INTERACTION BETWEEN CD36 AND OXIDIZED LDL MODULATES MACROPHAGE CYTOSKELETAL FUNCTIONS: A MECHANISM OF MACROPHAGE TRAPPING

by

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* We also certify that written approval has been obtained for any proprietary material contained therein.

Dedicated to my mother,

Kiyeol Sophia Kim

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Introduction

Chapter 1. *

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Both the happiest and the saddest moments in my life happened just one year apart while I studied in the Ph.D. program at Case Western Reserve University. During that time, my son, Ansel was born and soon after my mother passed away. This time in my life was not easy. I tried to be both a daughter of whom my mother might be proud as well as a good mother to my own son. Yoonsoo, my husband, was always in my corner. His help was enormous. He always encouraged me and gave me general advice being a scientist himself, majoring in economics. I must also give thanks to my family, my father and my two brothers. They helped me overcome the difficulties that occurred after I lost my mother. They always have trusted in me, which gave me the confidence that drove me to go forward. I also must thank my mother and father-in law who helped take care of Ansel when he was a baby. Thus, the power that drove me to perform research and get my doctoral degree started with the love and support of all my family.

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INTERACTION BETWEEN CD36 AND OXIDIZED LDL MODULATES MACROPHAGE CYTOSKELETAL FUNCTIONS: A MECHANISM OF MACROPHAGE TRAPPING

Abstract

by

YOUNG MI PARK

Trapping of lipid laden macrophages is a critical but reversible step of arterial and adipose tissue inflammation and contributes to atherosclerosis, obesity and insulin resistance. However, its mechanism is not clearly defined. We tested the hypothesis that CD36, a class B scavenger receptor expressed on macrophages, may be implicated in this process and revealed molecular mechanisms by which oxidized LDL (oxLDL) acts through CD36 and induce macrophage trapping. In vivo and in vitro assays showed that oxLDL but not native LDL inhibited migration of wild type macrophages in a CD36 dependent manner. OxLDL also induced rapid spreading and actin polymerization in CD36 positive, but not negative macrophages. The mechanism was shown to be dependent on oxLDL mediated CD36 signaling which results in sustained activation of focal adhesion kinase (FAK) and inactivation of src homology 2-containing phosphotyrosine phosphatase (SHP-2). The latter was due to generation of reactive oxygen species (ROS) with resulting oxidative inactivation of SHP-2. Macrophage

migration in the presence of oxLDL was partially restored by antioxidants or NADPH oxidase inhibitors which restored dynamic activation of FAK. In addition, oxLDL mediated CD36 signaling also induced loss of cell polarity in macrophages. Live cell imaging of macrophages showed that oxLDL, but not native LDL, actuated retraction of macrophage front end lamellipodia. *Cd36 null* macrophages and macrophages null for Vav, a guanine nucleotide exchange factor (GEF), did not show this effect. Migration velocity and dynamic movement of the macrophage membrane were decreased by oxLDL in a CD36 and Vav dependent manner. These findings were related to the activity of myosin II, a cell polarity determinant. OxLDL induced dephosphorylation of myosin regulatory light chain (MRLC) by increasing the activity of a small molecular weight G protein Rac. Six-thio-GTP which inhibits binding of Vav to Rac, abrogated the effect of oxLDL. Thus, the mechanism causing loss of cell polarity, is via Vav-dependent activation of Rac and dephosphorylation of MRLC. Activation of these pathways may induce trapping of macrophages in the arterial intima and adipose tissue and promote atherosclerosis and diabetic adipose tissue inflammation, two hallmarks of the metabolic syndrome.

INTRODUCTION

1. Role of Macrophages in the Pathogenesis of Atherosclerosis and Metabolic Syndrome

Cardiovascular disease is currently the leading cause of death in many developed countries and atherosclerosis is the most important underlying pathology (1). Atherosclerosis is a disease characterized by accumulation of lipids and an inflammatory response in the arterial intima, resulting in the formation of plaque that can lead to arterial narrowing and that is susceptible to rupture with acute thrombotic occlusion (2,3). In the initial stage of atherogenic inflammation, monocyte-derived macrophages perform a critical role by internalizing oxidatively modified low density lipoprotein (oxLDL) through scavenger receptors (4,5). This function of macrophages may be driven as a protective response to clear the pathogenic lipoproteins in the arterial intima. However, by promoting unregulated lipid uptake as well as further inflammatory responses, macrophages play critical pathologic roles in progression of atherosclerosis. A previous study supports this by showing that hypercholesterolemic mice that were bred to macrophage-deficient mice, were notably resistant to atherosclerosis (6). The initial atherosclerotic lesion, called a fatty streak, contains abundant lipid laden macrophages (7). The formation of fatty streaks is initiated by the adherence of circulating monocytes to activated endothelial

cells. These monocytes migrate into the arterial intima and differentiate to macrophages. Macrophages become foam cells by internalizing modified lipoproteins such as oxLDL through scavenger receptors (8). Foam cells contribute to a further complex lesion formation by secreting various cytokines that recruit T cells, macrophages and smooth muscle cells (9). Macrophages also function to promote LDL oxidation. They express lipoxygenases (LOs), myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and NADPH oxidases, all of which contribute to LDL oxidation (10). Thus, macrophages in atherosclerotic lesions accelerate foam cell formation and recruitment of other immune cells by expanding the pool of modified LDL, which is a vicious cycle.

A main feature of atherosclerotic inflammation is that it does not resolve and thereby leads to an irreversible remodeling of arteries. Perturbed function of macrophages is an attribute of atherogenic inflammation. Typically in acute inflammation, inflammatory responses end with resolution of infiltrated immune cells. Dying neutrophils are taken up by macrophages and macrophages emigrate from the inflammatory lesion to draining lymph nodes (11, 12). The atherogenic inflammatory process has distinct features compared to the classic inflammatory process. In atherogenic inflammation, a neutrophil-dominant phase is not seen in the early stage. Macrophages, not neutrophils, appear to be inflammation initiating effectors (13). In addition, macrophages are trapped in atherosclerotic lesions and keep propagating the inflammatory responses without resolution. However, previous observations showed that under certain conditions, atherosclerotic lesions can regress and that this occurs concurrent with macrophage emigration. (14-16). Llodra et al. induced regression of

atherosclerotic plaque by transplantation of arterial segments with atherosclerotic plaque from hypercholesterolemic mice into normal mice. These studies revealed the important role of macrophage neointimal trapping in lesion growth and macrophage emigration to regional lymph nodes in lesion regression (12-18). The emigrated macrophages from regressing plaques were found in both the hepatic and iliac lymph nodes, suggesting that macrophage emigration from plaque uses two routes including afferent lymphatic drainage as in other inflammations, and in a abluminal to luminal trafficking for direct re-entry into the bloodstream (16). This confirms the first report by Gerrity that foam cells traverse the aortic endothelium and go back to circulating blood (19).

The mechanisms by which macrophages lose their mobility in atherosclerotic lesions and how migrating ability is restored in healthy subjects are unknown. But the studies of Llodra *et al*. strongly suggest that the hyperlipidemic state and its associated oxidant stress may play a key role in limiting migration. One explanation for the limited egress of macrophages is that the atherosclerotic arterial intima is separated from the afferent lymphatic vessels, the typical route of macrophage egression from tissues (20). However, the anatomical limitation seems to be recoverable by eliminating the atherogenic conditions (16). There have been studies to reveal the mechanism of macrophage trapping. Previous studies showed that oxLDL changes macrophages to be more adherent. OxLDL or oxidized linoleic acids, components of oxLDL induce differentiation of monocytes to macrophages with lower expression of chemokine (C-C motif) receptor (CCR) 2 and higher expression of CX3CR1, which are more adherent to arterial smooth muscle cells (21-24). A

previous study also showed that cholesterol addition to plasma membrane inhibits macrophage migration (25). Thus, lipid mediated signals appear to be a strong candidate mechanism. Nevertheless, the mechanism of macrophage trapping within atherosclerotic lesions has not been clearly defined, yet represents a novel target for potential therapeutic intervention.

We hypothesize that lipid-derived signals generated by the pro-atherogenic state are strong candidates for the mechanism of macrophage trapping. This is supported by work from Steinberg's group nearly 25 years ago showing that oxLDL inhibits macrophage migration (26). Defining the mechanism of macrophage trapping may suggest novel approaches to re-mobilize lipid laden macrophages from the atherosclerotic lesion and facilitate regression of the disease. This could provide a new therapeutic strategy for treatment of atherosclerosis (summarized in Figure 1).

Mechanisms of macrophage trapping in atherosclerosis may also have important implications for metabolic syndrome. Metabolic syndrome is a multiplex set of risk factors for cardiovascular disease characterized by six major components, including abdominal obesity, atherogenic dyslipidemia, hypertension, insulin resistance, and a proinflammatory and prothrombotic state (27). Although there are other influencing factors such as genetic and aging factors, there is a consensus on the concept that obesity is the major factor that contributes to the other components of metabolic syndrome. Obesity and associated hyperlipidemia induce chronic inflammation in white adipose tissue and arterial intima and thus predispose to both insulin resistance and atherosclerosis (28-31).

As in atherosclerosis, adipose tissue inflammation in obese subjects is characterized by abundant macrophage infiltration, that is thought to be the main origin of the systemic inflammatory response (30). Adipocytes function in glucose uptake and inhibition of lipolysis. These functions are dependent on sensitivity of the adipocytes to insulin, and adipose tissue inflammation impairs the function of adipocytes and induces insulin resistance. Previous studies revealed important roles for macrophages in this process. Macrophages in adipose tissue alter expression of glucose transport protein 4 (GLUT4) and the insulin receptor substrate (IRS)-1 in co-cultured 3T3-L1 adipocytes (32). In addition, IL-1β secreted by macrophages downregulates IRS-1 and promotes insulin resistance (33). Although the mechanism by which macrophages are recruited into the adipose tissue of obese subjects is not clearly defined, it appears to have similarity to the atherogenic mechanism in arteries. Macrophages from arteries and adipose tissue of obese subjects have high lipid content and their accumulation is proportionate to the degree of inflammation (31,34-36). Interestingly, conditions that reverse disease progression concomitantly reduce the number of macrophages in the diseased tissues. Weight loss improves the adipose tissue inflammation and insulin resistance by reducing the number of adipose tissue macrophages (37-39). These observations suggest the dynamic nature of adipose tissue inflammation, and suggest that the mechanistic explanations for macrophage trapping may underlie a common pathogenic mechanism of atherosclerosis and adipose tissue inflammation, and thus lead to new therapeutic strategies.

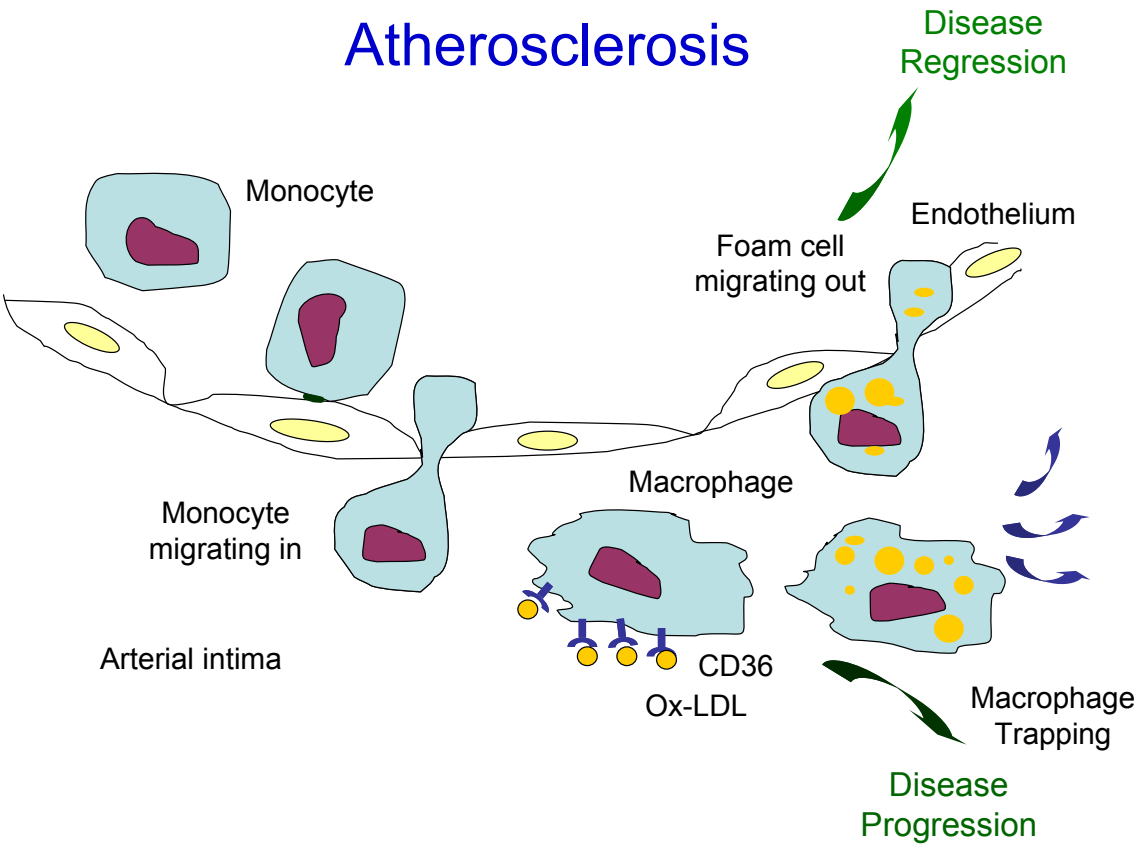


Figure 1. Macrophage trapping as a mechanism of atherosclerosis.

Atherosclerotic process is initiated by monocyte entry into arterial intima, followed by differentiation of these cells to macrophages. Macrophages internalize oxLDL through scavenger receptors like CD36, and are trapped in the arterial intima. They promote further inflammatory responses by secreting cytokines which recruit other immune cells to the lesion. Progression of atherosclerosis is related to macrophage accumulation while regression is induced by macrophage emigration. Thus, understanding mechanisms that regulate macrophage trapping in atherosclerotic lesions and macrophage emigration from lesions could lead development of new strategies for the treatement of atherosclerosis.

2. Macrophage Scavenger Receptor CD36

The focus of this thesis research is on the mechanistic role of the macrophage scavenger receptor CD36 in regulating migration. This hypothesis is based on previous observations that oxLDL inhibits macrophage migration (26) and CD36 is a major receptor that interacts with oxLDL in macrophages (40-42).

CD36 is a transmembrane glycoprotein receptor that is expressed on various cell types including monocyte/macrophage, dendritic cells, microglia, retinal pigment epithelium, erythroid precursors, microvascular endothelial cells, platelets, adipocytes, hepatocytes, cardiac and skeletal myocytes and epithelium of the breast, kidney, and gut (43). It is a member of class B scavenger receptor family and is a pattern-recognition receptor. Scavenger receptors including CD36, scavenger receptor A (SRA), lectin like oxidized LDL receptor (LOX)-1, Toll-like receptors (TLRs) and CD68, are involved in innate immunity by recognizing pathogens and aiding the functions of phagocytes that eliminate the pathogens. These receptors recognize specific molecular patterns of polyanionic macromolecules that are presented by pathogens or by infected cells (44,45). CD36 is known to recognize specific bacterial cell wall components of Staphylococcus and Mycobacterium, β-glycans of fungus, and Plasmodium falciparum infected erythrocytes (46-49). CD36 also functions in recognizing certain endogenous molecules or particles. These include apoptotic cells (50-52), photoreceptor outer segments (53,54),

fibrillar β-amyloid (55) and oxidatively modified LDL (40-42). In addition to this, CD36 binds to the extracellular matrix components containing so-called thrombospondin structural homology repeat (TSR), including thrombospondin-1 (TSP-1), thrombospondin-2 (TSP-2) and vasculostatin, and inhibits pro-angiogenic activity of endothelial cells (56-58). Therefore, the diverse functions of CD36 are derived from the ability of CD36 to bind to different classes of exogenous or endogenous ligands in different cellular contexts (summarized in Figure 2).

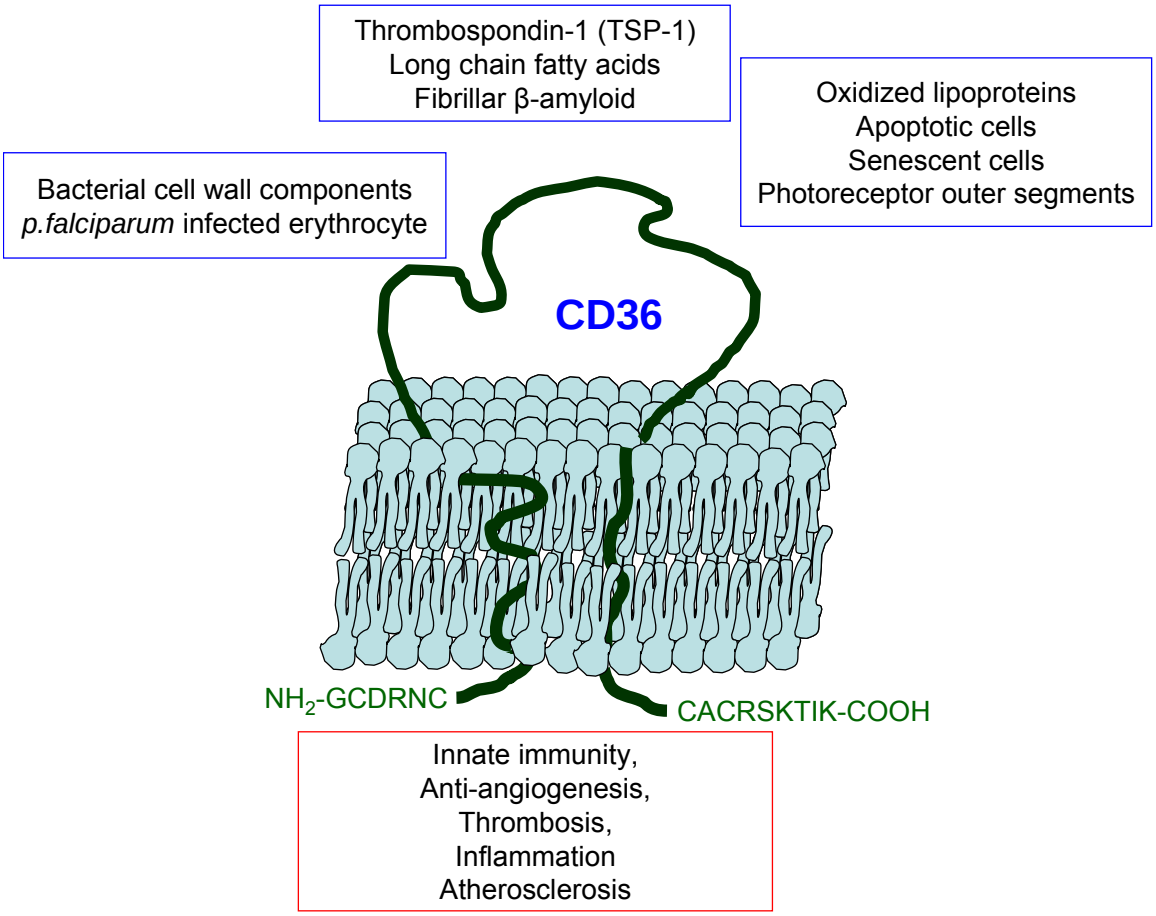
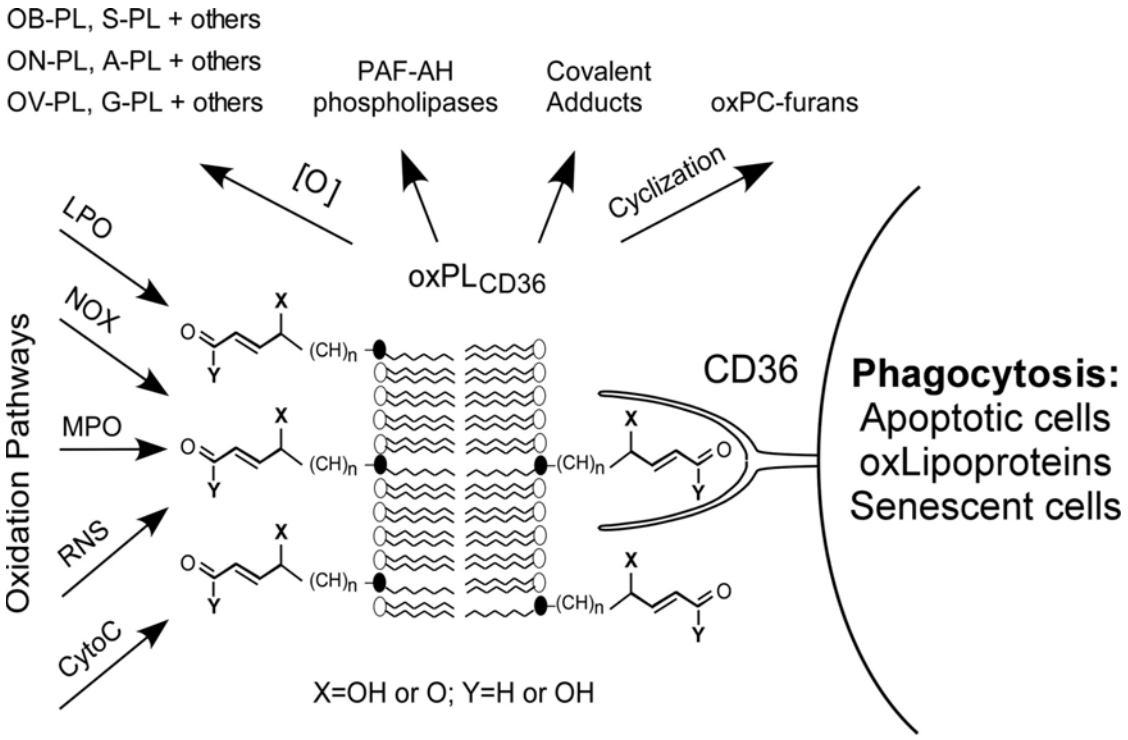


Figure 2. Various functions of CD36 depend on ligands.

CD36, expressed in monocyte/macrophages, microvascular endothelial cells, adipocytes, microglia and platelets, is implicated in functions related to common pathologic processes through binding to specific ligands.

Among the ligands for CD36, oxidized phospholipids are known to play an important role in atherosclerosis (59,60). Phospholipids are essential components of lipoproteins and cell membranes. They are composed of fatty acids bound to a glycerol backbone which contains a polar head group. Phospholipids, particularly those containing unsaturated fatty acids, are susceptible to free-radical or enzymatic oxidation by reactive oxygen and nitrogen species generated by myeloperoxidase, lipoxygenase, and other enzymes present in the vessel wall (9). Modification of phospholipids creates conformational changes including the protrusion of oxidized fatty acids from the hydrophobic membrane or lipoprotein interior into the more polar aqueous compartment. Thus, modified phospholipids gain accessibility to interact with pattern recognition receptor like CD36 (61). Accumulation of oxidized phospholipids is found in conditions having oxidative stress such as viral infections and inflammatory conditions like rheumatoid arthritis and atherosclerosis. Necrotic cells and apoptotic cells are also major generators of oxidized phospholipids (59,60). There have been extensive studies to reveal the chemical and structural characteristics of the oxidatively modified lipids on the surface of cell membranes and/or lipoprotein particles that serve as ligands for CD36. Early studies revealed that the extractable lipid portions of oxidized lipoproteins and senescent or apoptotic cells retained a significant portion of CD36 binding activity (41, 62-65) and that oxidized phosphatidylcholines (oxPC) are the primary ligands (62). Accordingly, Podrez et al. reported that LDL modified by myeloperoxidase-H_2O_2-nitrite system of monocytes, is a more physiologically relevant oxLDL, binding to CD36 with high affinity. They also found that the structural characteristics required for binding to CD36 are phospholipids with an sn-2 acyl group that incorporates a terminal γ-hydroxy

(or oxo)- α, β-unsaturated carbonyl ($oxPC_{CD36}$) (41). In addition, oxidized phospholipids with reactive groups bind covalently to proteins and form lipid-protein adducts. Both the free oxidized phospholipids and the adducted forms are recognized by CD36 (66) (summarized in Figure 3).



Hazen S.L. *J. Biol. Chem.* 2008;283:15527-15531

Figure 3. Formation of oxidized phospholipids that serve as high affinity ligands for CD36.

Phospholipids modified by various oxidation pathways share a characteristic for high affinity binding to CD36, which is an sn-2 acyl group that incorporates a terminal γ-hydroxy (or oxo)- α, β-unsaturated carbonyl (oxPC$_{CD36}$). By recognizing these ligands, macrophage CD36 mediates internalization of oxLDL, apoptotic cells and senescent cells. LPO, lipoxygenase; NOX, NAD(P)H oxidase; MPO, myeloperoxidase, RNS; reactive nitrogen species; CytoC, cytochrome C

Macrophage CD36 has been implicated in atherosclerosis, primarily by mediating uptake of oxLDL. Febbraio *et al.* revealed that CD36 deficient macrophages are profoundly defective in oxLDL uptake and *Cd36* null mice on the proatherogenic *Apoe*-null background showed significantly less atherosclerotic lesion formation than *Apoe*-null mice (67-69). A study using stem cell transplantation also revealed the macrophage-dependent pathogenic mechanism of atherosclerosis. Mice receiving *Cd36* null macrophages had profoundly less atherosclerotic lesion formation and re-introduction of macrophages with CD36 induced a 2 fold increase in atherosclerotic lesion area. In this study, no differences were found in engraftment, macrophage recruitment, glucose tolerance, weight, LDL, and high-density lipoprotein (HDL) cholesterol between mice receiving *Cd36* null macrophages and mice having macrophages with wild type CD36 (70). In addition, treatment with a competitive peptide ligand (EP80317) derived from growth hormone-releasing peptide family that blocks the oxLDL binding site of CD36, also reduced atherosclerotic lesion formation by 51% in *ApoE* null mice (71). These studies provide strong evidences that the role of macrophage CD36 is pro-atherogenic and blocking the function of CD36 prevents atherosclerosis. However, interpretation of the studies was complicated by a study with a different *Cd36* null mouse strain that showed *Apoe/Cd36* double null mice fed a high fat diet had modest reduction or even an increase in some atherosclerotic lesions compared to *Apoe* null mice. In this study, Moore *et al*. also reported reduced lipid accumulation in peritoneal macrophages coupled to significantly higher (40%) plasma cholesterol levels in male, but not female, *Apoe/Cd36* double null mice in comparison to *Apoe* null mice (72). The contradictory observations were originally highly controversial. As Witztum and Collot-Teixeira *et al.* pointed out,

the differences could have been the result of using two different mouse strains (73,74). A very slight genetic variation between the strains, including the number of backcrossing, induces differences and an undefined disease modifying gene may also intervene. The potential presence of pathogens in breeding rodent colonies cannot be ignored. Certain pathogens such as *Chlamydia pneumoniae* and Cytomegalovirus are known to increase atherosclerotic lesion size (75). Indeed, CD36 deficient mice are defective in phagocytosis of pathogenic bacteria like *Staphylococcus aureus* and mycobacterium (76). In addition, the investigators of two groups observed the atherosclerotic lesions in different areas of arteries at different time points on high fat diet. Moore *et al.* observed aortic valve lesions at 8 weeks after high fat diet while Febbraio *et al.* investigated aortic arch or aorta at 12 and 35 weeks (67, 68, 72). The apparent controversy about the role of CD36 in murine atherosclerosis has been tempered by recent studies from Febbraio's group showing significant atheroprotection in an additional *Cd36* null strain crossed the *Apoe* null strain and in a different atherosclerosis model, the LDL receptor deficient strain (70,71,77). Furthermore, two recent papers from Moore's group reported significant atheroprotection – approaching that seen in Febbraio's original reports – in the *Cd36/Apoe* null strain developed in their lab (78,79). It is also worthwhile to address a recent report of Van Eck *et al.* This study showed that the presence of class B, type I scavenger receptor (SR-BI) in bone marrow derived macrophages in *Ldlr* null mice was proatherogenic at the earliest phase of lesion formation (after 4 weeks on a Western-type diet) but having no effect on 6 weeks and protective in later stages of lesion formation (after 9 and 12 weeks) (80). These observations of possible differing effects of CD36 on atherosclerotic lesion formation at different time points may be explained by our

hypothesis about macrophage trapping. We hypothesized that interaction between CD36 and oxLDL may inhibit macrophage migration and induce macrophage trapping in atherosclerotic lesions. If it is true, at the early phase of atherosclerotic lesion formation where macrophages are infiltrated and accumulated without egress, the number of macrophages in the atherosclerotic lesions and the size of the lesion areas may not be different between *Apoe* null and *Apoe/Cd36* double null mice as Moore *et al.* observed. However, when the macrophage infiltration reaches a plateau, macrophages may start to egress and the rate of egression may determine the disease progression. Therefore, at the late phase of atherosclerotic lesion formation, *Apoe/Cd36* double null mice may have egression of macrophages at any extent and thus have reduction of macrophage accumulation and lesion size as Febbraio *et al.* observed. *Apoe* null mice may have progressive lesion formation since macrophage egression is inhibited by CD36/oxLDL interaction.

