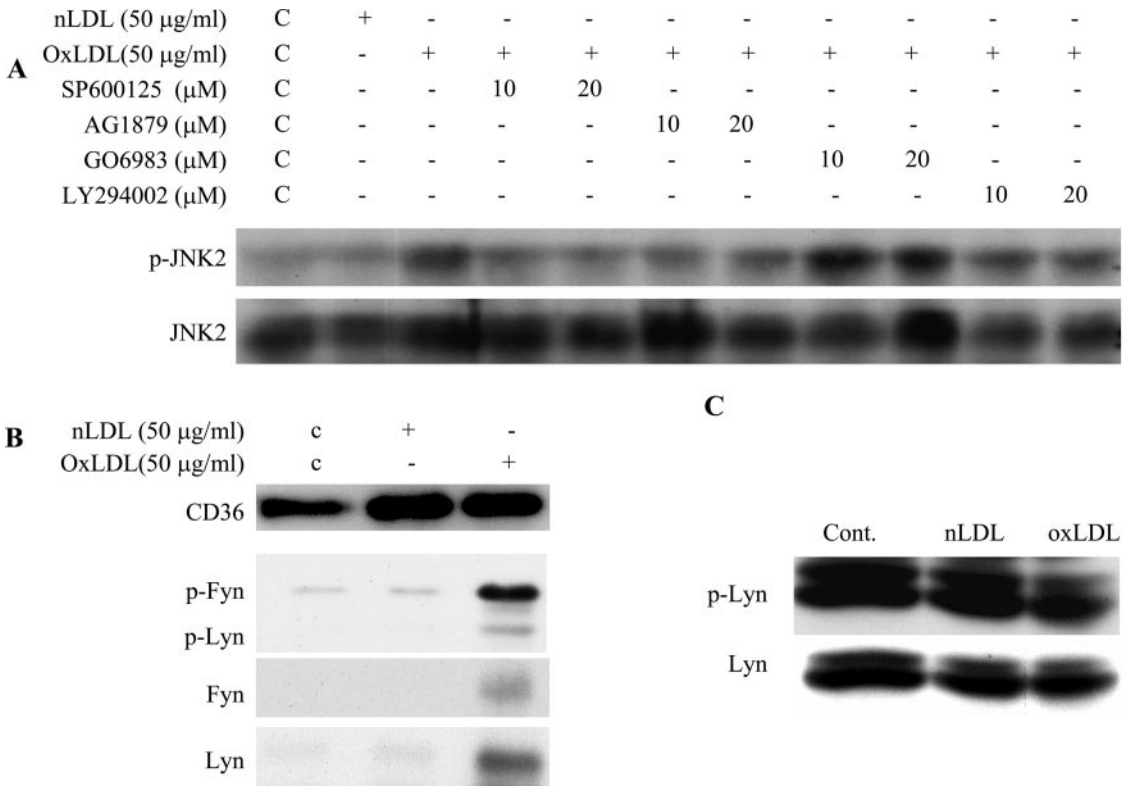

Figure 3. Recruitment of src family kinases to platelet CD36 is essential for oxLDL-mediated signaling. A, Human platelets were incubated with the JNK inhibitor SP600125, src inhibitor AG1879, PKC inhibitor GO6983, or PI3K inhibitor LY294002 for 30 minutes before incubation with 50 μg/mL oxLDL. The platelet lysates were then analyzed by immunoblot as in Figure 1 for JNK phosphorylation. B, Platelets were incubated with oxLDL or nLDL and then lysed. CD36 was precipitated by FA6 anti-CD36 IgG. Precipitates were analyzed by immunoblot with antibodies to CD36, phospho-src (Y416), Fyn, and Lyn. C, Platelets were incubated with 50 μg/mL oxLDL or nLDL and then lysed. Lyn was immunoprecipitated from lysates, and the precipitates were analyzed by immunoblot with a phosphotyrosine antibody (4G10) and Lyn antibody.

platelets (2-sample *t* test, $P=0.01$) but had no effect in mice transfused with *cd36*$^{-/-}$ platelets (2-sample *t* test, $P=0.37$). Supplemental Figure III shows representative fluorescence images from these studies. These data strongly suggest that CD36 mediated JNK signaling promotes platelet activation and thrombus formation in vivo.

