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ORIGINAL ARTICLE

Signaling role of CD36 in platelet activation and thrombus formation on immobilized thrombospondin or oxidized low-density lipoprotein

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Summary. *Background and Objective:* Platelets abundantly express glycoprotein CD36 with thrombospondin-1 (TSP1) and oxidized low-density lipoprotein (oxLDL) as proposed ligands. How these agents promote platelet activation is still poorly understood. *Methods and Results:* Both TSP1 and oxLDL caused limited activation of platelets in suspension. However, immobilized TSP1 and oxLDL, but not LDL, strongly supported platelet adhesion and spreading with a major role of CD36. Platelet spreading was accompanied by potent Ca^{2+} rises, and resulted in exposure of P-selectin and integrin activation, all in a CD36-dependent manner with additional contributions of $\alpha_{IIb}\beta_3$ and ADP receptor stimulation. Signaling responses via CD36 involved activation of the protein tyrosine kinase Syk. In whole blood perfusion, co-coating of TSP1 or oxLDL with collagen enhanced thrombus formation at high-shear flow conditions, with increased expression on platelets of activated $\alpha_{IIb}\beta_3$, P-selectin and phosphatidylserine, again in a CD36-dependent way. *Conclusions:* Immobilized TSP1 and oxLDL activate platelets partly via CD36 through a Syk kinase-dependent Ca^{2+} signaling mechanism, which enhances collagen-dependent thrombus formation under flow. These findings provide novel insight into the role of CD36 in hemostasis.

Keywords: CD36, outside-in signaling, oxidized low density lipoprotein, platelets, thrombospondin, thrombus.

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Introduction

The 80- to 90-kDa protein CD36, or glycoprotein (GP) IV, is a major platelet protein with 10 000–25 000 copies expressed on the platelet surface and additional copies present in the α-granules [1,2]. As a double membrane-spanning protein, CD36 has two short N- and C-terminal cytoplasmic domains and a large glycosylated extracellular domain with partly overlapping binding sites for thrombospondin-1 (TSP1), oxidized low-density lipoproteins (oxLDL), oxidized forms of phospholipids and long-chain fatty acids [3,4]. The various CD36 ligands are considered to bind at or near the CLESH-1 domain (for CD36 LIMP II Emp structural homology-1 domain), which contains two consensus glycosylation sites and is located next to a proline- and cystein-rich domain, determining the conformation of the extracellular loop [5].

In spite of its abundant presence, the function of platelet CD36 is still unclear. In the early days, CD36 was considered to be a collagen receptor [6,7], but it then appeared that platelets from CD36-deficient patients display normal collagen-induced responses [8–10]. Peptide studies with platelets and other cells have pointed to a role of CD36 in the binding of TSP1 [11,12]. For platelets, this might provide a mechanism for autocrine activation, as TSP1 similarly to CD36 is expressed in the α-granules [13,14]. Other papers have shown that TSP1 can also recognize other platelet membrane proteins in a Ca^{2+}-dependent way, including several integrins, integrin-associated protein (CD47) and GPIb [15–17]. In macrophages and other cell types, CD36 acts as a scavenging receptor for oxLDL and oxidized phospholipids [18,19]. Such role of CD36 has also been reported in platelets, next to other scavenger receptors such as SR-A [20,21].

The binding of oxLDL to platelets and other cells is considered to trigger intracellular signaling events, but the precise contribution of CD36 is a matter of debate [3,5]. One suggestion is that oxLDL induces or enhances activation of the platelet Src family and Syk kinases, accompanied by Ca^{2+} entry, which events have been attributed to stimulation of

lysophosphatidate receptors [22]. Other authors show that interaction of oxLDL with CD36 activates members of the mitogen-activated protein kinase family (MAPK: Erk2, p38, JNK) [20,23]. A similar uncertainly exists on the signal transduction triggered by TSP1 binding to platelets. Thrombospondin peptides can evoke, partly integrin $\alpha_{IIb}\beta_3$-independent, signaling via Src kinases, LAT, Syk and phospholipase Cγ2 [24,25]. Under conditions of shear stress, the binding of TSP1 to CD36 can activate platelets via the receptor complex GPIb–V–IX [17,26]. Another report proposes that TSP1 binding cancels the inhibiting effects of nitric oxide on platelets [27]. Furthermore, also integrin-dependent ways of TSP1 action on platelets have been reported [28]. Altogether, there appears to be a similarity in the action mechanisms of oxLDL and TSP1 on platelets, which raises the question whether this involves common signaling events via CD36.

In the present study, we investigated how TSP1 and oxLDL can stimulate platelets via interaction with CD36. When immobilized on a surface, both TSP1 and oxLDL appear to trigger a mechanism of outside-in signal transduction via CD36 with support of $\alpha_{IIb}\beta_3$, involving the tyrosine kinase Syk, Ca^{2+} rises, and an autocrine feed-forward loop relying on ADP receptor activation.

Materials and methods

Materials

Native LDL, freshly isolated from human plasma (Intracel, Frederick, MD, USA), was processed and used within 2 weeks. Human trimeric, platelet-derived TSP1 as well as PP1, PP2, PP3, Src-family kinase (SFK) inhibitor I, and Syk inhibitors II and IV were from Merck (Darmstadt, Germany). Human fibrinogen, MRS-2179, bovine serum albumin (BSA) and fucoidan from *Fucus vesiculosus* were from Sigma (St Louis MO, USA); H-Phe-Pro-Arg chloromethyl ketone (PPACK) from Calbiochem (La Jolla, CA, USA); and low-molecular-weight heparin (fragmin) from Pfizer. The $P2Y_{12}$ receptor antagonist, cangrelor (AR-C69931MX) was kindly provided by the Medicines Company (Parsippany, NJ, USA). Fibrillar Horm type I collagen was from Nycomed Pharma (Munich, Germany); and annexin A5 labeled with fluorescein isothiocyanate (FITC) from PharmaTarget (Maastricht, the Netherlands). Human fibrinogen labeled with Oregon green (OG)488, Alexa Fluor (AF)647-labeled annexin A5, pluronic F-127, dimethyl BAPTA (DM-BAPTA) and Fluo-4 acetoxymethyl esters came from Invitrogen (Leiden, the Netherlands). Anti-human CD36 mAb FA6-152, directed against the TSP1 and oxLDL binding site, was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), as well as 4D10 anti-Syk mAb. Human IgG1 isotype control was from Genway Biotech (San Diego, CA, USA). CD36 peptide P(93–110) and thrombin receptor-activating peptide SFLLRN were from Bachem (Bubendorf, Switzerland). FITC-labeled anti-CD62 mAb was from Immunotech (Marseille, France); FITC-PAC1 mAb from BD Biosciences (San Jose, CA, USA); anti-phospho-Syk (Tyr525/526) mAb C87C1 from Cell Signaling (Danvers, MA, USA); rabbit anti-α-tubulin Ab from Abcam (Cambridge, UK); horseradish peroxidase (HRP)-conjugated IgG ECL Ab from GE Healthcare (Hoevelaken, the Netherlands). Chrono-Lume luciferase-luciferin reagent was from Chronolog (Havertown, PA, USA). Other materials were from sources as described previously [29].

