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Type 2 scavenger receptor CD36 in platelet activation: the role of hyperlipemia and oxidative stress

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Abstract

Platelet hyper-reactivity and a systemic prothrombotic state are associated with atherosclerosis and other inflammatory conditions. CD36, a member of the Type 2 scavenger receptor family, is a multiligand pattern recognition receptor that recognizes specific oxidized phospholipids, molecules expressed on microbial pathogens, apoptotic cells, and cell-derived microparticles. Recent studies have demonstrated that CD36 binding to oxidized LDL or microparticles activates a specific signaling pathway that induces platelet activation. This pathway is activated *in vivo* in the setting of hyperlipidemia and oxidant stress. Genetic deletion of CD36 protects mice from pathological thrombosis associated with hyperlipidemia without any apparent effect on normal hemostasis. Targeting CD36 or its signaling pathway could potentially lead to the development of novel antithrombotic therapies for patients with atheroinflammatory disorders.

Keywords

CD36; microparticles; oxidized LDL; platelets; thrombosis

Platelets & hyperlipidemia

For many years platelet activation has been postulated to contribute to the pathogenesis of atherosclerosis and platelet hyper-reactivity has been thought to play a role in acute atherothrombotic disorders such as myocardial infarction and stroke. Clinical studies support an association between platelet reactivity and prognosis in patients with coronary disease [1] and numerous studies have linked hyperlipidemia to atherothrombotic risk [2]. In 1974, a potential connection between these two risk factors was identified by Carvalho *et al.*, who demonstrated that platelets from patients with familial hypercholesterolemia were more sensitive to activation by epinephrine or ADP, although the mechanism of this phenomenon was not explored [3]. Shortly thereafter, Shattil and colleagues demonstrated in a series of *in vitro* experiments that artificial loading of platelet membranes with cholesterol increased sensitivity to agonists, while cholesterol depletion reduced sensitivity [4]. They postulated that changes in membrane fluidity or dynamics might account for these differences and that similar changes could occur in the setting of hyperlipidemia [5]. In retrospect, it is more likely that

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these manipulations modulated the structure and function of cholesterol-rich membrane microdomains thereby nonspecifically altering cell signaling mediated by G-protein-coupled receptors (GPCR) and src-family kinases that reside in these domains.

In the 35-year period since these seminal observations, advances in cell biology and genome science coupled with the development of numerous mouse genetic knockout strains that have been tested in robust models of *in vivo* thrombosis have led to understanding specific mechanisms by which lipids interact with platelets. A critical event in the evolution of our knowledge arrived with the development of mouse models of hyperlipidemia, such as the *apoe*-null strain developed by the Breslow laboratory [6]. When fed a diet replicating a typical human high-fat western diet these mice develop severe hypercholesterolemia and atherosclerotic lesions that closely resemble those observed in humans. Importantly, Eitzman and colleagues demonstrated that *apoe*-null mice fed a western diet and then subjected to arterial injury displayed shortened time to form occlusive thrombi compared with wild-type mice [7]. These studies are consistent with the human clinical data and demonstrated that the *apoe*-null mouse model could be used to probe pathophysiologic mechanisms of hyperlipidemia-associated hypercoagulability.

Although increased plasma levels of plasminogen activator inhibitor-1 have been implicated in some aspects of the prothrombotic phenotype of the *apoe*-null mice [8], it is also clear that platelet hyperactivity plays a significant role. In that context much attention has been paid to identifying and characterizing receptors on the platelet surface that recognize specific classes of lipids and lipoproteins. In some cases the signaling pathways linked to these receptors have been partially characterized and shown to either activate or inhibit platelet function. The receptors fall into two main groups; pattern recognition receptors common to the innate immune system and GPCRs. Among the GPCRs are at least two members of the EDG/lysophosphatidic acid (LPA) family of receptors for LPA, the platelet-activating factor receptor, and the thromboxane receptor [9–11]. Among the pattern-recognition receptors are members of the Toll-like receptor (TLR) family, including TLR4 [12], which is the receptor for bacterial lipopolysacharide, members of the scavenger receptor (SR) B-1 family [13] (as well as CD36 [14]), and up to three splice variants of the LDL receptor-related protein-8 (LRP-8), also known as apoER2 [15]. This paper focuses mainly on CD36, a receptor for oxidized phospholipids that has been implicated by studies from our laboratory and others as a major modulator of platelet reactivity with particular relevance to hyperlipidemia and oxidant stress [16].

CD36, hyperlipidemia & oxidant stress

Phospholipid components of circulating lipoproteins [17–19], including HDL and LDL, are highly susceptible to oxidation *in vivo* and an abundance of experimental data link oxidized LDL (oxLDL) to important components of the pathogenesis of atherosclerosis and thrombosis. OxLDL can be detected in atherosclerotic plaque and in the circulating plasma of experimental animals and patients with atherosclerosis and hyperlipidemia [16,19]. Macrophages recognize oxLDL primarily via CD36 and SRA1 [20], leading to activation of signaling cascades that inhibit migration [21] and that promote internalization of the lipoprotein particles [22], formation of foam cells and a proinflammatory response [23,24]. Studies of mouse gene knockouts have shown that loss of CD36 confers substantial protection against atherosclerosis in the *apoe*-null model [25–28] and suggest that CD36 is the most relevant scavenger receptor in atherogenesis. Although much is known about the mechanism of oxLDL interactions with macrophages, only recently have studies demonstrated that CD36 also mediates platelet responses to oxLDL and contributes to the pro-coagulant state associated with hyperlipidemia and oxidative stress [16].