Discussion

Earlier studies demonstrated that engagement of platelet CD36 with oxLDL in vitro or with endogenous oxidized phospholipid ligands generated in vivo under hyperlipidemic conditions induced platelet activation,[17,33,34] but the mechanisms by which platelet CD36 acts as a modulator of platelet activity have not been defined. The studies reported here identify the MAP kinase JNK2 as a critical mediator of CD36-dependent platelet signaling. Our work also sheds light on the specific mechanisms linking CD36 and JNK. Earlier studies demonstrated that antibodies to platelet CD36 coprecipitated Fyn, Lyn, and Yes, suggesting a physical association with these nonreceptor protein tyrosine kinases.[18] Consistent with this work, we showed that pharmacological inhibition of src kinases abolished CD36-dependent JNK2 phosphorylation and subsequent platelet activation (Figures 3A and 4D). Furthermore, we showed that the active phosphorylated form of Fyn and Lyn were recruited to CD36 on oxLDL treatment (Figure 3B). There was no change in the levels of active Fyn or Lyn in the fractions not associated with CD36 (data not shown). These studies, thus, suggest that CD36 functions to assemble a signaling complex in a ligand-dependent manner.

Using *apoE*-null mice fed a high-fat western diet as a model of hyperlipidemia and oxidant stress, we showed that the CD36 signaling pathway was activated in vivo, leading to increased basal levels of JNK phosphorylation in resting platelets (Figure 2). With mesenteric and carotid thrombosis models, we previously demonstrated that the time to thrombotic occlusion after induction of injury was significantly shorter in hyperlipidemic *apoE*-null mice than in WT mice.[17] This hyperlipidemia-induced prothrombotic phenotype was rescued by genetic deletion of CD36 in the *apoE*-null background. We, thus, hypothesized that the increased basal JNK activity contributed to CD36-dependent platelet hyperreactivity associated with hyperlipidemia.[26] We also showed with a carotid injury model in chow-fed mice that phosphorylation of platelet JNK during thrombus formation in vivo was, in part, CD36-dependent and that pharmacological inhibition of platelet JNK produced a significant antithrombotic effect, supporting a role for the CD36-JNK signaling axis even under nonhyperlipidemia conditions (Figures 5 and 6). These latter studies suggest that CD36 ligands are generated during arterial injury and are consistent with recent data from our laboratory showing that *cd36*-null mice are less sensitive to carotid injury (ie, have longer times to thrombo-