Another interesting finding is that Moore *et al*. showed that despite no difference in atherosclerotic lesion formation, lipid accumulation in the peritoneal macrophages from *Apoe*/*Cd36* double null mice was less than that in the cells from *Apoe* null mice, which is consistent with the findings of Febbraio *et al.* Therefore, it seems that the function of lipid uptake, or intracellular lipid content of macrophages may not be the sole influencing factor for atherosclerosis.

OxLDL taken by macrophage scavenger receptors moves to lysosomes, where the cholesterol ester is hydrolyzed to be free cholesterol and fatty acids. Excess free cholesterol is re-esterified by acyl coenzyme A: acylcholesterol transferase (SOAT or

ACAT). It is likely that reducing the expression or activities of SOAT may affect the atherogenic inflammation by decreasing foam cell formation. However, both *Soat1-Apoe* and *Soat1-Ldlr* double null mice developed atherosclerosis to a similar extent as *Apoe* or *Ldlr* null mice with hypercholesterolemia (81). Moreover, *Ldlr* null mice receiving *Soat1* null macrophages had no difference in serum cholesterol levels and larger atherosclerotic lesions than *Ldlr* null mice receiving wild type macrophages (82). Based on these observations, it seems that it is not the intracellular lipid content in macrophages but the functional outcome derived by interaction between oxLDL and scavenger receptors that generates the major impact on atherogenesis. This observation is supported by the fact that intracellularly generated oxysterol activates a nuclear hormone receptor peroxisome proliferators-activated receptor γ (PPAR-γ) (83). PPAR-γ is known to increase transcription of CD36. Thus, oxLDL taken up by CD36 increases the atherogenic proinflammatory response by increasing CD36 and its interaction with oxLDL. In the setting of SOAT ablation, macrophages may retain the lipid as free cholesterol rather than cholesterol ester. However, by maintaing or increasing the ability of CD36 that interacts with oxLDL, macrophages may perform proinflammatory functions in atherosclerosis.

CD36 has other functions that may be relevant to atherosclerosis and obesity. It is a fatty acid transporter in adipocytes, myocytes, hepatocytes and enterocytes (84-89), and *Cd36* null mice demonstrate abnormal plasma lipid profile, which may be from impairment of uptake and utilization of fatty acids (86). A recent study showed that *Cd36*-null mice on a proatherogenic *Apoe*-null background had a lower degree of insulin resistance than *Apoe*-null mice, demonstrating less adipose tissue inflammation with less

infiltration of macrophages (90). The function of CD36 that increases intracellular lipids may induce lipotoxicity in adipocytes and result in insulin resistance . This is supported by an observation that CD36 ablation reduces lipid accumulation in cardiomyocytes and prevents cardiomyopathy (91). Nevertheless, the roles of macrophages in adipose tissue inflammation may also contribute to insulin resistance by generating an inflammatory milieu. A previous study showed adipocytes co-cultured with *Cd36* null macrophages had higher sensitivity to insulin than adipocytes co-cultured with wild type macrophages (90).

The addressed studies confirm that macrophage CD36 through interaction with oxLDL generates signals required for atherogenic inflammation. Therefore, we hypothesized that CD36 modulates macrophage cytoskeletal signaling in response to oxLDL and inhibits macrophage migration.

3. CD36 as a Signaling Molecule

CD36 functions as a ligand-dependent signaling molecule. That generates cell-specific functional outcomes including oxLDL uptake, proinflammatory cytokine release by macrophages, inhibition of pro-angiogenic endothelial cell functions and platelet activation (92-98). In macrophages, microglial cells, microvascular endothelial cells, and platelets, CD36 binding to its ligands such as oxLDL and thrombospondin-1 (TSP-1) induces phosphorylation of src-family kinases including Fyn and Lyn (99-101). These kinases are physically associated with CD36. In macrophages, oxLDL binding to CD36 induces phosphorylation of Lyn and this subsequently activates c-Jun N terminal kinase (JNK) 2. A function of activated JNK2 is facilitating uptake of oxLDL. A previous study showed that macrophages treated with pharmacological inhibitors of src kinases or JNK had defects in oxLDL uptake (95). In addition, oxLDL/CD36 interaction induces activation of transcription factor NFκB in macrophages through protein kinase C (PKC) (96) and triggers secretion of inflammatory cytokines such as TNFα/β, IL-1β, IL-6, and interferon beta and gamma (IFNβ/γ) whose expressions are significantly decreased in CD36-deficient macrophages (92,97,98). The downstream effectors, Lyn and JNK2 also function in platelets. OxLDL binding to CD36 on platelets facilitates platelet activation by inducing recruitment of Fyn and Lyn to the CD36 signaling complex, leading to JNK2 activation. Platelet activation by oxLDL is blocked by pharmacological inhibitors of src kinases or JNK (99). Fibrillar β amyloid binding to CD36 in microglial cells activates

Fyn, Lyn and a different mitogen activated protein kinase (MAPK) family member, p44/42 MAPK. Activation of these kinases mediate inflammatory responses of these cells to fibrillar β amyloid (100). In microvascular endothelial cells, Fyn is the prominent src family member and p38, the prominent MAPK activated by CD36, and apoptosis is the functional consequence(56, 101).

CD36 can diversify its downstream signaling pathways and functions by interacting with other membrane receptors, such as integrins, tetraspanins like CD9 (102) and toll-like receptors (TLRs) which sense microbial pathogens and initiate inflammatory responses (103) . Macropahge CD36 responds to lipotechoic aicd (LTA) on the membrane of *Staphylococcus aureus* and diacylated lipoproteins, depending on interactions with TLR2/6 (76, 104-106). CD36 also cooperates with TLR2 and its downstream effector, interleukin-1 receptor-associated kinase 4 (IRAK4) to respond to *Plasmodium falciparum* infected erythrocytes and secrete cytokines (103). CD36 mediated uptake of oxLDL is independent of TLR2 (95). However, a recent study revealed that CD36 induces assembly of TLR4/6 heterodimer, which is responsible for the responses to oxLDL and amyloid-β, including inflammatory gene expression, IL-1β release and NFκB activation (79). Several functions of CD36 are derived from its interaction with integrins, receptors that transduce signals from extracellular matrix to intracellular molecules. CD36 is physically associated with integrin β_1 and β_3 (107-109). Phagocytosis of apoptotic cells depends on interaction of CD36 with integrin α_v β_3 in macrophages, and α_v β_5 in dendritic cells and retinal pigment epithelial cells (50,52). A previous study also revealed that integrin α_v β_3 is involved in uptake of oxLDL in macrophages. Antonov *et al.* suggested a potential role of integrin α_v β_3 by showing that

macrophages incubated with oxLDL or isolated in atherosclerotic lesions expressed high level of expression of α_v β_3 and blocking the α_v β_3 reduces CD36 mediated uptake of oxLDL in macrophages (110).

The interaction between CD36 and other receptors and signaling molecules may be aided by its localization in membrane microdomains. CD36 is isolated from the detergent resistant membrane (DRM) fraction of cells, called a lipid raft. Lipid rafts are enriched in sphingolipids and cholesterol, and are known to retain various proteins including caveolins, flotillins, GPI-linked proteins, low molecular weight and heterotrimeric G proteins, src family kinases like Lyn and Syk, epidermal growth factor (EGF) receptors, platelet-derived growth factor (PDGF) receptors, endothelin receptors, the phosphotyrosine phosphatase syp, Grb2, Shc, MAP kinase (MAPK), protein kinase C, and the p85 subunit of PI 3-kinase (111). Previous studies showed that disruption of lipid rafts by cyclodextrin inhibits CD36 mediated uptake of long chain fatty acids in adipocytes (112). In macrophages, oxLDL is internalized by lipid raft pathway independent of caveolin-1, a constituent of caveolae that is a specialized subset of lipid raft (113).

The function of CD36 depends on specificities of ligands and cell types. Furthermore, its distinct signaling cascade for a specific molecule diversifies the function of that molecule. For example, TSP-1 binds to both the CD36 and calreticulin-low density lipoprotein receptor-related protein receptor complex (CRT-LRT complex). TSP-1 binding to CD36 inhibits migration of microvascular endothelial cells while its binding to CRT-LRT complex facilitates migration of fibroblasts and smooth muscle cells. The

mechanism for the TSP-1/CRT-LRT complex is the dynamic activation of focal adhesion kinase (FAK) and RhoA inactivation, resulting in disassembly of focal adhesions (101,114,115). In contrast, the role of CD36 in cell migration has not been clearly defined.

A recent study showed that cytoskeletal signaling including p130Cas and tyrosine kinase Pyk2, is affected by CD36/ fibrillar β amyloid interaction (116). In addition, guanine nucleotide exchange factor (GEF)s, Vav family proteins were found to be phosphorylated by fibrillar β amyloid binding to CD36 in microglial cells (117) and by oxLDL binding to CD36 in macrophages (118). Vavs are known substrates for Fyn and Lyn, and function in activation of small molecular weight G proteins such as Rac1 and RhoG. Vav proteins are known to regulate cytoskeletal reorganization in a small molecular weight G protein dependent or independent manner (119). It is also known that small molecular weight G proteins regulate various cytoskeletal and redox pathway (120).

Based on these studies, we hypothesize that a CD36 induced signaling cascade modulates macrophage cytoskeletal functions and results in macrophage trapping. A goal of this thesis research is to define the key elements of this cascade.

4. Cell Migration : Dynamic Integration of Signals

Cell migration is an extremely complex multistep dynamic process that regulates integration of multiple signaling cascades. Functionally, the first responses of a cell to a migration initiating cue are spreading and protrusion, followed by cell polarization. The protrusion from migrating cells forms a large and broad lamellipodia tipped with spike-like filopodial projections. This process is driven by actin polymerization and stabilized by generating focal adhesions that connect extracellular matrix (ECM) to the actin cytoskeleton. Cell polarization is a pre-requisite for migration, meaning that the molecular processes at the front and the back of a migrating cell must be different. A polarized cell morphology characterized as front end lamellipodia and rear end retraction fibers, is a mechanical outcome derived by retraction and detachment of the rear end from the ECM. However, the molecular processes that generate the front and the rear of a cell are distinct and the individual signaling cascade functions in advancing the cell body as well as trailing the rear end (121).

Small molecular weight G-proteins including Rho and Rac, are known to regulate cell protrusion and polarization. Their binding to guanosine triphosphate (GTP) or guanosine diphosphate (GDP) determines activity through conformational changes. GTP bound G-proteins interact with their downstream substrates including protein kinases, lipid-modifying enzymes and activators of the Arp2/3 complex (122). Rac facilitates actin polymerization and adhesion complex assembly by activating Wiskott-Aldrich syndrome protein-homologous protein (WAVE) and thus induces cell protrusion (120). Rho

stabilizes microtubules and contributes to rear end formation and focal adhesion turnover (123). The currently accepted model is that the mutual antagonism and the balance between Rac and Rho determine the polarized cell shape. Rac is more active at the front, inhibiting Rho activity. In contrast, Rho is more active at the sides and the rear part of a cell, inhibiting the function of Rac (124-128). However, in some studies, Rac functions in detachment at the rear of migrating cells (129). In addition, there has been a report that showed Rho induces Rac activation (130). Therefore, functions of Rac and Rho appear to be dependent on cell types and their functions in regulating cell polarity and migration are more complex.

Small molecular weight G proteins perform a function that couples the molecular processes to physical cytoskeletal functions by modulating acto-myosin contractiliy. Non-muscle myosin II is primarily regulated by small molecular weight G proteins. Rho activates myosin II by phosphorylation of myosin regulatory light chain (MRLC). Rho phosphorylates and inhibits the activity of myosin phosphatase and increases MRLC phosphorylation (131-133). In contrast, Rac decreases phosphorylation of MRLC and inhibits the activity of myosin II by inhibiting myosin light chain kinase (MLCK) activity (134). Myosin II binds to actin filaments and produce ATP-dependent motion. By pulling two actin filaments past one another, myosin II generates tension (135). At the front end lamellipodium of a cell, myosin II promotes retrograde actin flow and adhesion maturation, generating traction force (136). At the rear of a cell, myosin II is involved in adhesion disassembly and detachment (137). Thus, the coordinated functions of myosin II regulate cell migration and establish cell polarity (138).

Previous studies of CD36 signaling provide several lines of evidence, supporting the concept that CD36 induced signaling may affect cytoskeletal functions. In all cell types that express CD36, CD36 binding to ligands induces src-kinase activation (95, 99, 100). Many well known target proteins of src kinases are found at focal adhesions. They are key components in the integrin-mediated signal transduction and bound to actin or integrin. They include vinculin, cortactin, talin, paxillin, focal adhesion kinase (FAK), tensin, ezrin and p130cas (139). In addition, recent studies revealed that CD36 signals to Vav, a GEF that is known to regulate small molecular weight G proteins, the major regulator of non muscle myosin II (117,118). In addition, oxLDL/CD36 interaction activates Vav through src kinase activation (118). Vav family of proteins includes Vav1 that is specifically expressed in the hematopoietic system, and Vav2 and Vav3 that are more ubiquitously expressed (140). Previous studies showed that Vav1 is involved in cytoskeletal reorganization, as manifested by decreased actin cap formation and T cell receptor (TCR) clustering in *Vav* null T cells (141,142). In addition to a function as a GEF, Vav seems to directly modulate the cytoskeleton by binding to talin and vinculin, two proteins that anchor the actin cytoskeleton to the cell membrane, and zyxin, a focal adhesion component (117,118).

Therefore, we set out to test our hypothesis that interaction between CD36 and oxLDL may modulate cytoskeletal functions in macrophages. These studies thus provide a potential mechanism for macrophage trapping in atherosclerotic lesions (Figure 4).

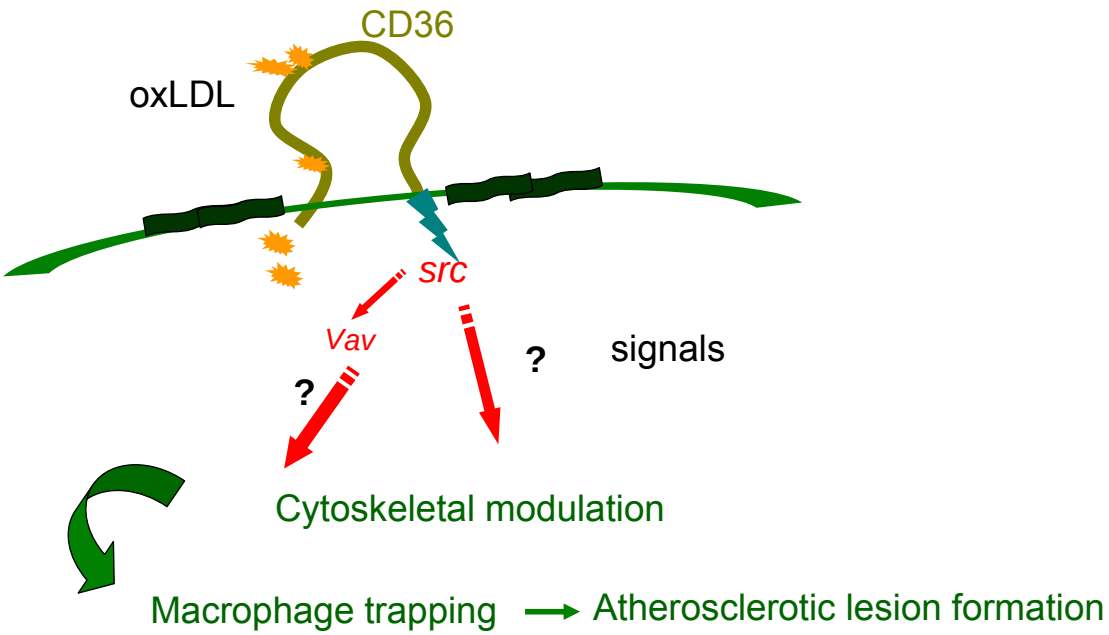


Figure 4. Model of the study

Interaction between CD36 and oxLDL activates src kinases and Vav, known effectors of cytoskeletal signaling. We hypothesize that oxLDL through CD36 may modulate cytoskeletal signaling in macrophages and inhibits migration.

CHAPTER 1.

CD36 modulates macrophage migration in response to oxidized LDL

INTRODUCTION

Macrophages have a crucial role in the development of atherosclerosis and are involved in all stages of plaque generation. The macrophage scavenger receptor CD36 is involved in atherogenesis in part by mediating uptake of oxLDL and subsequent foam cell formation. This function requires transmission of intracellular signals, including activation of src-family kinases. Macrophage retention in the inflamed atherogenic vessel wall also contributes to plaque progression and is caused by impaired migratory functions. Since src kinases and other CD36-mediated signals may also influence cytoskeletal function in response to oxLDL we hypothesize that CD36 modulates macrophage migration in response to oxLDL. In this chapter we report data testing this hypothesis using in vivo and in vitro models of migration, and we describe a novel CD36-mediated signaling pathway that contributes to oxLDL induced inhibition of migration.

METHODS

Reagents and antibodies

LDL prepared from human plasma by density gradient ultracentrifugation (144), was oxidatively modified using two different methods. NO_2LDL was generated by incubating LDL in a buffer containing 50mM sodium phosphate (pH 7.0) and 100μM DTPA with 30nM MPO, 100μg glucose, 20ng/ml glucose oxidase (grade II, Boehringer Mannheim Biochemicals) and 0.5mM $NaNO_2$ at 37ºC for 8 hours (41). Oxidation reaction was terminated by addition of 40μM butylated hydroxyl-toluene (BHT) and 300nM catalase to the reaction mixture. We also prepared a control, native LDL called NO2(-)LDL, by incubating LDL with all the components mentioned above but $NaNO_2$. Copper oxidized LDL was generated by dialysis with 5 μM $CuSO_4$ in PBS for 6 h at 37°C. Oxidation was terminated by dialysis against PBS containing EDTA (100uM). Acetylated LDL (Ac-LDL) and phorbol 12-myristate 13-acetate (PMA) were purchased from Invitrogen. Lipopolysaccharide (LPS), 5-fluorescein-iodoacetamide (5-F-IAA), N-acetyl cysteine (NAC), resveratrol, apocynin, 7-aminoactinomycin D (7-AAD) and diphenyleneiodonium-sulfate (DPI) and were purchased from Sigma-Aldrich. Antibodies against tyr 576/577 phosphorylated focal adhesion kinase (p-FAK), FAK, tyr 580 phosphorylated protein tyrosine phosphatase SHP-2 (p-SHP-2), and SHP-2 were purchased from Cell Signaling Technology. Anti–fluorescein antibody, fluorescein-conjugated phalloidin, and 5,6, carboxy-2'7' dichlorodihydro-fluorescein diacetate (carboxy-H_2DCFDA) were purchased from Molecular Probes. Anti-β-tubulin and PE-conjugated anti-Mac-1 antibodies were purchased from Abcam. Anti-neutrophil antibody

MCA771FA was purchased from AbD Serotec. PE-conjugated annexin-V apoptosis detection kit was purchased from BD Biosciences Pharmingen. FAK inhibitors (PF-573228 and PF-562271) were generously provided by Pfizer Inc. Src-kinase inhibitor, PP2 was purchased from Calbiochem. CNBr-activated sepharose 4B was purchased from GE healthcare. TSP-1 was given by Dr. Josephine Adams in the university of Bristol. DAPI containing cell mounting medium (Vectashield) was purchased from Vector laboratory.

Animals and cells

CD36 null mice generated by targeted homologous recombination were described previously (145) and have been backcrossed such that they are genetically greater than 98% C57BL/6. Littermate derived background matched mice were used as controls. Mouse peritoneal macrophages were collected by lavage 4 days after intraperitoneal injection of thioglycolate (1ml, 4%). Cells were cultured in RPMI containing 10% fetal bovine serum. The human monocyte cell line, THP-1, was obtained from American Type Culture Collection. Human monocytes were isolated from peripheral blood by centrifugation through Ficoll-hypaque and were cultured in RPMI containing human bovine serum (10%) for 7 days for macrophage differentiation.

In vivo migration assay

To demonstrate the effect of lipoproteins on mouse macrophage migration, we modified the macrophage efflux model previously described by Cao *et al*. (146). WT and CD36 null mice were injected intraperitoneally with 1 ml of 4% thioglycolate. After 5

days the mice were injected intraperitoneally with PBS, NO_2(-)LDL (50 μg), or NO_2LDL (50 μg) and 1 hr later they were injected with LPS (250 μl; 5 μg/ml). After 4 hr the peritoneal cells were collected by lavage and counted using a Beckman Coulter particle counter. The percentage of macrophages in the lavage was quantified by flow cytometry with PE-conjugated anti-Mac-1 antibody. Macrophage count was plotted as a migration index. Migration index is defined as [1- the peritoneal macrophage count from each animal divided by the average number of peritoneal macrophages of control group mice (mice that received thioglycolate only)] x 100 (%). Significance was determined using ANOVA followed by Bonferroni's multiple comparison test. Mac-1 staining polymorphonuclear neutrophils in the lavage were detected using a specific anti-neutrophil antibody and accounted for less than 1% of the total population.

In vitro migration assay (modified Boyden chamber migration assay)

Migration of mouse peritoneal macrophages, human peripheral blood-derived macrophages, and PMA treated THP-1 cells was measured in a modified Boyden Chamber migration assay using Transwell inserts with a 5 μm porous membrane (Corning) or the Chemicon Migration QCM 96 well 5 μm migration assay kit (Chemicon). Cells were loaded into the migration chamber with various lipoproteins including NO_2(-)LDL, NO_2LDL, and Cu^{2+}oxLDL. Media containing 10 ng/ml monocyte chemotactic protein-1 (MCP-1) was placed in the lower chamber in some studies. After allowing cell migration for 16 hr, cells were removed from the upper side of membranes and nuclei of migratory cells on the lower side of the membrane were stained with 4',6-diamidino-2-phenylindole (DAPI). The number of migratory cells was counted by

fluorescence microscopy. To evaluate the effect of antioxidants and NADPH oxidase inhibitors on macrophage migration, cells were pre-treated with NAC (20 mM), resveratrol (100 μM), DPI (0.5 μM, 2 μM, and 4 μM) or apocynin (2 μM, 10 μM and 100 μM) for 1 hr prior to loading onto the migration chamber.

In vitro migration assay (scratch wound closure assay)

Peritoneal macrophages (2 X 10^6 cells) from wild type and *Cd36* null mice were plated onto glass bottom 6 well plates. After 18 hours, confluent monolayers were scratched using a pipette tips and rinsed with PBS. RPMI media was added with or without NO_2LDL. Live cell imaging was used to record the migration of macrophages for 18 hours. Images were taken every 5 minutes and from 3 randomly chosen locations of each well.

Cell spreading assay

Mouse peritoneal macrophages were placed onto serum coated coverslips at 37ºC. After 30 min to allow macrophages to adhere to the surface, 50 μg/ml of NO_2(-)LDL or NO_2LDL was added. After the indicated incubation times, cells were fixed with 4% paraformaldehyde and stained with fluorescein-conjugated phalloidin. Spread cells were counted using a Leica TCS-SP2 Spectral laser confocal microscope (Leica Microsystems) and the cell surface areas were measured using Image-Pro Plus software (Mediacybernetics). In some studies macrophages were incubated with specific FAK inhibitors (PF-573228 and PF-562271) at 10μM for 1 hr prior to addition of NO_2LDL.

Flow cytometry assays

To quantify apoptosis in peritoneal macrophages in the in vivo migration studies 10^5 cells were rinsed with PBS, incubated with PE-annexin-V and 7-AAD at room temperature for 15 minutes in the dark and then counted by flow cytometry using a Becton Dickinson FACScan. Data were analyzed by Cell Quest V.3.3 (Becton Dickinson). To quantify neutrophils in the mouse peritoneal exudate, cells were co-incubated with FITC-conjugated anti-neutrophil antibody (MCA771FA) and PE-conjugated anti-Mac-1 antibody. MCA771FA positive cells were counted as above and were less than 1% of the total Mac-1 positive pool. To detect polymerized actin (F-actin), mouse peritoneal macrophages were incubated with NO_2(-)LDL, NO_2LDL or Cu^{2+}oxLDL at 37ºC. After the indicated times, cells were fixed with 4% paraformaldehyde and stained with fluorescein-conjugated phalloidin. Fluorescence intensity was quantified by flow cytometry using a Guava® PCA-96 system (Guava Technologies) and data were analyzed by FlowJo software (Tree Star).

Western blot analysis

Mouse peritoneal macrophages incubated with 50 μg/ml of NO_2(-)LDL or NO_2LDL for the indicated times were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate. Clarified lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were probed with antibodies to p-FAK or p-SHP-2. After chemiluminescence detection, membranes were stripped with 0.2 M sodium hydroxide and re-probed with antibodies to β-tubulin,

FAK or SHP-2 for normalization. Band intensities were quantified by ImageJ (NIH Image) and Gel-Pro analyzer (MediaCybernetics).

Immunoprecipitation

Mouse peritoneal macrophages were lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate. Sepharose beads (Amersham Pharmacia Biotech) were rinsed with 1mM hydrogen chloride, resuspended in antibody coupling buffer containing 0.1M $NaHCO_3$ and 0.5M NaCl in pH 8.3, and incubated with anti-CD36 antibody for 16 hours. Sepharose beads were rinsed, resuspended in 0.1M tris-HCl pH 8.0 for 2 hours and serially washed with 0.5M NaCl - 0.1M sodium acetate pH 4.0 solution and 0.5M NaCl - 0.1M tris-HCl pH 8.0 solution. The beads washed with PBS were incubated with macrophage lysates for 16 hours at 4ºC. After being rinsed 5 times, the beads were resuspended in 2X Laemmli sample buffer, and heated at 95ºC for 3 minutes. The supernatant collected from centrifugation was applied to SDS-PAGE gel for electrophoresis. The gel was transferred to PVDF membrane and immunoblotted for FAK and SHP-2.

Oxidative modification assay

Oxidative modification of the active site thiol group of SHP-2 was evaluated as described by Wu *et al.* (147). Mouse peritoneal macrophages incubated with NO_2(-)LDL or NO_2LDL were lysed as described above and 100 μg of protein from each lysate was exposed to 5-F-IAA for 1 hr at room temperature. For immunoprecipitation, agarose

beads (CNBr-activated sepharose 4B, GE Healthcare) were coupled with anti-SHP-2 antibody following the manufacturer's instruction. Proteins were incubated with anti-SHP-2 antibody coupled beads and separated by SDS-PAGE. Fluorescein bound SHP-2 was detected by fluorescence scanning of the gel (Typhoon Trio, GE Healthcare). The proteins in the gel were then transferred to a membrane and immunoblotted with an antibody to SHP-2.

Reactive oxygen species (ROS) detection

Mouse peritoneal macrophages were plated on coverslips or in 96 well plates and incubated with NO_2(-)LDL or NO_2LDL. After the indicated times cells were washed and stained with carboxy-H_2DCFDA (25 μM) for 30 min at 37ºC. Nuclei were counter-stained with Hoechst 33342. Fluorescently labeled cells were counted by microscopy (Leica DMR, Leica Microsystems) and fluorescence intensity was measured with a 96 well fluorescence plate reader (SpectraMaxGeminiEM, Molecular Devices).

RESULTS

OxLDL inhibits macrophage migration in vivo in a CD36-dependent manner

To evaluate the effect of oxLDL on macrophage migration in vivo, we measured lipopolysaccharide (LPS) induced efflux of murine macrophages from the peritoneal cavity using the method developed by Cao *et al*. (146). In this assay, the number of macrophages remaining in the peritoneal cavity after an intra-peritoneal injection of LPS is counted and compared to those remaining after PBS injection. We used a form of oxLDL generated by myeloperoxidase (MPO) and nitrite that we term NO_2LDL in these studies. This is a specific high affinity ligand for CD36 with little capacity to bind scavenger receptor A (42). We first confirmed the observation of Cao *et al.* that LPS induced efflux of macrophages from the peritoneal cavity; ~ 40% of macrophages migrated from the peritoneal cavity 4 hours after LPS injection. No efflux was observed, however, when mice were injected with NO_2LDL prior to LPS (Figure 1A). A control, non-oxidized LDL preparation that was exposed to all components of the MPO system except the oxidant (termed NO_2(-)LDL), had no such inhibitory effect on emigration. Intra-peritoneal injection of LPS in *Cd36* null mice induced a similar degree of macrophage efflux as in wild type (WT) mice, but unlike in the WT mice, NO_2LDL did not have an inhibitory effect (Figure 1B). These studies suggest that peritoneal macrophage trapping by NO_2LDL is dependent on CD36.