CD36 structure

CD36 was initially identified in platelets and was termed glycoprotein IV as it was the fourth major band observed on SDS-PAGE gels of platelet membranes [14]. It is sometimes referred to as glycoprotein IIIB in older literature, but most reports now use the CD designation, which was assigned when it was shown to be identical to the antigen recognized by the antimonocyte/macrophage monoclonal antibody OKM5 [29]. It is the defining member of a small gene family [30,31] that in vertebrates contains two other members, lysosomal integral membrane protein (LIMP)-2 and CLA-1 (CD36 and LIMP-2 analogs), which is also known as SRB-1. The primary structure of CD36 family members is conserved in mammals, and multiple ortho-logs have been identified in insects, nematodes, sponges and slime mold [32–36], suggesting that the common ancestral gene is very ancient.

CD36 is 88,000 Da in molecular weight and has two transmembrane domains, short intracytoplasmic domains of five to seven and 11–13 amino acids, and a large extracellular domain with six conserved cysteines linked in three disulfide bridges (Figure 1) [37,38]. The extracellular domain is glycosylated on at least nine of the ten asparagine residues, accounting for the observed molecular weight 30–40,000 Da greater than predicted by the cDNA. Recent studies suggest that glycosylation is necessary for correct trafficking to the plasma membrane [39]. The extracellular domain also contains a hydrophobic stretch that may be membrane-associated and a consensus protein kinase C phosphorylation site with a threonine residue at position 92 that may be phosphorylated on certain cells, including platelets [40,41]. Both of the intracellular domains contain paired cysteine residues that are lipid acylated and thus probably tightly associated with the inner leaflet of the plasma membrane [42]. CD36 can be ubiquitinated on two lysines (amino acids 469 and 472) in its C-terminal domain [43] and this provides a level of control of expression by targeting it to lysosomes; this pathway is sensitive to insulin and fatty acids.

It is now clear that CD36 is a true ‘multifunctional’ protein recognizing at least three classes of ligand; modified phospholipids [17,19,44]; a subset of proteins containing a structural domain termed the thrombospondin type I repeat (TSR) [30,45,46]; and free fatty acids [47, 48]. CD36 affects very different cellular processes depending on the nature of the ligand and the type and location of the cell that expresses it. Cross competition experiments suggest that the binding sites for the three types of ligand are independent. Studies with recombinant and synthetic peptides identified the linear sequence between amino acids 93 and 110 as the likely site for TSR protein binding [49,50]. This peptide contains a negatively charged sequence that is conserved among the three members of the human CD36 family, rodent CD36 and invertebrate orthologs. We termed this domain CD36, LIMP2, drosophila epithelial membrane protein and SRB-1 homology (CLESH) domain and demonstrated that it is present in several unrelated proteins where it also mediates TSR protein binding [51,52]. Interestingly, ectodomain phosphorylation of threonine 92 has been shown to regulate binding of thrombospondin-1 to platelet CD36 [40,41]. The oxidized phospholipid binding site on CD36 remains incompletely characterized, but recent studies from Podrez's group and others suggest that the region between amino acids 157–171 contains a major binding site and that lysine residues at positions 164 and 166 are critical [53,54].

CD36 expression

CD36 is present on many mammalian cell types, including platelets, erythroid precursors and microvascular endothelium; most ‘professional’ phagocytes (macrophages, dendritic cells, microglia and retinal pigment epithelium); hepatocyes; adipocytes; cardiac and skeletal myocytes; and epithelia of the gut, breasts and kidneys [55]. Expression in monocytes has been studied by our group and others and found to be highly regulated at the transcriptional level

[56]. Expression increases by as much as five- to ten-fold as peripheral blood monocytes differentiate in culture to macrophages or dendritic cells [57,58], and also in response to exposure to Il-4, macrophage colony-stimulating factor, granulocyte macrophage colony-stimulating factor, oxLDL and PPAR-γ agonists [56,59,60]. Expression decreases rapidly and dramatically after exposure to lipopolysaccharide, dexamethasone or TGF-β [56,61]. *In vivo*, monocyte CD36 expression levels increase in response to lipid infusion [62]. CD36 expression in macrophages is increased by exposure to hyperglycemia [63,64] and certain HIV protease inhibitor drugs [65]. The glucose effect is not transcriptional, but rather due to increased efficiency of CD36 mRNA translation [63]. In the liver, the lipogenic transcription factors PPAR-γ, liver X receptor (LXR) and pregnane X receptor (PXR) [59,61] cooperate to regulate CD36 expression [66].