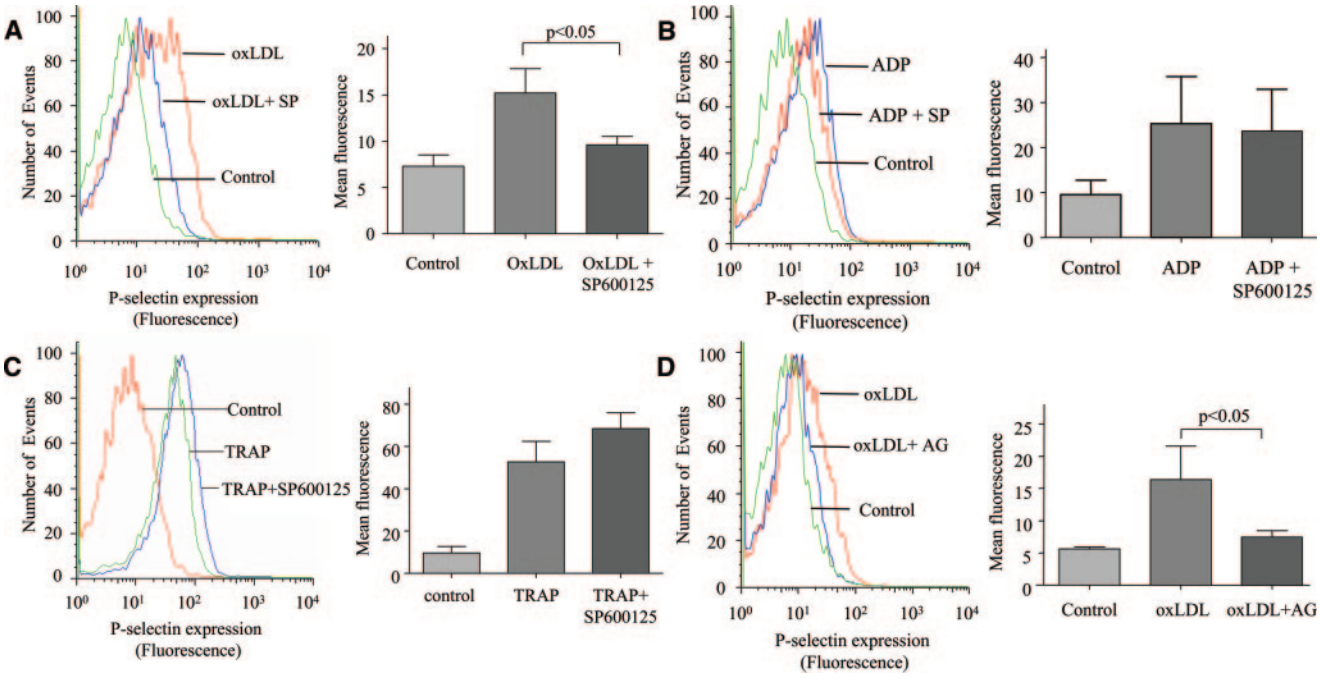

Figure 4. oxLDL-induced platelet activation of platelets is mediated by JNK and src kinases. A through C, Human platelets were incubated with the specific JNK inhibitor SP600125 (final concentration of 20 μmol/L) for 30 minutes before incubation with 50 μg/mL oxLDL (A), 10 μmol/L ADP (B), or 2 μmol/L TRAP (SFLLRN) (C) and analyzed by flow cytometry with phycoerythrin-labeled anti–P-selectin antibody. D, Platelets were incubated with src family kinase inhibitor AG1879 (10 μmol/L) for 30 minutes before incubation with 50 μg/mL oxLDL. Control platelets were treated with the vehicle, DMSO. Results are representative of at least 3 independent experiments from different donors. The bar graph represents mean fluorescence intensities measured with flow cytometry. The error bars are expressed as mean±SE (n=4 for A; n=3 for B through D).

sis).[28] The nature of the CD36 ligands remains to be defined, although recent studies suggest that endothelial cell–derived microparticles could function in this capacity.[28]

Our findings are consistent with recent studies from other laboratories showing that MAP kinases, including JNK, have a significant role in platelet biology.[35–39] Pharmacological inhibition of JNK in a model of arteriolar and venular thrombosis in mice suggested a role in arteriolar but not venular thrombosis.[39] It was recently demonstrated that JNK was activated after thrombin exposure and during collagen-induced platelet aggregation.[39,40] In the latter process, ADP release was required for JNK activation, although ADP alone was not sufficient to induce JNK activation. In contrast, we demonstrated that oxLDL-induced JNK activation was not dependent on ADP release (Figure 1D). Integrin outside-in signaling was also not required for JNK activation induced by oxLDL, suggesting oxLDL–platelet interactions directly trigger the signaling cascade. We also showed that although pharmacological inhibition of JNK blocked platelet activation by oxLDL, it did not inhibit ADP- or TRAP-induced activa-

