2007

Role of CD36 in Platelet Function

Arunima Ghosh
Cleveland State University

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ARUNIMA GHOSH

M.B.B.S
Calcutta University
October, 1998

Submitted in partial fulfillment of requirements for the degree
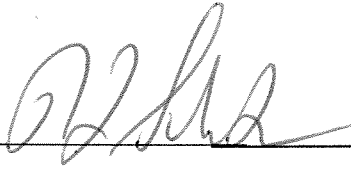
DOCTOR OF PHILOSOPHY IN REGULATORY BIOLOGY

at the

CLEVELAND STATE UNIVERSITY

December, 2007

This thesis/dissertation has been approved for

the Department of Biological, Geological,

and Environmental Sciences and for the

College of Graduate Studies of

Cleveland State University

by
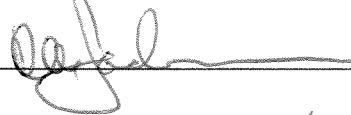
Dr. Roy Silverstein, Cell Biology, CCF_____ Date:_12 12.07_

Major Advisor
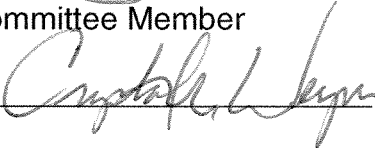
Dr. Don Jacobsen, Cell Biology, CCF_____ Date: 12-12-07

Advisory Committee Member

Dr. Crystal M Weyman, BGES, CSU_____ Date: 12/13/07

Advisory Committee Member

Dr. Christine Moravec, CCF_____ Date: 12-12-07

Advisory Committee Member

Dr. Jonathan Smith, Cell Biology, CCF_____ Date: 12.12.07

Internal Examiner

Dr. Keith McCrae, CASE _____ Date: 12/12/07

External Examiner

DEDICATION

To

Maa (Namita Ghosh)

Who has always believed in me

and

Reek, Raka, Gigi (my nephew and nieces)

Rai (my daughter)

Whom I believe in

ACKNOWLEDGMENTS

I would start off by thanking my mother whose inspiration has always been the driving force for anything I have achieved in life. I could never really express enough gratitude for all that she has done for me.

My other family members, my father (late) Dr. K.K.Ghosh, my sisters, Anasuya and Arundhati, my brother in laws Saumen and Bhaskar have also supported me in whatever I have done. In fact it was Arundhati and Saumen who motivated me into research and have stood by me like pillars in the fast few years. I would also like to thank my in laws, Nirodebaran and Chaitali Banerjee for all their encouragement and support over the years. Last but not the least, I thank my friend and husband Jashoman for always being there.

During my four years at the Cleveland State, I made a lot of new friends and acquaintances without whom life would have been different. I would like to thank them.

I would like to thank all my lab mates, who were just simply great. My buddy George, "PRP" Kan who is almost like a younger brother I never had, James whom I am never tired of mothering, "Papa" Phil and his "HRGP", Dave with his funny jokes and whole lot of positive energy, Wenxin, Jen and her chicken, "Mr JNK" Ohid, "kimchi" Young Mi, Lingyun, WeiLi, DiFernando, Sai and many more with whom I have interacted over the years.

I would like to thank my teachers at Cleveland State and CCF: Dr. Crystal Weyman, Dr. Chris Moravec, Dr. Jonathan Smith, Dr. Barsanjit Mazumdar, Dr.

Jahar Haque and Dr. Joseph Fontes. Their powerful teaching has left a deep impact in my mind.

Next, I would like to express gratitude towards my thesis committee, without whom this was not possible. Drs. Moravec, Weyman, Jacobsen, Smith, McCrae and Dean. Dr. Moravec was more than just a committee member, her friendly advice helped me survive a lot of "disasters."

I would like to thank our colleague Dr. McCrae, with whom we collaborated on the microparticle project, Dr. Qing Wang who helped us with genotyping and Drs. Barnard and Zhang, our biostatisticians whose discussions contributed to the understanding of this thesis.

Someone who I can never thank enough is Dr. Maria Febbraio. She is the heart of our lab. She is not only a kind-hearted person but a very good mentor. No matter how busy she was, she would always lend a hand and have good advice for me.

In the end, I would like to express my gratitude towards my advisor Dr. Roy Silverstein without whom I would not be here today. I don't think I have words enough to thank him. He is an excellent mentor; in spite of his very busy schedule, he would always find time for his students. His kind words of encouragement, supportive and caring nature would always make one feel that one is important and that has always been my strongest motivation to work and achieve in this lab. I am truly indebted to him.

ROLE OF CD36 IN PLATELET FUNCTION

ARUNIMA GHOSH

ABSTRACT

CD36 is a Class B scavenger receptor expressed on platelets, erythrocyte precursors, monocytes, microvascular endothelial cells, epithelial cells, adipocytes, and cardiac and skeletal myocytes. It recognizes multiple ligands including thrombospondins, oxidized phospholipids and apoptotic cells, and has been shown to play a role in phagocytosis, angiogenesis and atherosclerosis. The function of CD36 on platelets is incompletely characterized, but our group has recently identified CD36 on platelets as a signaling receptor which can modulate platelet function by binding to ligands such as oxidized LDL. Endothelial cell (EC) derived microparticles (MP) have been identified in the circulation of patients with diseases such as diabetes, anti-phospholipid syndrome and acute coronary syndrome in which patients are prone to arterial thrombosis, and thus platelet activation and aggregation play a pivotal role. Because EC MP express phosphatidyl serine (PS) on their surfaces, a potential CD36 ligand, we hypothesize that MP may bind to platelets via a PS-CD36 interaction and function to transmit an activating signal, thereby promoting a prothrombotic state.

To test this hypothesis, we first isolated EC-derived MP by stimulating human umbilical vein EC with TNFα and cyclohexamide according to a previously published protocol. MP were characterized and quantified by flow cytometry and shown to express CD105 and PS. Binding of MP to platelets was detected and quantified by flow cytometry and immunofluorescence microscopy. Platelet activation was assessed by aggregometry and flow cytometry. Washed human platelets (CD105 negative) were incubated with EC-derived MP at a ratio of 1:9 and analyzed by flow cytometry with a fluorescence tagged anti-CD105 dye) positive MP formed rosettes around (Calcein-Green Tagged) platelets. With both the flow cytometry and microscopy assays, platelet-MP association was inhibited by addition of anti-CD36 antibody or by using platelets from CD36 null donors. This inhibition by CD36 antibody was statistically significant (p=0.02). Furthermore, pretreatment of platelets with other CD36 ligands such as oxLDL inhibited MP-platelet association by more than 50%. Next we determined the functional effect of the MP-platelet association. We observed a significant increase in the rate and extent of platelet aggregation to low concentrations (2µM) of ADP and an increase in platelet secretion (measured as surface P-selectin expression) when platelets were incubated with EC-derived MP prior to addition of agonist. This effect was markedly diminished in platelets from CD36 null donors and also inhibited by pre-incubation with anti CD36 antibody. To test the MP-platelet interaction in vivo, carotid arteries were injured by $FeCl_3$ in wild type and CD36 knock out mice. The thrombosed arteries were sectioned and immunostained with an endothelial cell specific antibody to CD105 (red) and a

platelet specific antibody to CD61 (green). We reasoned that CD105 staining of thrombi would reflect incorporation of EC-derived MP into the thrombi. We observed significantly more CD105 staining within the thrombi from wild type mice compared to CD36 null mice,

We thus propose a model where CD36 ligands presented to platelets renders them "hyperactive" predisposing patients to pathological thrombosis. It is also possible that CD36 ligands such as EMPs, generated during an acute thrombotic event, could increase the thrombotic response in a CD36 dependent manner by signaling platelets in a positive feedback loop.