CD36 function may also be regulated posttranslationally by movement to and from the plasma membrane surface from an intracellular pool. In muscle cells, insulin, glucose, fatty acids, ischemia and contraction have been shown to regulate CD36 in this manner [67,68]. Regulation of CD36 expression in other cell types, including endothelial cells and megakaryocytes/platelets is not well studied, although one report suggests that platelet CD36 levels in humans may be influenced by lipid-lowering statin drugs [69].

Null mutations in the human *CD36* gene are surprisingly common. It has been estimated that 5–10% of Asians and Africans carry such mutations and therefore as many as 1% of these populations are *CD36* null [38]. CD36 function as a receptor for *Plasmodium falciparum* malaria-infected erythrocytes and have been postulated to be the selective pressure for these mutations. In addition to the *CD36*-null genotypes, approximately 7–10% of Japanese and other Asian populations carry the so-called Nak^{a-} blood group polymorphism associated with lack of platelet CD36 expression. Interestingly, CD36 expression is preserved on other cell types in these individuals. At present, there is no definitive phenotype associated with the absence of CD36, although studies of small numbers of subjects suggest defects in cardiac muscle uptake of fatty acids and perhaps an association with insulin resistance [70–73]. Bleeding or thrombotic diatheses have not been reported.

CD36 regulates angiogenesis

Several TSR-containing proteins, including thrombospondin-1 and -2, and vasculostatin are potent inhibitors of angiogenesis *in vivo* and of microvascular endothelial cell responses to angiogenic factors *in vitro* [74,75]. These proteins play important roles in regulating angiogenesis in pathological settings including cancer. Their antiangiogenic activity is contained in the TSR domains, and our group in collaboration with Bouck and colleagues identified CD36 as the endothelial cell receptor that mediates this activity [76]. CD36-mediated antiangiogenesis is caused by its ability to activate a specific signaling cascade that results in diversion of a proangiogenic response to an apoptotic response [77]. This cascade involves activation of specific members of the mitogen-activated protein kinase (MAPK) and Src kinase families (p38, c-jun N-terminal kinase [JNK] and fyn) resulting in direct activation of caspases as well as induction of expression of other proapoptotic proteins, such as Fas ligand and TNF receptor [78,79]. Recently, we identified a circulating protein, histidine-rich glycoprotein that contains a TSR-binding CLESH domain and that acts as a soluble decoy to block the antiangiogenic activities of TSPs, thereby promoting angiogenesis [74].

CD36 function in lipid metabolism

CD36 functions on adipocytes, muscle cells, enterocytes and hepatocytes as a facilitator of long-chain fatty acid transport [47,66,80–82]. In the gut, CD36 promotes absorption of long-chain fatty acids [81,82] and participates in carotenoid uptake for vitamin A metabolism [83]. In adipocytes, CD36 participates in lipid storage while in muscle it participates in lipid

oxidation. The mechanism by which CD36 performs these transport functions remain poorly understood [47,48] but *cd36*-null mice show abnormal plasma lipid and lipoprotein profiles and resting hypoglycemia [84], attributable partly to a marked impairment of fatty acid utilization in cardiac and striated muscle and fatty acid uptake by adipose tissues [80]. Studies in rodents and humans suggest that CD36 fatty acid interactions may contribute to the pathogenesis of metabolic disorders, such as insulin resistance, obesity and nonalcoholic hepatic steatosis [63,64,85–88]. Studies of rodent models also demonstrated that CD36 in taste bud cells mediates behavioral preferences for fatty food [89], implicating CD36 in a complex neurosignaling network. This is consistent with the findings that CD36 orthologs in sensory cells in insect antennae participate in behavioral responses to lipid pheromones [33–35].

CD36 scavenger functions

On professional phagocytes, CD36 functions as a pattern recognition/scavenger receptor; which is one of a group of structurally unrelated proteins that evolved with the innate immune system as primitive receptors involved in helping the organism eliminate 'foreign' agents (e.g., bacteria, parasites and viruses) during an infection [90,91]. The hallmark of these receptors is their ability to recognize specific classes of molecular patterns presented by pathogens or by pathogen-infected cells. CD36 recognizes specific lipid and lipoprotein components of bacterial cell walls [92], particularly those of staphylococcal and mycobacterial organisms [93], β-glucans on fungal species [94], and erythrocytes infected with *P. falciparum* malaria [30,95] and thereby triggers a reaction that results in opsonin-independent pathogen internalization. Scavenging is an evolutionarily ancient function of CD36; orthologs in *Drosophila* are involved in recognition and clearance of apoptotic cells and mycobacteria, and an ortholog on cells of the worm *Caenorhabditis elegans* participates in the innate immune response to fungi [94].