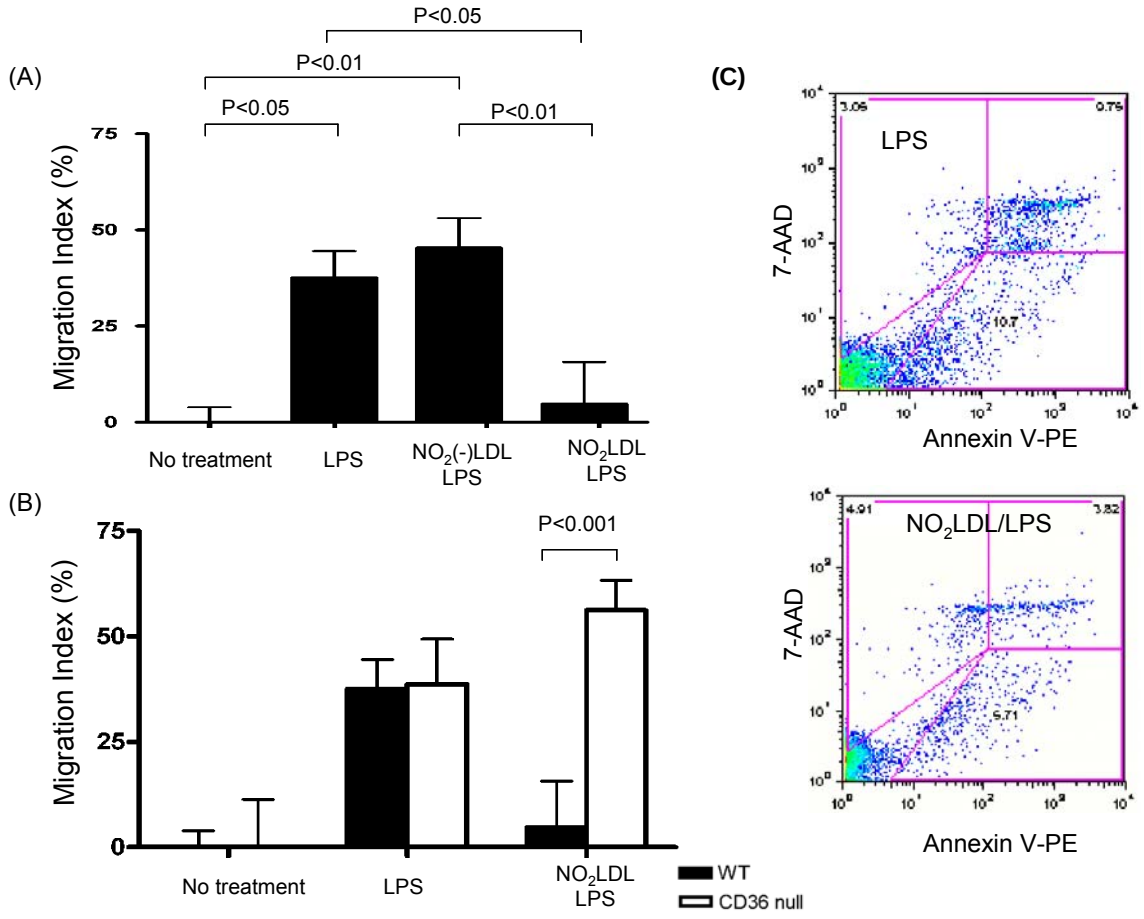


Figure 1. Macrophage migration in vivo is inhibited by NO_2LDL in a CD36 dependent manner.

(A) Thioglycollate elicited peritoneal macrophages were isolated by lavage and counted 4 hr after mice were injected intraperitoneally with LPS. In some cases mice were pre-treated with NO_2LDL or control LDL (NO_2(-)LDL) (50µg) prior to LPS injection. Data are plotted as the migration index defined as [1 - the ratio of the mean macrophage number harvested from the experimental animals to that from animals that did not receive LPS or lipoproteins] x 100 (%). (n=10-15 per group; significance was determined by ANOVA and Bonferroni's multiple comparison test). (B) Migration was evaluated as in (A), but in WT and CD36 null mice. (C) Peritoneal macrophages from LPS (upper panel) or LPS and NO_2LDL (lower panel) injected WT mice were stained with Annexin V and 7-AAD and subjected to analysis by flow cytometry.

To exclude the possibility that the inhibitory effect of NO_2LDL was due to induction of macrophage apoptosis, we analyzed the peritoneal cells by flow cytometry. As shown in Figure 1C, 10.7% of the macrophages taken from mice after intra-peritoneal injection of LPS stained positively with annexin-V indicating early apoptosis and 9.76% stained with both annexin V and 7-AAD indicating late apoptosis. Treatment with NO_2LDL prior to LPS did not influence the degree of apoptosis; 9.71% of the cells showed evidence of early apoptosis and 3.82% late apoptosis.

OxLDL inhibits macrophage migration in vitro in a CD36-dependent manner

Having shown a CD36-mediated inhibition of emigration by oxLDL in vivo we next showed that NO_2LDL inhibited migration of macrophages in vitro using a modified Boyden Chamber. Migration was quantified by staining the nuclei of the migratory cells on the lower side of insert membrane. Figure 2A shows that NO_2LDL inhibited both random and MCP-1 directed macrophage migration by more than 80%. We examined the effect of various other lipoproteins including HDL, NO_2(-)LDL, acetylated LDL (Ac-LDL), and copper-oxidized LDL (Cu^{2+}oxLDL). Cu^{2+}oxLDL inhibited macrophage migration to the same degree as NO_2LDL (Figure 2B), while NO_2(-)LDL, Ac-LDL and HDL had no effect. Using peritoneal macrophages from WT and *Cd36* null mice we showed that NO_2LDL inhibition of macrophage migration was CD36-dependent (Figure 3A). CD36 null macrophages migrated to the same extent as WT under basal conditions, but in the presence of NO_2LDL, WT cells were inhibited by >80%, whereas CD36 null macrophage migration was inhibited by <40%.

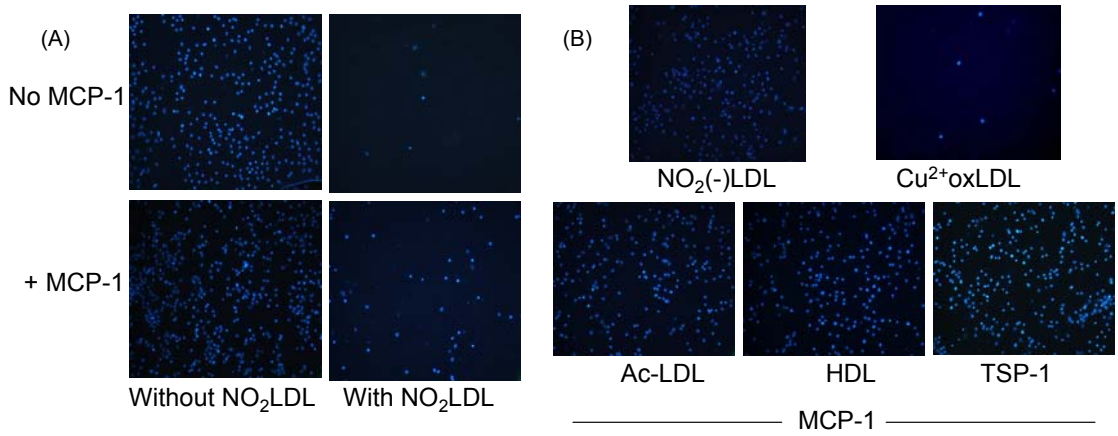


Figure 2. Murine macrophage migration in vitro is inhibited by oxLDL.

(A) Murine peritoneal macrophages were added to the upper chamber of the transwell with or without NO_2LDL (50 μg/ml) and allowed to migrate through the porous membrane into the lower chamber containing media alone or media with MCP-1 (10 ng/ml). Migrated cells on the lower side of the membrane were stained with DAPI and counted under a fluorescence microscope. (B) NO_2(-)LDL, Cu^{2+}oxLDL, AcLDL, HDL (50 μg/ml each) or TSP-1 (20 nM) was added to the migration chamber and migration quantified as above.

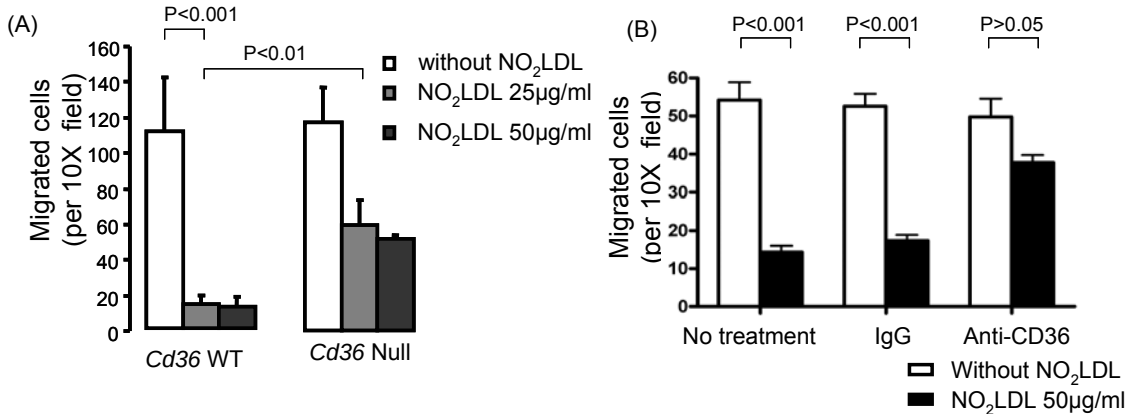


Figure 3. Inhibitory effect of NO_2LDL on macrophage migration is dependent on CD36. (A) Peritoneal macrophages from WT and CD36 null mice were exposed to 0, 25 and 50µg/ml NO_2LDL, and migrated cells quantified as in Figure 2. (B) Human peripheral blood monocyte-derived macrophages were treated with isotype matched control IgG or anti-CD36 monoclonal antibody (5 µg/ml) and were added to the migration chamber with or without NO_2LDL (50 µg/ml). Macrophage migration was quantified as in Figure 2.

Inhibition of macrophage migration by oxLDL was also observed with human peripheral blood monocyte-derived macrophages. NO_2LDL inhibited migration in the Boyden Chamber by ~75% (Figure 3B). The migration-inhibitory effect of NO_2LDL was prevented by pre-treatment of human monocyte-derived macrophages with a monoclonal antibody to CD36 but not by an isotype matched control IgG (Figure 3B).

In addition to the Boyden chamber migration assay, we performed scratch wound closure migration assays using peritoneal macrophages from WT and *Cd36* null mice. After 18 hours, wild type macrophages migrated into the scratched cell-free space and completely filled up the space. In the presence of NO_2LDL, however, the number of migrated macrophages was decreased by 50% (Figure 4A). *Cd36* null macrophages also migrated and filled the scratched space after 18 hours, but the inhibitory effect of NO_2LDL was less than 20% (Figure 4B).

We also examined the effect of thrombospondin-1 (TSP-1), on macrophage migration. TSP-1 is a CD36 ligand that mediates an anti-angiogenic function in microvascular endothelial cells (101, 148). TSP-1 did not inhibit macrophage migration in the Boyden Chamber (Figure 2B), suggesting that the migration inhibitory effect of NO_2LDL may be driven by the oxidized lipid moiety, or that additional TSP-mediated signals from other receptors may negate the CD36 effect.

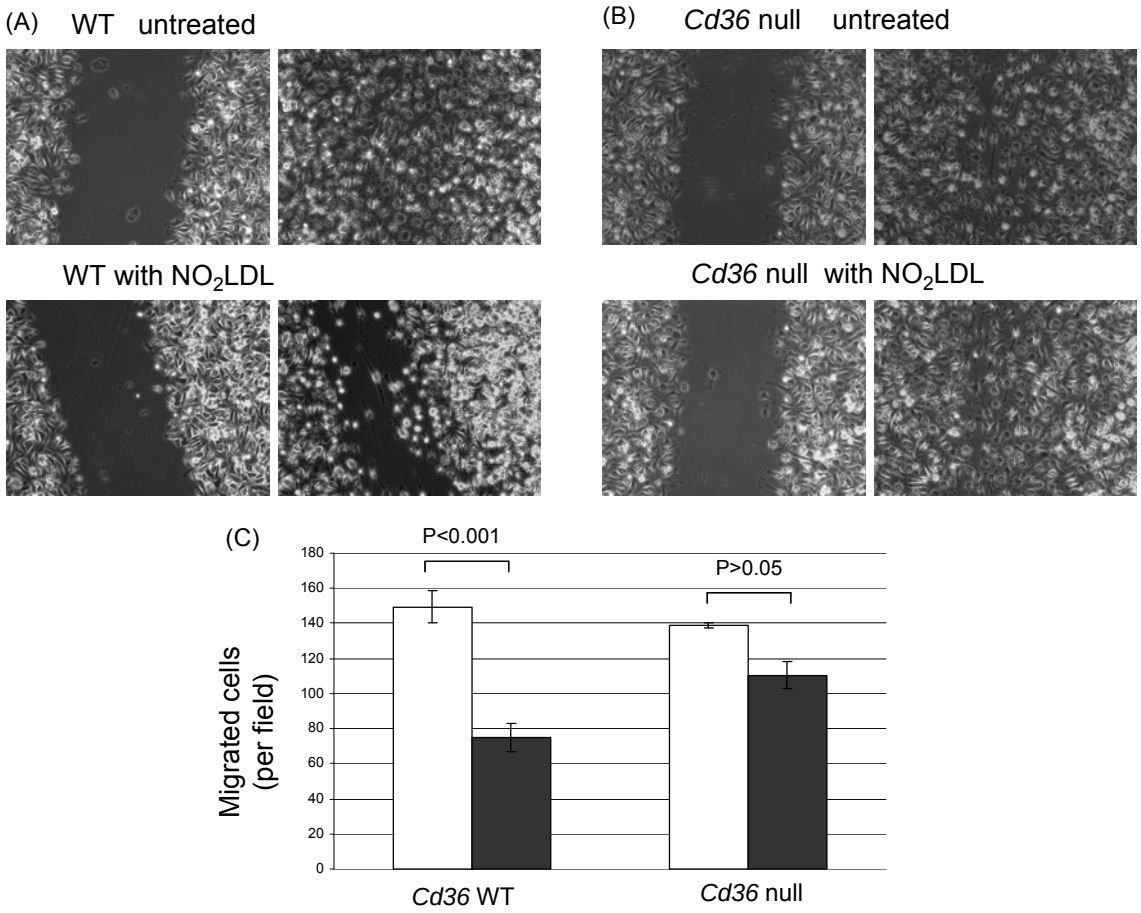


Figure 4. Scratch wound closure migration assay reveals the inhibitory effect of NO_2LDL on macrophage migration.

(A) Peritoneal macrophages from WT mice were plated onto a glass bottom plate. The confluent cell layer was scratched and treated with NO_2LDL or control LDL. Macrophages migrating into the free space were counted after 18 hours (right panels). (B) Macrophages from *Cd36* null mice were tested as described in (A). (C) Quantitative analysis of migratory cell count.

OxLDL induces rapid CD36-dependent microphage spreading and actin polymerization

Cell migration is a complex process that involves cellular spreading, disruption of existing focal contacts, and formation of new focal contacts (149). We thus examined the effect of oxLDL on macrophage spreading as the first step of migration. NO_2LDL induced rapid spreading of WT macrophages on serum-coated glass cover slips, while NO_2(-)LDL did not (Figure 5A). After 5 minutes exposure to NO_2LDL, the number of spread WT cells was 3 times greater than spread *Cd36* null cells (Figure 5B). The mean cellular area of WT cells was rapidly increased by NO_2LDL while CD36 null cells showed a pronounced blunting and delay in response (Figure 5C).

Since cell spreading requires actin polymerization to form lamellipodia (150) we examined the effect of NO_2LDL on the actin cytoskeleton. Macrophages from WT and *Cd36* null mice were treated with NO_2LDL and then stained with fluorescein-conjugated phalloidin to detect polymerized actin. Flow cytometric analysis showed that the amount of polymerized actin was increased by NO_2LDL in WT but not *Cd36* null cells (Figure 6).

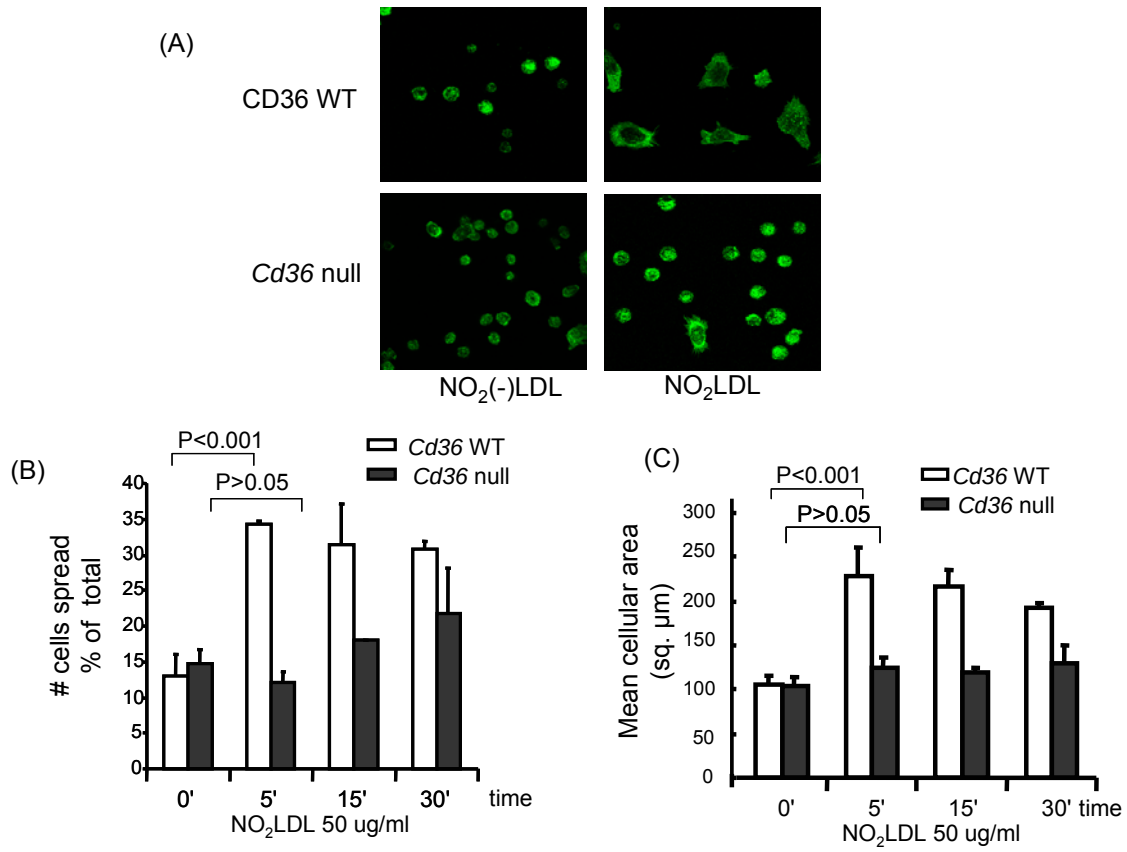


Figure 5. NO_2LDL induces rapid macrophage spreading in a CD36-dependent manner. (A) Macrophages from WT and *Cd36* null mice were plated on serum-coated glass coverslips, incubated with NO_2(-)LDL or NO_2LDL (50 µg/ml) at 37°C, and then photographed after 5 min. (B) Quantitative comparison of cell spreading was obtained by calculating the percentage of spread cells at each time point. (C) Mean cell surface areas were measured by confocal microscopy and quantitative comparisons obtained between macrophages from WT and *Cd36* null mice.

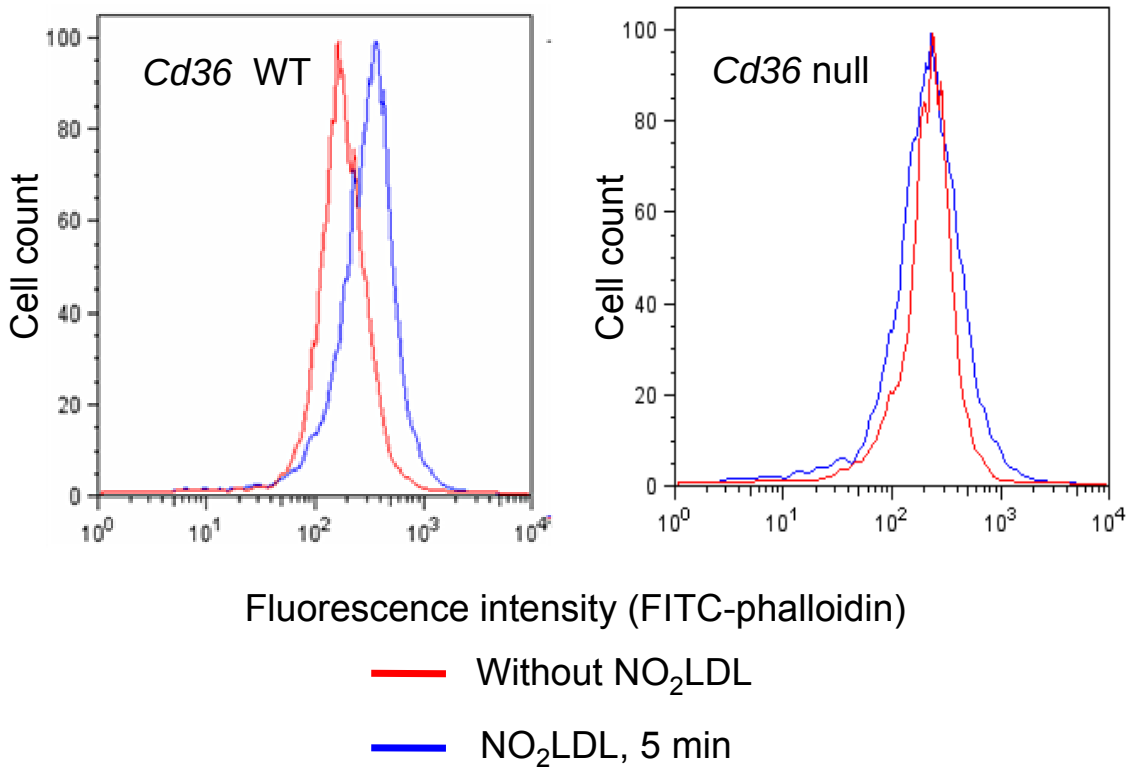


Figure 6. Actin polymerization is induced by NO_2LDL in a CD36-dependent manner. Peritoneal macrophages from WT and *Cd36* null mice were exposed to 50 µg/ml of NO_2LDL and then stained with fluorescein-phalloidin to detect polymerized actin. Fluorescence intensity was assayed by flow cytometry. The left histogram shows fluorescence intensity in WT cells with (blue) or without (red) exposure to NO_2LDL, while the right histogram shows fluorescence intensity in *Cd36* null cells.

OxLDL induces CD36-dependent phosphorylation of Focal Adhesion Kinase (FAK)

Since the signaling pathway required for actin polymerization includes activation of FAK (151) we evaluated the state of FAK phosphorylation after macrophage exposure to oxLDL. Western blot analyses revealed that NO_2LDL increased tyr 576/577 phosphorylation of FAK in WT but not in CD36 null cells (Figure 7A). Phosphorylation could have resulted from either direct CD36 signaling or from CD36-mediated uptake of biologically active oxidized lipids. We thus incubated peritoneal macrophages from WT mice with NO_2LDL at 37°C, a temperature at which NO_2LDL uptake proceeds, or at 4°C, at which NO_2LDL binds but is not internalized (95). Phosphorylation of FAK was observed at 4°C although it was slower than at 37°C (Figure 7B). These studies show that internalization of oxLDL is not required for phosphorylation of FAK, and suggest a role for direct signaling via CD36.

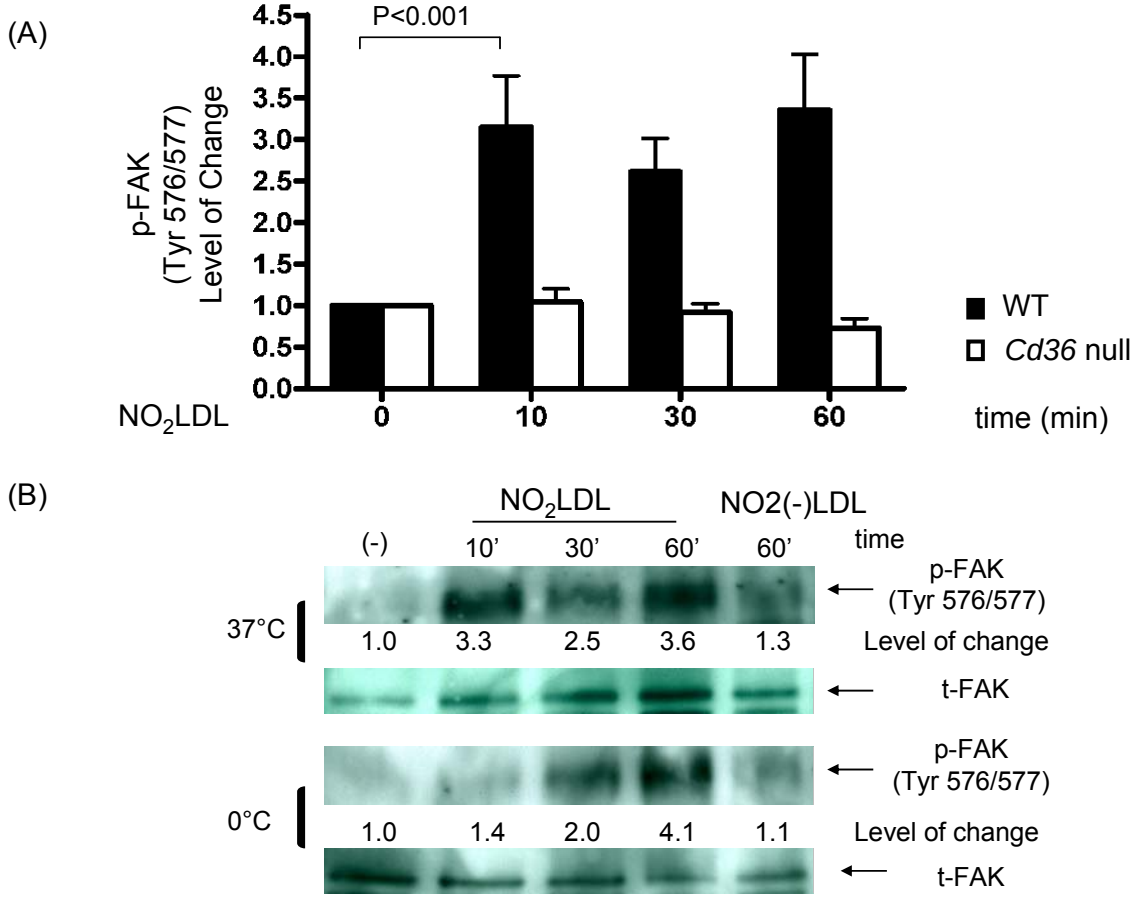


Figure 7. NO_2LDL induces phosphorylation of macrophage focal adhesion kinase (FAK) in a CD36 dependent manner.

(A) WT and *Cd36* null mouse peritoneal macrophages were exposed to 50 µg/ml NO_2LDL for the indicated times at 37ºC and then lysates subjected to Western blot analysis to detect levels of FAK tyr 576/577 phosphorylation using an antibody specific for the phosphorylated form. Immunoblotting with anti-FAK antibody was used for loading control and fold changes were calculated from scanned images. WT macrophages are shown with black bars and *Cd36* null macrophages with open bars. (B) WT mouse peritoneal macrophages were exposed to NO_2LDL or NO_2(-)LDL (50 µg/ml) at 0ºC or 37ºC and examined as in panel A.

Since Rahaman et al. previously demonstrated that oxLDL-CD36 binding activated the src-kinase lyn in macrophages (95) and since FAK is known to be a src kinase substrate, we evaluated if oxLDL-induced FAK phosphorylation was mediated by src-kinases. Macrophages treated with the src-kinase inhibitor PP2, but not a vehicle control, did not show phosphorylation of FAK in response to NO_2LDL (Figure 8). We also performed immunoprecipitation of macrophage lysates using an anti-CD36 antibody and showed by immunoblot that FAK specifically co-precipitated with CD36 (Figure 9).

To test the effect of FAK on macrophage spreading induced by NO_2LDL, we treated mouse peritoneal macrophages with two chemically distinct pharmacological inhibitors of FAK, PF-573228 and PF-562271, and showed that both compounds completely blocked the increased spreading response to NO_2LDL (Figure 10A and B; $p<0.001$). The inhibitors did not affect cell viability. In sum, these data show a direct link between oxLDL induced activation of a CD36 signaling complex and macrophage function.