CD36 expression levels have been reported to vary significantly among normal human subjects. We thus hypothesized that levels of expression in an individual donor would correlate with platelet reactivity in response to CD36 ligands. We developed a quantitative flow cytometric technique to measure CD36 surface expression on platelets and studied 32 normal healthy volunteers. We found that expression levels were highly variable ranging from as low as less than 2000 molecules per platelet to more than 14000 molecules per platelet. We then replicated this study in a larger population (567) of subjects who came for screening at the Cleveland Clinic Cardiac Catheterization lab. To assess whether this variability of CD36 expression had a functional effect, donors were selected from high, medium and low expressing groups, and their platelet reactivity to oxLDL was analyzed by flow cytometric techniques. Results showed a very good correlation of platelet activation by oxidized LDL with levels of CD36 expression.

Having shown that CD36 expression levels varied among individuals but was consistent over time in any single donor we hypothesized that a component of the variance was heritable. We identified 10 tagged SNPs in the CD36 gene from the International HapMap consortium website. DNA from the 550 subjects described above were then genotyped for the tagged SNPs and 3 SNPs were found to be significantly associated with expression level (rs3211864, p value 0.023, OR=0.55; rs3211932, p value=0.02, OR=0.617 and rs1537593, p value=0.03, OR=1.067). For all of these SNPs the minor allele was associated with lower levels of CD36 expression. These data suggest that the variability of CD36 expression on platelets is at least in part genetically determined and together this phenotype-genotype can affect platelet function.

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LIST OF ABBREVIATIONS

ADP   Adenosine diphosphate
AGE   Advanced Glycation End products
ANOVA   Analysis of Variance
ApoE   Apolipoprotein E
BMIPP   β methyl-iodophenyl-pentadecanoic acid)
BSA   Bovine Serum Albumin
CD   Cluster differentiation
CLESH   CD36 LIMPII Emp sequence homology
CI   Confidence Interval
DAG   diacyl glycerol
DNA   deoxyribonucleic acid
EMP   endothelial derived microparticles
FDR   false discovery rate
FITC   Fluorescein isothiocyanate
GP   glycoprotein
5HT   5 hydroxy tryptamine
HODE   hydroxydecanoic acid
HUVEC   human unbillical vein endothelial cells
IL-4/10   interleukin 4/10
IP   inositol triphosphate
JAM   junctional adhesion molecule
LIMPII   Lysosomal integral membrane protein II
LPS   lipopolysaccharide
LCFA   long chain fatty acids
M-CSF   monocyte-cell stimulating factor
MAF   minor allele frequency
MFI   mean fluorescence intensity
MP   microparticle
NO   nitric oxide
oxLDL   oxidized LDL
OCS   open canalicular system
PAR1/4   proteinase activated receptor
PGI2/H2   prostaglandin I2/H2
PDGF   platelet derived growth factor
PS   phosphatidylserine
PECAM   Platelet-endothelial cell adhesion molecule
PCR   Polymerase chain reaction
PI3K   PI3 kinase
PRP   platelet rich plasma
PPP   platelet poor plasma
PE   Phycoerythrin

PVDF	Polyvinylidene difluoride
PBS	phosphate buffered saline
SNP	single nucleotide polymorphism
SHR	Spontaneous hypertensive rat
Sema4D	Semaphorin 4D
SRB1	Scavenger receptor B1
SDS –PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD	Standard deviation
SE	Standard error
TTP	Thrombotic Thrombocytopenic purpura
TG repeats	Thymidine-guanosine repeats
TGFβ	Transforming Growth Factor β
TSP1	Thrombospondin1
TxA2	Thromboxane A2
TPPFP	Tail probability of false positive
VLDL	very low density lipoprotein
VEGF	vascular endothelial growth factor
vWF	vonWillebrand factor

CHAPTER I

INTRODUCTION

<u>**PLATELETS**</u>

The mammalian hemostatic system is a defense mechanism whose function is to preserve the integrity of the high-pressure circulatory system. An intact vessel wall lined by endothelial cells maintains an inert surface that helps to traffic blood flow without interaction of the blood cell components (erythrocytes, leukocytes and platelets), or the soluble plasma proteins required for blood coagulation with the endothelium. Platelets are anucleated cells that originate from their precursors, the megakaryocytes and "circulate in the blood surveying the integrity of the vascular system" (Ruggeri, 2000). When the lining of the vessel wall is disrupted, platelets are engaged by the body's defense mechanism on the exposed subendothelium to form a clot and thereby seal the leak in the blood vessel. However platelets cannot distinguish between traumatic wounds in the vessel lining and other potentially pathogenic "lesions" on the vessel wall and can form thrombi at sites of ruptured atherosclerotic plaque. The

otherwise beneficial defense mechanism, now becomes an important cause of heart attacks, strokes and peripheral vascular diseases.

<u>Platelet adhesion:</u>

      The first step in hemostasis is platelet adhesion which is highlighted by the platelet-subendothelial matrix interaction. The constituents of the exposed sub endothelium including collagen, von Willebrand factor (vWF), fibronectin, laminin and thrombospondin mediate this interaction. Platelets bind to vWF via platelet membrane glycoproteins GP1b-IX-V complex (Fig 1). The history of this discovery dates back to 1948 when Bernard and Soulier reported two children of consanguineous parents with a mucocutaneous bleeding disorder, giant sized platelets and thrombocytopenia (Lopez et al., 1998). Later, based on the pioneering studies by Nurden and Caen that identified a defect in platelet GP1b as a cause of Bernard Soulier syndrome (Caen et al., 1976; Nurden and Caen, 1975), many others have provided important information about the interactions between platelet glycoprotein receptors GP1b and GPIIbIIIa ($\alpha_{2b}\beta_3$) with vWF. vWF-GP1b interaction can transmit signals that initiate $\alpha_{2b}\beta_3$ activation (De Marco et al., 1985; Kasirer-Friede et al., 2004). This receptor complex allows firm anchoring of platelet monolayer to the vessel wall. Also, it has been shown that in conditions of high shear as seen in arterioles or larger arteries, it is vWF that mediates stable surface adhesion (Savage et al., 1996). The effect of shear may explain both the very large size and the multimeric structure of vWF. Collagen