As with many other scavenger receptors, CD36 also recognizes endogenously derived ligands, including apoptotic cells [58,96,97], cell-derived microparticles (MP) [98], shed photoreceptor outer segments [44,99], oxidized lipoproteins [17,18], glycated proteins and amyloid-forming peptides [100,101]. Research carried out in Hazen's laboratory utilizing a combination of HPLC, mass spectrometry and synthetic chemistry identified the structural characteristics of the oxidized phospholipids in oxLDL that are recognized by CD36. Truncated oxidized unsaturated fatty acids in the *sn*-2 position of glycerol-phospholipids that contain a terminal aldehyde or carboxylic acid, a double bond in the β position, and a ketone or alcohol in the γ position are high affinity ligands for CD36 (Figure 2) [19]. These structures, termed $oxPC_{CD36}$, are present in atheroma, in oxLDL circulating in patients and mice with hyperlipidemia, and on the surface of apoptotic cells [16,19,44]. Structural modeling based on hydrogen-deuterium mass spectrometry suggests that the CD36 ligand structures protrude from the lipoprotein particle or apoptotic cell surface into the plasma milieu where they can be recognized by CD36-bearing cells [102].

Studies with the *cd36*-null mouse strain generated in our laboratory have led to considerable insight into the function of CD36 scavenging function *in vivo*. Our group demonstrated that CD36 plays a role in retinal pigment epithelial cell phagocytosis of shed photoreceptor outer segments [99], antigen presentation [58] by dendritic cells, and ischemia–reperfusion injury in the brain [103]. Loss of CD36 confers protection from diet-induced atherosclerosis [25] and limits inflammation and tissue infarction associated with acute cerebrovascular occlusion [103], but may increase susceptibility to certain infections [92]. The atheroprotective role of CD36 deficiency has been controversial [104–106], but the preponderance of evidence supports an important role for CD36 in mediating proatherogenic macrophage responses, including foam cell formation [22] and inhibition of migration [21], and studies in several *in*

vivo model systems often show dramatically smaller plaque lesions [25–28] and/or less complex lesions with less aortic cholesterol in the absence of CD36 [106].

CD36 mediates platelet hyperreactivity induced by hyperlipidemia & oxidant stress

Although CD36 has been recognized as a major platelet surface glycoprotein for more than 30 years [14], only recently have functional roles been identified. As early as 1999, Volf *et al.* noted that oxLDL could bind to platelet surfaces and induce activation [107], but mechanisms were not identified. Later studies using competitive ligands for different scavenger receptors suggested that SRA1 was not a likely candidate to serve as the platelet oxLDL receptor [108]. The development of highly specific ligands for CD36 along with mouse models of *cd36* deficiency has now helped clarify this field by demonstrating a central role for platelet CD36. In collaboration with Podrez, Byzova and Hazen, our laboratory definitively identified CD36 as the platelet receptor for oxLDL and demonstrated that oxLDL induced platelet activation in a CD36-dependent manner [16]. OxLDL bound to the surface of platelets in a saturable, concentration and time-dependent manner that was inhibited by monoclonal antibodies to CD36 but not to SRA1. Importantly, specific binding was not observed with platelets from CD36-negative humans or mice. Unilammelar phospholipid liposomes containing the synthetic $oxPC_{CD36}$ species previously described also bound platelets in a CD36-dependent manner. At saturating concentrations, oxLDL or $oxPC_{CD36}$ induced activation of platelets detected by flow cytometry assays for expression of the specific activation markers P-selectin and activated integrin $\alpha_{IIb}\beta_3$. These agents did not induce activation of platelets from *cd36*-null mice or from human CD36-null donors. These observations are consistent with pharmacologic studies from Göpfert *et al.* demonstrating that platelet activation by specific oxidized phospholipids was not mediated by platelet-activating factor receptor, thromboxane receptor or LPA receptors [109]. At lower concentrations (e.g., observed in individuals with atherosclerosis), we demonstrated that oxLDL augmented platelet aggregation responses to low doses of ‘classic’ platelet agonists, such as ADP and collagen (Figure 3) [16,110]. Based on these observations we hypothesized that CD36, owing to its unique specificity for ligands generated during host responses to atheroinflammatory diseases (that is oxidized phospholipids and apoptotic cells), may function as a ‘disease sensor’ capable of triggering a signaling cascade that could influence platelet responses and contribute to disease pathology. To test this hypothesis and show *in vivo* relevance of the studies described previously, we studied western diet-fed *apoe*-null mice as a model of the hyperlipidemic, atherogenic, prothrombotic state [16]. Platelet-rich plasma from these mice showed enhanced aggregation responses to low doses of ADP, and when washed platelets from wild-type mice were resuspended in plasma obtained from the western diet-fed *apoe*-null mice, they showed the same increase in sensitivity to low doses of ADP as did platelets incubated with oxLDL. Platelets from *cd36*-null mice did not show a proaggregation response to oxLDL or hyperlipidemic plasma. As noted previously, *apoe*-null mice fed the high-fat ‘western’ diet have shortened tail vein bleeding times (an assay for ‘normal’ hemostasis) and shortened times to form occlusive thrombi in carotid arteries or mesenteric arterioles and venules in response to chemical injury (an assay for pathological thrombosis) [7]. When *cd36*-null mice were bred into the *apoe*-null background to create a double null strain, this prothrombotic phenotype disappeared (i.e., the bleeding times and occlusion times returned to that observed in wild-type mice) [16]. Associated with this phenotypic ‘rescue’, we also demonstrated that the platelet hyper-reactivity *in vitro* disappeared. These studies thus defined CD36 as a receptor that provides a mechanistic link between oxidant stress, hyperlipidemia and thrombosis.