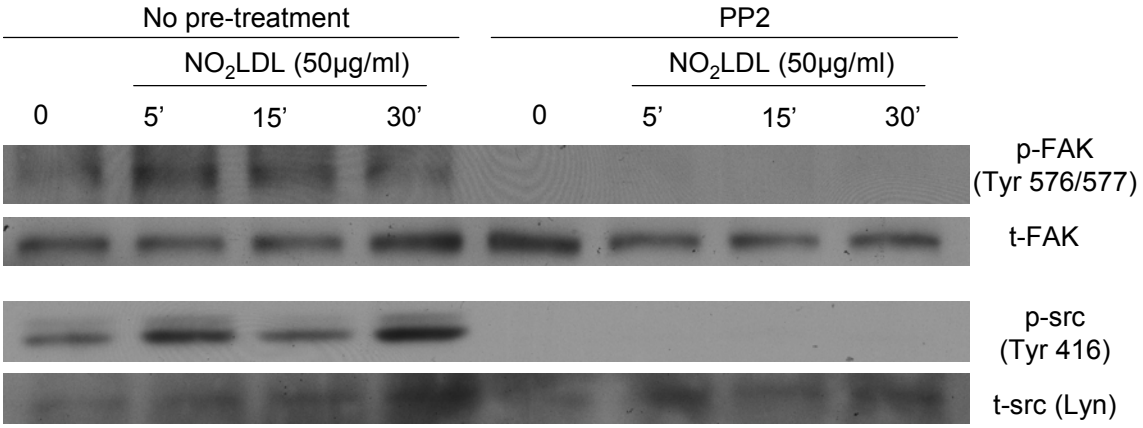


Figure 8. FAK activation by NO_2LDL is dependent on src kinase activity.

Peritoneal macrophages were pre-incubated with src kinase inhibitor PP2 (10µM) or vehicle control along with 50 µg/ml NO_2LDL for indicated times. Cells were then lysed and subjected to immunoblotting to detect FAK tyr 576/577 phosphorylation and src-kinase tyr 416 phosphorylation.

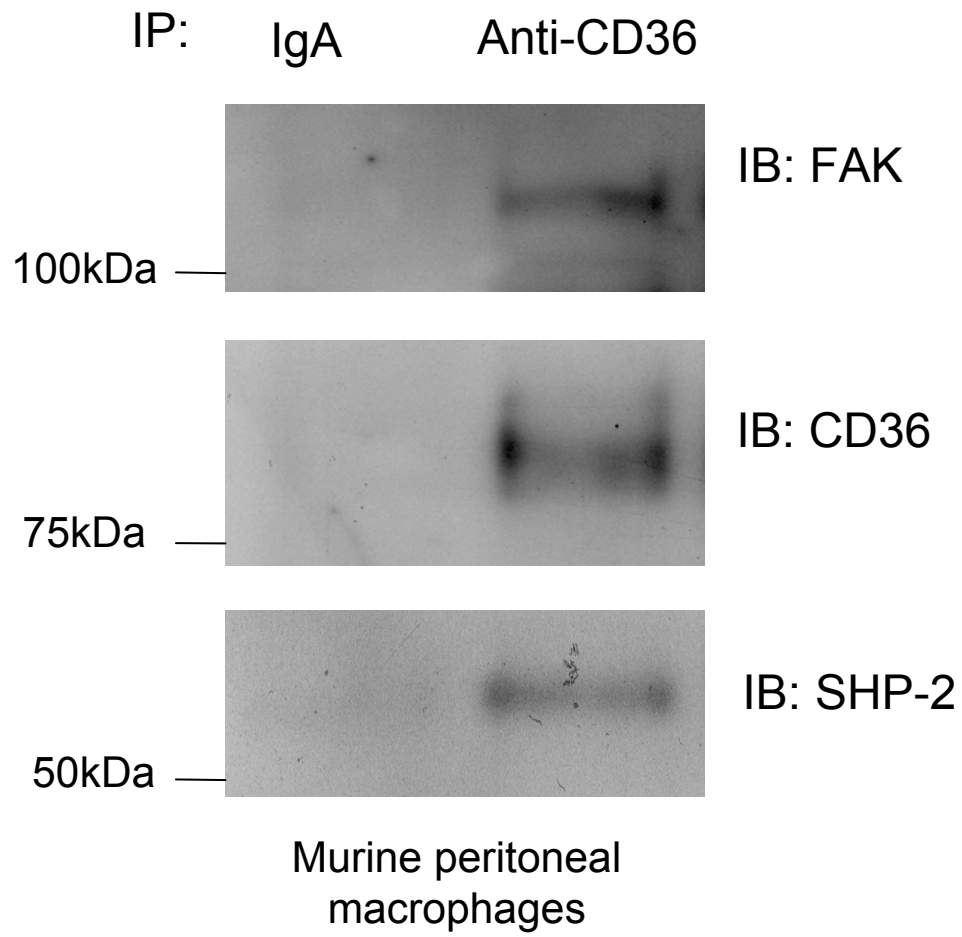


Figure 9. CD36 is physically associated with FAK and SHP-2.

Peritoneal macrophages were immunoprecipitated with anti-CD36 antibody or non-immune IgA and the precipitates then analyzed by immunoblot using anti-FAK (top), anti-CD36 (middle), and anti-SHP-2 antibodies (bottom).

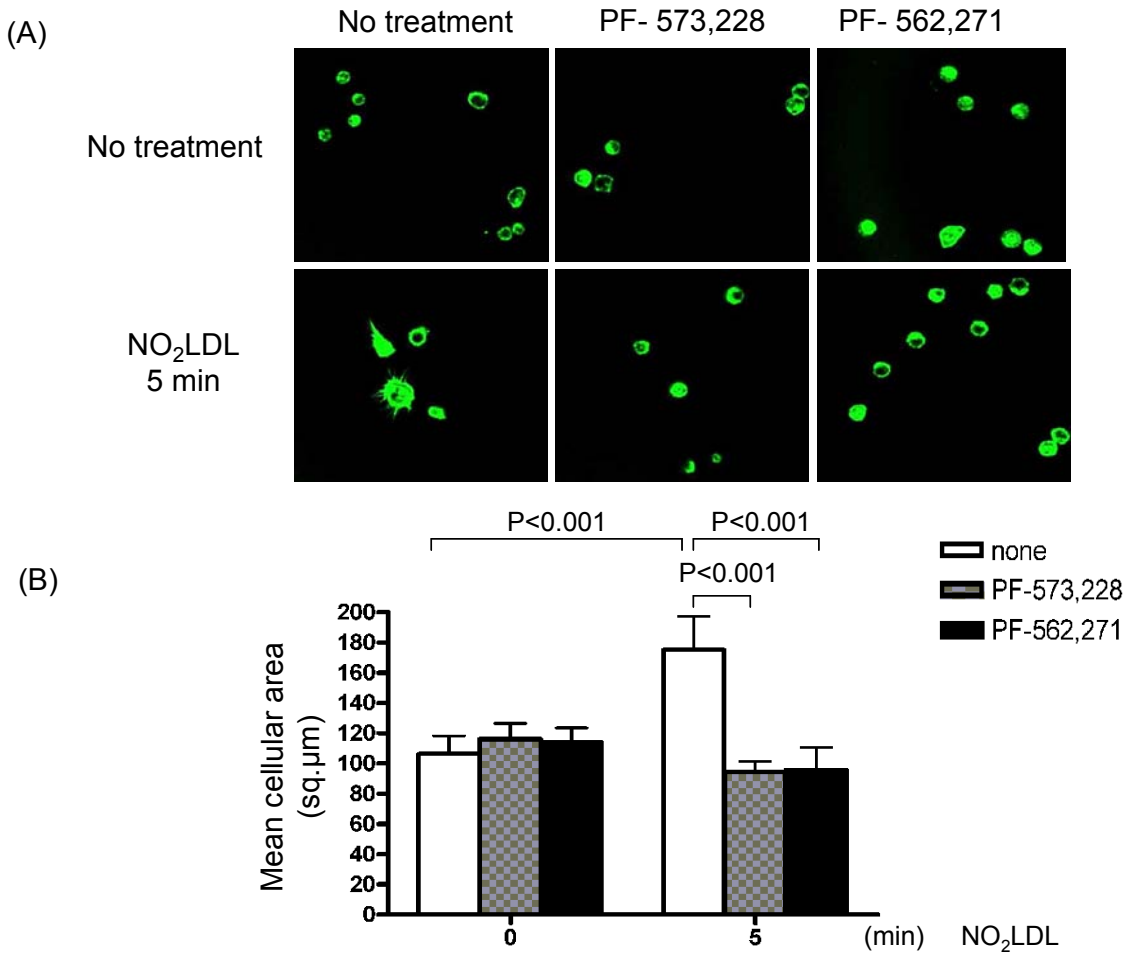


Figure 10. FAK mediates macrophage spreading in response to NO_2LDL. (A) Mouse peritoneal macrophages were pre-incubated with FAK inhibitors (PF-573228 and PF-562271, 10µM for each) for 1 hour and incubated with or without NO_2LDL at 37°C. Cells were photographed after 5 min. (B) Mean cellular area was measured by confocal microscopy and quantitative comparisons were obtained between FAK inhibitor treated and untreated cells.

OxLDL induces CD36-dependent inactivation of src homology 2-containing phosphotyrosine phosphatase (SHP-2) via generation of reactive oxygen species

Kinetic studies showed that NO_2LDL induced sustained phosphorylation of macrophage FAK that persisted for at least two hours, while TSP-1, a known FAK activator that did not inhibit migration (152) induced transient phosphorylation lasting less than 30 minutes (Figure 11). Since the prominent effect of CD36 signaling on FAK was kinetic, we explored the role of the protein tyrosine phosphatase SHP-2 which is known to be a critical regulator of FAK phorphorylation dynamics (153). We first showed that similar to FAK, SHP-2 was specifically co-precipitated from macrophage lysates by anti-CD36 antibody (Figure 9). Next we determined if sustained phosphorylation of FAK by NO_2LDL was associated with loss of SHP-2 activity. Phosphorylation of SHP-2 at tyr580 relieves basal inhibition and stimulates phosphatase activity (154). Using an antibody specific for phospho-tyr580 we showed by western blot that exposure of macrophages to NO_2LDL led to rapid and sustained dephosphorylation of phospho-tyr580 in WT, but not CD36 null cells. Specific phosphorylation was reduced by >75% within 10 minutes and remained low for at least 60 minutes (Figure 12A and B).

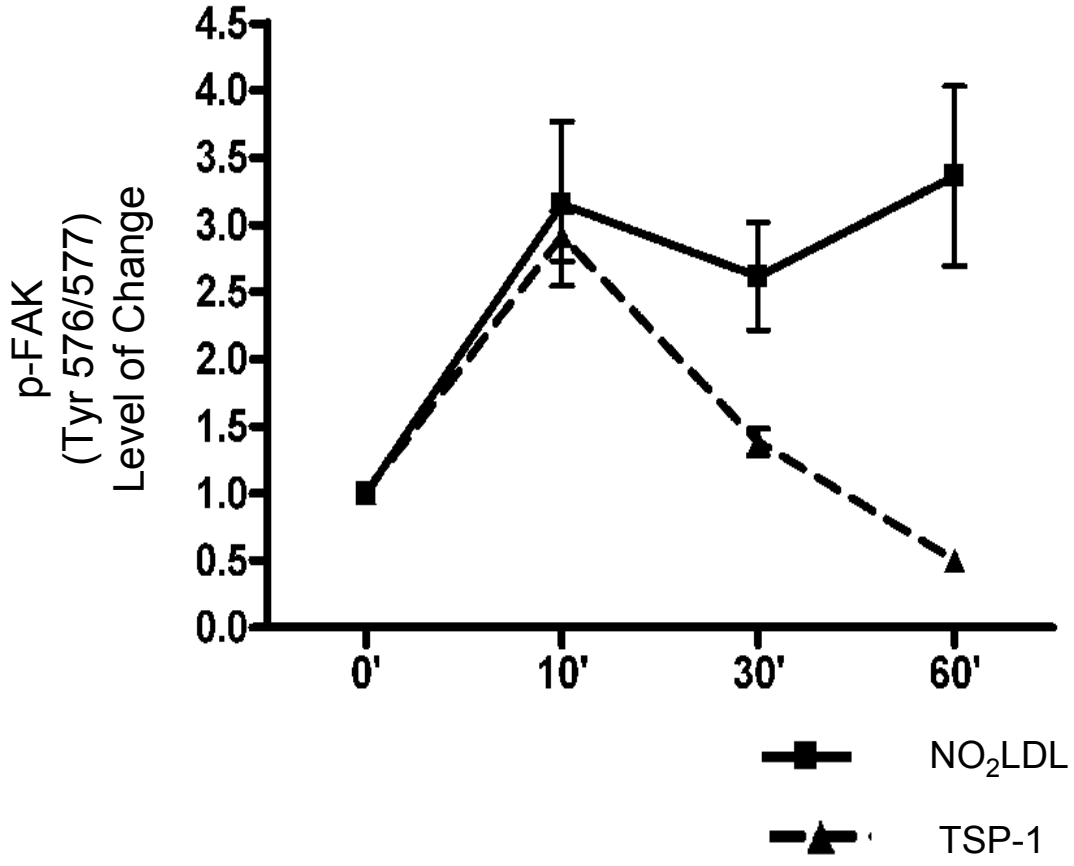


Figure 11. NO_2LDL induces sustained activation of FAK.

Comparison of FAK tyr 576/577 phosphorylation kinetics between NO_2LDL (solid line) and TSP-1 treated cells (dashed line).

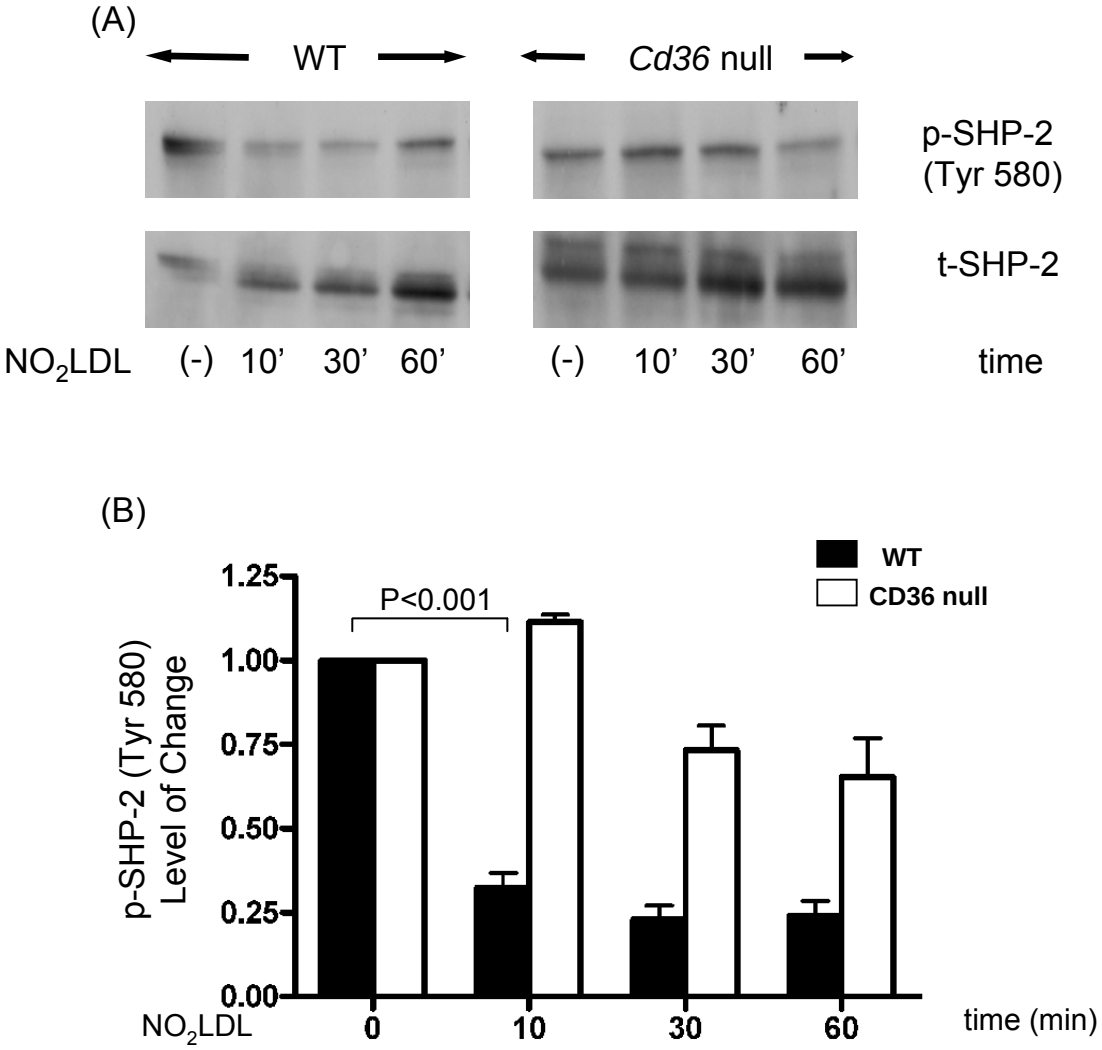


Figure 12. Inactivation of src homology (SH) 2-containing phosphotyrosine phosphatase, SHP-2, by NO_2LDL.

(A) Peritoneal macrophages from WT and *Cd36* null mice were exposed to 50 μg/ml NO_2LDL for the indicated times. Western blot analysis of lysates was performed with an antibody against phospho-SHP-2 (tyr 580) (upper blots) and total SHP-2 (lower blots). Dephosphorylation of SHP-2 was rapid and sustained in WT but not CD36 null cells. (B) Quantitative analysis of phosphorylated SHP-2 band intensities from the western blot in (A).

Protein tyrosine phosphatases, including SHP-2, have a critical cysteine residue in their catalytic site that is subject to thiol oxidation. Such oxidation inactivates the enzyme and is a major pathway of regulation. We measured the oxidation status of SHP-2 after exposure to NO_2LDL using an assay developed by Wu and Terada (155) in which active enzyme is detected by its ability to be cysteine-acetylated by fluorescein-conjugated iodoacetamide. Fluorescein-bound SHP-2 was detected in anti-SHP-2 immunoprecipitates by fluorescence image scanning and showed a marked decrease 5 minutes after cell incubation with NO_2LDL and near complete disappearance at 15 minutes (Figure 13). These data demonstrate that NO_2LDL induced oxidative modification and inactivation of SHP-2.

Since oxidative modification of protein tyrosine phosphatases is induced by ROS (156), we incubated mouse peritoneal macrophages with NO_2(-)LDL or NO_2LDL and measured ROS generation by staining with 2',7'-dichlorodihydrofluorescein (DCF). The number of DCF positive cells was significantly increased by exposure to NO_2LDL but not NO_2(-)LDL (Figure 14A and B). Spectrometric quantification of fluorescence staining also revealed that macrophages from WT mice showed significantly more ROS generation in response to NO_2LDL than macrophages from *Cd36* null mice (Figure 14C). These studies show that oxLDL induced ROS generation was mediated by CD36.

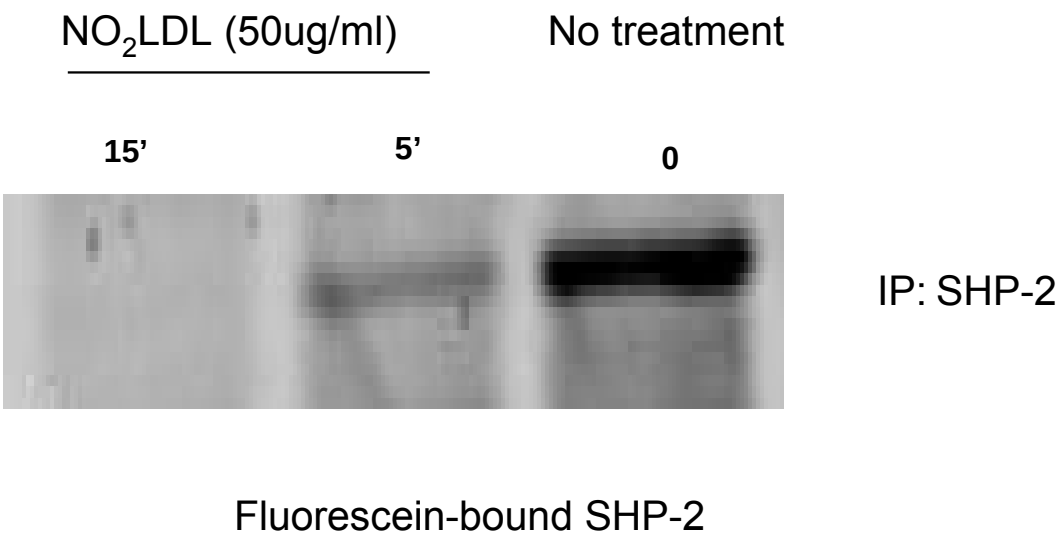


Figure 13. NO_2LDL induces oxidative modification of SHP-2.

Lysates from macrophages incubated with NO_2LDL or NO_2(-)LDL for indicated time periods were incubated with 5-IAA to acetylate available cysteine residues. Cell lysates were then immunoprecipiptated with anti-SHP-2 antibody and then analyzed by SDS-PAGE. The fluoresceinated SHP-2 band was detected by *in situ* fluorescence scanning of the gel. Decreased band intensity of fluoresceinated SHP-2 indicates time-dependent oxidation of the active site cysteine and inactivation of SHP-2.

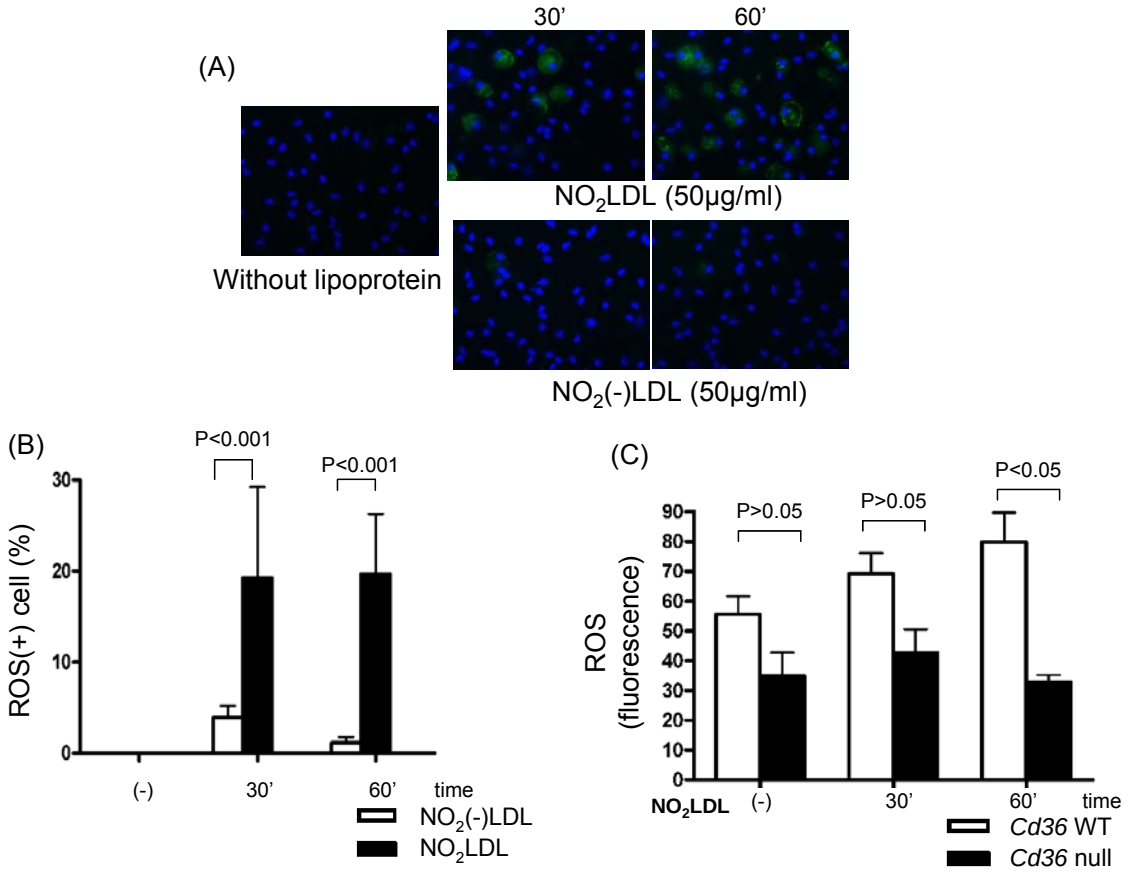


Figure 14. Macrophage exposure to NO_2LDL induces generation of reactive oxygen species (ROS) in a CD36-dependent manner. (A) Peritoneal macrophages were exposed to 50 µg/ml NO_2LDL or NO_2(-)LDL for 30 min and 60 min. ROS were then detected with the fluorescent probe carboxy-H_2DCFDA by fluorescence microscopy. Quantification by counting fluorescent cells is shown in (B). NO_2(-)LDL treated cells are shown with open bars and NO_2LDL treated cells with black bars. (C) Fluorimetric quantification of ROS generation in a separate study comparing macrophages from WT (open bars) with those from CD36 null (black bars) mice.

Inhibition of macrophage ROS generation restores dynamic phosphorylation of FAK and macrophage migration in the presence of oxLDL.

To determine if ROS generation impacts macrophage migration we tested the effect of anti-oxidants and NADPH oxidase inhibitors. First, we showed that these agents inhibited macrophage ROS generation induced by NO_2LDL. Macrophages pre-incubated with anti-oxidants N-acetyl-cysteine (NAC) and resveratrol, and NADPH oxidase inhibitors apocynin and DPI, showed >90% inhibition of DCF staining (Figure 15) compared to control cells. Second, we performed immunoblots for phosphorylated FAK (tyr576/577) using macrophages pre-incubated with NAC and apocynin before NO_2LDL treatment and found that both NAC and apocynin-treated cells showed a brief increase in phosphorylation of FAK while untreated macrophages had sustained phosphorylation (Figure 16). Third, we found that macrophage migration in the Boyden chamber in the presence of NO_2LDL was partially restored when cells were pretreated with antioxidants or NADPH oxidase inhibitors to restore dynamic FAK activation (Figure 17).

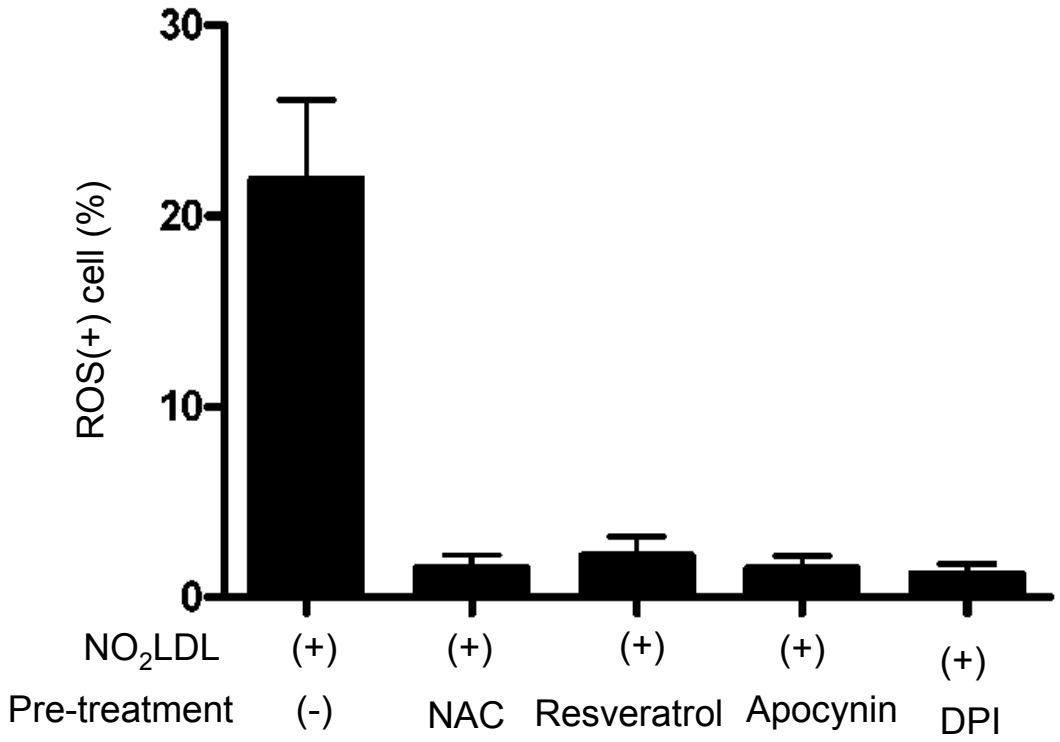


Figure 15. Anti-oxidants and NADPH oxidase inhibitors block the NO_2LDL induced ROS generation. Peritoneal macrophages were pre-treated with anti-oxidants (NAC 20mM and resveratrol 100μM) or NADPH oxidase inhibitors (apocynin 10μM and DPI 4μM) and then exposed to 50 μg/ml NO_2LDL. ROS were detected as in Figure 14.