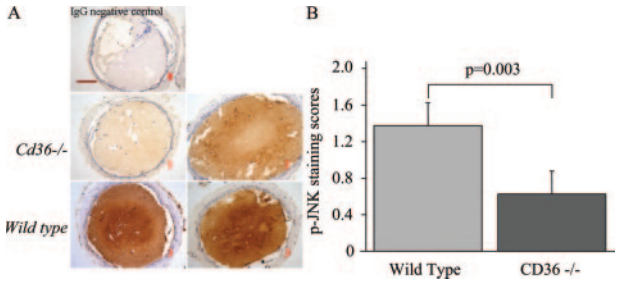

Figure 5. JNK is phosphorylated during thrombus formation in a CD36-dependent manner. A, Representative images of Immunohistochemical detection of phosphorylated JNK (p-JNK) in carotid thrombi from WT and *cd36*$^{-/-}$ mice. No staining with nonimmune IgG control showed specificity. Brown indicated positive staining. Scale bar=100 μm. B, The bar graph represents mean±SE of a staining score of 5 sections from 3 thrombi in each group.

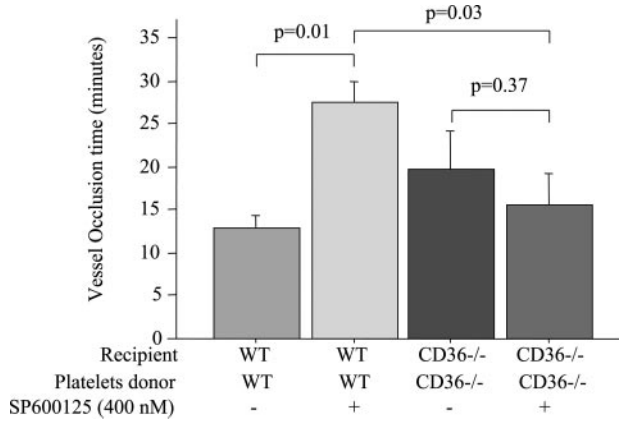

Figure 6. JNK inhibition prolongs occlusion times in a CD36-dependent manner in vivo after carotid artery injury. Carotid thrombosis times were assessed by intravital video microscopy in irradiated thrombocytopenic WT and *cd36*$^{-/-}$ mice transfused with platelets from donor animals of identical genotype. Arteries were injured by topical application of $FeCl_3$ (12.5%). Platelets were incubated with the JNK inhibitor SP600125 (400 nmol/L) or vehicle control for 30 minutes before transfusion. Bar graph represents occlusion time. Data are expressed as means±SE (n=5).

tion (Figure 4A through 4C), suggesting that JNK may be specific to CD36 signaling. Our studies (data not shown) and those of others have shown that another member of the MAP kinase family, p38, is phosphorylated in platelets after exposure to oxLDL.[33,41,42] Whether p38 and JNK work synergistically or independently in the process of platelet activation by oxLDL remains to be determined. The precise function of JNK in platelet biology also remains to be determined. It is known to be involved in a wide variety of diverse cellular processes through transcription-dependent and transcription-independent mechanisms. Because platelets are anucleate cells, it is unlikely that JNK action would be transcription-dependent. It will be important to define substrates of JNK in platelets to further define its functional role.

Several studies have shown that LDL subjected to various methods of in vitro oxidation, including by exposure to metal ions or hydrochlorous acid, can influence platelet function.[41–44] Our data clearly identify a central role for the interaction of CD36 with specific oxidized phospholipids within oxLDL in platelet signaling. CD36-specific ligands are generated when LDL is oxidized in vitro and in vivo and have been shown to accumulate in atherosclerotic plaque and to circulate in the blood of patients with hyperlipidemia and atherosclerosis.[29,45,46] oxLDL, however, is a complex particle and can contain a variety of biologically active lipids other than CD36 ligands, including lysophosphatidylcholine, platelet-activating factor, lysophosphatidic acid (LPA), and 9- and 13-HODE.[47] The role of these other lipids in JNK activation remains unknown. Others have shown that LPA in oxLDL can induce platelet shape change via a specific G protein–coupled LPA receptor.[44,48] The signaling pathways triggered by LPA involve tyrosine phosphorylation of specific proteins including Syk and an increase of cytosolic Ca^{2+}.[49,50] It is also possible that LPA or platelet-activating factor receptors may work in a synergic way with CD36 and contribute to JNK activation.

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Disclosures

None.