These studies do not imply that CD36 is the only functional receptor on platelets for lipoproteins or modified lipoproteins. Studies using so-called ‘minimally’ oxidized LDL implicated LPA as a platelet reactive substance in oxLDL [9–11,111,112]. While these nonoxidized, modified

phospholipids can be detected in atherosclerotic plaque and some oxLDL preparations [113], and platelets are known to express at least three different G-protein-coupled LPA receptors [9,11], the *in vivo* relevance of this system has not been demonstrated. However, *ex vivo* studies demonstrated that minimally oxidized LDL at high concentrations could induce platelet shape change and sensitize platelets to aggregate in response to other agonists [112].

Studies of HDL effects on platelet function have been inconsistent, with some reports showing inhibition [114,115] and others showing activation [116]. LRP-8 (particularly a splice variant known as apoER2′) binds lipidated apoE resulting in enhanced nitric oxide production and inhibition of platelet activation by ADP and other agonists [117–119]. Platelets from *lrp8*-null mice or normal platelets treated with a general LRP antagonist showed partially abrogation of this effect [15]. On the other hand, platelets from mice haplo-insufficient for *lrp8* showed lower reactivity to ADP and the mice displayed prolongation of thrombosis times after carotid injury [15]. These inconsistencies may be related to the relative low affinity of platelets for HDL, the ability of HDL particles to ‘carry’ a wide array of bioactive molecules, and also to the capacity of LRP-8 to bind apoB-containing lipoproteins [120,121]. The latter has been shown to result in thromboxane generation and sensitization of platelets to other agonists. Another potential confounder is that HDL is at least as susceptible to oxidation *in vitro* and *in vivo* as LDL. The Podrez laboratory demonstrated that oxHDL, but not native HDL inhibits platelet reactivity to multiple agonists, including ADP, collagen and thrombin [122]. Using antibodies and null mice, they demonstrated that this inhibitory effect was mediated by SRB1 (not CD36) and was not dependent on nitric oxide generation. They hypothesized that the balance between oxLDL and oxHDL may determine platelet reactivity in patients with hyperlipidemia and oxidant stress. However, in contrast to these studies, Assinger *et al.* demonstrated that oxHDL-activated platelets in a manner that was inhibited by antibodies to CD36 [116].

CD36 modulates thrombosis in the absence of hyperlipidemia: role of microparticles

Although neither CD36-deficient mice nor humans have a noticeable bleeding diathesis, we observed a subtle hemostatic phenotype in *cd36*-null mice subjected to models of *in vivo* vascular injury and thrombosis, even in the absence of hyperlipidemia. When carotid arteries in *cd36*-null mice were subjected to oxidant-induced injury with a low concentration of ferric chloride (7.5% compared with the ‘standard’ 10–12.5% concentrations used in most of the published literature) the time to form an occlusive thrombus was nearly doubled compared with wild-type mice [98]. Thrombosis times were also significantly prolonged in mesenteric arterioles and venules injured with the lower dose. Direct visualization of thrombus formation *in vivo* using video microscopy and measurement of blood flow with a Doppler probe suggested that thrombi formed more slowly in *cd36*-null mice, but were not less stable when they finally formed. To determine the mechanism of these observations, we demonstrated that platelets from CD36-defcient human subjects had a subtle defect in activation, detected as a rightward shift of dose–response curves to agonists such as ADP.

Based on these discoveries, we suggested that CD36 must recognize endogenous ligands generated during vascular injury and hypothesized that cell-derived MP could be one such ligand. Previous studies from our laboratory and others demonstrated that CD36-dependent phagocyte recognition and uptake of apoptotic cells and/or shed photoreceptor outer segments was mediated by binding of CD36 to phosphatidylserine (PS) and/or oxPS on their surfaces [44,99]. A characteristic feature of MP generation is the loss of membrane asymmetry resulting in surface expression of PS. Thus, we hypothesized that PS or oxPS on MP might also act as a ligand for platelet CD36 and thereby promote platelet activation.

Microparticles are vesicular fragments that bud off cells during either activation or apoptosis [123]. They are 200–1000 nm in size and possess different antigenic properties depending on the type of cell from which they are derived. MP can be generated from platelets, monocytes, erythrocytes, leukocytes and endothelial cells during vascular injury and have been shown to become incorporated in developing thrombi *in vivo* [124]. MP, mostly of platelet origin, can be detected in the circulation of normal human subjects, but markedly increased numbers of circulating MP have been reported in patients with a variety of inflammatory and prothrombotic disorders including cancer, acute coronary syndrome [125], sickle cell disease [126], diabetes mellitus [127,128], thrombotic thrombocytopenic purpura [129], vasculitis, antiphospholipid antibody syndrome [130], hypertension [131] and hematopoietic stem cell transplantation [132]. MPs have been postulated to participate in thrombus formation as PS on their surfaces can be a site for catalytic assembly of the prothrombinase complex [133] and because MP derived from some cells (e.g., monocytes and tumor cells) are a source of circulating tissue factor [134]. In our studies, we used MP derived *in vitro* from cultured endothelial cells, purified human monocytes or platelets, or isolated from plasma of normal human subjects as model systems and showed using fluorescence-based assays that they bound specifically to platelets in a concentration-dependent manner. Binding was inhibited by anti-CD36 antibody or competitive CD36 ligands (e.g., oxLDL), not observed with platelets from CD36-null donors, and inhibited by blockade of exposed PS on the MP surface by annexin V or a monoclonal anti-PS IgM antibody [98].