Blood collection and platelet preparation

Donors gave full informed consent according to the Helsinki declaration, and had not taken medication for 2 weeks. Blood was collected into acid citrate dextrose (85 mM trisodium citrate, 67 mM glucose, 42 mM citric acid) for the preparation of platelet-rich plasma and washed platelets. For flow perfusion, blood was collected into 40 μM PPACK and 20 U mL^{-1} fragmin; additional 10 μM PPACK was added after 1 h [30]. Washed platelets were resuspended in Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM glucose and 0.1% BSA) in the presence of 0.1 U mL^{-1} apyrase. Where indicated, platelets were loaded with 8 μM Fluo-4 acetoxymethyl ester and/or 50 μM DM-BAPTA acetoxymethyl ester in the presence of 0.4 mg mL^{-1} pluronic F-127 [31].

LDL oxidation

Native LDL (1.0 g mL^{-1}) was dialyzed against phosphate-buffered saline (PBS) at 4 °C, and oxidized under controlled conditions, as described [32]. Briefly, LDL was incubated with 25 μM $CuSO_4$ at 37 °C during 8 h, after which oxidation was stopped with 50 μM EDTA. Preparations were immediately dialyzed against PBS with 10 μM EDTA (4 °C), and were checked for oxidation by electrophoresis with 1% agarose gels. Only preparations were used that were oxidized for ~90% and had a relative electrophoretic mobility vs. LDL of ~3.5. These were stored for a maximum of 2 weeks under N_2 at 4 °C. Over-oxidized preparations were not used. For specific experiments, native LDL was oxidized for 48–72 h with 5 μM $FeSO_4$ at 4 °C, and dialyzed as described previously [33].

Flow cytometry

Washed platelets (2×10^8 mL^{-1}) in Hepes buffer containing 2 mM $CaCl_2$ were activated with ADP or SFLLRN in the presence or absence of TSP1 (2.5 μg mL^{-1}) or oxLDL (250 μg mL^{-1}) without stirring. After 10 min, activation of integrin $\alpha_{IIb}\beta_3$ and α-granule secretion was detected with OG488-fibrinogen (150 μg mL^{-1}) and FITC-labeled anti-CD62 mAb (1:50), respectively. Fluorescence was measured with a FACScan flow cytometer counting a minimum of 10 000 events [29].

Platelet aggregation and adhesion

Aggregation of platelets in plasma was determined by light transmission aggregometry [34]. For static adhesion assays,

96-well plates were coated with human fibrinogen, TSP1 or oxLDL (100 μg mL^{-1} each), and then blocked with saline containing 1% BSA. Platelets (1.5×10^8 mL^{-1}) in Hepes buffer plus 2 mM $CaCl_2$ were pretreated with indicated inhibitors for 10 min, and then allowed to adhere to the coated wells. After 45 min, unbound platelets were washed out, and adherent platelets were solubilized with Triton X-100. Acid phosphatase activity was determined in lysates from the cleavage of *p*-nitrophenyl-phosphate at 405 nm using a microplate reader [35]. Numbers of adhered platelets per well were calculated by comparing with platelet suspensions of known counts.

Spreading and activation responses of adhered platelets

Glass coverslips were coated with fibrinogen, TSP1, LDL or oxLDL (100 μg mL^{-1} each), and blocked with Hepes buffer plus 1% BSA. Coated proteins on the coverslip surface were verified by immune-fluorescence microscopy. After blocking with BSA (1%), the coverslips were mounted in open chambers, and incubated with platelets (1.5×10^8 mL^{-1}) in Hepes buffer containing 2 mM $CaCl_2$ and inhibitors, as indicated. Spreading of adhered platelets was monitored by real-time capturing of microscopic brightfield contrast images [36]. Surface expression of activation markers on adhered platelets, that is P-selectin (FITC-anti-CD62 mAb), activated $\alpha_{IIb}\beta_3$ integrin (FITC-PAC1 mAb) and phosphatidylserine (AF647-annexin A5), was measured by two-color confocal microscopy and differential interference contrast imaging [29], using a Leica TCS SP5 multiphoton system (Rijswijk, the Netherlands). Images were analyzed with the software package Metamorph (MDS, Sunnyvale CA, USA). For ATP measurements, coated six-well plates were incubated with 500 μL platelets in Hepes buffer (1.5×10^8 mL^{-1}), and supernatants were collected after 45 min for analysis with luciferase-luciferin reagent using a Spectramax luminescence microplate reader and ATP standards (Molecular Devices, Downingtown, PA, USA).

To determine Ca^{2+} responses during adhesion, coated coverslips were incubated with Fluo-4-loaded platelets (5×10^7 mL^{-1}) in Hepes buffer with 2 mM $CaCl_2$. Fluorescence changes of adhering platelets were recorded in real-time at 5 Hz using a sensitive EM-CCD camera system, controlled by VISITECH software (Sunderland, UK) [34]. Single-cell traces of nanomolar Ca^{2+} concentrations were obtained by off-line analysis of selected regions-of-interest, each representing one adhered platelet, using pseudo-ratio calibration [37].

Thrombus formation under flow

Glass coverslips were coated with fibrillar type I collagen, TSP1, LDL or oxLDL, either alone or in combinations (all applied at 100 μg mL^{-1}) [30]. For co-coatings, collagen was allowed to form fibers, after which TSP1, oxLDL or LDL was post-coated. The presence of TSP1 or (ox)LDL on coverslips was confirmed by immuno-fluorescence microscopy. After blocking with 1% BSA, coverslips were mounted into a transparent parallel-plate flow chamber (depth 50 μm, width 3 mm). Chambers were perfused with PPACK/fragmin-anticoagulated blood at a shear rate of 1000 s^{-1}, and microscopic brightfield and fluorescence images of thrombus formation were taken [36]. Expression of activation markers on platelets in thrombi was analyzed with probes as described above and the Leica TCS SP5 multiphoton system.

Gel electrophoresis and western blotting

Tyrosine phosphorylation was measured by western blot analysis of lysates from suspended or adhered platelets. Six well plates were coated with TSP1 or oxLDL and incubated for 45 min with 500 μL platelets in BSA-free Hepes buffer (5×10^8 mL^{-1}). After washing, the surface-adhered platelets were lysed with ice-cold NP-40-based lysis buffer pH 7.45 in the presence of protease and phosphatase inhibitors [38]. Protein in lysates was quantified with a BioRad DC protein kit (Veenendaal, the Netherlands). Samples with equal protein amounts were separated on 10% SDS-PAGE gels, and transferred to blotting membranes by semi-dry transfer. The membranes were immuno-stained with anti-phospho-Syk (Tyr525/526) mAb (1:1000) and colored with HRP-coupled anti-rabbit Ab (1:5000), using ECL detection technology [29]. Membranes were reprobed with anti-Syk mAb (1:1000) and coloring with anti-mouse HRP. Duplicate blots were stained with anti-α-tubulin Ab (1 μg mL^{-1}) as sample loading control. Analysis of stained blots was by densitometry [38].

Statistical analysis

Significance of differences was determined with a parametric *t*-test or a non-parametric Mann–Whitney *U*-test, as appropriate, using the statistical package for social sciences (SPSS 15.0, Chicago, IL, USA).