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A Specific CD36-Dependent Signaling Pathway Is Required for Platelet Activation by Oxidized Low-Density Lipoprotein

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Supplementary Information

Materials and Methods

Materials

Anti-CD36 antibody FA6-152 was obtained from Abcam (Cambridge, MA). The blotting antibody for Lyn, PE-conjugated anti-CD62P, FITC-conjugated PAC-1 and mouse anti-CD41 antibody were from BD Biosciences (San Jose, CA). Antibodies to Fyn and Lyn were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-phospho-tyrosine antibody (4G10) was from Upstate Biotechnology, Inc (Norwalk, CT). Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-rat IgG antibody were from Invitrogen (Carlsbad, CA). All other antibodies were obtained from Cell Signaling Technology, Inc (Danvers, MA). The DAB visualization system for immunohistochemistry (EnVision™ Doublestain Kit) was from DAKO (Carpinteria, CA). Thrombin receptor activated peptide (SFLLRN) was from Bachem (Torrance, CA). SP600125, LY294002, Go6983 and AG1879 were purchased from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

LDL was isolated from fresh plasma as previously described and stored under N_2 until use[1]. LDL protein concentration was determined by the Lowry assay. All LDL concentrations were given in terms of their protein content. Native LDL （nLDL）was oxidized with $CuSO_4$ for 24 hours at 37 °C and extent

of oxidation was determined by measuring malondialdehyde (MDA) formation as thiobarbituric acid reacting substances (TBARS). MDA values were given as nmol/mg LDL protein. The amount of MDA in the starting nLDL material was between 0.6-0.8 nmol/mg LDL protein. OxLDL with final MDA values between 2-10 nmol/mg were used in these studies to avoid any potential disruption of the LDL particles.

Human platelet preparation and flow cytometry

Whole blood was collected from healthy human volunteers in 0.109 M sodium citrate (1:9 dilution) in accordance with the Cleveland Clinic Institutional Review Board (IRB). These donors were not taking aspirin, non-steroidal anti-inflammatory drugs or any other medication. Platelet rich plasma (PRP) was obtained by centrifugation at 100 g for 12 minutes at room temperature. Washed platelets were prepared by centrifuging PRP at 600 g for 10 minutes in the presence of 100 nM prostaglandin E_1 (PGE_1). The platelet pellet was washed twice with Pipes/saline/glucose (5 mM Pipes, 145 mM NaCl, 4 mM KCl, 50 μM Na_2HPO_4, 1 mM $MgCl_2$- $6H_2O$, and 5.5 mM glucose) in the presence of 100 nM PGE_1 and then resuspended in Modifed Tyrode's Buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5 mM HEPES, 0.1% glucose and 0.35% bovine serum albumin, pH 7.2). Platelet concentration was adjusted to 2×10^8 platelets/ml, unless otherwise indicated. $CaCl_2$ (final concentration of 2 mM) and $MgCl_2$ (final concentration of 1 mM) were added immediately before platelet stimulation.

For flow cytometry analysis, resting washed platelets or washed platelets activated by various agonists under non-stirring conditions were incubated with PE-conjugated anti-CD62P antibody or FITC- conjugated PAC-1 antibody for 30 minutes and analyzed using a Guava PCA-96 instrument (Guava Technologies, Inc) and FlowJo software (Treestar, Inc). Platelets from 3-4 different donors were used for all studies.

Immunoprecipitation and Immunoblotting

For immunoblotting, platelets were lysed in 2 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin, and protein concentrations were measured with a Bio-Rad Protein Assay Kit based on the method of Bradford. Immunoblotting were performed following standard protocols. After chemiluminescence detection of phosphorylated proteins, blots were stripped and re-probed with antibodies to total relevant proteins for normalization. For analysis of JNK activation in murine platelets, 10 μg of total proteins was electrophoresized and blotted as above, the membrane was blot with mouse anti p-JNK (Cell signaling) and rabbit anti-total JNK simultaneously at 4°C for overnight. Membrane was then incubated with Odyssey goat anti-mouse IRDye 800 and donkey anti-Rabbit IRDye 680 secondary antibodies (LI-COR biosciences, Lincoln NE) for 1 hour at room temperature in dark, washed and then developed with Li-Cor Odyssey Infrared Imaging System. For immunoprecipitation, pre-cleared lysates