The interaction of MP with platelets has important functional implications. Similar to the effect of oxLDL, preincubation of platelets with MP led to CD36-dependent augmentation of platelet activation in response to low doses of ADP, assessed by measuring integrin $\alpha_{2b}\beta_3$ activation, P-selectin expression and aggregation. Immunofluorescence confocal microscpopic analysis of murine cartotid thrombi from CD36-null mice showed a significant decrement in endothelial antigen accumulation, suggesting that CD36 plays a role in MP recruitment into thrombi [98]. Importantly, the platelet-activating effect of MP was independent of tissue factor, and thus these data define a novel role for CD36 in thrombosis that may be particularly relevant to the increased risk of pathological thrombosis observed in settings where circulating MP levels are known to be increased.

Mechanisms of CD36 signal transduction in platelets

The mechanisms by which CD36, a receptor with minimal intracellular presence, no intrinsic kinase or phosphatase activity, no known intracellular scaffolding domain(s), and no direct link to GTPases, activates multiple signaling pathways remain incompletely understood, but are under intense study. CD36 signaling in response to its ligands leads to multiple outcomes, including triggering phagocytes to internalize (scavenge) bound ligands [58,95–97], inducing leukocyte proinflammatory responses [21,23,24,101,103], inducing microvascular endothelial antiangiogenic responses [77–79], and promoting platelet granule secretion and integrin activation [16,18,110]. In all cases studied to date, the intracellular signals involve recruitment and activation of Src family nonreceptor protein tyrosine kinases and serine/threonine kinases of the MAPK family, but the specific signaling partners differ depending on the cellular context; for example, the Src family member Lyn and the MAPK JNK2 are critical effectors of macrophage responses [22] while the Src family member Fyn and p38 MAPK are the primary mediators of endothelial cell responses [77].

Korporaal and colleagues recently demonstrated that oxLDL-induced phosphorylation of p38 MAPK in platelets in a pathway that involved both SRA1 and CD36 [135]. We demonstrated by immunoprecipitation that the active phosphorylated forms of Fyn and Lyn were recruited to CD36 in platelets exposed to oxLDL [110]. We also demonstrated that JNK2 and its upstream activator MAPK kinase-4 were phosphorylated after exposure to oxLDL; this was

not observed in platelets from CD36-null donors, showing that the effect was CD36 dependent. Pharmacological inhibition of Src kinases reduced JNK phosphorylation by oxLDL and inhibition of either JNK or Src abolished platelet activation by oxLDL *in vitro.* We also found that JNK was constitutively phosphorylated in 'resting' platelets from western diet-fed *apoe*-null mice, but not from *cd36/apoe*-double-null mice. When carotid artery thrombi induced by ferric chloride injury were analyzed by immunohistochemistry, we found significantly less phosphorylated JNK in the thrombi formed in *cd36*-null mice than the wild-type. Importantly, pharmacological inhibition of JNK prolonged thrombosis times in wild-type but not *cd36*-null mice *in vivo* [110]. These findings demonstrate that a specific CD36-dependent signaling pathway is activated in platelets by oxLDL.

It is highly likely that the C-terminal cytoplasmic domain of CD36 directs its associations with Src and MAPKs. Point mutations of specific tyrosine or cysteine residues (Y^{463} or C^{464}) in this domain [136] result in the loss of response to ligands in cell lines and a recombinant protein containing this cytoplasmic domain was shown to precipitate a multiprotein complex from monocyte lysates that contained Lyn, a MAPK kinase, and several as yet unidentified proteins [22]. As CD36 resides in cholesterol-rich, detergent-insoluble lipid raft domains and co-purifies with caveolae from some tissues [137–140], it is possible that CD36 signaling relates to its localization in these membrane regions in which signaling molecules, such as Src, accumulate. It is also likely that CD36 may affect signal transduction, in part, by interacting with other membrane receptors, such as integrins, tetraspanins [141] and TLR [142]. The latter was elegantly demonstrated in studies showing cooperation between CD36 and TLR2 or TLR6 in macrophage recognition and response to some bacteria and bacterial cell wall components [142,143]. However, uptake and proinflammatory responses in response to others did not require TLR-mediated activation of NF-κB and was entirely dependent on CD36-JNK signaling [144,145]. Similarly, TLRs are not required for CD36-dependent uptake of oxLDL [22] or apoptotic cells. Several CD36 functions require coexpression of integrins and both β_2[100,146] and β_3[141] integrins coimmunoprecipitate with anti-CD36 antibodies. Internalization of apoptotic cells and photoreceptor outer segments require integrins – $\alpha_v\beta_3$ in macrophages [96] and $\alpha_v\beta_5$ in dendritic cells [58] and retinal pigment epithelia [147]. Microglial responses to αβ amyloid require β_2 integrins and the spreading of brain tumor cells on thrombospondin-1 seems controlled by a functional interaction between β_1 integrins and CD36 [148]. The potential role of integrins, TLRs, tetraspanins and membrane microdomains in platelet CD36 signaling has not been studied.