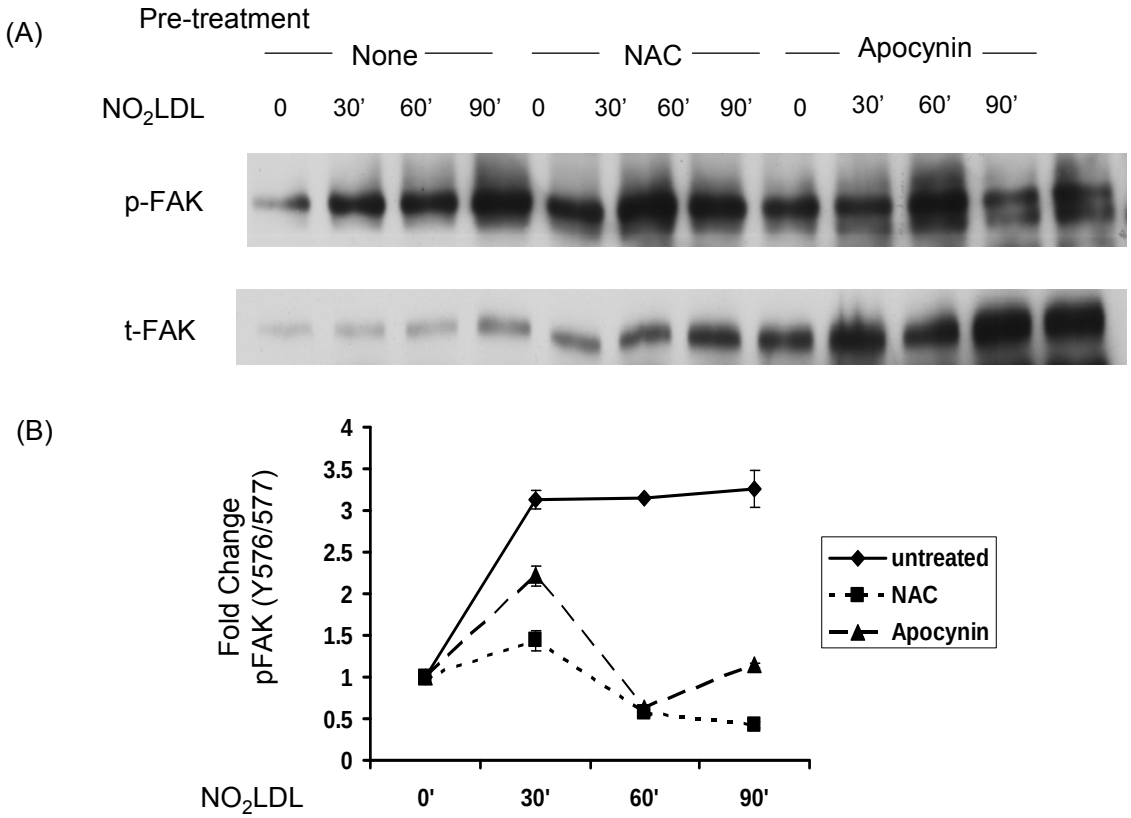


Figure 16. Anti-oxidants and NADPH oxidase inhibitors restore dynamic activation of FAK. (A) Peritoneal macrophages were pre-treated with anti-oxidant (NAC 20mM) or NADPH oxidase inhibitor (apocynin 10μM) and incubated with 50 μg/ml NO_2LDL for indicated times. Cells were then lysed and subjected to immunoblotting to detect FAK tyr 576/577 phosphorylation. (B) Quantitative and kinetic analysis of FAK phosphorylation.

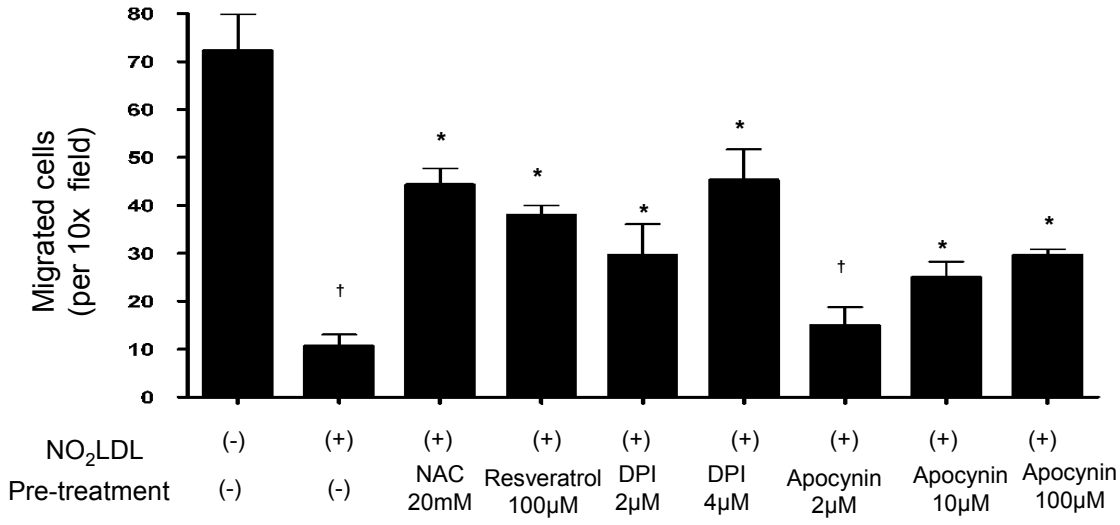


Figure 17. Anti-oxidants and NADPH oxidase inhibitors restore macrophage migration in the presence of NO_2LDL. Peritoneal macrophages were pre-treated with anti-oxidants or NADPH oxidase inhibitors and then loaded into a Boyden chamber with NO_2LDL. Migrated macrophages were counted after 16 hr as described in Figure 2.

(† $P<0.05$, * $P>0.05$, when compared to 'no treatment')

SUMMARY

In sum, the studies described in this chapter show that oxLDL inhibits macrophage migration in vivo and in vitro. Using cells from CD36 null mice we showed that the inhibtion was CD36-dependent. A mechanism by which this occurs is CD36 mediated sustained activation of FAK. Interaction between CD36 and oxLDL induces ROS generation that inhibits the activity of SHP-2, a major inactivator for FAK. Thus, oxLDL perturbs the kinetics of FAK activation and results in sustained activation of FAK. The net effect of this is increased actin polymerization, facilitated cell spreading, and inhibition of migration.

CHAPTER 2.

Interaction between CD36 and oxidized LDL induces loss of cell polarity and inhibits macrophage locomotion

INTRODUCTION

We have shown an inhibitory effect of oxLDL on macrophage migration and demonstrated a role for CD36 regulation of actin polymerization via src-family kinase activation, ROS generation, FAK activation and SHP-2 inactivation in this process. Blockade of the pathway such as by treatment with pharmacological inhibitors of ROS, did not block the effect of oxLDL completely. Thus, it is likely that oxLDL inhibits macrophage migration through multiple pathways. In chapter 2, we used live cell imaging to characterize the effects of oxLDL on macrophage polarity and dynamic membrane movement and to demonstrate roles for Vav, a guanine nucleotide exchange factor (GEF), Rac1, and myosin light chain kinase in these processes.

METHODS

Reagents and antibodies

Antibodies for phosphorylated myosin regulatory light chain (MRLC) (T18/S19) and phosphorylated MRLC (S19) were purchased from Cell Signaling Technology. Antibodies for actin and α-tubulin were purchased from Santa-Cruz Biotechnology. COS-7 and HeLA cell lysates, anti-myosin IIa and anti-myosin IIb antibodies, ML-7, and NSC23766 were provided by Dr. Thomas Egelhoff in the Cleveland Clinic. 6-thio GTP was purchased from Zena Biosciences.

Animals and cells

Vav1 null mice were provided by Dr. J. Rivera (NIH, Bethesda, MD) and *Vav1/3* double null mice were obtained from Dr. W. Swat (Washington Univ. school of Medicine, St. Loius, MO). Background-matched mice were used as controls. Resident peritoneal macrophages were also collected by lavage without prior thioglycollate injection and selected by removing unbound cells 30 minute after plating onto surfaces devoid of matrix protein. Macrophages were cultured in RPMI containing 10% FBS. Human monocytes were isolated from peripheral blood by Ficoll-Hypaque centrifugation and were cultured in RPMI containing human AB serum (10%) for 7 days for macrophage differentiation.

Live cell imaging

Live cell imaging of single cells was performed using a TIRF (Total Internal Reflection Fluorescence) microscopy (Leica AM TIRF MC System equipped with HCX Plan Apo 100X/1.46NA Objective Lens, Leica microsystems). Mouse peritoneal macrophages were plated on a serum-coated glass bottom dish and visualized by transmitted light differential interference contrast (DIC) imaging. ImageEM C9100-13 EMCCD camera (Hamamatsu) captured an image every 15 seconds for 1 hour before the addition of NO_2LDL and for another 1 hour after the addition of NO_2LDL.

Live cell imaging for cell migration assays was performed using Leica DMIRB inverted microscope with 10X objective lens (Leica microsystem).

Cell migration assay (Scratch wound closure migration assay)

Peritoneal macrophages (2 X 10^6 cells) from wild type, *Vav1* null or *Vav 1, 3* double null mice were plated onto glass bottom 6 well or 12 well plates. After 18 hours, confluent monolayers were scratched using a pipette tip and rinsed with PBS. RPMI media was added with or without NO_2LDL. Live cell imaging was used to record the migration of macrophages for 18 hours. Images were taken every 5 minutes and from 3 randomly chosen locations of each well.

Image analysis

The images taken by live cell imaging were analyzed by Image-Pro Plus software. (Media Cybernetics). We counted retraction fibers around the front end lamellipodia using this program. We also measured the ruffle area as a parameter to evaluate dynamic membrane movement. The ruffle area was defined as the mean instant protrusive area from the prior cellular margin. The protrusive areas were counted every 15 seconds for 1 hour before and after the addition of NO_2LDL. We merged the pictures from live cell imaging and generated video clips using this software.

Western blot analysis

Mouse peritoneal macrophages incubated with 50μg/ml of NO_2LDL or NO_2(-)LDL for the indicated times were lysed with sample buffer containing 4% sodium dodecyl sulfate (SDS) after treating the cells with 10% trichloroacetic acid (TCA). Lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were probed with antibodies against phosphorylated MRLC, actin, or α-tubulin for normalization. Band intensities were quantified by ImageJ (http://rsbweb.nih.gov/ij/) and Gel-Pro Analyzer (MediaCybernetics). Immunoblotting for non-muscle myosin IIa and IIb were performed as described above using anti-myosin IIa and anti-myosin IIb antibodies. COS-7 and HeLa cell lysates were added to the gel as positive or negative controls for myosin IIa or IIb.

Immunoprecipitation

Mouse peritoneal macrophages were lysed in buffer consisting of 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% triton-X 100, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, and 1mM sodium orthovanadate. Cell lysates were added to protein A/G sepharose beads (Santa-Cruz biotechnology, Inc.) conjugated with anti-Rac antibody (abCam) and incubated overnight at 4ºC. The sepharose beads were rinsed, resuspended with 2X Laemli sample buffer (Bio-Rad) and heated at 100ºC. After centrifugation, the supernatants were loaded onto SDS-PAGE and transferred to PVDF membrane. Immunoblotting for Vav was performed and ExactaCruz^{TM} C (Santa Cruz biotechnology, Inc.) was used for detection.

Rac and RhoA activity assay

GTP-bound Rac and RhoA were detected by Rac G-LISA activation assay kit and RhoA G-LISA activation assay kit from Cytoskeleton, Inc. In these assays, mouse peritoneal macrophages treated with or without NO_2LDL were lysed. 5-10μg of cell lysate protein was applied to a 96 well plate coated with p21 binding domain (PBD) of p21 activated kinase (PAK) or Rho binding domain (RBD) of Rhotekin, which are known to specifically bind to GTP bound Rac and RhoA, respectively (157,158). After a 30 minute incubation, we rinsed the plates and added anti-Rac or anti-RhoA antibody. HRP conjugated secondary antibody was added 1 hour later and the level of antibody binding was measured by a colorimetric method.

RESULTS

OxLDL inhibits murine macrophage locomotion by inducing loss of cell polarity

We investigated the effect of oxLDL on macrophage cytoskeletal functions by using live cell imaging. Resident peritoneal macrophages from wild type, *Cd36* null and *Vav* null mice plated on serum-coated glass coverslips made protrusions and then spontaneously polarized. Polarized macrophages protruded broad lamellipodia on their front ends and started to move by retracting their rear ends, leaving retraction fibers on the rear ends (Figure 1). After the addition of NO_2LDL, macrophages from different mouse strains showed different responses. Macrophages from wild type mice retracted their front end lamellipodia and generated retraction fibers around the front end. Since the front end lamellipodia acquired the function of the rear end, macrophages lost their cell polarity as well as the ability of the cell to advance (Figure 1). Macrophages from *Cd36* null mice did not show lamellipodial retraction, nor retraction fiber formation around the front end, and thus, maintained ability to migrate in the presence of NO_2LDL (Figure 2). We then tested macrophages from mice null for Vav1, a GEF recently shown to be a downstream effector of CD36 (117,118). As *Cd36* null cells, macrophages from *Vav1* null mice did not show lamellipodial retraction in response to NO_2LDL (Figure 3).

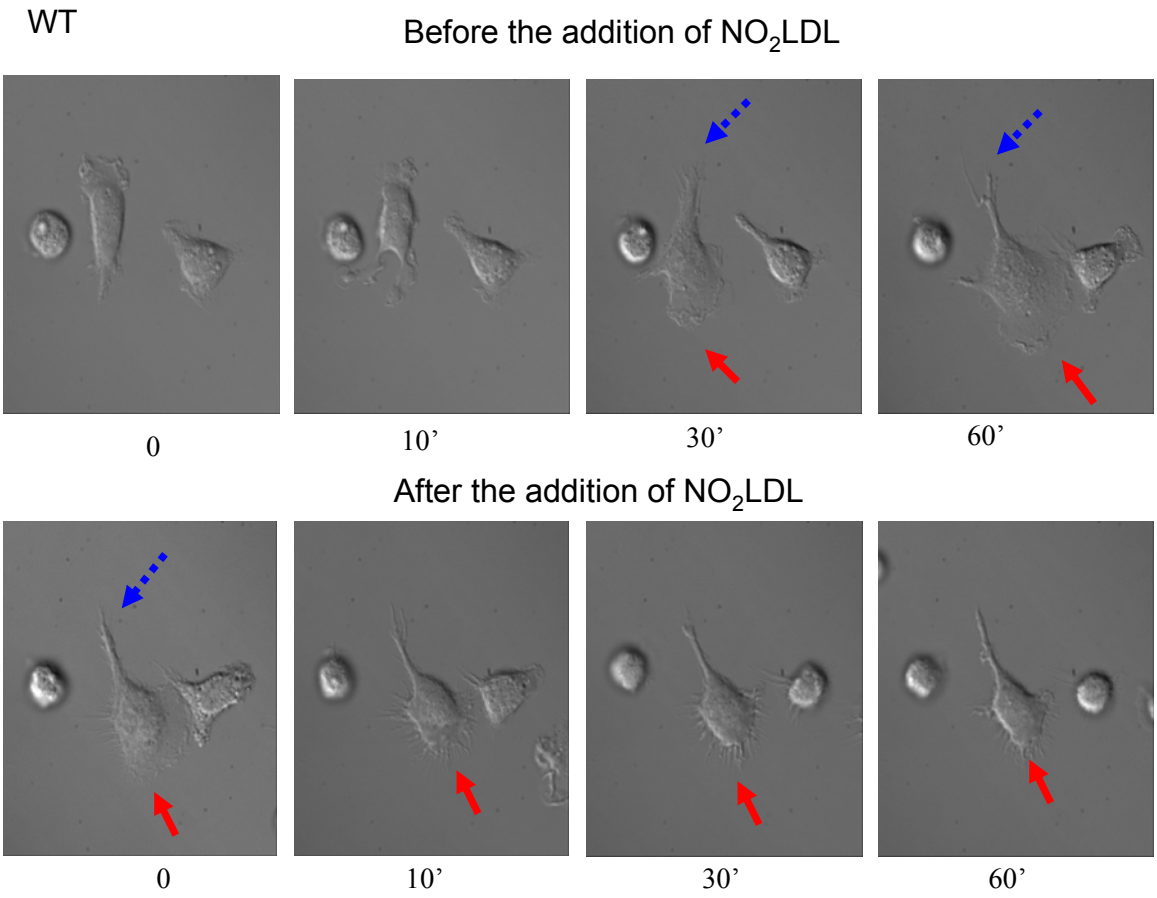


Figure 1. NO_2LDL induces retraction of lamellipodia and loss of cell polarity. Resident peritoneal macrophages from wild type mice were plated onto a serum coated glass bottom dish and allowed to spontaneously polarize. Time lapse images were taken every 15 seconds for 1 hour before and after the addition of NO_2LDL (50µg/ml). Red solid arrows indicate the front end lamellipodia and blue dashed arrows indicate the rear end.

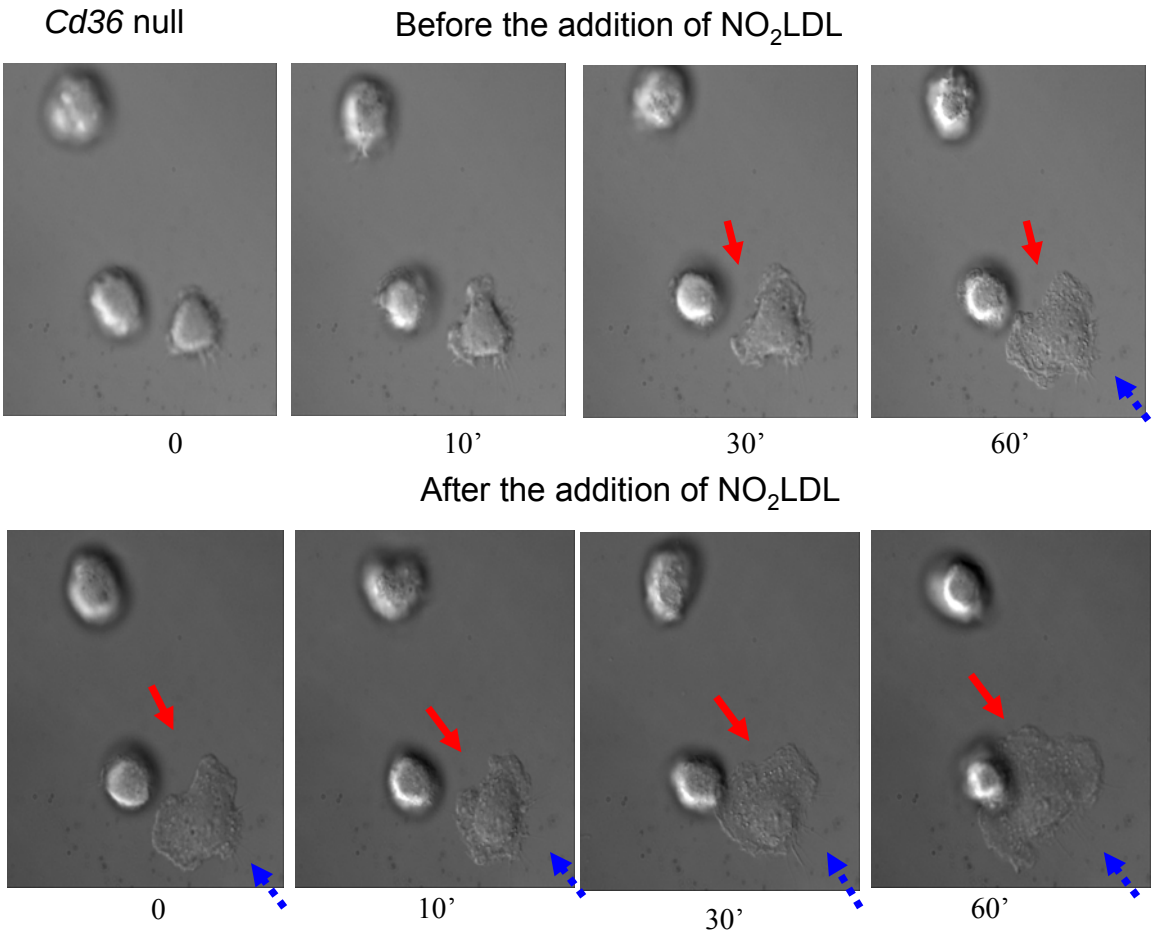


Figure 2. Cell polarity of *Cd36* null macrophages is not affected by NO_2LDL. Macrophages from *Cd36* null mice were tested as described in Fig.1. Red arrows indicate the front end and blue arrows indicate the rear end.

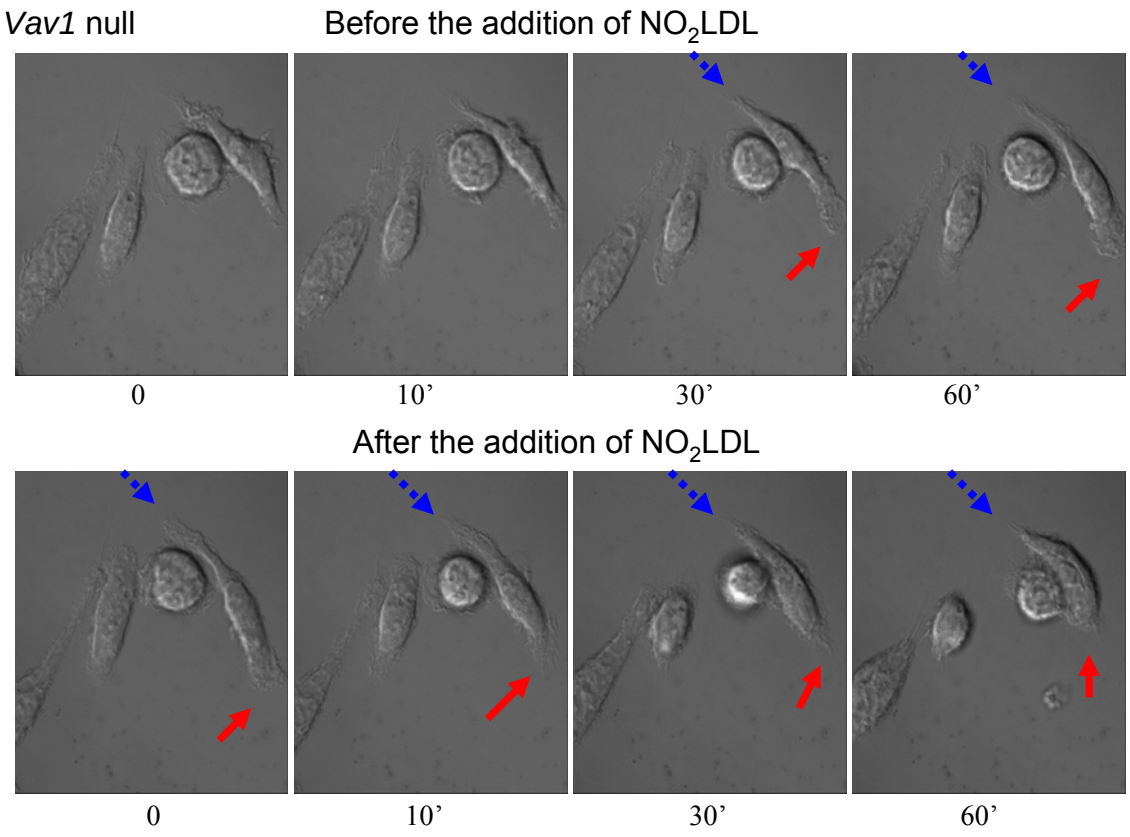
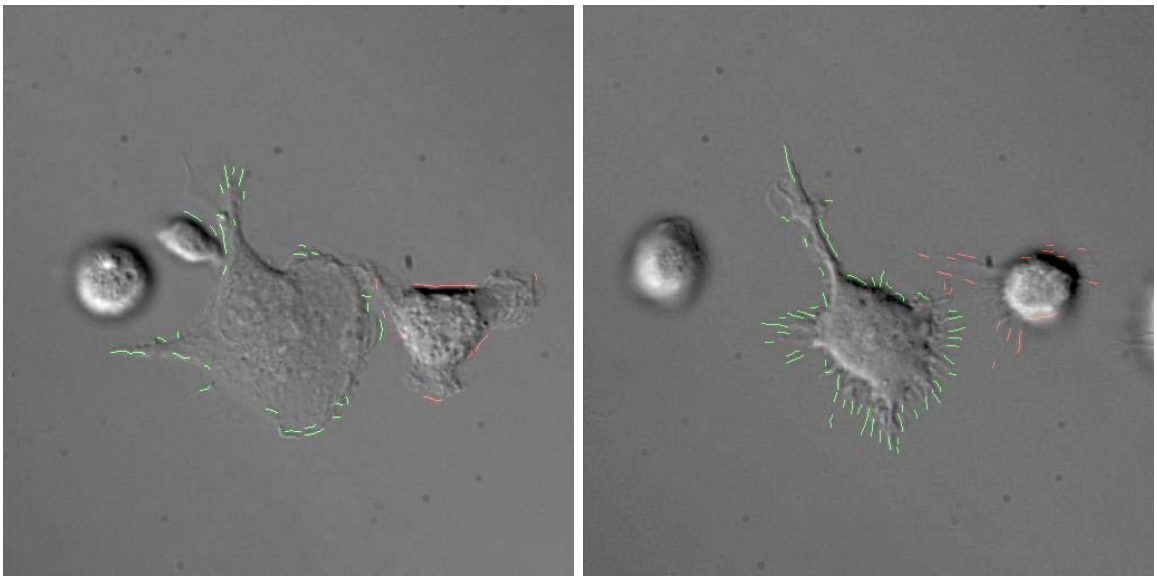


Figure 3. Cell polarity of *Vav1* null macrophages is not affected by NO_2LDL. Macrophages from *Vav1* null mice were tested as described in Fig.1. Red arrows indicate the front end and blue arrows indicate the rear end.

Quantitative analysis of the live cell imaging studies was performed using several different parameters. Counting the number of retraction fibers showed that NO_2LDL increased the retraction fibers by 1.5 fold in WT macrophages but not in *Cd36* null and *Vav1* null macrophages (Figure 4 and 6A). Dynamic movement of the macrophage membrane was assessed by measuring ruffle area, defined as a mean instant protrusive area from the prior margin of a cell. NO_2LDL decreased the ruffle area in wild type but not *Cd36* null macrophages. The response in *Vav1* null cells was intermediate that of WT and *Cd36* null cells (Fig.5 and 6B). Macrophage velocity was measured as start-to-end travel distance in 1 hour and was decreased by NO_2LDL in WT but not in *Cd36* null or *Vav1* null cells (Figure 6C). These studies, in sum, show that NO_2LDL inhibited directional cell movement in wild type macrophages.

In all studies, NO_2(-)LDL, the control LDL that was exposed to all the components of the MPO system except the oxidant, had no effect (Figure 7).

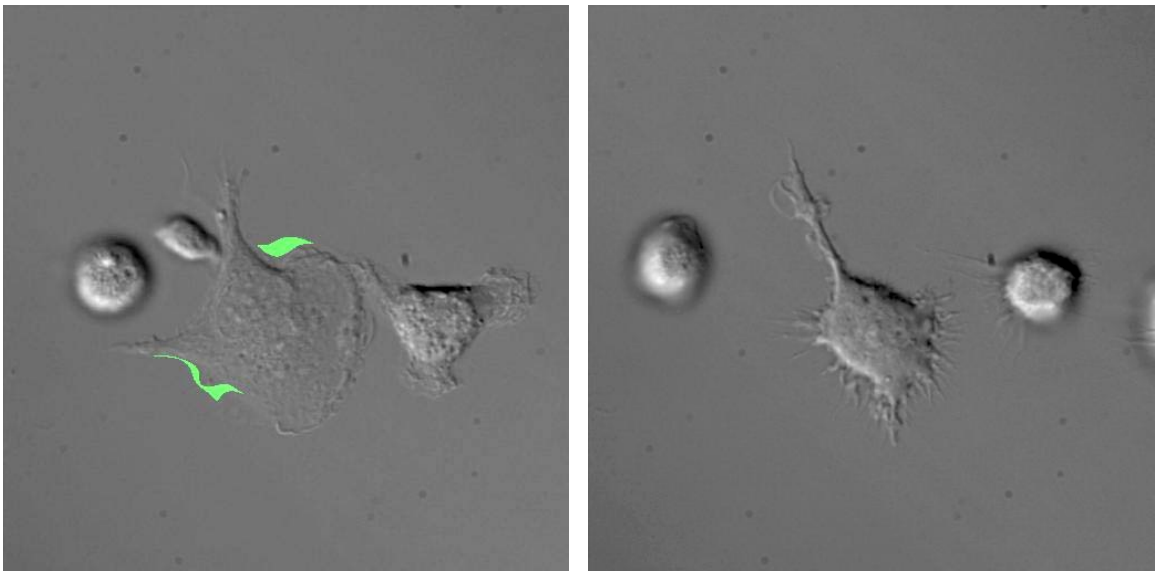
We also tested thioglycollate-elicited macrophages using the live cell imaging. Macrophages from WT mice injected with thioglycollate also showed lamellipodial retraction with increased number of retraction fibers in response to NO_2LDL as resident macrophages (Figure 8 and 9A). The dynamic movement of macrophage membrane assessed by ruffle area was also decreased by NO_2LDL in thioglycollate-elicited macrophages (Figure 9B).