containing 500 µg proteins were incubated with 3 µg of antibodies to Lyn immobilized on protein-A-agarose beads overnight at 4°C. Beads were washed, boiled in 2 x SDS-PAGE loading buffer, and the bound material was analyzed by immunoblotting. For co-immunoprecipitation studies, cells were treated with Dithiobis-succinimidyl-propionate (DSP) before lysis in buffer containing 1% CHAPS. Immunoprecipitation was performed with antibody to CD36 as previously described[1]. Band intensities were quantified with ImageJ software (Bethesda, MD).

Murine platelet studies

cd36 null, *apoe* null, *apoe;cd36* double null, and their appropriate C57Bl/6 matched control strains were generated as described previously[2, 3]. To isolate platelets, mice were anesthetized with kitamine (90 mg/kg) and xylazine (15 mg/kg) and 600 µl blood obtained by cardiac puncture into syringes containing 100 µl acid citrate dextrose (ACD; 75 mM trisodium citrate dehydrate, 38 mM citric acid monohydrate and 100 mM glucose) and 500 µl Ca^{2+}- and Mg^{2+}-free PBS. Blood was centrifuged at 100 g for 10 minutes to collect PRP. PRP was then subjected to centrifugation at 325 g for 5 minutes in the presence of PGE_1 to pellet platelets.

Immunofluorescence

Experimental groups consisted of 6 WT and 3 *apoe* null mice on chow diet and 3 *apoe* null and 3 *apoe;cd36* double null mice on high-fat diet (HF). PRP

from mice blood was spread on silanized slides (DAKO, Carpinteria, CA) and platelets were allowed to adhere for 30 minutes at room temperature. It was confirmed by P-selectin staining that silane coated glass slide did not activate platelets. Cells were then fixed in acetone at -20°C for 5 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes. After blocking non-specific binding with 5% bovine serum albumin, slides were incubated with a rabbit antibody to phosphorylated JNK (final 1:25 dilution) and a rat antibody to CD41 (final 1:100 dilution) in PBS overnight at 4°C. Cells were then incubated with Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-rat IgG antibodies for 1 hour at room temperature. Coverslips were mounted with VectaShield (Vector Laboratories) and fluorescence images were taken with a Leica TCS-SP2 AOBS upright confocal microscope. Fluorescence intensities of 30 randomly selected platelets from 3 different mice were analyzed by Image J software (Bethesda, MD). The platelet area was identified by CD41 staining, and the mean fluorescence intensity per platelet was calculated

Murine carotid artery thrombosis

Twelve week old wild type (WT) mice and *cd36* null (*cd36-/-*) mice matched for body weight were anesthetized with ketamine (90 mg/kg)/xylazine (15 mg/kg) and the right jugular veins and the left carotid artery exposed via a middle cervical incision. For some studies that do not need platelet transfusion, 100 µl of rhodamine 6G (0.5 mg/ml) was injected directly into the right jugular

vein to label platelets. The left carotid artery was stripped of adventitia and a piece of black plastic was placed under the vessel to reduce the background fluorescence. A 1x2 mm piece of filter paper saturated with 12.5% $FeCl_3$ solution was applied to the carotid artery for 1 minute. The filter paper was removed, and the vessel was rinsed with saline. Thrombus formation was observed in real-time under a Leica DMLFS fluorescent microscope with an attached Gibraltar Platform (EXFO, Quebec, Canada) and a water immersion objective at 10x magnification. Time to thombosis was determined through visual inspection using real time video image capture with a QImaging Retigo Exi 12-bit mono digital camera (Surrey, Canada) and Streampix version 3.17.2 software (Norpix, Montreal, Canada). The end points were set as either cessation of blood flow for >30 seconds or no occlusion after 30 minutes, in which case the time was recorded as 30 minutes.