Results

Role of CD36 in platelet adhesion and spreading on immobilized TSP1 or oxLDL

Initially, platelets in suspension were stimulated with the two alleged CD36 ligands, TSP1 and oxLDL. We employed a commercial preparation of TSP1, isolated from human platelets. However, oxLDL was prepared freshly from LDL by controlled oxidation [32]. Using flow cytometry, TSP1 (2.5 μg mL^{-1}) did not significantly influence ADP- or SFLLRN-induced integrin $\alpha_{IIb}\beta_3$ activation or α-granule secretion, which was measured as fibrinogen binding and P-selectin expression, respectively (Fig. S1A,B). On the other hand, oxLDL (250 μg mL^{-1}) moderately stimulated integrin activation and granule secretion, but did not enhance the effects of ADP or SFLLRN. The blocking anti-CD36 mAb, FA6-152, antagonized the stimulating effects of oxLDL (not shown, but see below). Together, these results confirmed that oxidation-modified LDL has a mild activating effect on platelets in suspension via CD36 [20,39].

The matrix of a (damaged) vessel wall contains surface-immobilized forms of TSP1 and oxLDL [13,40,41]. Knowing that surface-bound fibrinogen can activate platelets via outside-in $\alpha_{IIb}\beta_3$ signaling [1], we evaluated whether immobilized TSP1 and oxLDL can activate platelets via CD36. As determined in a well-plate assay, the surface coating of fibrinogen, TSP1 or oxLDL, but not of native LDL, provoked high platelet adhesion (Fig. 1). Involvement of CD36 in platelet adhesion was demonstrated with two inhibitors: the blocking FA6-152 mAb, directed against the TSP1 and oxLDL binding sites on CD36 [42]; and the CD36-derived peptide, P(93–110), corresponding to theTSP1 binding site and competing with CD36-TSP1 interaction [11]. Control measurements showed that neither the FA6-152 mAb nor the P(93–110) peptide affected platelet adhesion to fibrinogen (Fig. 1A). However, both inhibitors substantially reduced adhesion to TSP1, whereas only FA6-152 mAb interfered with adhesion to oxLDL, confirming the specificity of P(93–110) for TSP1 binding. Isotype control IgG1 did not influence adhesion. With either immobilized TSP1 or oxLDL, platelet adhesion was partly reduced by blocking the $P2Y_1$ and $P2Y_{12}$ receptors for ADP (MRS-2179 and AR-C69931MX, respectively), or by blocking the $\alpha_{IIb}\beta_3$ integrin with tirofiban (Fig. 1B). Similarly, the loading of platelets with intracellular Ca^{2+} chelating agent, DM-BAPTA, resulted in a partly reduced adhesion. Tirofiban did not increase the effect of anti-CD36 mAb (not shown, but see below).

Time-lapse video microscopy was applied to monitor the morphological changes of platelets adhering to the coated surfaces. As reported before [43,44], platelets on fibrinogen formed filopods and lamellipods, causing a > six-fold increase in surface area coverage in 45 min (Fig. 2A). Strikingly, also platelets adhering to immobilized TSP1 or oxLDL actively produced filopods and lamellipods (Fig. 2B,C). In contrast, immobilized LDL did neither provoke stable adhesion nor changes in morphology (Fig. 2D).

Both CD36 inhibitors, FA6-152 mAb and P(93–110) peptide, suppressed the spreading of platelets on TSP1 by 50%, but only the antibody reduced the spreading on oxLDL by 70% (Fig. 3A,B). Again, the isotype control IgG1 was without effect, while the loading of platelets with DM-BAPTA fully abrogated spreading on TSP1 or oxLDL. As established

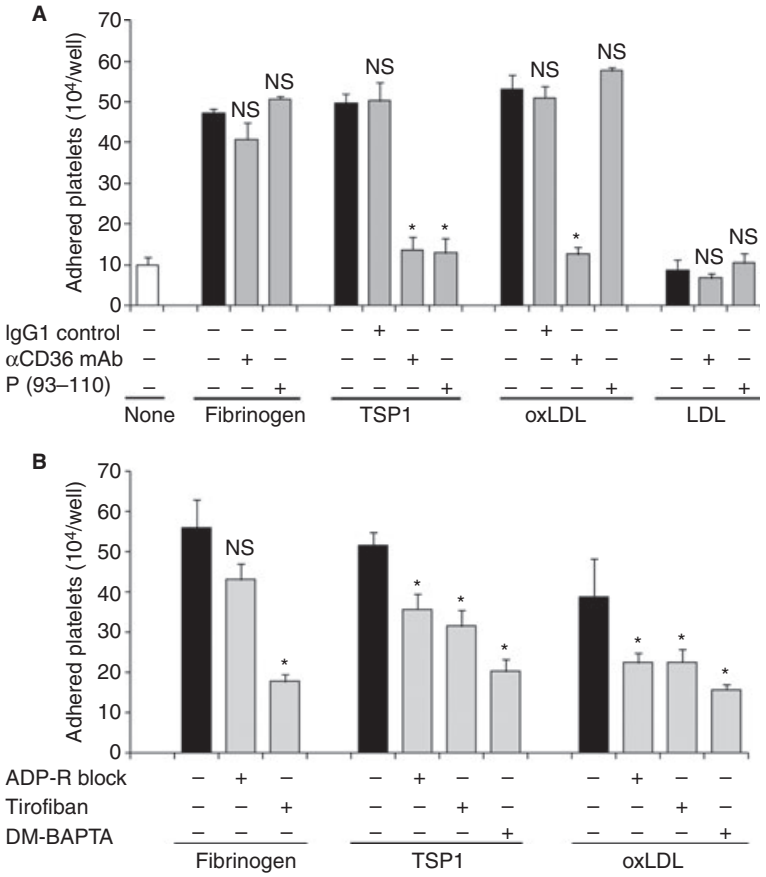

Fig. 1. Role of CD36 in platelet adhesion to immobilized thrombospondin-1 (TSP1) and oxidized low-density lipoprotein (oxLDL). Washed platelets (pretreated as indicated) were added to immobilized fibrinogen, TSP1, oxLDL or LDL in well plates during 45 min. Non-adherent platelets were removed by a rinse, and remaining cells were lysed to measure acid phosphatase activity. (A) Platelets were incubated with vehicle buffer, IgG1 isotype control (2 μg mL^{-1}), anti-CD36 FA6-152 mAb (2 μg mL^{-1}) or CD36 peptide P(93–110) (50 μg mL^{-1}). (B) Platelets were pretreated with ADP receptor blockers (10 μM cangrelor and 100 μM MRS-2179), tirofiban (2 μg mL^{-1}) or were preloaded with dimethyl BAPTA (DM-BAPTA). Data are means ± SEM (n = 3–4), NS, not significant, *P < 0.05 vs. vehicle control.