Before the addition of NO_2LDL

After the addition of NO_2LDL

Figure 4. Retraction fiber formation around lamellipodia is induced by NO_2LDL.
Images from the time lapse microscopy were analyzed. All protrusions are indicated and counted by Image-Pro software (Media Cybernetics). Green or yellow lines indicate protrusions. We used indicator lines with different colors to record protrusion count of different cells.



Before the addition of NO_2LDL After the addition of NO_2LDL

Figure 5. NO_2LDL decreases ruffle formation of macrophages.

Images from the time lapse microscopy were analyzed. Ruffle area, defined as an instant protrusion from the prior cell margin, was indicated and measured by Image-Pro software (Media Cybernetics). Area filled with green color is the newly formed protrusion from the prior cell margin taken before 15 second.

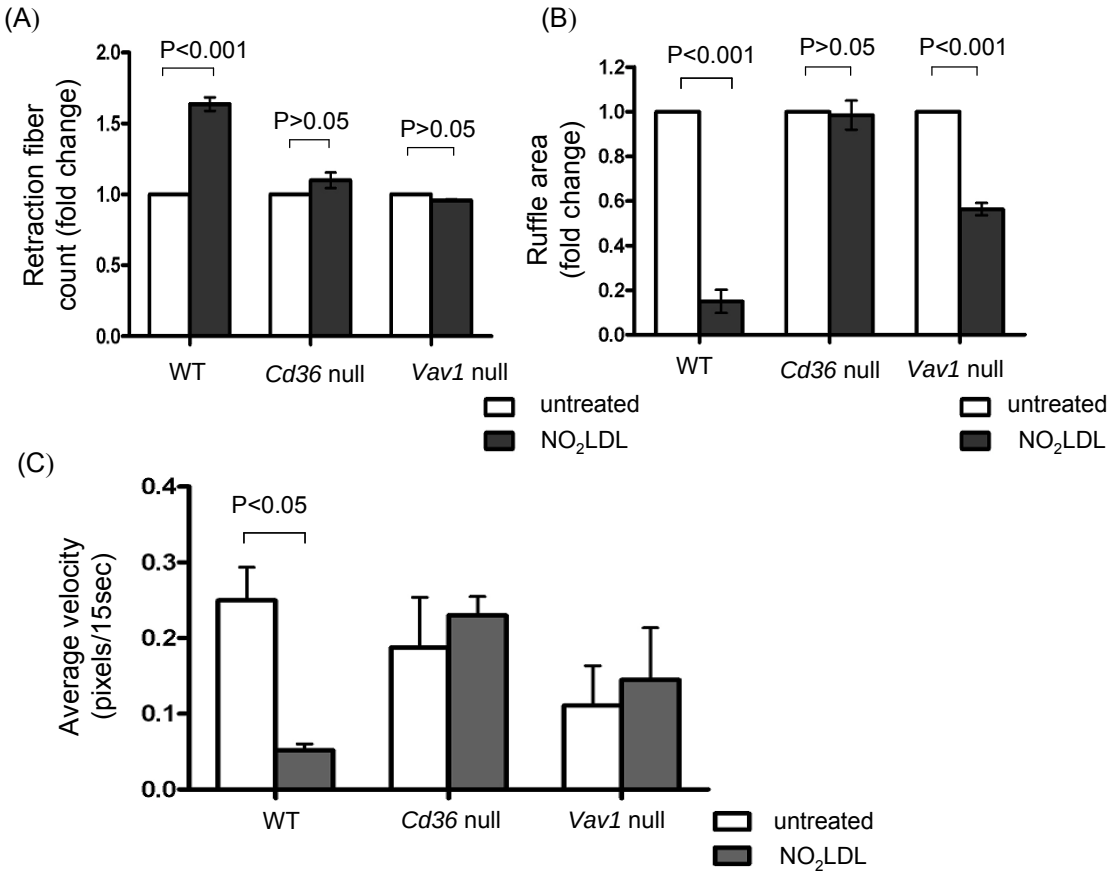


Figure 6. Quantitative analyses of live cell imaging data.

Live cell imaging data shown in Figure 1-3 were analysed by Image-Pro software. (A) Retraction fibers were counted and compared among WT, *Cd36* null and *Vav1* null macrophages. (B) Ruffle area defined as a protrusive area from the initial margin of a cell, was counted and compared among WT, *Cd36* null and *Vav1* null macrophages. (C) Average velocity measured as start-end travel distance, was compared among WT, *Cd36* null and *Vav1* null macrophages.

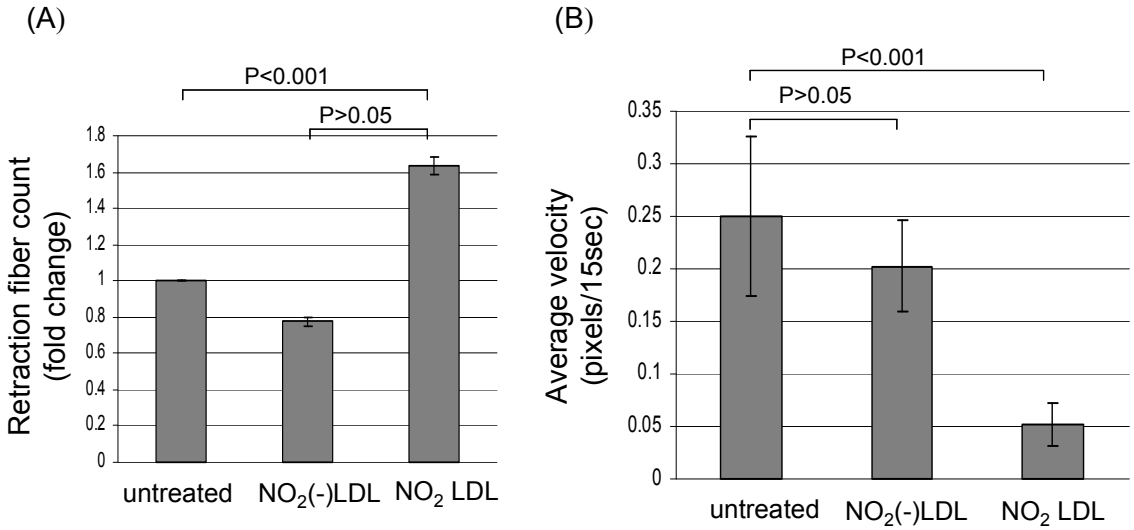


Figure 7. NO_2LDL, but not NO_2(-)LDL induces lamellipodial retraction.
WT macrophages were untreated or treated with NO_2LDL or NO_2(-)LDL and applied to live cell imaging as described in Figure 1. The data from the live cell imaging were analyzed. (A) Retraction fiber count was compared among macrophages untreated or treated with NO_2LDL or NO_2(-)LDL. (B) Average velocity of macrophages, untreated or treated with NO_2LDL or NO_2(-)LDL, were compared.

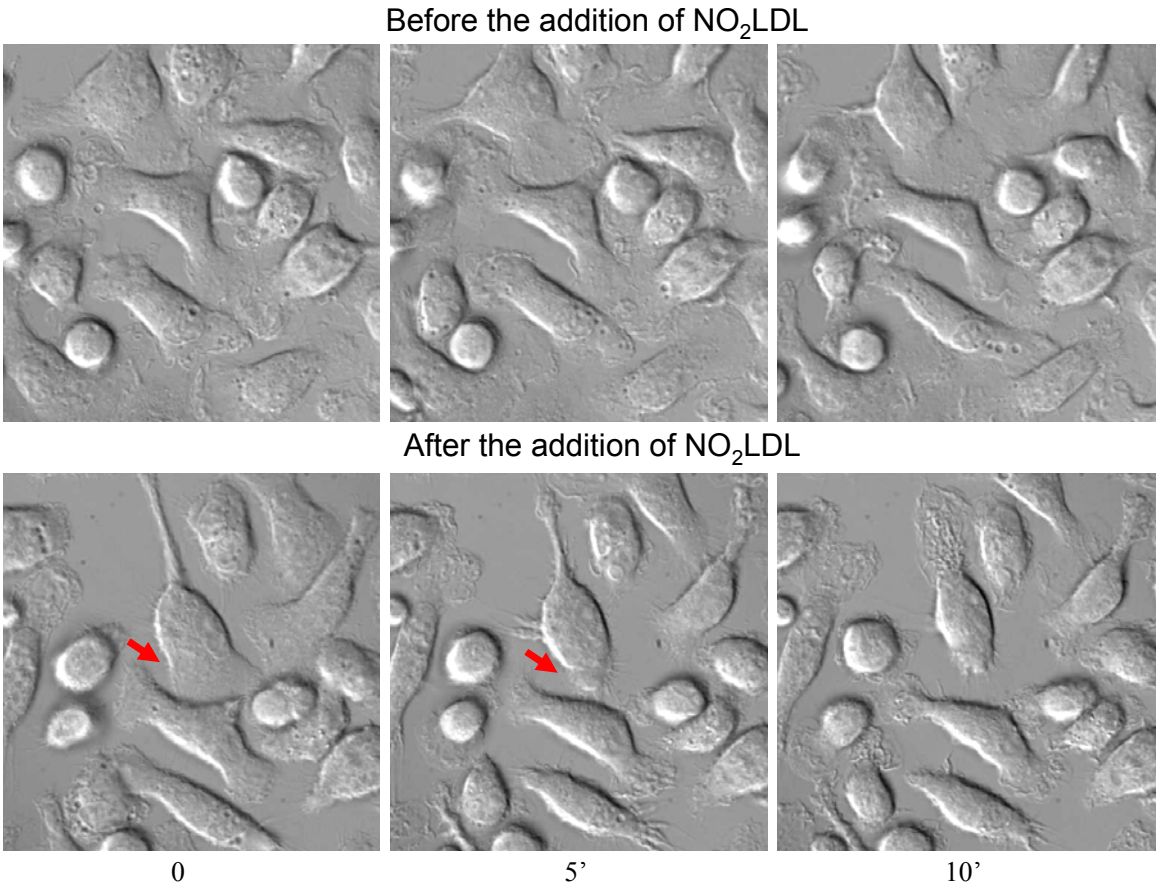


Figure 8. NO_2LDL induces lamellipodial retraction in thioglycollate-elicited macrophages.

Peritoneal macrophages from WT mice injected with thioglycollate were plated on glass bottom dish and cellular movement was recorded by live cell imaging as described in Figure 1. Upper panels and lower panels show macrophages before and after the addition of NO_2LDL, respectively. Red arrows indicate lamellipodia.

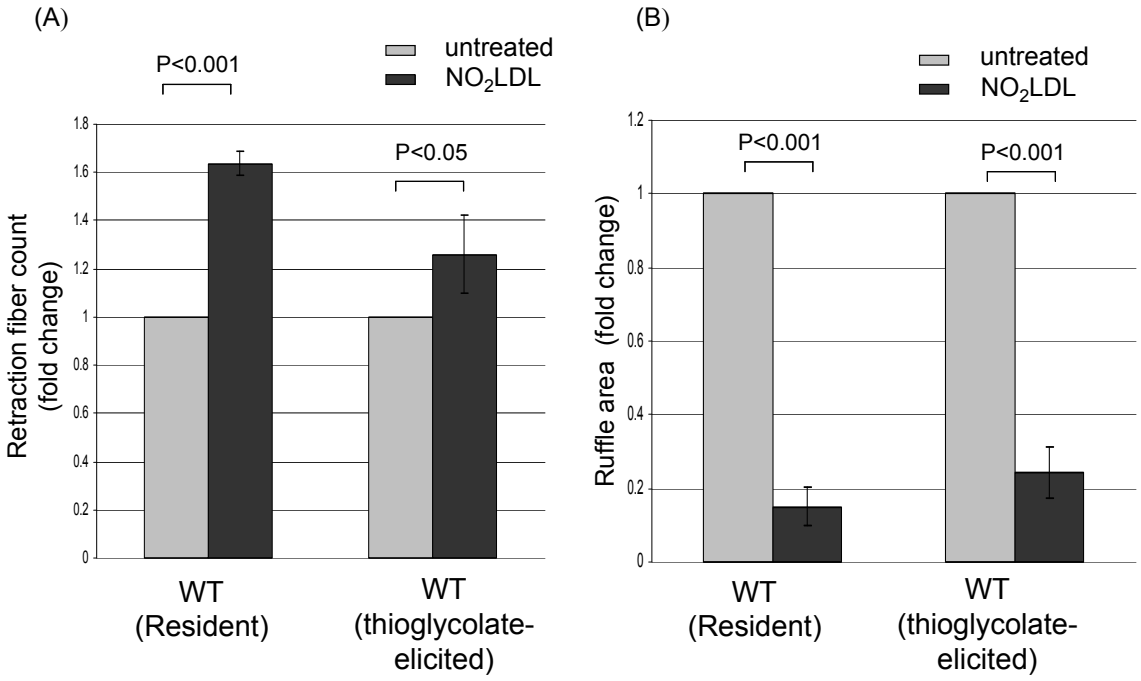


Figure 9. NO_2LDL induces retraction fiber formation and decreased ruffle formation in thioglycollate-elicited macrophages.

Macrophages from WT mice injected with thioglycollate were treated with or without NO_2LDL and applied to live cell imaging as described in Figure 1. The data from live cell imaging were analyzed. (A) Retraction fiber count was compared between resident or thioglycollate-elicited macrophages treated with or without NO_2LDL. (B) Average ruffle area of resident or thioglycollate-elicited macrophages was compared beween resident or thioglycollate-elicited macrophages treated with or without NO_2LDL.

OxLDL induced inhibition of macrophage migration depends on CD36 and Vav family guanine nucleotide exchange facctors

Scratch wound closure migration assays were performed using confluent cultures of macrophages from WT, *Cd36* null and *Vav1* null mice. After 19 hours, wild type macrophages migrated into the scratched cell-free space and completely filled up the space (Figure 10A and 11). As reported in previous studies, migration of *Vav* null macrophages was slower than WT macrophages under basal conditions (159,160). However, NO_2LDL treatment had significantly less impact on migration of *Vav1* null macrophages compared to WT (Figure 10B and 11). Since macrophages also express Vav3 (118), we tested *Vav1,3* double null macrophages in the migration assay and found that like *Cd36* null cells, *Vav1,3* double null macrophages were not inhibited by NO_2LDL in migration (Figure 10C and 11).

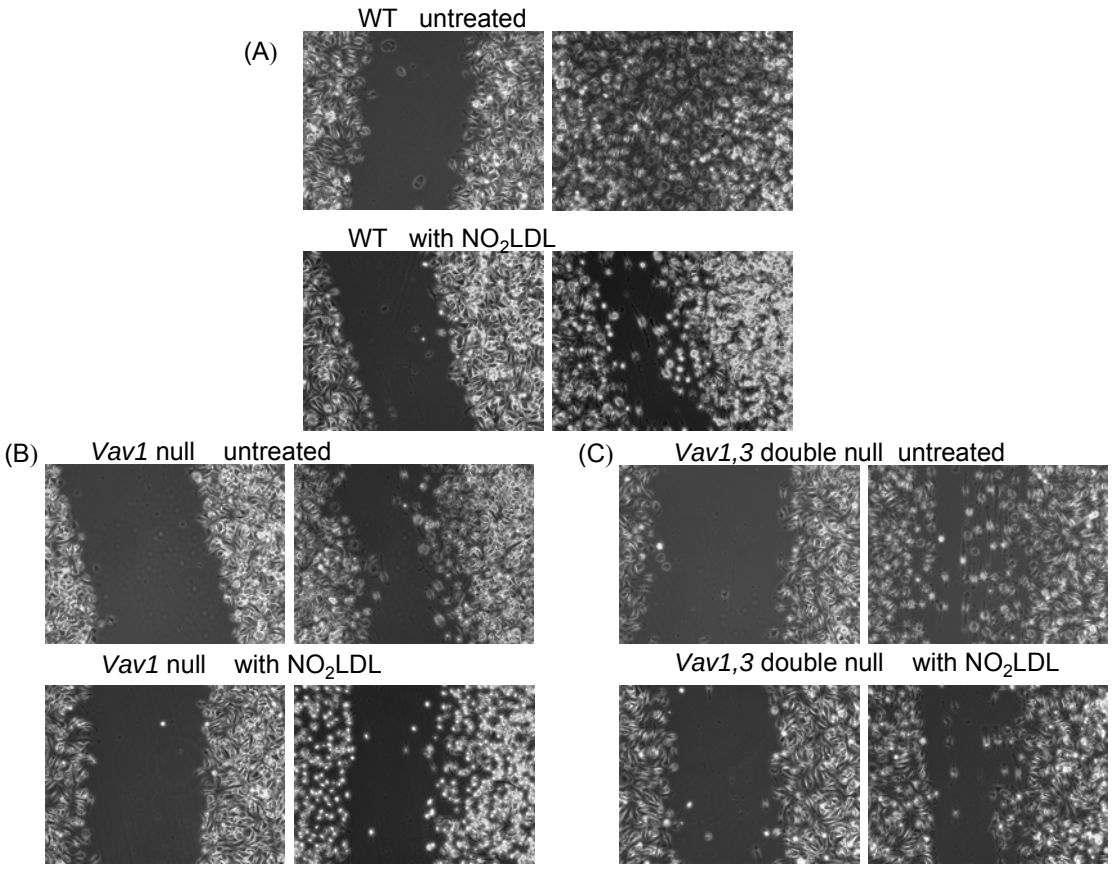


Figure 10. Scratch wound closure migration assay. (A) macrophages from wild type mice were plated onto a glass bottom dish. After 18hours, the confluent cell layer was scratched and treated with NO_2LDL or control LDL. Macrophages migrating into the free space were counted after 19 hours (right panels). (B) Macrophages from *Vav1* null mice were tested as described in (A). (C) Macrophages from *Vav 1,3* double null mice were tested as described in (A).

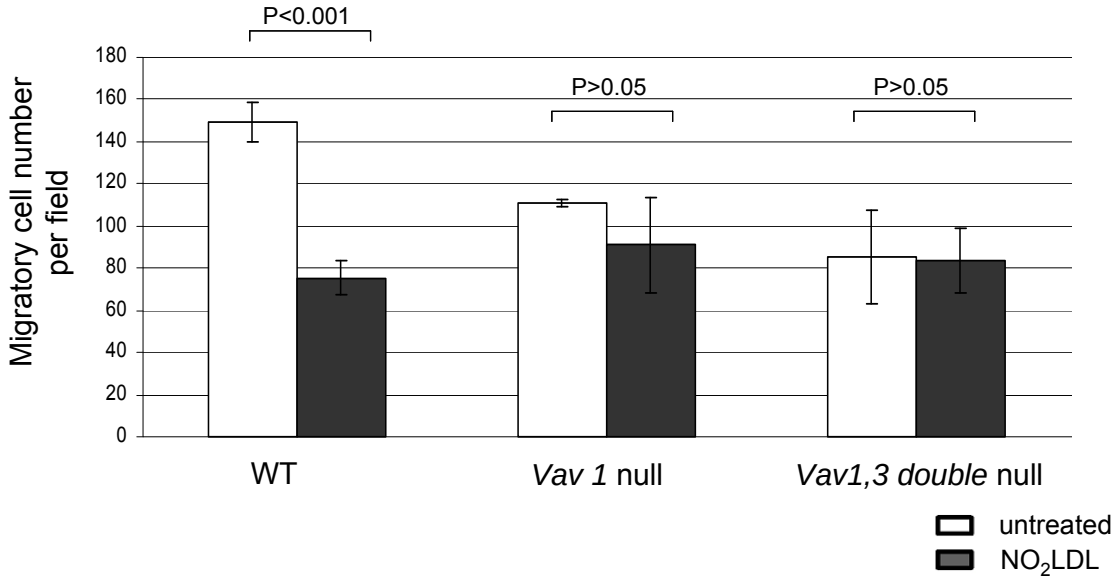


Figure 11. NO_2LDL induced inhibition of macrophage migration is dependent on Vav1 and Vav3. Quantitative analysis of the scratch wound closure migration assay was performed as described in Figure 6. Migratory cell number was compared among WT, *Vav1* null and *Vav1,3* double null macrophages. Open bars are migratory cell number of untreated macrophages and filled bars are migratory cell number of NO_2LDL treated macrophages. Blocked lines indicate S.D.

OxLDL induces myosin regulatory light chain dephosphorylation

To evaluate mechanism by which NO_2LDL induces lamellipodial retraction and loss of cell polarity, we tested if NO_2LDL affects the activity of non-muscle myosin II, which was recently proposed to be a cell polarity determinant and is known to generate lamellipodia traction force (136,161,162).

Macrophages treated with NO_2LDL or control LDL were lysed and analyzed by western blot to detect phosphorylated (T18/S19) myosin regulatory light chain (MRLC) which determines the functional activity of non-muscle myosin II (163-165). The western blot , repeated more than 6 times, shows that NO_2LDL treatment decreased phosphorylated MRLC by 60% in WT cells but not in macrophages from *Cd36* null or *Vav1* null mice (Figure 12A). NO_2LDL also induced 60% decrease in phosphorylation of MRLC in human peripheral blood monocyte derived macrophages. (Figure 12B). NO_2(-)LDL control did not induce dephosphorylation of MRLC (Fig.13).

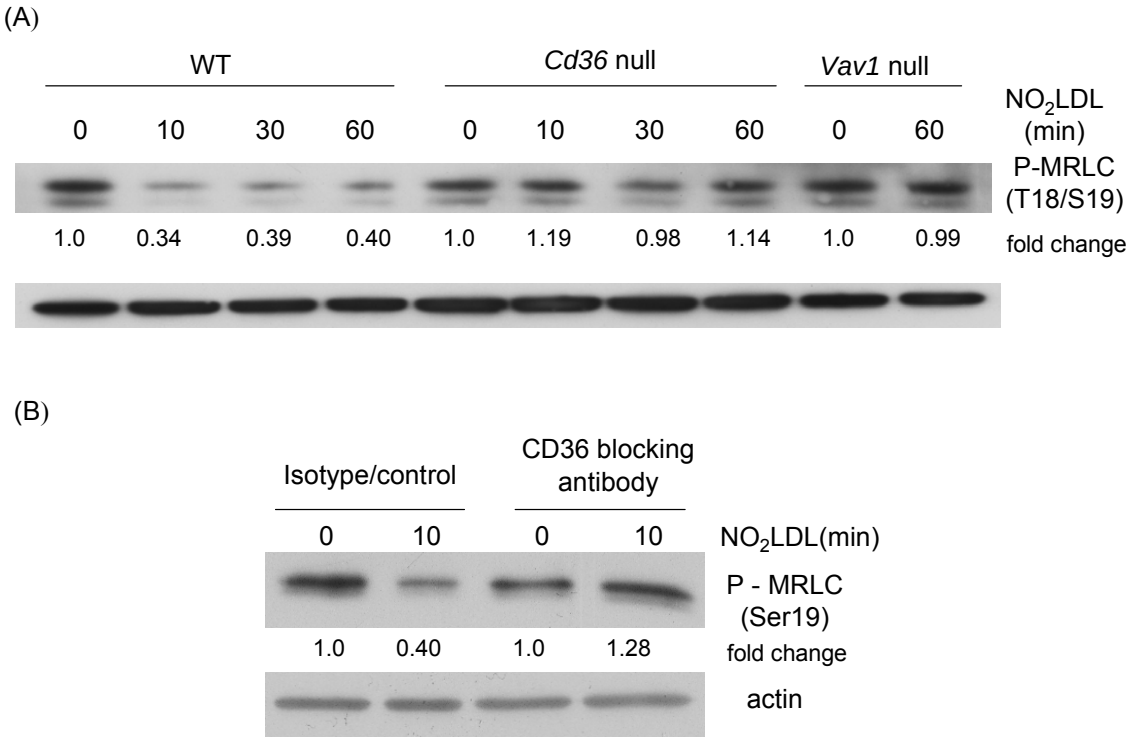


Figure 12. NO_2LDL inhibits non-muscle myosin II activity by dephosphorylating MRLC. (A) WT, *Cd36* null, and *Vav* null macrophages were incubated with or without NO_2LDL for the indicated times and lysed. The lysates were applied to a western blot to detect phosphorylated MRLC (Thr-18/ Ser-19). (B) Human peripheral monocyte derived macrophages were treated with an isotype control IgG and CD36 blocking antibody. After incubating with NO_2LDL, macrophages were lysed and applied to a western blot to detect phosphorylated MRLC (Ser-19).

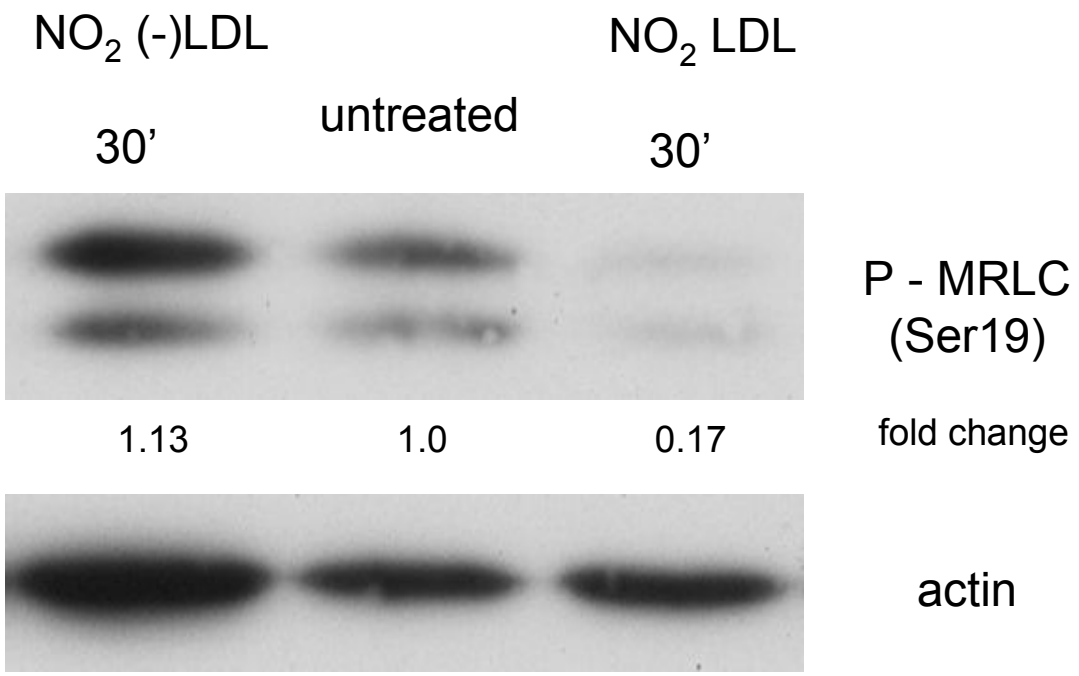


Figure 13. NO_2LDL, not NO_2(-)LDL inhibits phosphorylation of MRLC. Phosphorylated MRLC (Ser-19) was measured in macrophages treated with NO_2LDL, NO_2(-)LDL or media alone.

Non-muscle myosin II has 2 different heavy chain isoforms, myosin IIa and IIb (166). To detect which isoform of non-muscle myosin II is most affected by NO_2LDL, we determined which isoform is expressed in macrophages. Macophages expressed high levels of non-muscle myosin IIa and very low levels of myosin IIb (Fig.14). Therefore, the cytoskeletal changes derived from myosin inactivation in macrophages are likely due to perturbed function of myosin IIa.

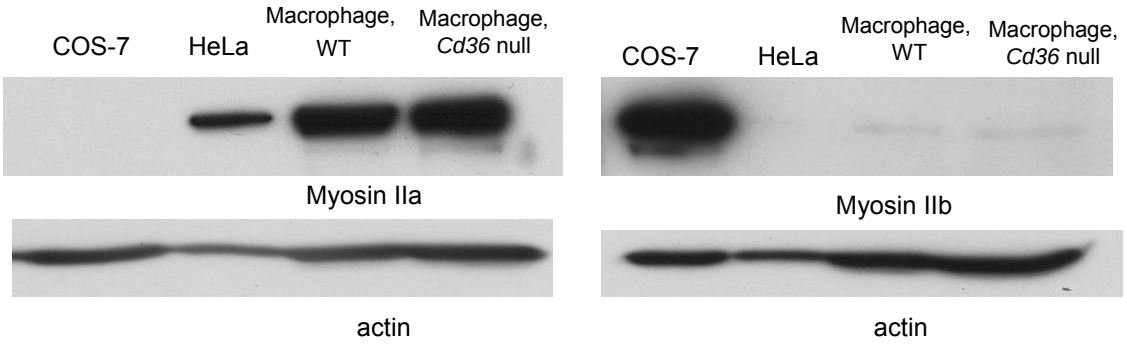


Figure 14. Macrophages have high level of expression of myosin IIa.
Myosin IIa and myosin IIb expressions of macrophages, COS-7 and HeLa cells were tested by western blot.