At the end of the experiment the carotid arteries were harvested, fixed in 4% formaldehyde and embedded in paraffin. Cross sections (4-6 μm) were cut through the thrombi and analyzed by immunohistochemical staining with an antibody to phospho-JNK. Sections were counter stained with hematoxylin and images taken with a Leica DMR upright microscope equipped with a Retiga Exi Cooled CCD camera with a Liquid Crystal Tunable RGB filter (Qimaging, Surry, BC Canada). Experimental groups consisted of 3 WT mice and 3 *cd36-/-* mice. For quantification, images were scored on the basis of staining intensity by a blind observer. Scores 0-3 were allocated as follows: 0,

negative; 1, weak staining; 2, moderate staining and 3, intense staining[4]. For immunoblot analysis, thrombi from 9 WT and *cd36-/-* mice were pooled and lysed.

Platelet transfusion studies

Experimental groups consisted of 10 WT and 10 *cd36-/-* mice. Recipient WT and *cd36-/-* mice were exposed to 11Gy of external beam irradiation from a Cesium 137 source to induce thrombocytopenia with platelet counts <5% of normal after 5 days as previously described[2]. Donor platelets were then isolated from wild type and *cd36-/-* donor mice as mentioned above, re-suspended in 500 µl Ca^{2+} and Mg^{2+} free PBS, and incubated with Calcein AM (final concentration of 0.5 mg/ml) in the presence or absence of the JNK inhibitor SP6000125 (final concentration of 400 nM) for 30 minutes at room temperature. 2×10^9 donor platelets were then injected through the jugular vein of thrombocytopenic mice 10 minutes prior to carotid injury with 12.5% $FeCl_3$ to allow the transfused platelets to reach equilibrium in the circulation.

Statistical analysis

Each experiment was repeated at least 3 times with different platelet donor or different mice. Values are expressed as Mean ± SE. The statistical significance was evaluated using an unpaired t test or ANOVA or Mann whitney if appropriate and considered statistically significant when $p < 0.05$

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Online Figures

Supplemental Figure S1

OxLDL-induced platelet activation is CD36-dependent. Platelets were incubated with 50 μg/ml oxLDL or native LDL and then analyzed by flow cytometry for binding of PE-labeled anti-CD62P antibody to detect α-granule secretion (A) or FITC-labeled PAC-1 antibody to detect $\alpha_{IIb}\beta_3$ activation (B). Platelets from a CD36 null donor were incubated with oxLDL (Ci) or 10 μM ADP (Cii) and then analyzed by flow cytometry with PE-labeled anti-CD62P antibody. The bar graph represents mean fluorescence intensities from three independent experiments. The error bars are expressed as Mean ± SE for n=3.