also contributes to platelet adhesion by binding to $\alpha_2\beta_1$ and GPVI (Clemetson and Clemetson, 2001).
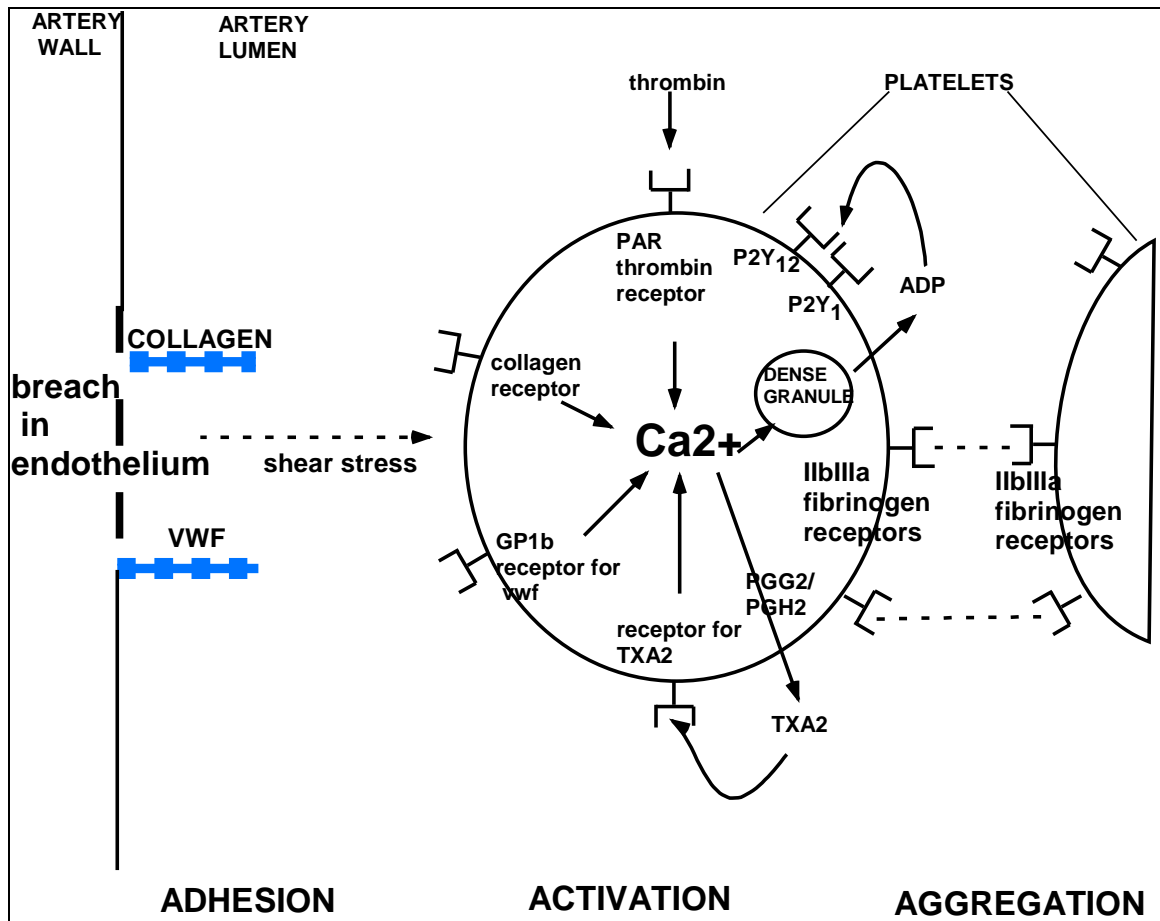
Fig 1. **Schematic diagram showing the classical platelet adhesion, activation and aggregation**. Adhesion involves interaction of the specific platelet receptors with collagen and vWF. Adhesion leads to platelet shape change and release of granules (ADP, TxA2) and activation. There are specific G-protein coupled receptors for ADP (P2Y12 and P2Y1) and TxA2. Finally, platelet aggregation is a result of the cross-linking of activated $\alpha_{2b}\beta_3$ molecules expressed on two different platelets by fibrinogen or vWF (Adapted from "Antiplatelet drugs" G.J. Hankey and J.W. Eikelboom, The Medical Journal of Australia)

Platelet activation: shape change:

The adhered platelets undergo **shape change** and activation. Shape change is typically described as a change from the discoid form to a spherical form. The platelet granules then retreat towards the center and finger like filopods are extended from the surface leading to spreading of the platelets (Hartwig, 2006). Reorganization of the actin cytoskeleton and intracellular signaling drive the alterations in the platelet morphology (Maxwell et al., 2006). Shape change is very rapid, beginning within seconds and completed within a minute after exposure to agonists.

Platelet activation: secretion:

The initial platelet "plug" now undergoes "extension" by recruitment of more platelets (Fig 3). Recruitment is mediated by the local accumulation of molecules that are secreted or released from activated platelets such as Adenosine diphosphate (ADP) and TxA2 (Thromboxane A2). A large number of granule contents (from alpha and dense granules) are released from activated platelets and these can significantly modify platelet activation. The dense granules of platelets contain important agonists like ADP and serotonin. "ADP is predicted to be the prominent amplifier of initial platelet aggregation" (Gachet, 2001). It interacts with specific extracellular membrane receptors to induce intracellular signaling. When platelets are activated, diacylglycerol (DAG) and inositol triphosphate (IP3) cause release of calcium from the dense tubular system. Intracellular calcium levels increase and this causes exocytosis of

platelet granules. There are two classes of receptors for ADP, $P2Y_1$ and $P2Y_{12}$ which belong to the seven transmembrane receptor family (Dorsam and Kunapuli, 2004; Fabre et al., 1999) and $P2X_1$ which belongs to the ADP/ATP driven calcium channel family of purinergic receptors (Sun et al., 1998). ADP interacts with $P2Y_1$ to mobilize calcium and shape change and "transient aggregation" (Fig 1) (Fabre, 1999 #320). $P2Y_{12}$ receptor is believed to potentiate platelet secretion and to be involved in "sustained irreversible platelet aggregation" (Dorsam and Kunapuli, 2004).

Serotonin is another bioactive substance released from the dense granules. It is a vasoconstrictor and by binding to G-protein coupled receptor 5HT2A, it exerts its action locally (Baumgartner and Born, 1968). Interestingly, Dale et al described a new concept in platelet biology called the "coat platelets." These are platelets activated by thrombin and collagen that bind serotonin to retain certain procoagulant protein such as alpha granule protein factor V, fibrinogen and thrombospondin on their surface (Dale et al., 2002).