The signaling partners downstream of Src and MAP kinases in platelets are not well described. In other cells, studies have implicated focal adhesion components, including the tyrosine kinases Pyk2 and focal adhesion kinase and the adaptor proteins $p130^{cas}$ and paxillin, in CD36 signaling [21,149]. For example, after macrophage exposure to oxLDL, focal adhesion kinase undergoes prolonged phosphorylation and activation [21] owing to direct activation by Src family kinases coupled with inactivation of a specific phosphatase src homology (SH) domain 2 containing tyrosine phosphatase-2 (SHP-2). The latter was the result of intracellular generation of reactive oxygen species and subsequent oxidative inactivation of a critical cysteine residue in the enzymatic active site. The net result of these intracellular events was enhanced actin polymerization, increased cellular spreading and inhibition of migration. The Vav family of proteins may also link CD36 to downstream events [146]. These proteins are known substrates for Fyn and Lyn and when phosphorylated function as guanine nucleotide exchange factors for Rho and Rac GTPases. Vavs are large multidomain proteins that contain SH3 domains flanking a single SH2 domain and, thus, they function as scaffolds as well as guanine nucleotide exchange factors. Vavs are phosphorylated in macrophages, microglial cells and platelets in a CD36-dependent manner and may be an important link between CD36 and responses requiring small molecular weight G-proteins.

Conclusion & future perspective

The studies reviewed in this paper provide convincing evidence of a significant role for platelet CD36 in modulating platelet reactivity and for promoting a prothrombotic state in settings where CD36 ligands are present. These ligands, such as oxLDL, MP and apoptotic cells are generated *in vivo* by oxidant stress, hyperlipidemia, inflammation and cancer; and thus, targeting CD36 and/or its signaling pathway may provide a novel approach for development of antithrombotic therapies relevant to specific high-risk states. Importantly, CD36 deficiency in humans is not rare and is not associated with any significant pathologic phenotype, including excess bleeding. Similarly, *cd36*-null mice are phenotypically normal under most conditions. These observations strongly suggest that targeting CD36 pharmacologically is feasible. As CD36 is expressed on many different cell types, further study of CD36 signaling in platelets is warranted to identify unique features that could be targeted to 'spare' the effects of blocking CD36 globally.

Executive summary

Platelet CD36 is a receptor for oxLDL & cell-derived microparticles

- Platelet CD36 activates a signaling cascade involving fyn and lyn kinase, JNK2 and Vav, resulting in platelet activation.
- Platelet CD36 signaling cascade is activated in vivo by hyperlipidemia and oxidant stress.

CD36 is thromboprotective in mice

- Genetic deletion of CD36 abrogates the hypercoagulable state induced by hyperlipidemia and oxidant stress and protects against formation of occlusive thrombi induced by chemical vascular injury.
- CD36 deficiency is not associated with a bleeding diathesis.

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▪ of interest

▪▪ of considerable interest

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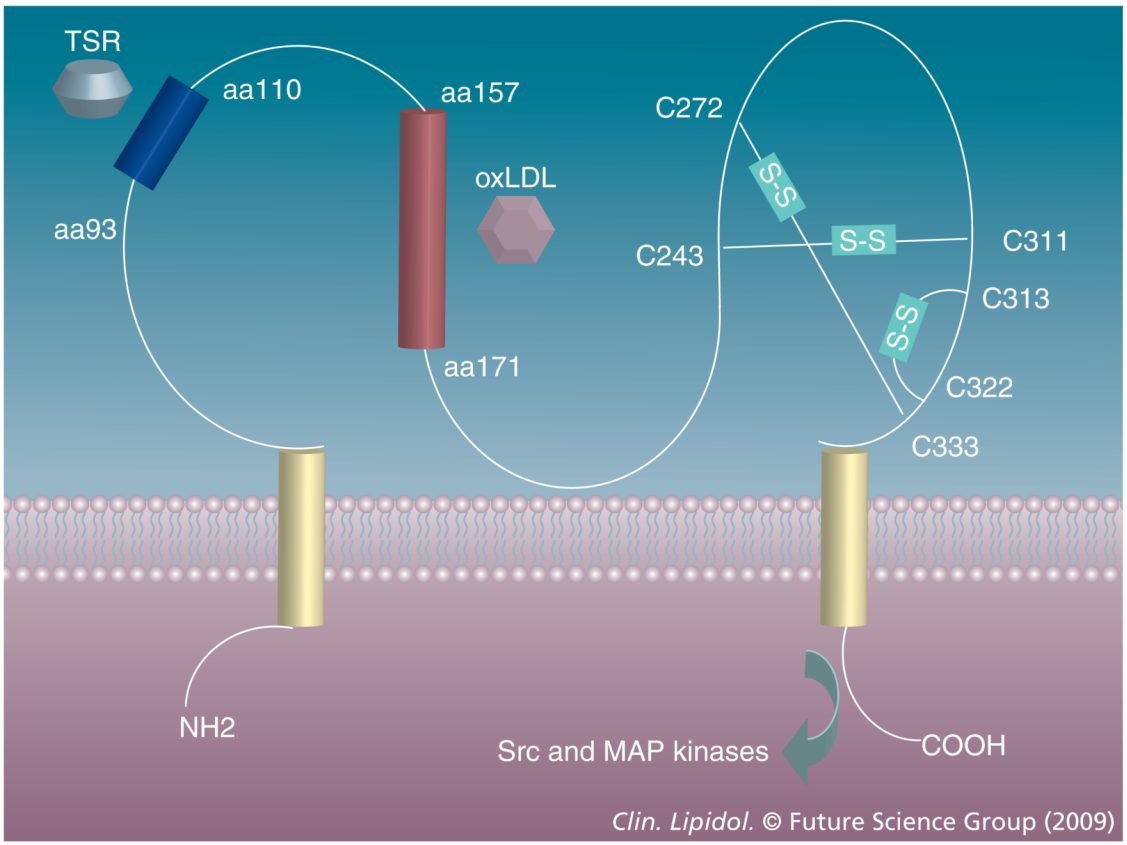