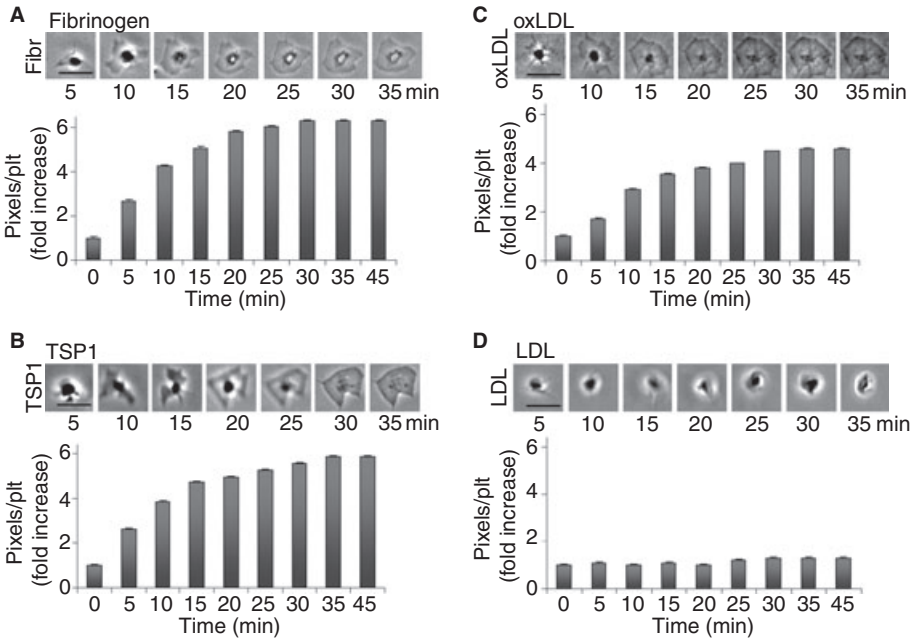

Fig. 2. Spreading of platelets on immobilized fibrinogen, thrombospondin-1 (TSP1) and oxidized low-density lipoprotein (oxLDL). Washed platelets were allowed to adhere for 45 min to immobilized fibrinogen (A), TSP1 (B), oxLDL (C) or LDL (D). Indicated are representative microscopic phase contrast images from single adhered platelets, captured at various time intervals (bars, 10 μm). Graphs show increases in surface area coverage per platelet in time, expressed as fold pixel increase. Means ± SEM (n = 4–6 experiments, 10 cells/experiment).

for fibrinogen-adhered platelets [45], blocking of the platelet ADP receptors (MRS-2179 and AR-C69931MX) or $\alpha_{IIb}\beta_3$ (tirofiban) diminished the spreading on TSP1 or oxLDL (Fig. 3A,B). Combined blocking of CD36, ADP receptors and $\alpha_{IIb}\beta_3$ nearly eliminated spreading on TSP1, but not on oxLDL. Post-addition of anti-CD36 mAb, but not of isotype control IgG1, after 10 min resulted in a halt of spreading or even in detachment of partially spread platelets from the surface (not shown). This could explain the major reduction by FA6-152 mAb in the platelet adhesion assay (Fig. 1A).

The potential involvement of SR-A as oxLDL receptor was investigated using the SR-A antagonist fucoidan [20]. By itself this compound (50 μg mL^{-1}) was inactive on platelet spreading. However, in combination with FA6-152 mAb, fucoidan reduced the size of platelets on oxLDL with 28 ± 2% (mean ± SEM, n = 3), approaching the pixel size of resting, non-adhered platelets. Together, these data point to CD36 as a main activating receptor for platelet spreading on immobilized TSP1 and oxLDL, with additional effects of ADP receptor and $\alpha_{IIb}\beta_3$ activation. The contribution of $\alpha_{IIb}\beta_3$ is in agreement with evidence for a functional association of CD36 with $\alpha_{IIb}\beta_3$ and the tetraspannin CD9 in platelet membranes [46].

Role of CD36 in platelet activation on TSP1 or oxLDL surfaces

Studies were carried out to determine the CD36-dependent signaling events in spreading platelets. Real-time measurement of Ca^{2+} fluxes in single, Fluo-4-loaded platelets showed high-amplitude, repetitive spiking rises in cytosolic $[Ca^{2+}]_i$, which started after several minutes of platelet adhesion to TSP1or oxLDL (Fig. 4A). The Ca^{2+} spikes decreased in amplitude, but were not abolished with anti-CD36 FA6-152 mAb. The TSP1 peptide P(93–110) reduced only the Ca^{2+} rises in TSP1-adhered platelets, but not those in oxLDL-adhered platelets. Recording of fluorescence images at a low frequency of 0.25 Hz during longer time periods (30 min) indicated that the Ca^{2+} rises persisted during the whole period of spreading. Control studies with LDL oxidized for 72 h with $FeSO_4$ [33] provoked similar long-term rises in Ca^{2+} (data not shown).

Quantitative analysis indicated that the blockade of CD36 (FA6-152 mAb) caused a 40–50% reduction in mean Ca^{2+} rises after stable platelet adhesion to TSP1 or oxLDL (Fig. 4B). Control IgG1 was without effect, while platelet treatment with DM-BAPTA completely suppressed the Ca^{2+} signal. Tirofiban alone suppressed the mean Ca^{2+} rises with ~30%, while also blockage of ADP receptors caused a partial reduction in the absence and complete reduction in the presence of FA6-152 mAb (Fig. 4C,D). Combined application of tirofiban and FA6-152 mAb prevented the Ca^{2+} rises of platelets on TSP1, but not on oxLDL (Fig. 4D). Together, this pointed to additional roles of $\alpha_{IIb}\beta_3$ and ADP receptors, next to CD36 in platelet Ca^{2+} signal generation.

Using confocal microscopy with differential interference contrast optics, activation-dependent surface characteristics were determined of the adhered platelets. Spreading platelets were labeled with AF647-annexin A5 to probe phosphatidylserine exposure, and with FITC-anti-CD62 mAb to detect P-selectin exposure, both of which are Ca^{2+} -dependent responses. Quantitative analysis of overlay images showed that the majority (81%) of TSP1-adhered platelets had P-selectin