Platelet alpha granules are the most abundant secretory granules in platelets (Sixma et al., 1989) and release a large number of adhesive proteins (vWF, thrombospondin, fibronectin); mitogenic factors (PDGF, TGFβ, VEGF); coagulation factors (factors V, VII, XI, XIII). Some glycoproteins such as Pselectin (CD62P) is localized to the surface of the alpha granule membrane and are trafficked during platelet secretion to be exposed on the surface of the activated platelet. In recent years a number of important functions have been attributed to P selectin and these include platelet binding to neutrophils and

monocytes (Singbartl et al., 2001) or leukocyte rolling on platelets (Furie et al., 2001). Patients with alpha storage pool disorder have diminished or absent alpha granules and a variable bleeding diathesis (Weiss et al., 1979).

Platelets also synthesize **thromboxane A2** (TxA2) which is produced from arachidonic acid in platelets. Arachidonate is derived from phospholipids by the enzyme phospholipase C, it is converted to TxA2 by the cycloxygenase pathway, of which prostaglandin H2 (PGH_2) is an important intermediate (Fig 1). Once formed, TxA2 can diffuse across the plasma membrane and like ADP can activate and recruit other platelets. TxA2 receptors on platelets are physically associated with G protein family (Fig 1). TxA2 and ADP together with the other agonists produced at the site of vascular damage (thrombin) modulate platelet adhesion and activation.

Platelet-coagulation protein interactions:

Another important step in platelet activation is localization of subsequent procoagulant events to the injury site. Activation of platelets leads to assembly of a prothrombinase complex on their surface (Fig 2). A very important mechanism for formation of the prothrombinase complex on the surface of the platelets is reversal of membrane asymmetry. Once there is rise in intracellular calcium levels in the ADP or thrombin activated platelet, an enzyme called the phospholipid scramblase (Williamson et al., 1992; Zwaal et al., 1993) causes rapid flip-flop of all the major internal phospholipids such as phosphatidylserine (PS) and phosphatidylethnolamine to exteriorize (Fig 2). This is vital for the

assembly of the prothrombinase complex on the platelet surface (Zwaal, 1978). The prothrombinase complex is capable of converting large amounts of prothrombin into thrombin to form a consolidate platelet-fibrin thrombus (Fig 2).
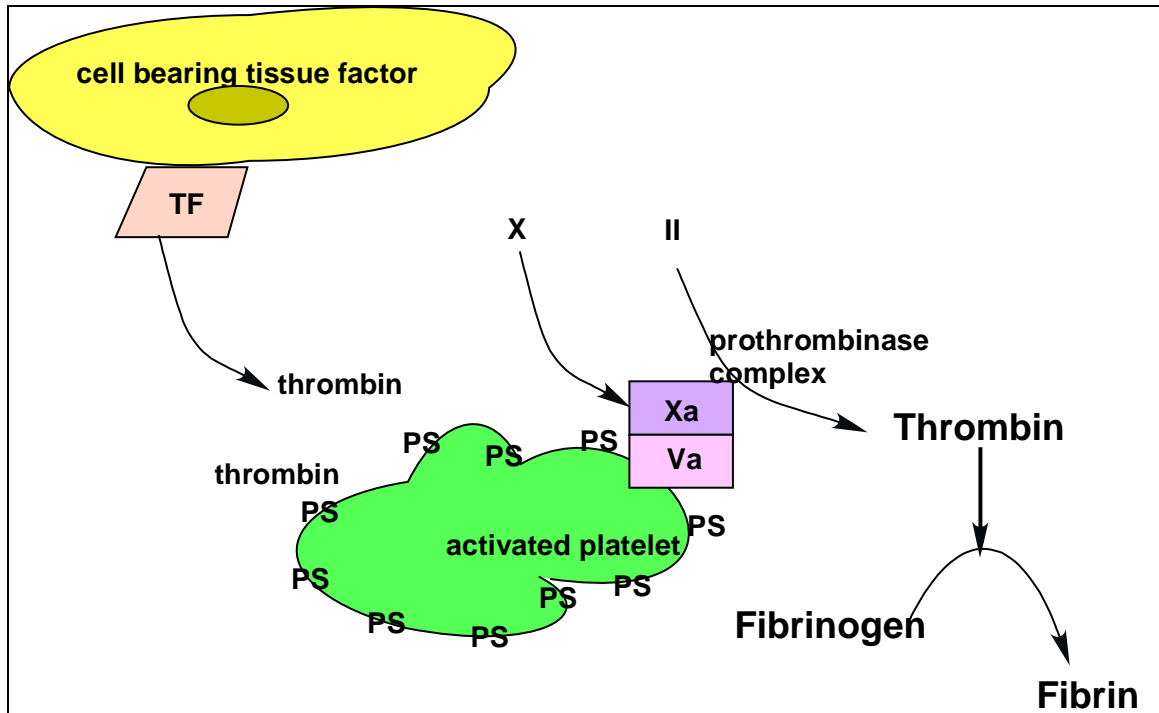
Fig 2. **Platelets and formation of prothrombinase complex**: The activated platelet expresses PS (phosphatidylserine) on their surface due to reversal of membrane asymmetry. This allows activation of several blood coagulation factors on the platelet surface. Activated Factor Xa binds to Factor V on the platelet surface to form the prothrombinase complex. This prothrombinase complex is capable of converting prothrombin into thrombin which in turn can clot fibrinogen. Tissue factor (TF) is expressed on the surrounding extravascular cells and TF can generate minute quantities of thrombin which can initiate the coagulation pathway by activating factor XI on platelets. (adapted and modified from Current concepts of hemostasis, Richard B. Weiskopf, Anaesthesiology 2004; 100:711-30).

Platelet aggregation:

The aggregation of platelets results in the accumulation of platelets into a hemostatic plug. Platelet aggregation is a result of the cross-linking of activated $\alpha_{2b}\beta_3$ molecules expressed on two different platelets by fibrinogen (Fig 1) (Marguerie et al., 1979; Peerschke et al., 1980) at low shear rates or vWF at high shear rates (Savage et al., 1996; Weiss et al., 1989). $\alpha_{2b}\beta_3$ is a platelet membrane protein of the integrin family and is composed of heterodimers of α_{2b} and β_3 expressed on resting platelets in an inactive conformation. Activation of integrin $\alpha_{2b}\beta_3$ is brought about by **inside-out signals** that modulate $\alpha_{2b}\beta_3$ affinity by regulating its interaction with intracellular proteins including talin. (Ulmer et al., 2003; Vinogradova et al., 2000; Vinogradova et al., 2004; Vinogradova et al., 2002). This transmits information to the cytoplasmic domain of $\alpha_{2b}\beta_3$ leading to a cascade of signaling events which includes the phosphorylation of tyrosine residues Y747 and Y759 in the β3 cytoplasmic domain. $\alpha_{2b}\beta_3$ undergoes a Ca^{2+} dependent conformational change so that it becomes "fit" to bind fibrinogen. Inside-out signaling is initiated either by agonists such as ADP or TxA2 through the G-protein coupled receptors (Brass et al., 1997; Eckly et al., 2001), or by the adhesive proteins vWF and collagen which interact with GP1b-IX-V or collagen receptors GPVI and $\alpha_2\beta_1$ respectively.