The small molecular weight G-protein, Rac, is activated by oxLDL

The activity of non-muscle myosin II is regulated by small-molecular weight G-proteins, including Rac and RhoA. Activated Rac decreases MRLC phosphorylation by inhibiting myosin light chain kinase (MLCK) activity while active RhoA increases MRLC phosphorylation by activating Rho kinase which inhibits myosin phosphatase activity (167) (schematized in Figure 15). To evaluate the mechanism by which MRLC dephosphorylation was induced by NO_2LDL, we tested if Rac or RhoA were activated by NO_2LDL. ELISA assays were used to detect active, a GTP-bound form of Rac and RhoA, and showed that NO_2LDL induced a dynamic increase in GTP-bound Rac in wild type macrophages, but not in *Cd36* null or *Vav1* null cells (Figure 16). GTP-bound RhoA was not affected by NO_2LDL (Figure 17). Active RhoA is known to phosphorylate the myosin binding subunit (MBS) of myosin phosphatase (MP) (168,169).

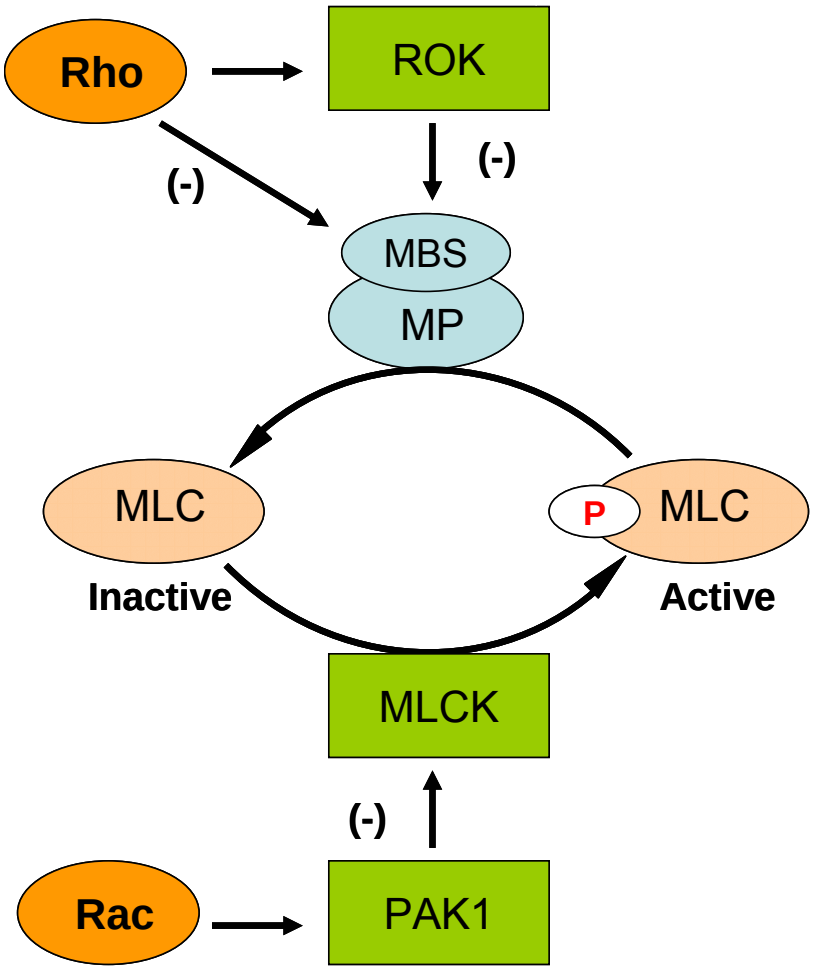


Figure 15. MRLC phosphorylation is regulated by coordinated activities of small molecular weight G-proteins including Rac and Rho. Rho increases MRLC phosphorylation by inhibiting MP dicrectly or indirectly through Rho kinase (ROK). Rac decreases phosphorylation of MRLC by inhibiting MLCK activity.

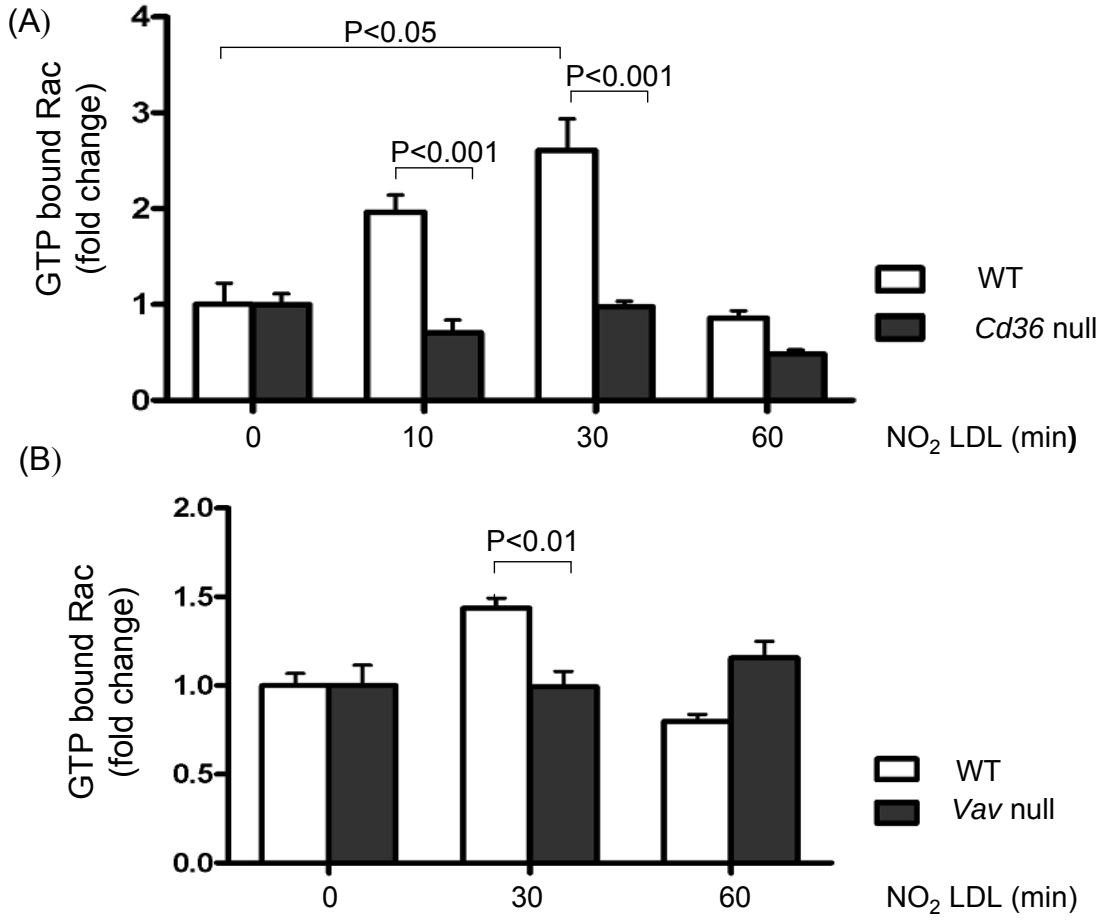


Figure 16. NO_2LDL induces Vav mediated Rac activation. (A) GTP-bound Rac was measured by ELISA in wild type and *Cd36* null macrophages treated or untreated with NO_2LDL for the indicated times. (B) GTP-bound Rac was measured in wild type and *Vav* null macrophages as described in (A).

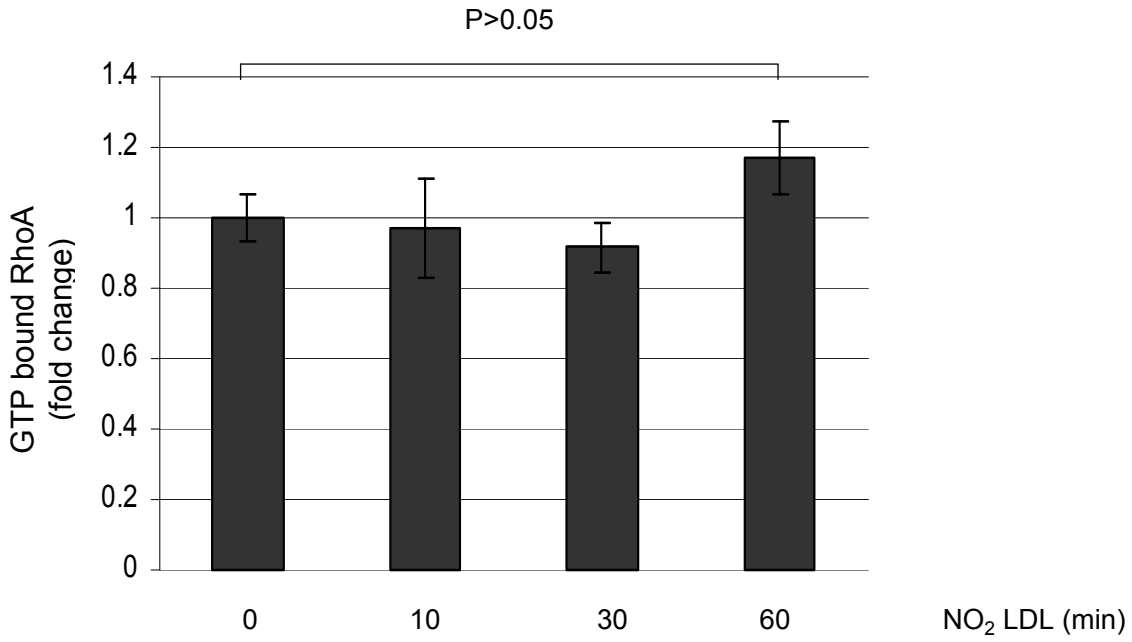


Figure 17. RhoA activity is not affected by NO_2LDL.

GTP-bound Rho was measured by ELISA in wild type and *Cd36* null macrophages with or without NO_2LDL.

We performed immunoprecipitation of macrophage cell lysates with anti-Rac antibody to assess the physical association of Vav and Rac. Firuge 18 shows that anti-Rac antibody co-precipitates Vav. After treatment with NO_2LDL, the amount of Vav co-precipitates was increased by 2 fold within 2 minutes. The immunoprecipitation was repeated 3 times and these experiments consistently show increase of the association between Vav and Rac after treatment with NO_2LDL (Figure 18A and B).

These data suggest that NO_2LDL may function through a Rac-mediated pathway that regulates MRLC phosphorylation.

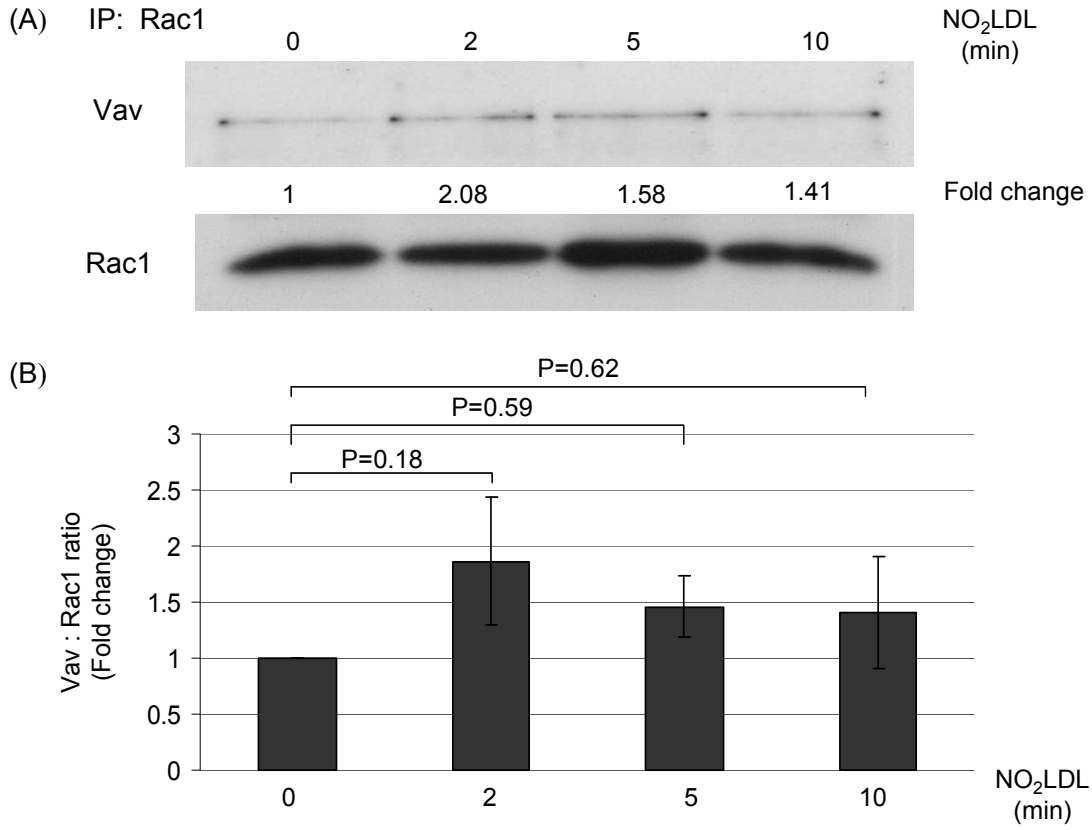


Figure 18. NO_2LDL increases the physical association between Vav1 and Rac1. Rac was pulled down from wild type macrophages treated with or without NO_2LDL for indicated times. (A) Immunoprecipitate was applied to a western blot to detect Vav. (B) Quatitative analysis of the immunoblots for Vav in Rac1 immunoprecipitates, summarized from 3 separate experiments.

OxLDL inhibits myosin activity through Rac

We used a pharmacological approach to confirm that NO_2LDL inactivates non-muscle myosin II by activating Rac. Calyculin-A is a myosin activator that increases MRLC phosphorylation by inhibiting myosin phosphatase (170-172). Thus, calyculin-A blocks the effect of myosin inhibitors such as Y27632 that act upsteam of the phosphatase by inhibiting Rho and/or Rho kinase (173). Calyculin A, however, does not block myosin inhibitors such as ML-7 which act through the MLCK pathway (174). (Schematized in Figure 19).

We thus tested if calyculin-A could block the effect of NO_2LDL on MRLC dephosphorylation. Macrophages from wild type mice were incubated with myosin inhibitors Y27632, ML-7 and NO_2LDL with or without calyculin-A. The western blot (Figure 20) shows that calyculin-A, as expected, blocked the effect of Y27632 on MRLC dephosphorylation (red arrow), but in contrast, did not block the MRLC dephosphorylating effect of ML-7 (blue arrow) or NO_2LDL (yellow arrow). Therefore, we conclude that the activity of NO_2LDL is most likely mediated by inbhition of the MLCK pathway, as would be expected of an agent that activates Rac.

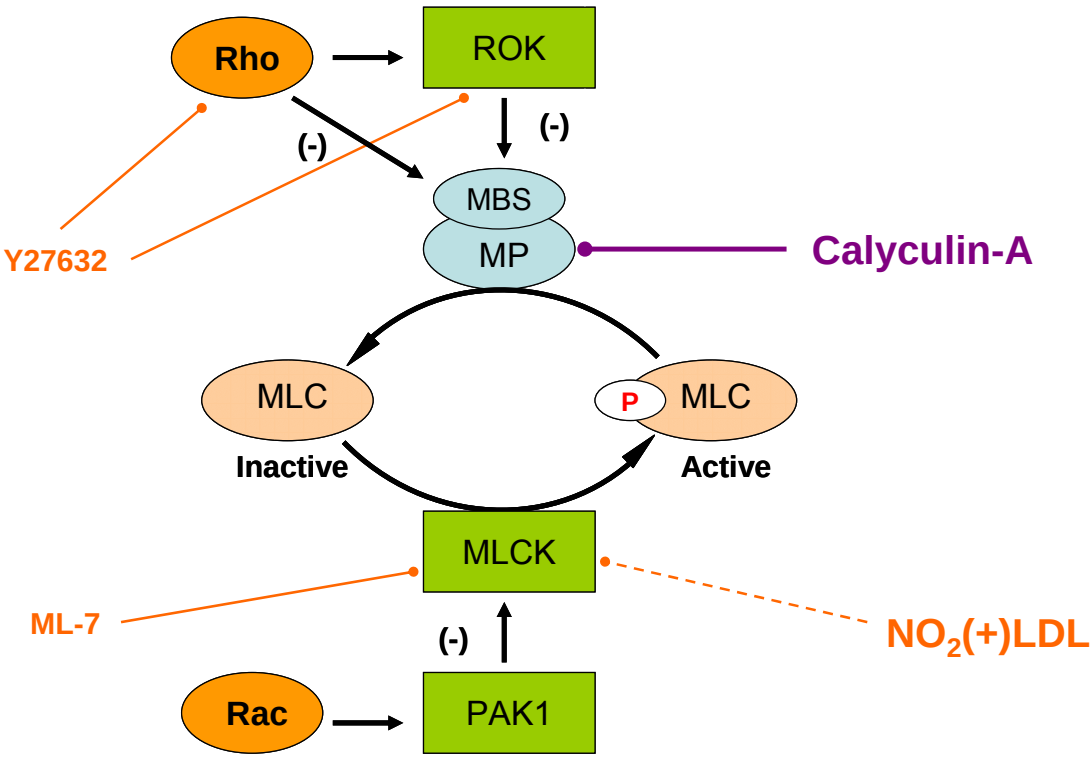


Figure 19. Model of the assay to reveal the pathway modulated by NO_2LDL. MRLC phosphorylation is regulated by balanced effects of MLCK and MP. Y23632 inhibits myosin through inhibition of Rho and Rho kinase (ROK) and ML-7 works through MLCK. Calyculin-A, a MP inhibitor reverse the effect of myosin inhibitors that function through Rho or ROK, but not the effect of myosin inhibitors working through MLCK.

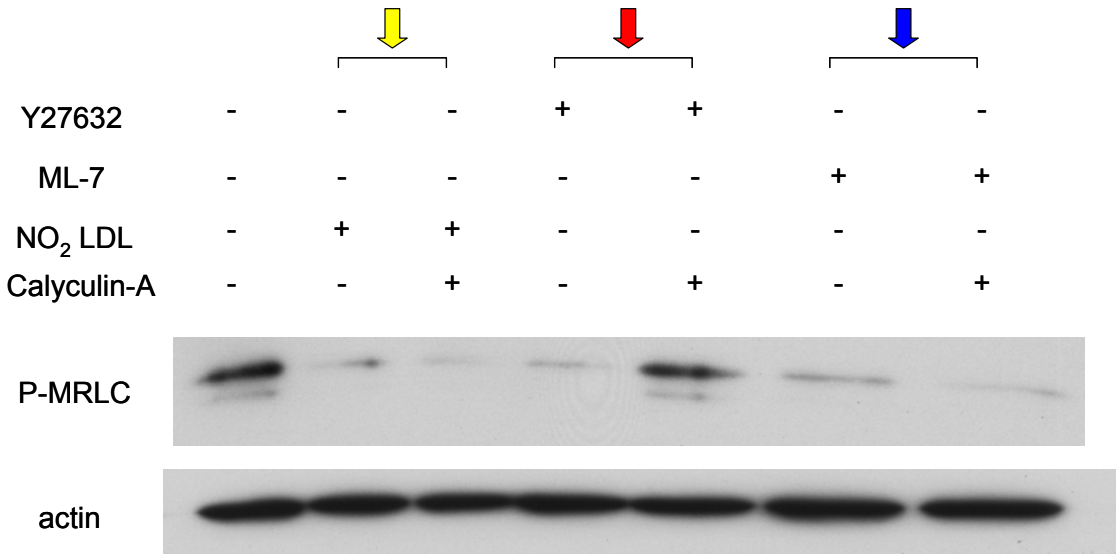


Figure 20. NO_2LDL dephosphorylates MRLC, consistent with a MLCK inhibitor. Macrophages from wild type mice were treated with Y27632 (2μM), ML-7 (15μM), and NO_2LDL (50μg/ml). And then calyculin-A was added. Cell lysates were applied to a western blot for detection of phosphorylated MRLC (Ser-19).

6-thio GTP blocks oxLDL induced Rac activation and non-muscle myosin II inhibition

We next evaluated if blockade of Rac activation blocked the effect of NO_2LDL on MRLC dephosphoryaltion. Six-thio GTP is a Rac inhibitor that inhibits Vav binding to Rac but does not affect other GEFs (175). Thus, we measured Rac activity in macrophages after incubation with 6-thio GTP. Unlike untreated cells, 6-thio GTP pre-treated macrophages did not show an increase in GTP-bound Rac after NO_2LDL treatment (Figure 21). Furthermore, western blots for phosphorylated (S19) MRLC showed that NO_2LDL failed to induce dephosphorylation of MRLC in 6-thio GTP pre-treated macrophages (Figure 22A and B). The blocking effect of 6-thio GTP was time dependent. Four hour incubation with 6-thio GTP incompletely blocked MRLC dephosphorylation, however, 16 hour of incubation completely blocked the effect of NO_2LDL (Fig.22B).

To determine the role of Vav in NO_2LDL- induced Rac activation and MRLC dephosphorylation, macrophages were incubated with NSC23766, a Rac inhibitor that does not influence Vav but inhibits binding of alternative GEFs, including Tiam-1 and Trio(176). Unlike 6-thio GTP, NSC23766 did not block MRLC dephosphorylation by NO_2LDL, further supporting a key role of Vav in NO_2LDL induced dephosphorylation of MRLC (Figure 22A).

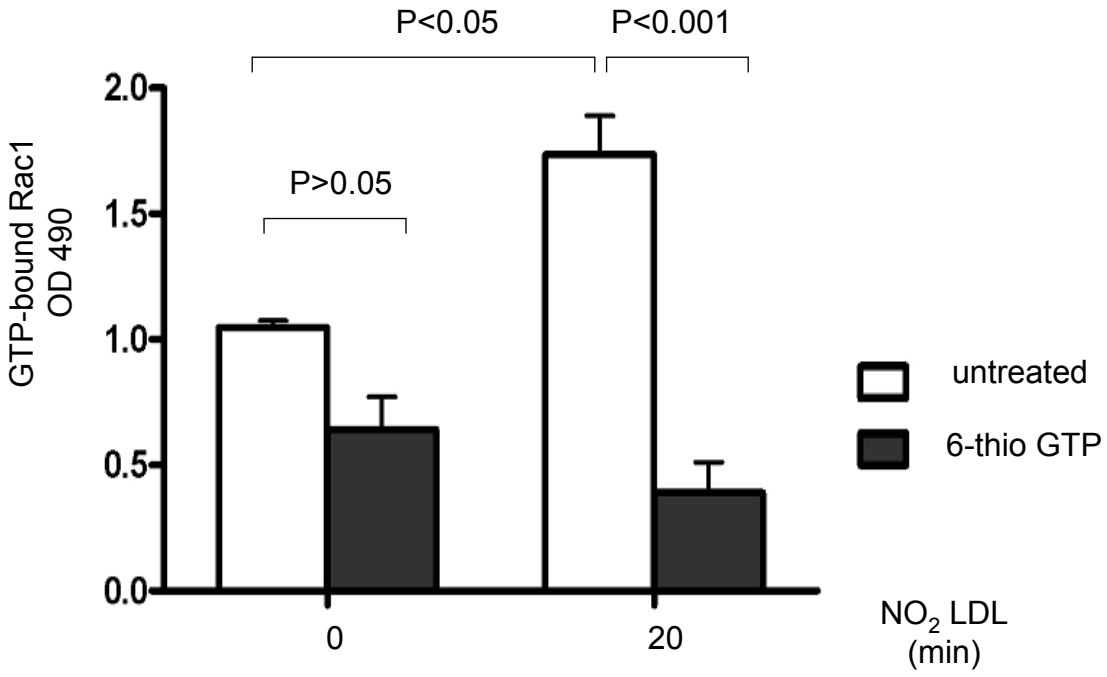


Figure 21. Six-thio GTP blocked the effects of NO_2LDL on Rac activation. Wild type macrophages were incubated with 6-thio GTP (5μM) and treated with NO_2LDL. GTP-bound Rac was measured as described in Figure 16.

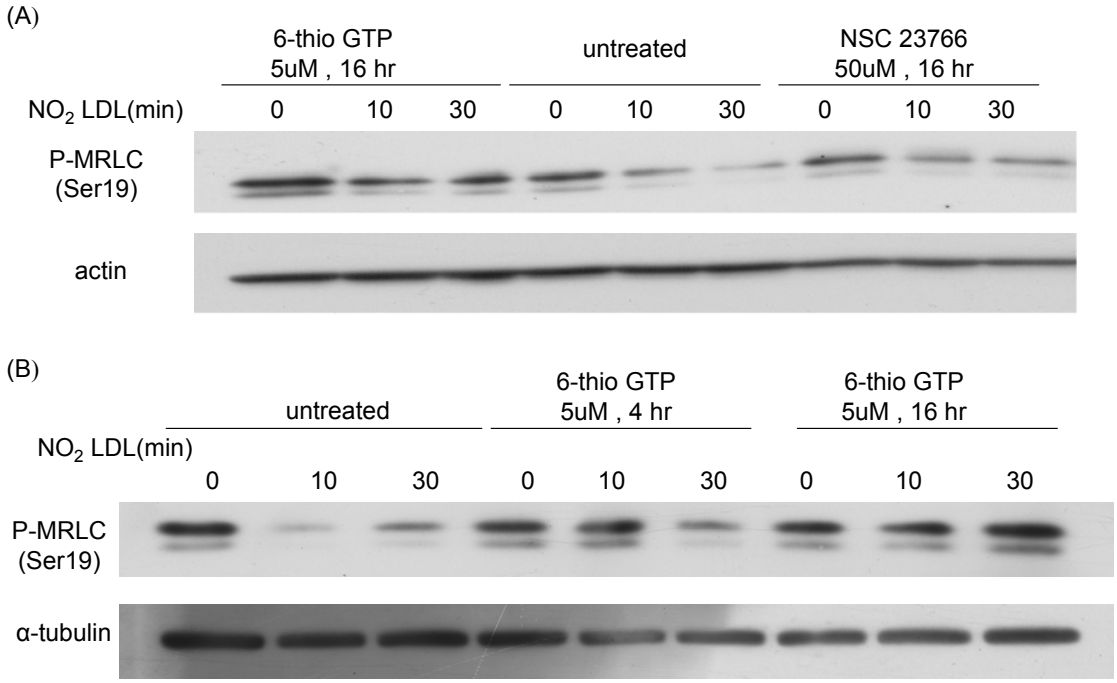


Figure 22. Six-thio GTP blocks NO_2LDL induced dephosphorylation of MRLC. (A) Macrophages were treated or untreated with 6-thio GTP and then treated with NO_2LDL. Phosphorylated MRLC (Ser-19) was measured using these cells. (B) Macrophages were incubated with 6-thio GTP for the indicated times, treated with NO_2LDL and lysed. The lysates were applied to a western blot for detection of phosphorylated MRLC (Ser-19).

Six-thio GTP blocks the effect of oxLDL on cell polarity and restores macrophage migration

Having shown that 6-thio GTP blocks the biochemical signaling effects of NO_2LDL on Rac activation and MRLC dephosphorylation, we next used live cell imaging to evaluate if 6-thio GTP concurrently blocks the effects of NO_2LDL on cell polarity. Macrophages pre-treated with 6-thio GTP showed spontaneous polarization and locomoting ability that was identical to untreated macrophages (Figure 23). NO_2LDL did not induce lamellipodial retraction and retraction fiber formation in 6-thio GTP treated macrophages (Figure 23 and Figure 24A). Dynamic movement of membrane and cell velocity were similarly not affected by NO_2LDL when macrophages were treated with 6-thio GTP (Figure 24B and C). The scratch wound migration assay also showed that migration of 6-thio GTP treated macrophages was not inhibited by NO_2LDL (Figure 25). Six-thio GTP treated macrophages thus maintained cell polarity and migrating ability despite the presence of NO_2LDL (Figure 23 and 25).

We conclude from all studies that NO_2LDL- induced cytoskeletal changes can be restored by the blockade of Vav and Rac interaction.