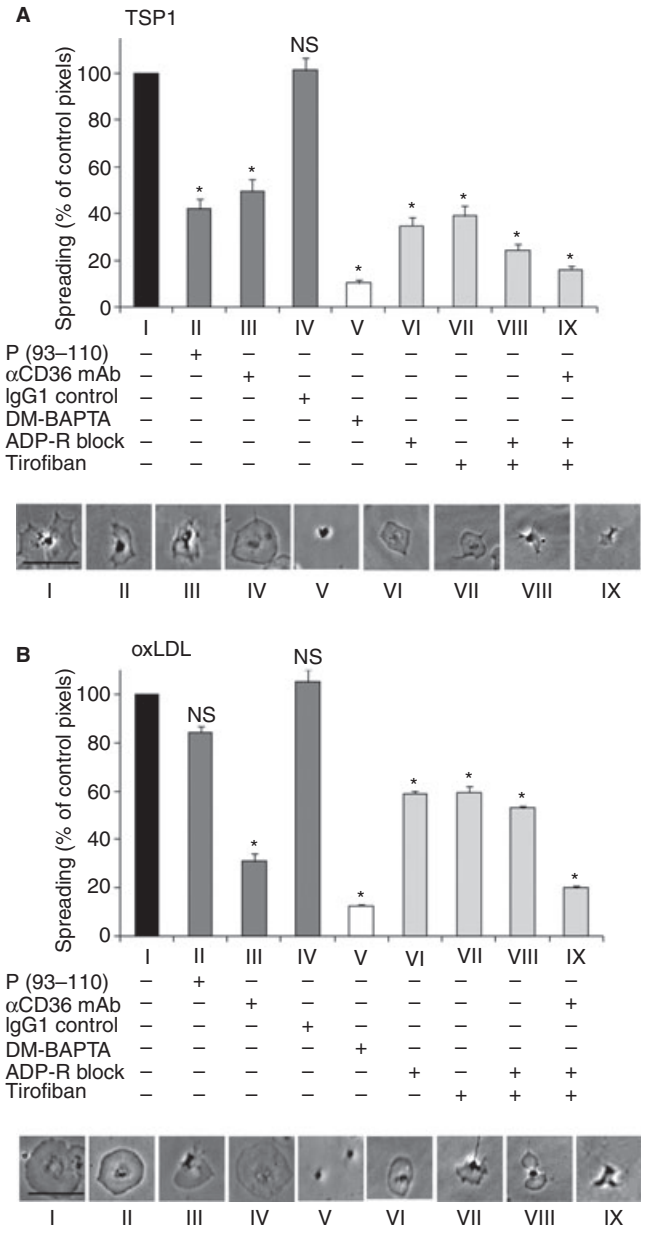

Fig. 3. Contribution of CD36 to platelet spreading on immobilized thrombospondin-1 (TSP1) and oxidized low-density lipoprotein (oxLDL). Platelets were incubated with vehicle buffer, P(93–110) peptide (50 μg mL^{-1}), FA6-152 anti-CD36 mAb (2 μg mL^{-1}), IgG1 isotype control (2 μg mL^{-1}), DM-BAPTA (preloading), ADP receptor blockers (10 μM cangrelor and 100 μM MRS-2179) or tirofiban (2 μg mL^{-1}), as indicated. Microscopic images were taken after 45 min of platelet interaction with immobilized TSP1 (A) or oxLDL (B). Graphs indicate inhibitor effects on platelet spreading (in pixels numbers relative to control condition). Images at bottom show one representative platelet per condition (bars, 10 μm). Means ± SEM (n = 6–8); *P < 0.05 vs. vehicle control.

expressed, whereas a smaller fraction (17%) exposed phosphatidylserine (Fig. 5A). Staining with FITC-PAC1 mAb indicated that most TSP1-adhered platelets had activated $\alpha_{IIb}\beta_3$ integrins (74%). Blockage of CD36 with either FA6-152 mAb or P(93–110) reduced the staining for P-selectin and activated $\alpha_{IIb}\beta_3$ (Fig. 5C). In comparison, platelets on oxLDL were slightly lower in activation markers (Fig. 5B,D). In this case only the FA6-152 mAb was inhibitory.

Combined application of FA6-152 mAb and ADP receptor blockers further reduced the fractions of adhered platelets staining for P-selectin and activated $\alpha_{IIb}\beta_3$ to 10%–15% (TSP1) or 8%–10% (oxLDL) of control. These results thus support the concept that CD36-mediated adhesion to TSP1 or oxLDL triggers Ca^{2+} -dependent secretion, which in combination with ADP receptor activation promotes the integrin activation and spreading process.

Integrin $\alpha_{IIb}\beta_3$-induced signaling in fibrinogen-adhered platelets is mediated by activation of Src-family and Syk protein tyrosine kinases [1,44]. We investigated a possible role of these kinases in the adhesion and spreading on TSP1 and oxLDL. Platelet pretreatment with Src-family kinase inhibitors (PP1, PP2, SKF inhibitor I) or Syk inhibitors II or IV, but not with the control substance PP3, greatly reduced adhesion to either surface (Fig. 6A,B). In the presence of the inhibitors, PP1 and Syk inhibitor II, spreading of the remaining adhered platelets was diminished with > 40% on TSP1 and with > 60% on oxLDL (Fig. 6C,D).

To verify the roles of CD36 and $\alpha_{IIb}\beta_3$ in protein tyrosine kinase activation, we evaluated the phosphorylation of Syk at Tyr525/526 in platelets adhered to TSP1 or oxLDL. Western blots from lysates, prepared after 45 min of adhesion, were probed with anti-phospho-Syk mAb and showed a 72-kDa band corresponding to the band detected with mAb against total Syk (Fig. 7A,B). For the platelets adhered to TSP1 or oxLDL, Syk phosphorylation partly reduced upon blocking of $\alpha_{IIb}\beta_3$ (tirofiban), but it almost completely abrogated with Syk inhibitor II or blocking anti-CD36 mAb, FA6-152.

A role of Syk was confirmed by measuring the Ca^{2+} rises in Fluo-4-loaded platelets. While CD36 blockage suppressed 45 ± 4% and 39 ± 3% of the mean Ca^{2+} signal of platelets on TSP1 and oxLDL, respectively, the combined presence of FA6-152 (2 μg mL^{-1}) plus Syk inhibitor II (1 μM) suppressed this response with 91 ± 4% and 79 ± 6% (mean values ± SEM, n > 10). Syk inhibitor II, with our without tirofiban, reduced these Ca^{2+} rises with 87 ± 4% and 85 ± 6%, respectively, the remainder relying on ADP. In agreement with this, the inhibitor suppressed the majority of ATP secreted by the surface-adhered platelets (Fig. S2). Taken together, these results indicate that both CD36 and $\alpha_{IIb}\beta_3$ contribute to tyrosine kinase activation, likely via stimulated Src-family and Syk kinases and Ca^{2+} signaling of platelets on TSP1 and oxLDL.

Role of CD36 in collagen-dependent thrombus formation in combination with TSP1 or oxLDL

Immobilized collagen promotes thrombus formation at high shear flow conditions [29,31]. We investigated whether also immobilized TSP1 and oxLDL could contribute to this process. Whole blood was thus perfused at an intermediate shear rate of 1000 s^{-1} over surfaces, consisting of collagen,