Once ligands bind to $\alpha_{2b}\beta_3$ receptor, it initiates a signaling event from the integrin into the cell and this is known as **outside in signaling**. This in turn can promote firm platelet adhesion, fibrin clot retraction and development of platelet procoagulant activity and microparticle generation (Kasirer-Friede et al., 2007).

Recent technical advances have provided a more detailed analysis of platelet aggregation and the factors involved in the process. Rate of blood flow is one of the factors that can influence platelet aggregation under different shear conditions. In conditions of low shear rate, such as veins, fibrinogen binding to $\alpha_{2b}\beta_3$ is the only appropriate mechanism for aggregation; whereas in conditions of high shear as seen in arterioles or larger arteries, vWF also participates in adhesion (Savage et al., 1996) or aggregation of platelets (Goto et al., 1998).

<u>Post–aggregation events in platelets:</u>

Formation of a platelet plug at the site of endothelial injury is essential for hemostasis and can also trigger pathological thrombosis. After the "initiation" of platelet plug formation by vWF and collagen (Fig 3), extension of the platelet plug is mediated by agonists released from the platelet granules such as ADP and TxA2 or thrombin. These agonists by signaling through their respective G-protein coupled receptors promote platelet aggregation by activating $\alpha_{2b}\beta_3$ on the platelet surface. These bridges can lead to sustained contacts between platelets and eventual "perpetuation" of the platelet plug (Fig 3). In this context, the phrase "contact dependent signaling" refers to "intercellular signaling events initiated by the binding of the proteins on the surface of one platelet to proteins on the surface of an adjacent platelet, either directly or indirectly" (Brass et al., 2006).
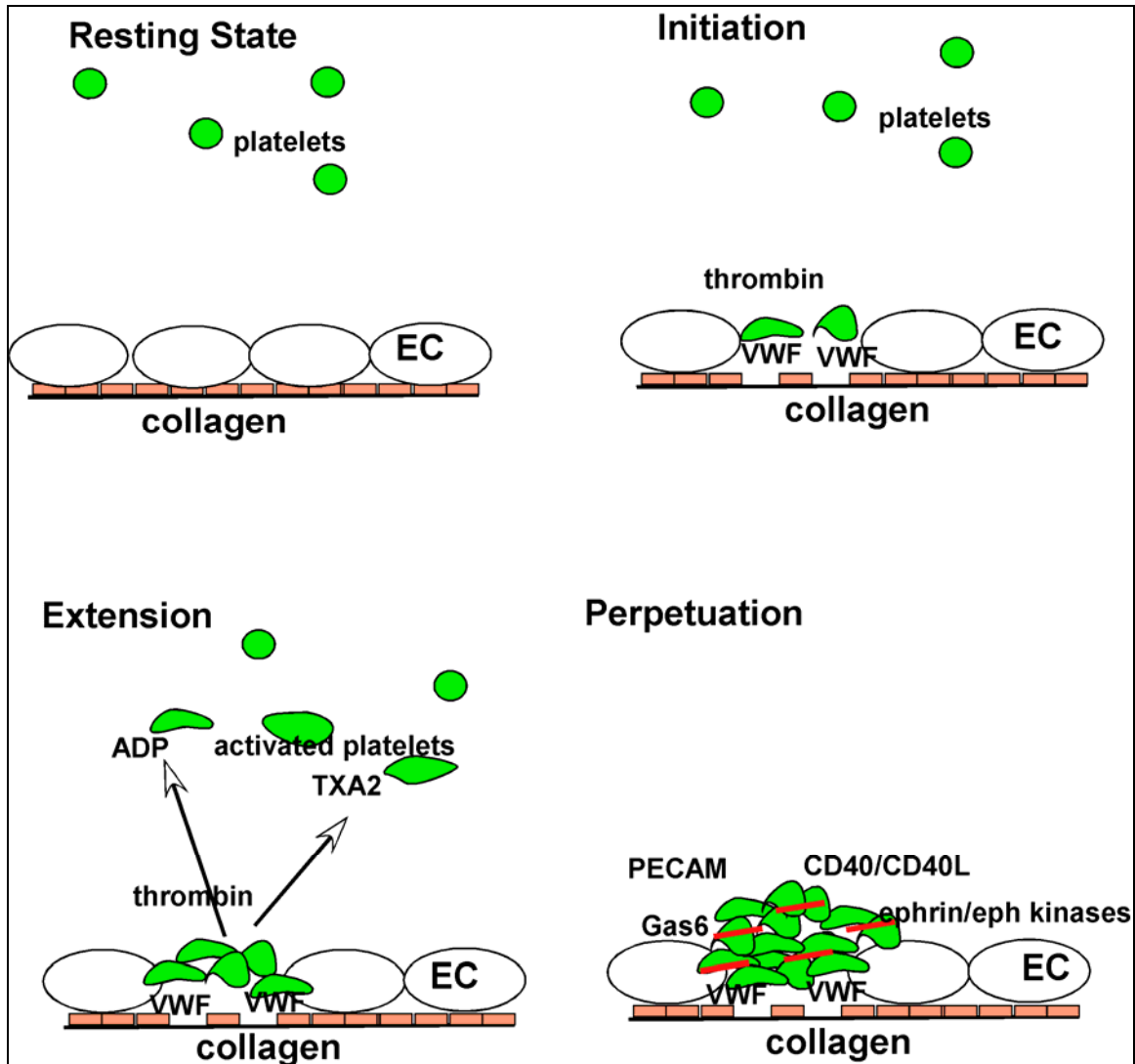
Fig 3. **Steps in platelet plug formation**. A) Resting state where PGI2 and NO form an inert endothelial surface. B) Platelet plug formation is initiated by exposure of collagen and vWF that causes platelets to adhere to the matrix. C) platelet plug is extended as more platelets are activated and recruited via the release of ADP, TxA2 and D) close contacts between platelets and contact dependent signaling help to stabilize the clot (Adapted from *Platelets*, Alan D. Michelson, Academic Press).

Among the assortment of proteins and signaling molecules involved in "contact dependent signaling" the most well studied molecule is $\alpha_{2b}\beta_3$. Adhesive proteins can bridge $\alpha_{2b}\beta_3$ of one platelet to another, and in the process, not only strengthens the adhesion but also initiates $\alpha_{2b}\beta_3$ mediated outside-in signaling. However, several other molecules play an important role in this "platelet synapse" (Fig 4) (Brass et al., 2006). These include receptor tyrosine kinases such as Ephrins and Eph kinases (Prevost et al., 2005; Prevost et al., 2004); Gas6 and its tyrosine kinase receptors, mer, tyro3 and axl (Angelillo-Scherrer et al., 2005; Angelillo-Scherrer et al., 2001); adhesive molecules such as PECAM (Falati et al., 2006); JAM-1 (Naik et al., 2001); CD40 and its corresponding ligand CD40L (Andre et al., 2002; Henn et al., 2001) and semaphorins (Zhu et al., 2007) (Fig 4).