Supplemental Figure S2

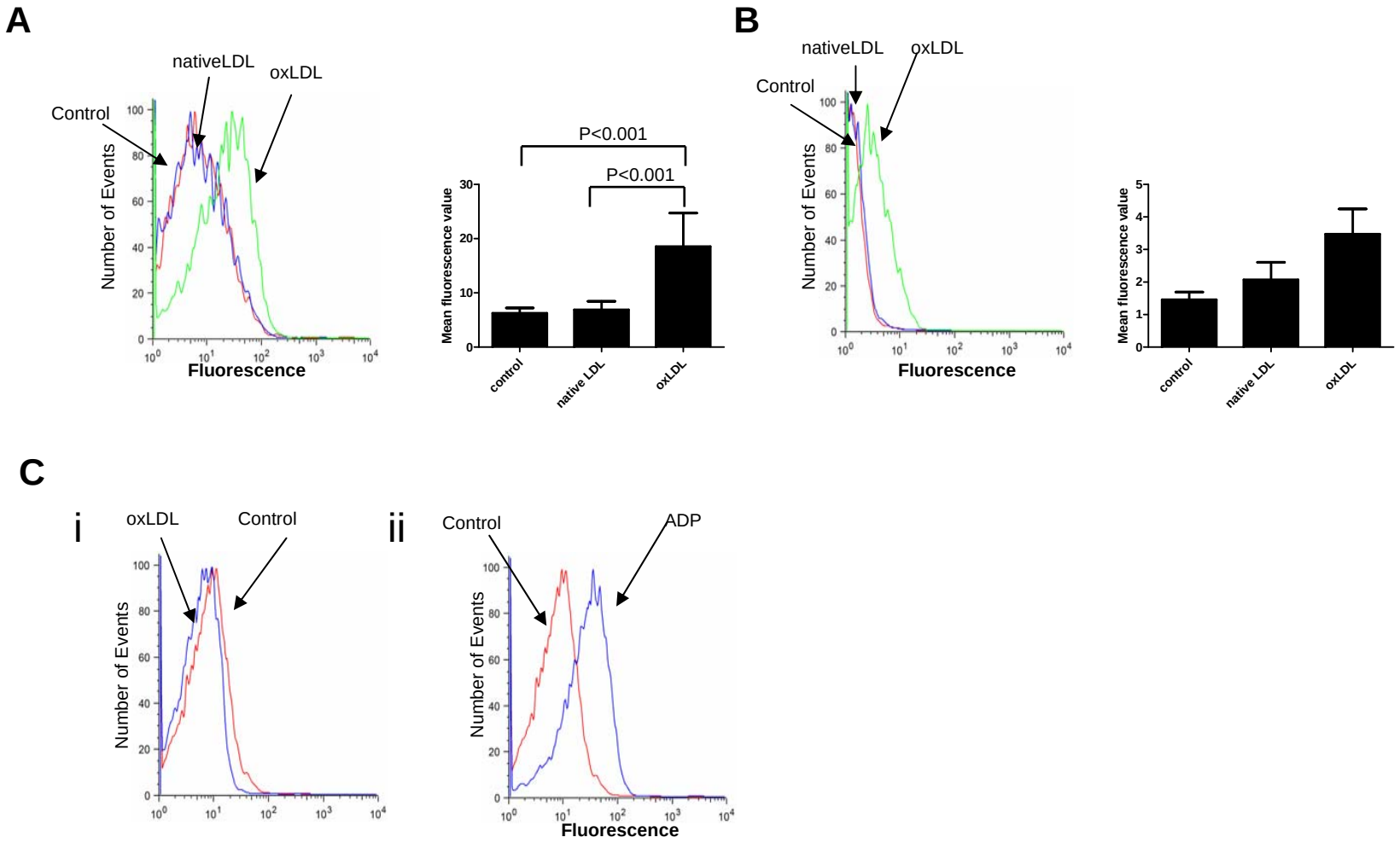
JNK is phosphorylated during thrombus formation in a CD36-dependent manner. Lysates of 9 pooled carotid artery thrombi

dissected from WT or *cd36-/-* mice were analyzed by immunoblot for phospho-JNK. The membrane was then stripped and re-probed with antibodies to the total JNK to normalize the protein loaded. The gel was scanned and band intensities quantified with NIH ImagePro software.

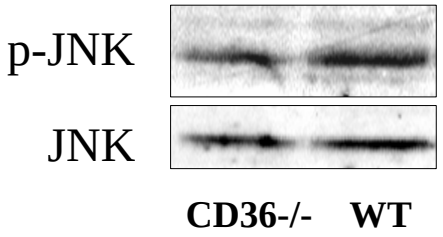
Supplemental Figure S3

JNK inhibition prolongs occlusion times in a CD36 dependent manner *in vivo* after carotid artery injury. Carotid thrombosis times were assessed by intravital video microscopy in irradiated thrombocytopenic WT and *cd36-/-* mice transfused with platelets from donor animals of identical genotype. Arteries were injured by topical application of $FeCl_3$ (12.5%). Platelets were incubated with the JNK inhibitor SP600125 (400 nM) or vehicle control for 30 minutes prior to transfusion. Representative fluorescence photograph of the time course of thrombus formation with WT and *cd36-/-* platelets treated with SP600125 and vehicle control.

Supplement Figure S1



Supplement Figure S2



Supplement Figure S3