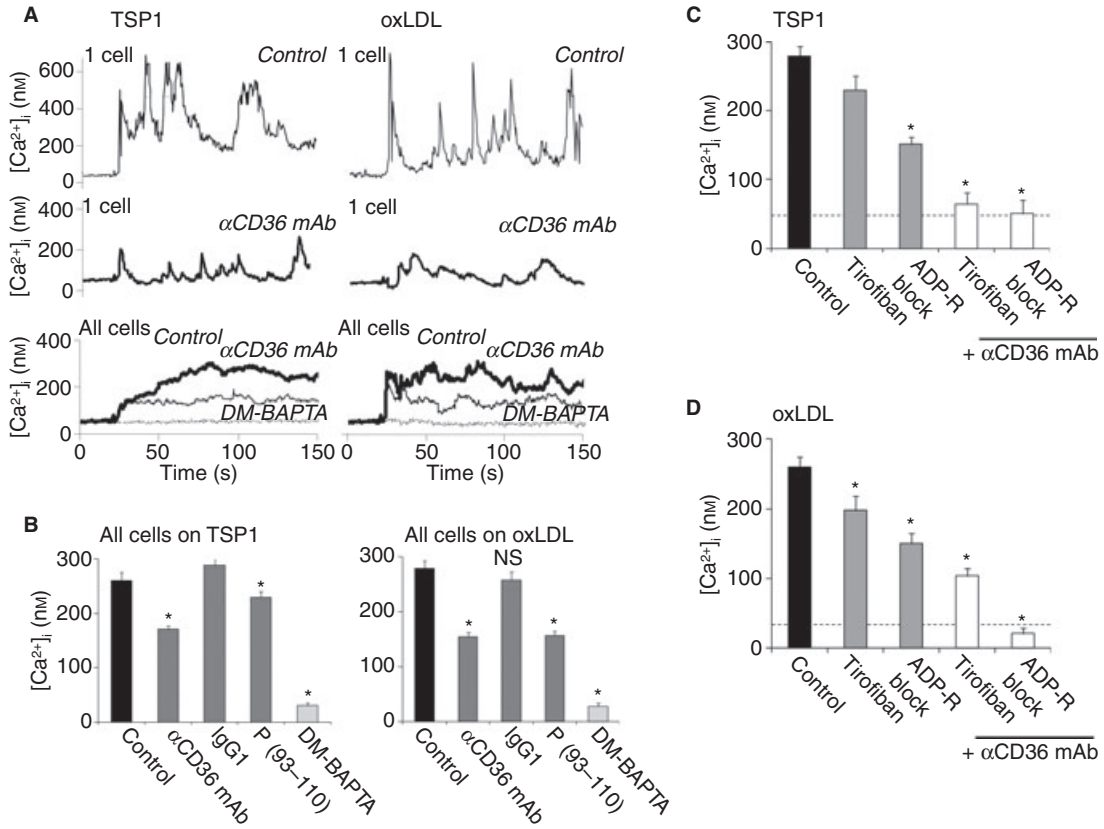

Fig. 4. Contribution of CD36 to Ca^{2+} responses of platelets adhered to thrombospondin-1 (TSP1) or oxidized low-density lipoprotein (oxLDL). Fluo-4-loaded platelets were incubated with vehicle buffer, FA6-152 anti-CD36 mAb (2 μg mL^{-1}), IgG1 isotype control (2 μg mL^{-1}), P(93–110) CD36 peptide (50 μg mL^{-1}), tirofiban (2 μg mL^{-1}) or ADP receptor blockers (10 μM cangrelor and 100 μM MRS-2179). Aliquots were preloaded with Fluo-4 and dimethyl BAPTA (DM-BAPTA), as indicated. (A, B) Ca^{2+} responses in platelets upon adhesion to immobilized TSP1 (left) or oxLDL (right). Traces represent rises in Ca^{2+} of single platelets and averaged overlays from > 50 platelets per condition (t = 0, stable adhesion). (B) Average Ca^{2+} rises during an interval of 60–120 s after initial. (C, D) Contribution of $\alpha_{IIb}\beta_3$ and ADP receptors to Ca^{2+} responses of adhered platelets, measured during 60–120 s after initial adhesion. Horizontal lines give basal Ca^{2+} level. Data are means ± SEM (n = 50–55); *P < 0.05 vs. vehicle control.

TSP1, oxLDL or LDL, or over collagen surfaces that were post-coated with these proteins. Immobilized TSP1 supported adhesion of only single platelets, but TSP1 in combination with collagen markedly increased platelet deposition and formation of large-sized platelet aggregates, when compared with collagen alone (Fig. 8A,B). Immobilized LDL (as control) or oxLDL were unable to form such aggregates. However, oxLDL (but not LDL) in combination with collagen significantly increased the aggregate formation. The enhanced thrombus formation with post-coated TSP1 or oxLDL was annulled in the presence of anti-CD36 mAb (Fig. 8C). Confocal fluorescence microscopy showed increased staining for P-selectin, $\alpha_{IIb}\beta_3$ activation, phosphatidylserine exposure of the thrombi that were formed on collagen/TSP1 or collagen/oxLDL (Fig. S3A,B). Accordingly, we concluded that immobilized TSP1 and oxLDL increase collagen-dependent thrombus growth and platelet activation under flow conditions.

Discussion

The present study provides novel evidence that surface-immobilized TSP1 and oxLDL can act as strong platelet-stimulating agents. This is a relevant finding, since both TSP1 and oxLDL accumulate in the healthy and atherosclerotic vessel walls and, hence, may contribute to the thrombogenic response upon vascular endothelial dysfunction [13,40,47]. Similarly to fibrinogen surfaces [44], it appears that immobilized TSP1 and oxLDL trigger outside-in signaling events in platelets with a major role herein of CD36. Signaling responses of platelets in contact with TSP1 or oxLDL – inhibitable by blockage of CD36 or tyrosine kinases – are pseudopod formation, Ca^{2+} rises, integrin $\alpha_{IIb}\beta_3$ activation, granule secretion to produce autocrine mediators and limited phosphatidylserine exposure. Furthermore, immobilized TSP1 and oxLDL appear to enhance the potency of collagen surfaces to form thrombi under flow conditions. Jointly, these results reveal a so far unknown way of outside-in signaling by surface-bound TSP1 and oxLDL and a novel mechanism of CD36 signaling in the adhered platelets.

Other authors have reported small activating effects of oxLDL on platelets in suspension stimulated with ADP and SFLLRN [21–23]. LDL oxidized under controlled conditions has also been found to activate the pathway of p38

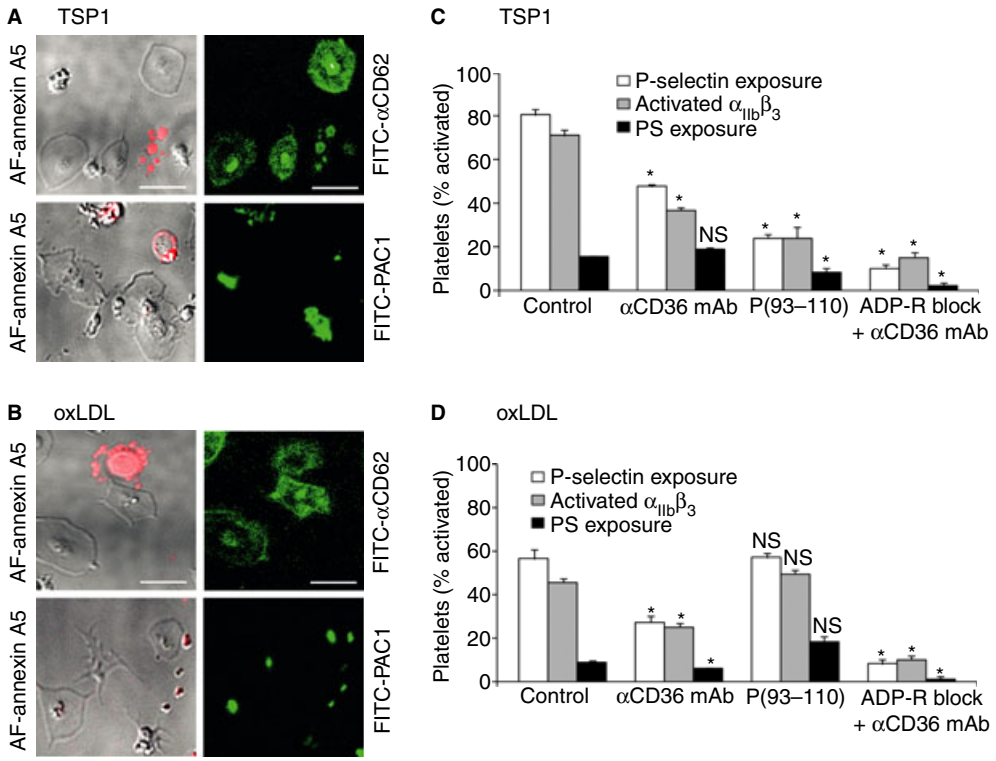

Fig. 5. Role of CD36 in activation of platelets adhered to thrombospondin-1 (TSP1) or oxidized low-density lipoprotein (oxLDL). Platelets were incubated with vehicle, anti-CD36 mAb FA6-152 (2 μg mL^{-1}), P(93–110) peptide (50 μg mL^{-1}) and/or ADP receptor blockers (10 μM cangrelor and 100 μM MRS-2179), and allowed to adhere to immobilized TSP1 or oxLDL for 45 min. Adhered platelets were stained for P-selectin (FITC anti-CD62P mAb, green) or activated integrin $\alpha_{IIb}\beta_3$ (FITC-PAC1, green) in combination with AF647-annexin A5 (red). (A, B) Representative confocal images of green and red fluorescence, overlaid with differential interference contrast images (bars, 10 μm). (C, D) Fractions of adhered platelets exposing P-selectin, active $\alpha_{IIb}\beta_3$ integrins and phosphatidylserine upon adhesion to TSP1 or oxLDL. Means ± SEM (n = 3–4); *P < 0.05 vs. vehicle control.