Figure 1. CD36 has two transmembrane domains (yellow), two short cytoplasmic domains and a large extracellular domain

The latter contains three disulfide bonds clustered near the N-terminal transmembrane domain and independent binding sites for TSR-containing proteins (blue) and oxLDL (red) on the N-terminal half. The C-terminal cytoplasmic domains directs intracellular signaling via activation of specific Src-family and MAP kinases. TSR: Thrombospondin type I repeat.

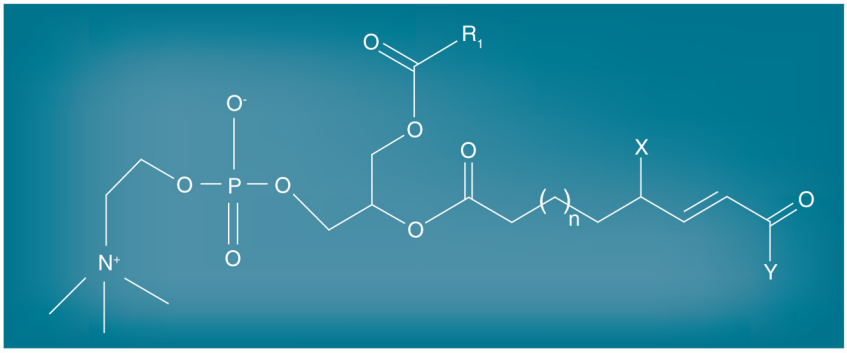

Figure 2. oxPCCD36 structure

Truncated oxidized unsaturated fatty acids in the sn-2 position of glycerol-phospholipids containing a terminal aldehyde or carboxylic acid, a double bond in the β position, and a ketone or alcohol in the γ position are ligands for CD36. X is OH or =O; Y is H or OH.

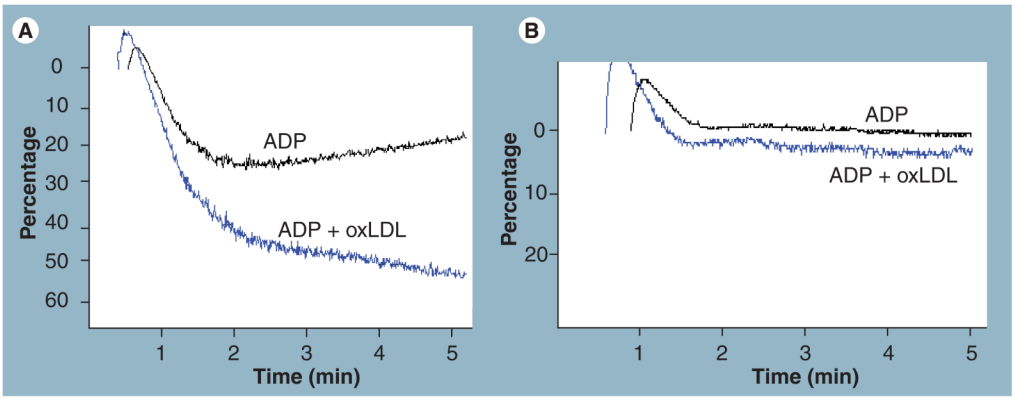

Figure 3. Oxidized LDL sensitizes platelets in plasma to activation by low doses of physiologic agonists

Washed platelets were incubated with 50 μM copper oxidized LDL (blue tracings) or native LDL (black tracings) for 30 min and then ADP added at 1 μM at 37°C under stirring conditions. Aggregation was detected as increase in light transmission over time. **(A)** OxLDL enhanced the aggregation response in CD36-positive donors, **(B)** but not CD36-negative donors.