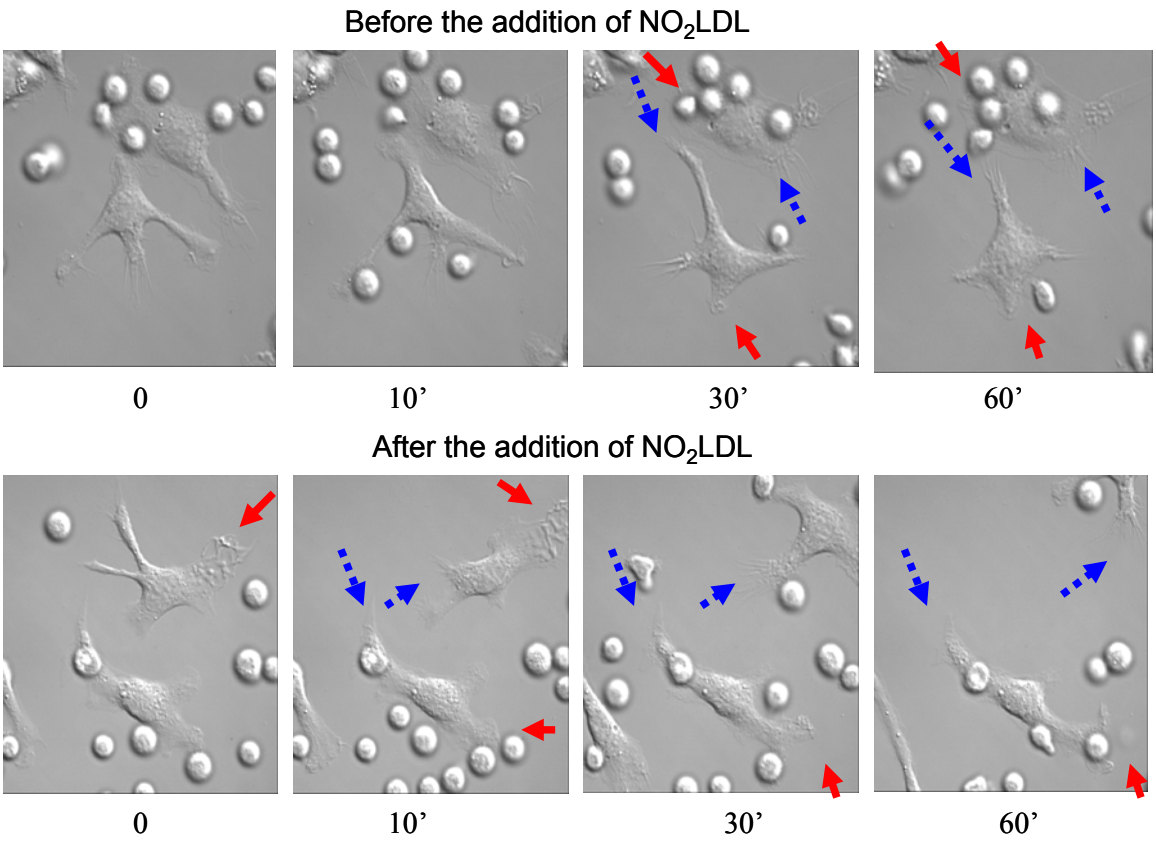


Figure 23. 6-thio GTP blocks the effect of NO_2LDL on cell polarity.

Wild type macrophages were incubated with 6-thio GTP and treated with NO_2LDL. Time lapse images were taken as described in Figure 1. Red solid arrows indicate the front end and blue dashed arrows indicate the rear end.

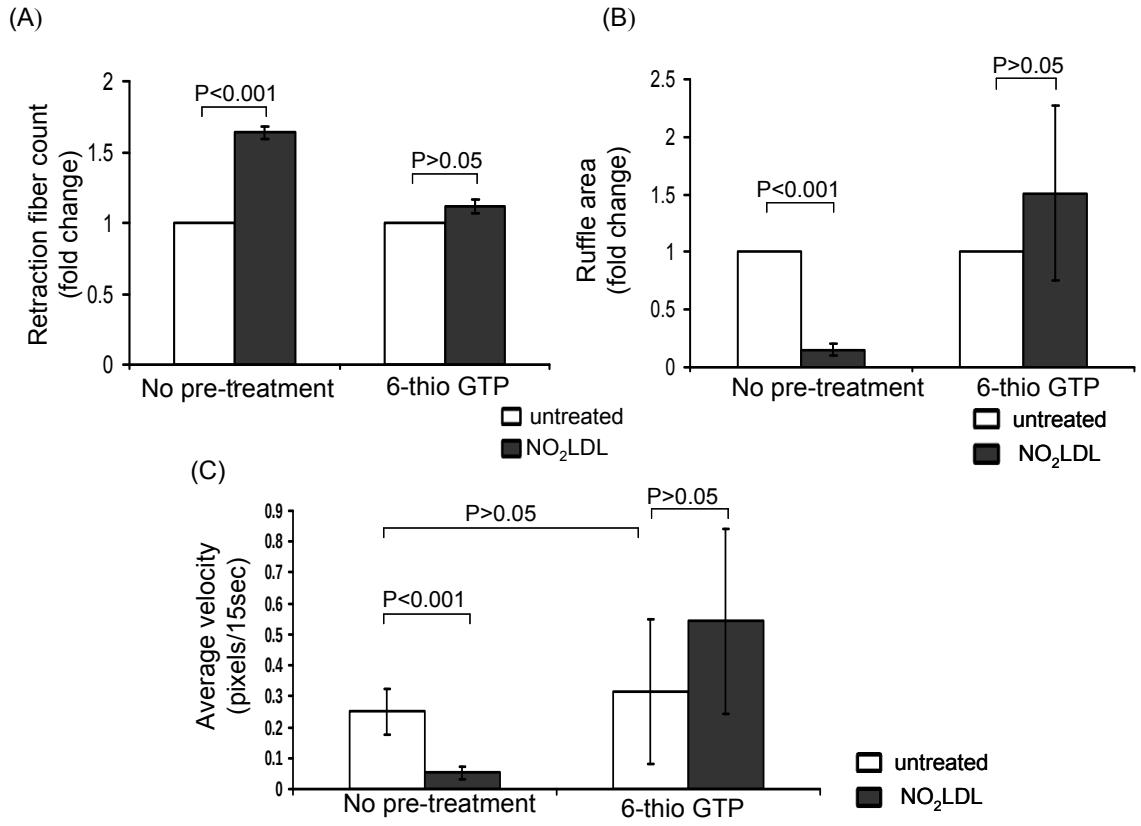


Figure 24. 6-thio GTP blocks the effects of NO_2LDL.

Live cell imaging with macrophages treated with or without 6-thio GTP and NO_2LDL was analyzed as described in Figure 2. (A)Retraction fibers of macrophages were counted before and after the addition of NO_2LDL. (B) Ruffle areas were counted before and after the addition of NO_2LDL. (C) Macrophage average velocity was caculated by measuring the start-to-end traveling distance before and after the addition of NO_2LDL.

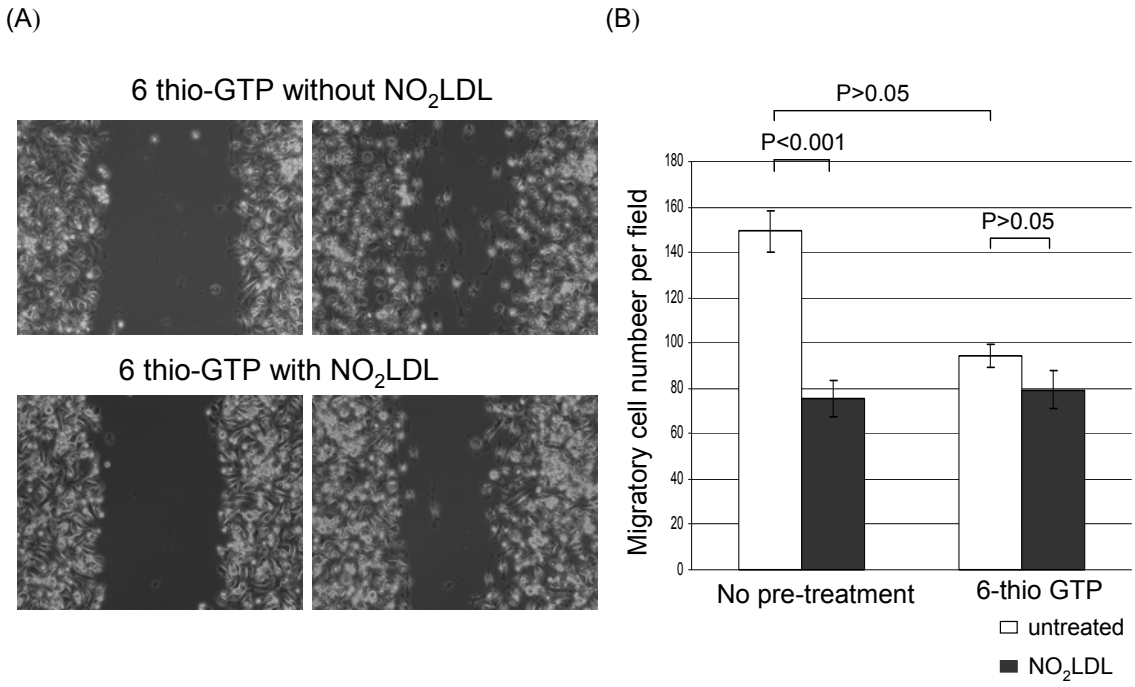


Figure 25. 6-thio GTP treated macrophage migration is not inhibited by NO_2LDL. (A) Confluent cell layers of macrophages were pre-treated with 6-thio GTP, scratched and loaded with NO_2LDL . (B) Quantitative analysis of the migration assay

SUMMARY

Live cell imaging analysis showed that oxLDL induced lamellipodial retraction of macrophages in CD36 and Vav dependent manners. Lamellipodial retraction along with retraction fiber formation in the cellular front induces loss of cell polarity, a pre-requisite for cell migration. The lamellipodial retraction by oxLDL also greatly limits the dynamic movement of macrophage membrane. These phenotypic changes are linked to intracellular signaling generated by interaction between CD36 and oxLDL leading to activation of Vav family GEFs. Vav subsequently activates the small molecular weight G-protein Rac. Activated Rac dephosphorylates MRLC and inhibits the activity of non-muscle myosin II, a cytoskeletal protein that conveys tensile force to lamellipodial protrusion. Inhibition of this pathway using 6-thio GTP, a Rac inhibitor that blocks binding of Vav to Rac, blocked the effect of oxLDL on macrophage migration.

DISCUSSION

In this thesis, we revealed mechanisms of macrophage trapping which are mediated by CD36. Our in vivo and in vitro migration assays showed that interaction between CD36 and oxLDL inhibits macrophage migration by modulating cytoskeletal signals. One mechanism is disturbed kinetics of FAK activation by direct phosphorylation by src-family kinases and also by inactivation of SHP-2, a major phosphatase for FAK. The inactivation of SHP-2 is due to oxidative modification of its active residue by ROS. In addition, oxLDL via CD36 also induces loss of cell polarity by inducing retraction of the front end lamelliipodia. This finding is related to CD36 mediated activation of the Vav – Rac pathway. Activation of this pathway induces failure of tensile force generation in lamellipodia by inhibiting myosin II, and thus results in retraction and loss of migratory ability. Blockade of any step in CD36 mediated pathways including CD36 deletion, generation of ROS, Vav deletion and/or inactivation of Rac prevented the effect of oxLDL. We conclude that CD36 may promote atherosclerosis by modulating macrophage cytoskeletal functions, thereby inhibiting emigration from lesions. Therefore, this study may suggest a novel strategy to reverse atherosclerosis (schematized in Figure).

We showed that oxLDL inhibits macrophage migration using both in vivo and in vitro migration assays. The in vivo macrophage peritoneal efflux model used in this study is an appropriate system to probe the effects of oxLDL on macrophage emigration from

inflammatory lesions for several reasons. First, factors influencing monocyte recruitment are eliminated; all studies begin with similar number of cells in the peritoneal cavity. Second, as with emigration of macrophages from atherosclerotic lesions, in this model the fate of the emigrating peritoneal cells is transit to regional lymph nodes or systemic circulation (146). Cao *et al.*, by combining this peritoneal migration model with cell tracker-green fluorescence protein (GFP) tagging marophages, showed that 30% of the injected GFP positive macrophages are found in regional lymph nodes and 25% in circulating blood (146). Thus, this trafficking model is similar to macrophage emigration from atherosclerotic plaque that use two routes including afferent lymphatic drainage and in a abluminal-luminal trafficking. However, migration studies such as with the transplant of atherosclerotic arteries in normocholesterolemic mice need to be performed to confirm the relevance of our observation. There have been several approaches to track macrophage movement in atherosclerotic arteries. Harb *et al.* used indium labeled macrophage injection to *Apoe* null mice treated with or without EP80317, a competitive ligand for CD36. They observed atherosclerotic lesions 48 hours after the injection with macrophages and found 43% less accumulation of macrophages in the atherosclerotic lesion of the mice treated with EP80317. This finding was related to decreased phosphorylation of protein tyrosin kinase 2 (Pyk2), a member of the FAK subfamily of protein tyrosine kinases, in macrophages from the EP80317 treated mice (177). This study did not showed the emigration kinetics of macrophages, but it supports in part, our hypothesis that macrophage trafficking and the role of CD36 in this process are an important regulator of atherosclerotic lesion progression. To monitor the trafficking of macrophages in atherosclerosis, fluorescent latex beads or nano-particles can be also used

as a tracker. Tacke *et al.* applied this technique to see different levels of accumulation of different monocyte subsets such as C-C motif chemokine receptor 2 (CCR2) or CCR5 positive or negative subsets in atherosclerotic lesions (178). Therefore, we may be able to apply these tracking techniques in continuing studies and provide further knowledge about the mechanism of macrophage trapping.

Our study showed that abrogation of CD36 only partially restored macrophage migration in the presence of oxLDL in the Boyden chamber migration assays while it completely restored migration in the scratch wound closure migration assay and in vivo model. This suggests macrophage migration through a porous membrane may be different from that on a 2 dimensional surface and inside tissues, and that the impact of oxLDL may be greater in the latter conditions.

Although CD36 has extremely short cytoplasmic domains, it has been shown to function as a signaling molecule in macrophages (95, 179-181), microglial cells (100), endothelial cells (101) and platelets (182-184). In these cells, CD36 ligands, such as oxLDL, TSP-1, bacterial cell wall components, and amyloid Aβ, have been shown to induce signaling cascades that involve recruitment and activation of the src-family tyrosine kinases fyn and/or lyn, with subsequent activation of specific MAP kinases including p38 and jnk2. In this study, we showed that oxLDL mediated CD36 signals led to enhanced actin polymerization associated with sustained phosphorylation of tyrosines 576/577 in the active site of FAK. Direct phosphorylation by src-family kinases likely contributes to FAK activation in this setting (139) as our experiments revealed that src-kinase inhibitors blocked FAK activation by oxLDL. Since previous reports revealed

that CD36 physically associates with integrins and may form a functional receptor complex (108,109), it is possible that integrins are also involved in FAK activation by oxLDL/CD36 interaction. We also showed that oxLDL interactions with CD36 led to inactivation of SHP-2, the major inactivating phosphatase for FAK. The mechanism of SHP-2 inactivation was shown to be due to oxidative modification of the catalytic site. Previous reports have shown that oxidative inactivation of protein tyrosine phosphatases, including protein tyrosine phosphatase-1B (PTP1B), SHP-2, phosphatase and tensin homologue (PTEN) and low molecular weight protein tyrosine phosphatase (LM-PTP), could be induced by intracellular ROS generated in response to specific receptor-mediated signaling pathways, such as those initiated by the epidermal growth factor receptor (EGFR), the platelet derived growth factor receptor (PDGFR), and the T cell receptor (147,185-188). Our studies showed that oxLDL/CD36 interactions in macrophages led to NADPH oxidase mediated generation of intracellular ROS, and that pharmacologic blockade of NADPH oxidase or treatment with anti-oxidants restored the dynamic activation of FAK and thus abrogated the anti-migratory effect of CD36. These findings suggest that CD36 ligands generate downstream signals via intracellularly generated ROS.

Loss of tyrosine phosphorylation of FAK is temporally associated with disruption of focal adhesions and acquisition of a motile phenotype (189) suggesting that agonists that induce rapid short-lived activation of FAK will promote migration (190), while agonists that result in sustained activation (such as oxLDL) may inhibit migration. Our data are consistent with previous reports which revealed that FAK hyper-phosphorylation in cells deficient in SHP-2 activity was associated with impaired migration (154,191).

By showing that the migration-inhibitory function of oxLDL followed from perturbation of reversible phosphorylation of FAK we confirmed the importance of highly coordinated dynamic cytoskeletal signaling in regulating cell migration. Our studies with FAK and ROS inhibitors also suggest that functional changes induced by oxLDL/CD36 interaction were directly related to the CD36-mediated signaling. The newly described effectors of this pathway, FAK and SHP-2 appear to exert their effect through physical association with CD36. This pathway involves NADPH oxidase mediated generation of ROS that in turn lead to oxidative inactivation of SHP-2. SHP-2 inactivation coupled with src family kinase activation results in sustained phosphorylation of FAK and increased actin polymerization. Thus the net effect of this pathologic CD36 signaling cascade is enhancement of cell spreading and inhibition of migration.

We have shown that disturbance of dynamic activation of FAK inhibits dynamic assembly and disassembly of cytoskeleton and suggested that as a mechanism of macrophage trapping. However, blockade of the pathway using inhibitors for ROS partially restored macrophage migration while blockade of CD36 completely restored macrophages migration. Thus, it appears that oxLDL/CD36 interaction modulates macrophage cytoskeletal functions through multiple pathways. Indeed we found another fundamental mechanism by which oxLDL/CD36 interaction inhibits macrophage migration. Our live cell imaging data revealed that oxLDL induced retraction of front-end lamellipodia of polarized macrophages. This process leaves retraction fibers behind as the membrane moves toward the cell body. This phenomenon was observed both in the resident murine peritoneal macrophages and thioglycollate-elicited peritoneal

macrophages. The retraction of lamellipodia induces loss of cell polarity and stops the locomotion of macrophages.

Although the mechanism of cell polarization has been intensively studied for decades, the role of relationships between all participating molecules remains incompletely understood. It has been found that small molecular weight G proteins including Rho, Rac and Cdc42 participate in this process (124,192). There is abundant evidence that active Rac initiates and maintains directional protrusion, responsible for the front end generation (125-127). In contrast, Rho is more active at the sides and the rear of the cell, antagonizing the function of Rac (124, 128, 193). However, our data suggest that this simplified view of cell polarity may in fact be more complex. We showed that activated Rac, induced by oxLDL, breaks the cellular asymmetry at the front by inducing lamellipodial retraction. This effect was derived from non-muscle myosin II inhibition by activated Rac. Previous studies showed that inhibition of Rho or Rho kinase, induced extended tail formation (128,194) and this may have been the result of a reduction in myosin- mediated actomyosin contractility of the cell. Thus, our results are similar to previous reports that showed that inhibition of myosin II enhances retraction process in the trailing edge. Interestingly, in our study, oxLDL brings this process to the front end of macrophages.

Recent studies have revealed that myosin IIa and IIb have distinct functions (195,196). The proposed effect of myosin in our study seems to be from myosin IIa, the major isoform of myosin II in macrophages. Even though myosin II has been suggested as a determinant of cell polarity (195,196), the mechanism was not clear. Our study, by using live cell imaging, shows the process of how inactivation of myosin II causes loss of

cell polarity. It is remarkable that retraction of pre-formed protrusions can be induced in polarized macrophages. In our study, myosin IIa appears to function in maintaining lamellipodial protrusion and conservation of cell polarity in macrophages. It also showed that cell spreading and protrusion of round macrophages were facilitated by oxLDL/CD36 interaction. Activated FAK and resultant actin polymerization is one mechanism for this finding. However, as shown in previous studies, it may also be caused by activated Rac. Thus, while myosin II appears to have a minimal effect on initial spreading of round macrophages, our data suggest a significant role in generating traction force to maintain lamellipodial structure and advance forward movement.

Activity of myosin II is regulated by coordinated activities of small molecular weight G proteins including Rho and Rac (167). Rho activates myosin II by inhibiting myosin phosphatase directly (131) or through Rho kinase (132,133) and increases phosphorylated myosin regulatory light chain. However, there have been controversial studies about the effect of Rac on myosin activity. Rac is known to activate p21-activated kinase (PAK) (134). There have been two opposing groups of studies regarding the effect of PAK on MRLC phosphorylation. Previous studies showed that PAK activated by Rac could phosphorylate MRLC (Ser-19) (197, 198). It was also reported that Rac could phosphorylate and activate MLCK through a kinase cascade that activates extracellular signal-regulated kinase (ERK) (199). Nevertheless, there have been contradictory reports that revealed PAK inhibited MLCK activity and decreased phosphorylation of MRLC (200). Our data suggest that oxLDL decreases the activity of non-muscle myosin II by Rac mediated dephosphorylation of MRLC. Most studies about the effect of Rac on MRLC phosphorylation used transfection of constitutively active or

dominant negative mutant Rac or PAK. Our study using oxLDL as an endogenous inhibitor of this pathway, gives an example of how this pathway derives functional outcome in the cytoskeleton. In comparison with other known myosin inhibitors, the effect of oxLDL was consistent with a MLCK inhibitor. Previous studies showed that MLCK regulates MRLC phosphorylation at the cellular periphery while ROCK functions at the center of cells (201,202). Our live cell imaging showed that oxLDL induced retraction is localized at the lamellipodial edges, also suggesting that oxLDL functions through inhibiting MLCK activity. However, direct measurement of MLCK activity in macrophages treated with or without oxLDL should be followed to confirm this.

We found that Vav, a guanine nucleotide exchange factor, mediates ox-LDL induced Rac activation. The Vav family proteins have activities as signaling adapters and GEFs for small molecular weight G proteins. Recent studies revealed that Vav is a downstream effector of CD36 signaling which is activated by src kinases (117,118). In the current study, we showed that oxLDL/CD36 interaction activates Rac by increasing the physical association between Vav and Rac. To block the effect of oxLDL, we treated macrophages with 6-thio GTP, which is a metabolite of an immunosuppressant, azathioprine and known to inhibit binding of Vav to Rac without affecting other GEFs (175). We found that blockade of Vav mediated Rac activation prevents the effect of oxLDL on cell polarity and MRLC dephosphorylation. Another Rac inhibitor, NSC-23766, which is known to block Tiam-1 and Trio but not Vav did not block the effect of oxLDL. This result confirms that oxLDL modulates the cytoskeleton through Vav.

Migration of *Vav* null macrophages was slower than WT macrophages, which was reported in other studies (160). A previous study revealed that Vav deficient macrophages have reduced spreading compared to WT and defects in adhesion-induced Rac and Rho activation. It also showed that Vav deficient macrophages have more directionality in random or chemotactic migration (203). In our study, we found that macrophages treated with oxLDL that activates Vav, showed reduced velocity in directional migration. It is the opposite characteristic to the *Vav* null macrophages, suggesting the role of oxLDL as an activator for Vav. Vav family proteins, Vav1 and Vav3 are known to have distinct functions and also responsible for redundancy in several functions including ROS generation, cell spreading and phagocytosis (119, 204, 205). Our study shows that Vav3 partially compensates functional deficiency of Vav1 in *Vav1* null macrophages. *Vav1* null macrophages showed delayed response to oxLDL and showed minimal reduction of dynamic membrane movement. Migration of Vav1 macrophages was also minimally inhibited by oxLDL and this minimal inhibition was abrogated by Vav3 deletion.

The cell migration process is composed of actin polymerization-driven lamellipodia extension, disruption of existing focal contacts, and formation of new focal contacts (112). Thus a regulated balance between cytoskeletal assembly and disassembly must be maintained to ensure cell movement. Cell polarization is also an important pre-requisite for migration, which means molecular dynamics are different in the front end and rear end (83). Complex process of cell migration can be thus easily impeded by modulation of various pathways. In our study, we revealed that oxLDL inhibits

macrophage migration by modulating two distinct aspects of migration mechanism. We found that oxLDL through CD36 modulates kinetics of cytoskeletal signals, disrupts the regulated balance between cytoskeletal assembly and disassembly and thus inhibits migration of macrophages. The second proposed mechanism of oxLDL-induced inhibition of macrophage migration is that signals generated by the interaction between CD36 and oxLDL induce loss of cell polarity by actuating lamellipodial retraction. These two pathways and functions modulated by CD36 seem to be distinct but may also be possibly linked, regarding the complex interplay between molecules in cytoskeletal signaling. ROS mediated interruption of dynamic activation of FAK is possibly linked with the pathway regulated by Vav-Rac pathway, since Rac is known to be an upstream regulator of NADPH oxidase (206). In addition, regarding the fact that myosin is an important regulator of focal adhesion dynamics, net effect of oxLDL on macrophage adhesion may be affected by both the sustained activation of FAK and inhibited myosin. Therefore, studies to reveal a possible link between these pathways should be followed to complete the map of cytoskeletal signaling cascade generated by oxLDL/CD36 interaction.

Our studies have defined a previously unknown macrophage signaling pathway triggered by oxLDL interaction with CD36. We also propose an example which shows disturbance of cytoskeletal signal kinetics results in inhibition of cell migration and a model demonstrating how the complex interplay among FAK, Vav, Rac and myosin II, the molecules that participate in normal cell migration, contributes to a pathologic process. In addition, these studies also provide additional mechanistic support for the

athero-protective effect of anti-oxidants (207, 208) and CD36 deficiency (67-71). As a whole, the thesis provide a mechanism by which oxLDL induces macrophage trapping in atherogenic inflammation and suggest novel ways to promote mobilization of lipid laden macrophages and induce regression of atherosclerosis.

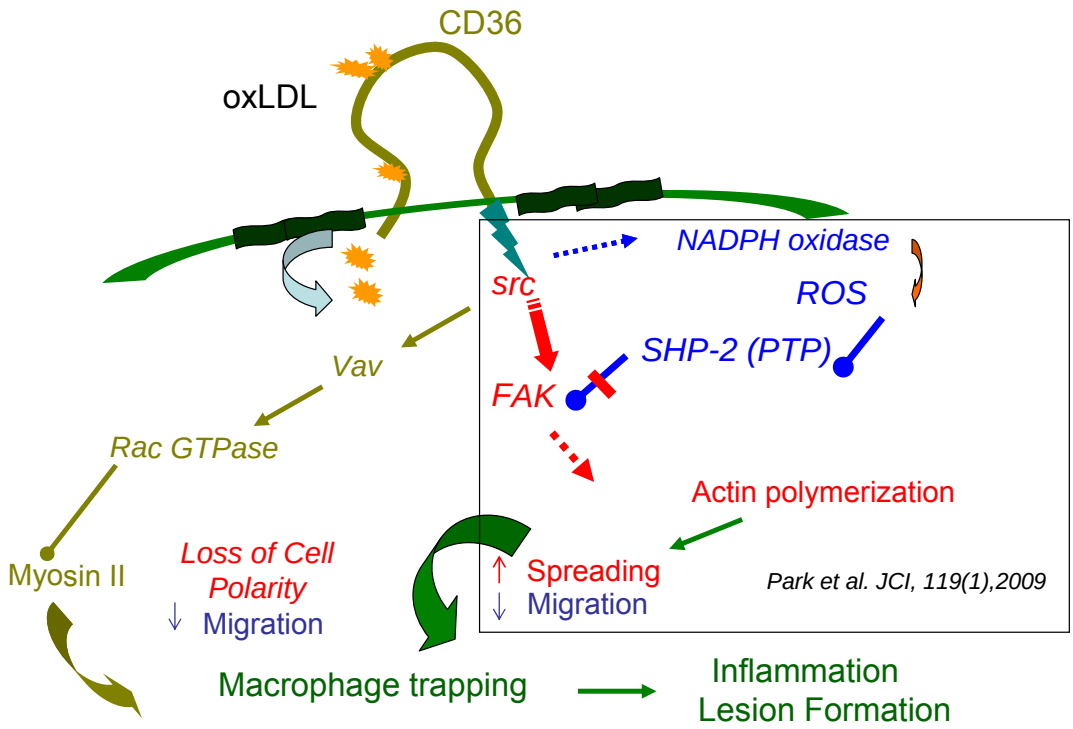


Figure. Model depicting CD36-dependent mechanism of macrophage trapping in the arterial intima.

OxLDL interacts with CD36 to induce a signaling cascade that leads to activation of specific src kinases (e.g. lyn) which in turn phosphorylate and activate FAK and lead to actin polymerization. OxLDL interactions with CD36 also lead to NADPH oxidase mediated generation of intracellular reactive oxygen species (ROS) which in turn induce oxidative inactivation of SHP-2 resulting in sustained FAK activation that perturbs cytoskeletal disassembly. The net effects are enhancement of cell spreading with concomitant inhibition of migration and therefore trapping of cells in the neointima. (The cascade in the boxed area, reported in the *J Clin Invest*, 119(1), 2009.)

Ox-LDL also induces loss of cell polarity and inhibits macrophage locomotion in a CD36 dependent manner. OxLDL/CD36 interaction activates Rac through Vav. Activated Rac then inhibits non-muscle myosin II by inducing dephosphorylation of MRLC. Inactivated myosin II cannot generate traction force of lamellipodia and therefore results in lamellipodial retraction. Lamellipodial retraction leads to a loss of cell polarity that is essential for macrophage migration.

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