mitogen-activated protein kinase in part via CD36 [20]. Reports on TSP1-induced activation of suspended platelets are mostly limited to the use of C-terminal TSP1 peptides, which trigger platelet aggregation via Fc receptor γ-chain phosphorylation, protein tyrosine kinases and autocrine-dependent events [24,25]. In our hands, the full TSP1 protein did not evoke platelet aggregation up to a dose of 2.5 μg mL^{-1}. However, when immobilized, TSP1 as well as oxLDL were more potent in platelet activation than when used as soluble agonists. This is suggestive either for a conformation change of the immobilized proteins, for example in a way resembling that of surface-bound fibrinogen [43], or even for a receptor (CD36?) cross-linking effect such as was reported for collagen-related peptide [48]. Other authors also showed that TSP1 can trap platelets under shear, which was ascribed to adhesion via GPIb or von Willebrand factor next to CD36 [17,40].

Our data point to a major role of CD36 in the spreading and Ca^{2+} responses of platelets on TSP1 or oxLDL, such in junction with signaling via autocrine stimulation via ADP receptors and mechanisms involving $\alpha_{IIb}\beta_3$ activation. Responses of platelets on TSP1 were markedly suppressed by the CD36 peptide P(93–110), representing the TSP binding site, and by the blocking mAb FA6-152. For platelets on oxLDL only the FA6-152 mAb was effective. This is in good agreement with the non-identical (but adjacent) ligand binding sites for TSP1 and oxLDL on CD36 [3,5]. Importantly, ADP receptor inhibitors and integrin blockers were both active in reducing platelet responses on both surfaces. Involvement of integrin activation, likely in cross-talk with ADP-mediated events [49], can be explained by the reported linkage of CD36 with CD9 and $\alpha_{IIb}\beta_3$ in the platelet membrane [46]. Cross-over effects from CD36 to integrin signaling may explain why immobilized TSP1 (this paper) and TSP1 peptides [24,25] seem to trigger similar Src-family and Syk kinase signaling events as those arising from $\alpha_{IIb}\beta_3$ outside-in signaling of platelets on fibrinogen [44,50].

The present studies furthermore shed new light on the early reports of CD36 as a platelet collagen receptor, which were corroborated by a reduced platelet-collagen interaction in patients with CD36 deficiencies [7,51]. The co-coating of TSP1 or oxLDL enhances collagen-dependent thrombus formation via CD36. This implies that under flow platelet interaction on these surfaces in fact occurs via multiple adhesive receptors. Hence, under physiological conditions where (platelet-derived) TSP1 or (plasma-derived) oxLDL is expected to bind to collagen, CD36 is likely to play a role in platelet adhesion and activation.

Taken together, in this report we propose a model for platelet activation by immobilized TSP1 or oxLDL via

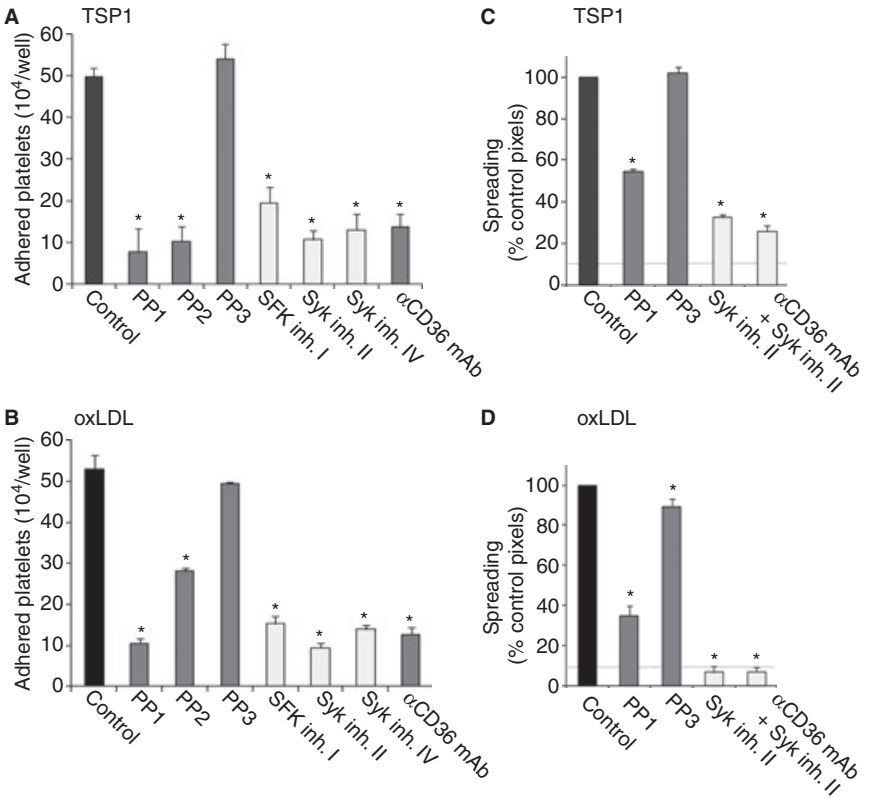

Fig. 6. Role of tyrosine kinases in platelet adhesion and spreading on thrombospondin-1 (TSP1) or oxidized low-density lipoprotein (oxLDL). Platelets were incubated for 10 min with vehicle, PP1 (20 μM), PP2 (40 μM), PP3 (40 μM), SFK inhibitor I (10 μM), Syk inhibitor II (1 μM), Syk inhibitor IV (10 μM) or FA6-152 anti-CD36 mAb (2 μg mL^{-1}), as indicated. (A, B) Inhibitor effects on platelet adhesion to TSP1 or oxLDL, determined after 45 min, as in Fig. 3. (C,D) Inhibitor effects on platelet spreading after 45 min.