An example of this contact dependent signaling is Eph kinase/ ephrin interactions. Eph kinases are receptor tyrosine kinases with an extracellular binding domain and an intracellular kinase domain. Eph kinases and their ligands, known as ephrins are known play a role in axon guidance and development of the vascular system (Adams and Klein, 2000). Human platelets express eph kinases Eph A4, Eph B1 and the Eph kinase ligand, ephrin B1 (Prevost et al., 2002). Studies have reported that in platelets Eph A4 and ephrin B1 interaction modulate platelet-platelet interaction: "forced clustering" of ephrin B1 or Eph kinases promotes platelet shape change, secretion, adhesion and aggregation , whereas inhibition of Eph/ephrine interactions leads to "premature disaggregation" at low agonist concentrations (Prevost et al., 2002). Other

studies by the same group have concluded that activation of Eph A4 and ephrin B1 on the platelet surface can support the growth of a stable platelet plug under flow (Prevost et al., 2003) and at the same time contribute to outside in signaling through $\alpha_{2b}\beta_3$ by assisting tyrosine phosphorylation of $\beta3$ chain and probably, a sustained activation of the integrin (Prevost et al., 2005). This provides considerable support to the growth and stability of the platelet plug (Prevost et al., 2005).

In addition to the Eph/ephrin interactions, other laboratories have shown that platelets express receptors for Gas6, a vitamin K–dependent protein that It can bind to receptor tyrosine kinases Axl, tyro3, and Mer (Stitt et al., 1995; Varnum et al., 1995). Gas6 is released form α granules upon initial stimulation of platelets by agonists. After release, Gas6 could significantly enhance the formation of stable platelet macro-aggregates by amplifying fibrinogen induced platelet spreading and irreversible platelet aggregation. Gas6 amplifies by signaling through its receptors. Activation of Gas6 receptor by Gas6 activates PI3kinase, granule secretion and stimulates tyrosine phosphorylation of $\beta3$ integrin. PI3K in turn, plays an important role in amplifying platelet aggregation (Kovacsovics et al., 1995; Trumel et al., 1999). Absence of Gas6 or any of its receptors can protect mice from thrombosis (Angelillo-Scherrer et al., 2001). Gas6 thus constitutes an important amplification system in pathological conditions.

Another important bioactive substance is CD40L which is a transmembrane protein that is shed following platelet stimulation (Henn et al.,

1998). Studies have shown that proteolytic cleavage of CD40L from platelets is stimulated by its binding to CD40 which is expressed "constitutively on platelets" (Henn et al., 2001). It was also reported that the soluble CD40L alone could potentiate integrin mediated platelet aggregation in high shear conditions (Andre et al., 2002). CD40L null mice exhibited delayed vessel occlusion and abnormally small platelet thrombi with risks of frequent embolization compared to the wild type mice (Andre et al., 2002; Crow et al., 2003).

In a continuing search for molecules that might contribute towards contact-dependent signaling events during thrombus formation, Zhu et al reported the role of Semaphorin D (sema4D), an integral membrane protein and the ligand of two receptors, CD72 and plexin B1. Platelets were shown to express both the ligand and its receptors, expression of both increased during platelet activation followed by gradual shedding of sema4D extracellular domain. Platelets from mice that lack sema4D show impaired collagen induced aggregation responses in vitro and after vascular injury, showed delayed arterial occlusion in vivo (Zhu et al., 2007). In the context of the contact dependent signaling, as platelet activation begins, sema4D that is on the platelet surface is able to interact directly with receptors on nearby platelets, promoting thrombus formation on exposed collagen.

Fig 4. "**Platelet synapse" formation and its importance**. The initiation of aggregation brings platelets into close contact with each other so that integrins and other cell molecules can interact. The space between platelets also provides a protective environment in which soluble agonists for G protein-coupled receptors (ADP, thrombin, TxA2) and receptor tyrosine kinases (Gas-6) and the proteolytically shed bioactive molecules of platelet surface proteins (CD40L and sema4D) can accumulate (Brass et al., 2006).

Once thrombus formation begins, contacts between platelets become increasingly stable and the spaces between the platelets become increasingly constrained. This provides a protective shelter (Fig 4) for not only the bioactive molecules shed from the platelet granules (such as ADP and Gas6) but also those released from the surface of activated platelets (such as CD40L or sema4D) which can locally accumulate at the site of platelet-platelet contact. All these contribute to promote the "stability of the hemostatic plug, support clot retraction and help to maintain the plug till wound healing is complete or at least under way" (Brass et al., 2004; Prevost et al., 2003).

CD36

CD36 is an 88kDa glycoprotein belonging to Scavenger Receptor family Type B (Tandon et al., 1989). It is a member of a family of related genes including SRBI (scavenger receptor B1) and LIMPII (lysosomal integral membrane protein). CD36 was identified several decades ago as a protease resistant platelet surface glycoprotein and named glycoprotein IV for its migration on SDS-PAGE gel (Clemetson et al., 1977). It was later found to be identical to the leukocyte differentiation antigen, CD36 a marker for blood monocytes and tissue macrophages. The CD36 protein sequence is highly conserved across species. There are several CD36-family homologs in Drosophila; *Croquemort*, epithelial membrane protein (*emp*), sensory neuron membrane protein (*SNMP*). *Croquemort* ("catcher of death") is expressed on macrophages and hemocytes, where it is essential for phagocytosis of apoptotic cells (Franc et al., 1996). *Emp*, which is expressed "in precursor cells for adult epidermal structures", has 32% homology to human CD36 and 34% homology to human LIMP-II (Hart and Wilcox, 1993). Recently, *SNMP* was described as another CD36 homologue in Drosophila and implicated in pheromone detection (Benton et al., 2007).

CD36 expression and its regulation:

CD36 is expressed on a diverse array of cells and tissues including platelets (Bolin, 1981), mature monocytes (Talle et al., 1983), endothelial cells of microvasculature (Greenwalt et al., 1990; Swerlick et al., 1992), erythroblasts

(van Schravendijk et al., 1992), epithelial cells of breast (Greenwalt and Mather, 1985), gut and kidney (Greenwalt and Mather, 1985; Greenwalt et al., 1995; Susztak et al., 2005) and skeletal muscles (Greenwalt and Mather, 1985). It is also expressed on phagocytic cells including dendritic cells (Albert et al., 1998), microglia (Husemann et al., 2002), monocytes and macrophages (Endemann et al., 1993) and retinal pigment epithelium (Ryeom et al., 1996). CD36 expression in monocytes has been shown to be highly regulated. It can be upregulated by adhesion (Huh et al., 1995), M-CSF and IL4 (Yesner et al., 1996) (Huh et al., 1996), native and oxidized LDL (Feng et al., 2000; Huh et al., 1996), glucose (Griffin et al., 2001) and endothelin1 (Kwok et al., 2007). CD36 is downregulated by cholesterol efflux (Han et al., 1999), TGF-β1 (Han et al., 2000), lipopolysaccharide (LPS) (Yesner et al., 1996), IL10 (Rubic and Lorenz, 2006), alpha tocopherol (Devaraj et al., 2001; Venugopal et al., 2004). Important observations by Tontonoz suggested that PPARγ activation in a macrophage cell line could increase CD36 expression (Tontonoz et al., 1998). These authors have suggested a mechanism of CD36 induction by PPARγ. In the presence of oxLDL, CD36 expression on a macrophage leads to intracellular accumulation of oxidized ligands (9- and 13-hydroxyoctadecadienoic acid [HODE], prostaglandin J2 [PGJ2]; which in turn activate PPARγ and this cycle continues in a "feed-forward loop" (Febbraio et al., 2001) ultimately leading to the conversion of macrophage into a foam cell.