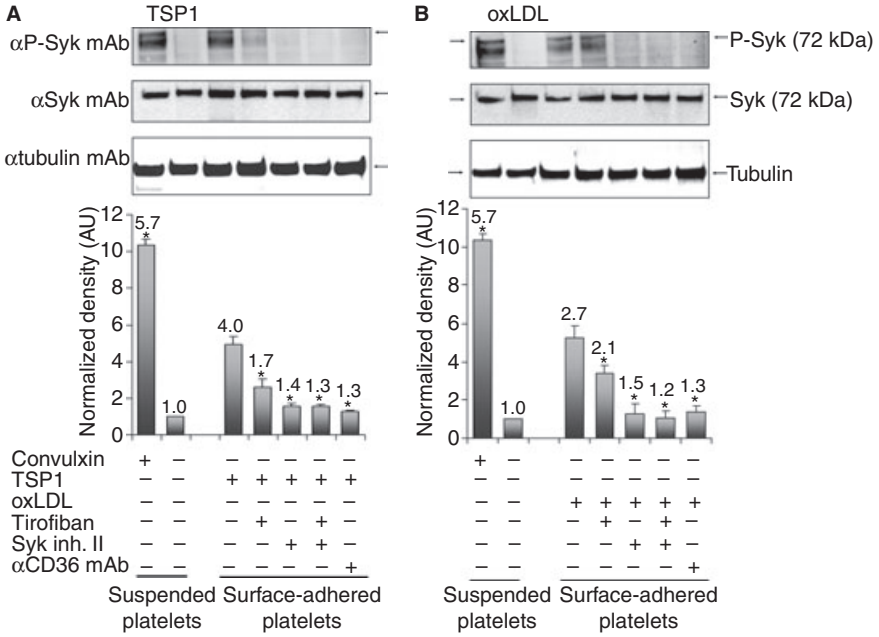

Fig. 7. Role of CD36 in Syk phosphorylation of platelets adhered to thrombospondin-1 (TSP1) or oxidized low-density lipoprotein (oxLDL). Washed platelets were incubated with inhibitors as in Fig. 6 or tirofiban (2 μg mL^{-1}), and allowed to adhere for 45 min to immobilized TSP1 (A) or oxLDL (B). Surface-adhered platelets were collected, lysed and subjected to gel electrophoresis. As positive control condition, platelets in suspension were stimulated with convulxin (80 ng mL^{-1}). Shown are western blots representative for three independent experiments, after staining for P-Syk (upper lanes, arrows indicating phospho-Syk band), total Syk (middle lanes) or α-tubulin (lower lanes). Graph bars represent normalized density of P-Syk vs. α-tubulin bands; italic numbers represent normalized density of P-Syk vs. Syk bands. Means ± SEM ($n = 3$); *$P < 0.05$ vs. vehicle control.

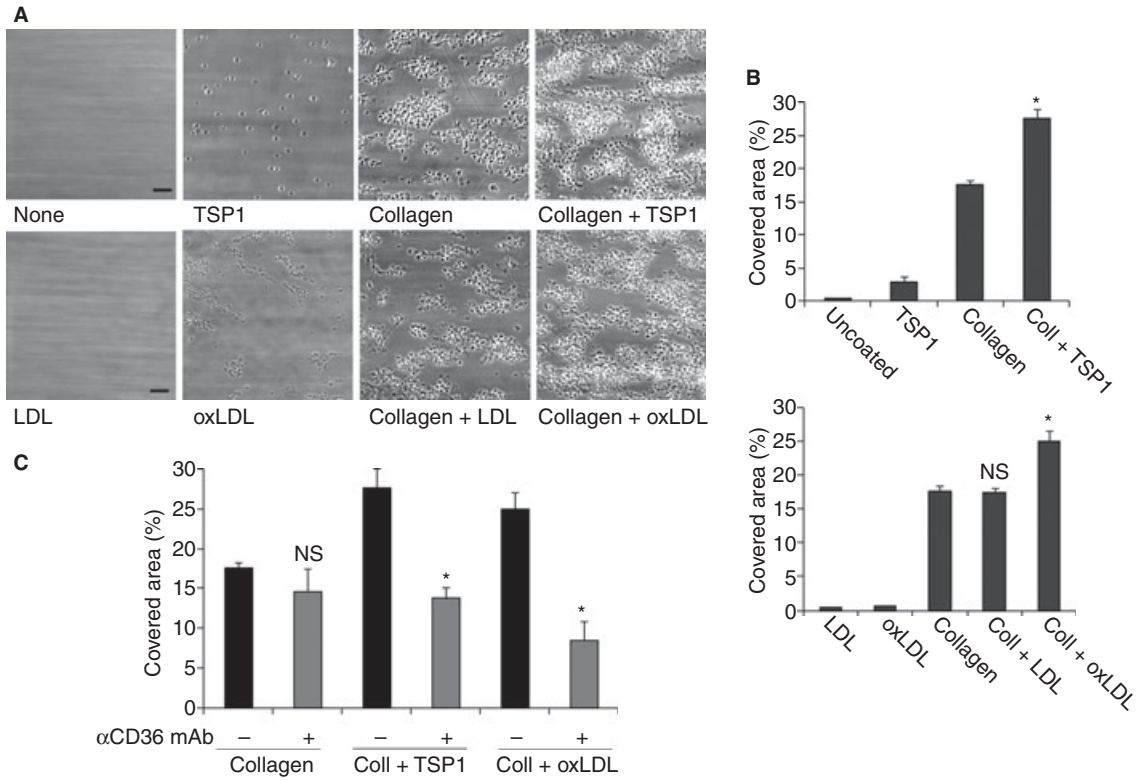

Fig. 8. Potentiation of collagen-induced thrombus formation by co-immobilized thrombospondin-1 (TSP1) and oxidized low-density lipoprotein (ox-LDL). PPACK/fragmin-anticoagulated blood, incubated with vehicle or FA6-152 anti-CD36 mAb (4 μg mL^{-1}), was flowed during 4 min at a shear rate of 1000 s^{-1} over surfaces coated with TSP1, collagen, LDL, oxLDL or combinations of proteins, as indicated. Surfaces were blocked with bovine serum albumin (BSA) before use. (A) Representative phase contrast images after 4 min of blood flow over various surfaces (bars, 10 μm). (B) Extent of thrombus formation on surfaces (% area coverage with platelets). (C) Contribution of CD36 to thrombus formation. Means ± SEM (n = 4–6); *P < 0.05 vs. vehicle control.

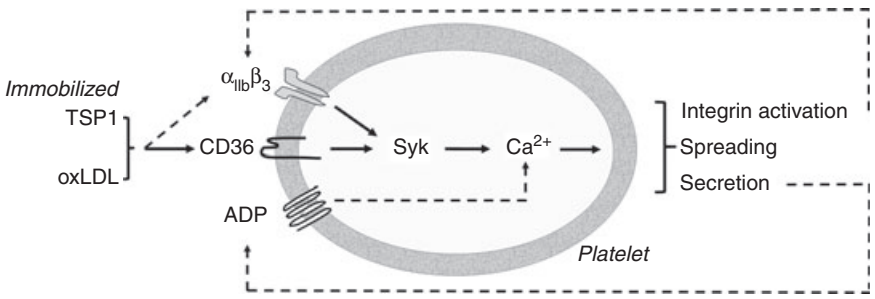

Fig. 9. Proposed role of CD36 in thrombospondin-1 (TSP1)- and oxidized low-density lipoprotein (oxLDL)-induced activation and spreading of platelets. Immobilized TSP1 and oxLDL induce initially via CD36 an activation pathway involving Syk kinase and Ca^{2+} signaling, which causes $\alpha_{IIb}\beta_3$ activation, spreading of platelets and secretion. Autocrine produced ADP and outside-in signaling via $\alpha_{IIb}\beta_3$ enhance these responses (dotted lines). See also text.

CD36-dependent outside-in signaling to protein tyrosine kinases, in particular Syk, resulting in Ca^{2+} rises and other downstream platelet responses (Fig. 9). This pathway is enforced by autocrine ADP-mediated activation, integrin activation and ensuing signaling events. In cases of deposited TSP1 and oxLDL, this CD36 route will enhance collagen-dependent thrombus formation.

Addendum

R.N.-U. designed, performed and analyzed experiments and wrote the manuscript. M.L. and R.v.K assisted with experiments and data analysis. M.K., J.G., J.C. and J.L. interpreted the data and participated in discussions. J.H. designed the experiments and wrote the manuscript.

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Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Limited platelet activation by soluble oxLDL but not TSP1.
Figure S2. Secretion of ATP by platelets adhered to TSP1 or oxLDL.
Figure S3. Co-immobilized TSP1 and oxLDL increase platelet activation in thrombus formation.

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