CD36: Structure:

In human, mice and rats CD36 consists of 472 amino acids with a predicted molecular weight of about 53 kDa. However, CD36 is extensively glycosylated and its apparent Molecular weight on SDS PAGE gel is about 88kDa (Greenwalt et al., 1990; Oquendo et al., 1989). It has 10 potential N glycosylation sites (Fig 5) which are processed differently in different cell types. This modification protects the protein from being degraded by cell specific proteases or during inflammatory processes (Febbraio et al., 2001). Phosphorylation is another post-translational modification reported in CD36. Asch et al showed by site-directed mutagenesis that CD36 was phosphorylated in the extracellular loop at position Thr92 (Asch et al., 1993).
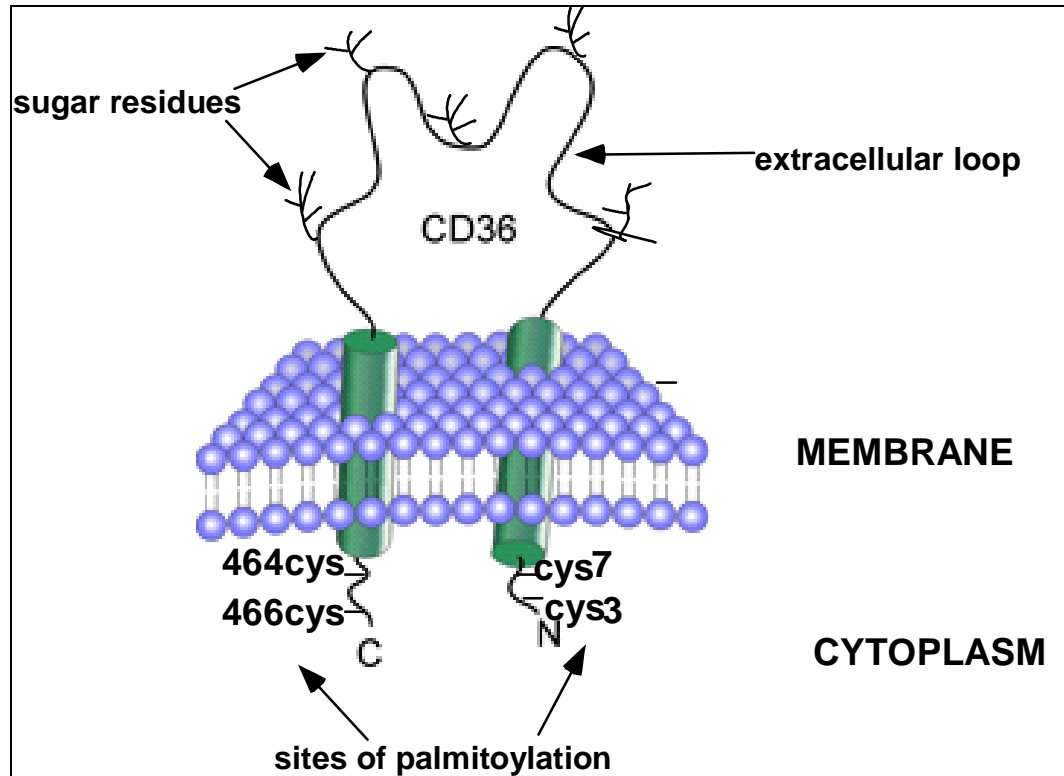
Fig 5. **Cartoon showing CD36 structure.** CD36 consists of a large, heavily glycosylated extracellular loop, two trans-membrane domains and two short (α helical) cytoplasmic tails. The cysteine residues at positions 3, 7, 464 and 466 at the N and C termini are palmitoylated and help anchoring the protein to the membrane. Phosphorylation occurs extracellularly at Thr92 residue.

CD36 consists of a hairpin like structure with a large glycosylated extracellular loop, two α helical transmembrane domains and two short cytoplasmic tails (Fig 5)(extending residues 1-6 and 167-172 respectively) (Oquendo et al., 1989; Vega et al., 1991). Very little is known about the secondary structure of the extracellular loop. The central hydrophilic domain, rich in N glycosylation sites and in monocloclal antibody epitopes (Daviet et al., 1995a; Daviet et al., 1995b) lies extracellularly but its exact topology is unknown.

However, six cysteines residues clustered around the C terminal half of this loop are proposed to be essential for the maturation and transport of the protein (Gruarin et al., 2000). A number of studies have contributed significantly towards our understanding of the CD36 topology. Tao et al reported that CD36 is palmitoylated at the membrane proximal cysteine residues (positions 3, 7, 464 and 466) in the N and C terminal cytoplasmic tails (Fig 5) suggesting that both N and C termini are cytoplasmic (Tao et al., 1996). In the same study a C-terminal truncation mutant of CD36 was made which deleted the C-terminal transmembrane domain. This mutant was found to be membrane-bound when expressed in human embryonic kidney 293 cells, indicating that the "N-terminal hydrophobic domain serves as a transmembrane anchor, and thus supporting a CD36 topology with two transmembrane domains" (Fig 5) (Tao et al., 1996).

Another study by Gruarin et al generated a panel of mutants lacking either one or both hydrophobic regions and analyzed their folding and transport in COS-7 cells. The N and the C-terminal hydrophobic regions were both sufficient to anchor CD36 in the membrane. These results indicated that CD36 adopts a "ditopic configuration" (Gruarin et al., 2000) as shown in Fig 5.

Binding sites for some of the different ligands have been mapped on CD36 molecule. Crombie et al have proposed a binding site for TSP-1 termed the CLESH domain (CD36 LIMP-II Emp sequence homology) (Crombie and Silverstein, 1998) on CD36 in the region of amino acids 93–120. High affinity binding sites for oxidized LDL (oxLDL) have been mapped on amino acids 28-93

(Pearce et al., 1998) whereas another binding site on amino acids 155-183 has also been reported (Puente Navazo et al., 1996).

CD36: Functions:

CD36 is a multi-functional molecule. It has independent binding sites for different classes of ligands such as modified phospholipids, thrombospondins, and free fatty acids. This enables CD36 responsible for several different cellular processes depending on the nature of the ligand and the type and location of the cell on which it is expressed. On phagocytes CD36 functions as a scavenger receptor helping in recognition and internalization of apoptotic cells (Albert et al., 1998; Fadok et al., 1998b; Greenberg et al., 2006; Ren et al., 1995), Falciparum malaria infected erythrocytes (Aitman et al., 2000; McGilvray et al., 2000; Omi et al., 2003; Pain et al., 2001b; Patel et al., 2004; Smith et al., 2003), photoreceptor outer segments (Ryeom et al., 1996), Staphylococcus aureus (Stuart et al., 2005), oxidized lipoproteins (Endemann et al., 1993; Febbraio et al., 2000; Kunjathoor et al., 2002; Rahaman et al., 2006) and non-enzymatically glycated end products (AGE) (Kuniyasu et al., 2003; Ohgami et al., 2001; Ohgami et al., 2002).

On adipocytes CD36 was identified as a long chain fatty acid transporter (Abumrad et al., 1993). It was later found that the C terminus of CD36 is required for its ability to enhance long chain fatty acid uptake. (Abumrad et al., 1993). Adipocytes in CD36 null mice failed to accumulate radiolabeled oleate or palmitate (Febbraio et al., 1999).

CD36 recognition, binding and uptake of oxidized LDL have been shown to be critical in cholesterol accumulation and foam cell formation. Cells expressing CD36 bound and took up oxLDL (Endemann et al., 1993) whereas CD36-deficient macrophages were resistant to foam cell formation (Febbraio et al., 2000). The expression of CD36 also could be upregulated by its ligand oxLDL (Han et al., 1997). A specific region of CD36 in the N-terminal region is said to contribute to this oxLDL recognition by CD36 (Pearce et al., 1998). In successive years Podrez et al, in collaboration with our group, have reported that LDL modified by the myeloperoxidase (MPO) system is highly specific for CD36 binding (Podrez et al., 1999). The MPO system is comprised of MPO generated reactive nitrogen species. This MPO-modified LDL could form foam cells which could be significantly inhibited by mAb against CD36 (Podrez et al., 2000). Our group also identified a highly specific oxidized phosphatidylcholine (oxPC) possessing a sn-2 acyl group (oxPC$_{CD36}$) that acts as a high affinity ligand on oxLDL for recognition by CD36 (Podrez et al., 2002). Other studies by our group have been instrumental in showing that CD36 is the major receptor on macrophages for binding and internalization of oxLDL and that although ApoE mice were pro-atherogenic, ApoE-CD36 double knock out mice were protected from developing atherosclerotic lesions by about 70-80% (Febbraio et al., 2000).

CD36 also has a very important function in endothelial cell biology. On microvascular endothelial cells, CD36 is a receptor for thrombospondin 1(Asch et al., 1987) and 2 (TSP-1 and 2) and mediates their anti-angiogenic activity (Dawson et al., 1997; Simantov et al., 2005). TSP1 inhibits angiogenesis by

inducing apoptosis in activated endothelial cells and this requires activation of CD36, p59fyn, caspase like proteases and p38 MAPK (mitogen–activated protein kinases) (Jimenez et al., 2000). Elucidation of this signaling pathway would help plan interventional strategies to inhibit tumor angiogenesis and hence tumor growth.

CD36 is also expressed on skeletal and cardiac muscles where it facilitates uptake of fatty acids. In CD36 null mice, injection of a radionuclide labeled long chain fatty acid (LCFA) analog, such as BMIPP (β methyl-iodophenyl-pentadecanoic-acid) showed that there was a significant decrease in fatty acid uptake in skeletal and cardiac muscles compared to wild type (Coburn et al., 2000). There have been reports describing an increased prevalence of CD36 deficiency in hereditary hypertrophic cardiomyopathy (Tanaka et al., 1997b) suggesting an important role of CD36 in fatty acid metabolism in the heart.

CD36 also functions as an adhesion molecule. Oquendo et al identified CD36 as the receptor that helps in cytoadherence of Plasmodium Falciparum parasitized erythrocytes (Oquendo et al., 1989). Pain et al reported that CD36 on platelet mediates clumping of P falciparum infested erythrocytes is strongly associated with severe malaria (Pain et al., 2001a). In contrast, CD36 on monocytes or macrophages can help phagocytosis of falciparum infested erythrocytes (McGilvray et al., 2000). Thus the location of CD36 receptor can regulate the severity of malarial disease.

Several studies have suggested an important role of CD36 in phagocytic clearance of apoptotic and senescent cells. In vitro, expression of CD36 on fibroblasts and melanoma cells (Ren et al., 1995) confers phagocytic activity by recognition of apoptotic cells. In vivo, CD36 mediated phagocytosis of apoptotic cells have observed in photoreceptor outer segments of retinal pigment epithelium (RPE) (Ryeom et al., 1996) or in dendritic cells (Albert et al., 1998). Earlier studies have reported interactions between phosphatidylserine (PS) (Fadok et al., 1998a; Pittoni and Valesini, 2002; Ryeom et al., 1996) on the outer surface of the apoptotic cells that acts as a recognition ligand for CD36. Recently we showed that similar to the sn-2 acyl group on oxPC$_{CD36}$ that are highly specific ligands for CD36, the sn-2 acyl groups on oxPS could be a highly specific ligand on apoptotic cells for recognition by CD36 (Greenberg et al., 2006).

CD36 signaling has been very well studied in macrophages where CD36 signals have been known to regulate reactive oxygen species (ROS) formation (Maxeiner et al., 1998). CD36 probably localizes in lipid rafts (Ehehalt et al., 2006; Pohl et al., 2005; Ring et al., 2006) with other signaling molecules such as CD9 (Miao et al., 2001) or integrin (Thorne et al., 2000). Jiminez et al showed that CD36 dependent fyn phosphorylation together with activation of p38 MAPK was required for anti-angiogenic activity of TSP-1 on capillary endothelial cells (Jimenez et al., 2000). Moore et al reported that β amyloid could induce association of CD36 with lyn and activate a signaling cascade involving the Src kinase fyn, p44/42 MAPK which in turn induced a macrophage inflammatory response. Inhibition of lyn kinase inhibited the recruitment of microglial cells to β

amyloid (Moore et al., 2002). More recently work by Rahaman et al in our lab illustrated the CD36 signaling in monocytes and macrophages by showing that CD36-lyn was engaged in a complex with MEKK2 and that uptake of pro-atherogenic oxLDL was associated with Jnk activation (Rahaman et al., 2006).

CD36 gene:

The CD36 gene extends 32kb on the q11.2 band of chromosome 7 (Armesilla and Vega, 1994). It consists of 15 exons (11 exons in the extracellular loop) of which 12 are coding and 3 non-coding exons. The regular translation initiation codon is located at position +290 (exon 3). There is also an ATG codon within the first exon at position +62 (Armesilla et al., 1996). This ATG codon might play a role in transcriptional control by acting as a binding site for transcriptional factors PEPB2/CBF (Armesilla et al., 1996). Sato et al identified three independent promoters for CD36 gene (Sato et al., 2002; Sato et al., 2007); CD36 has a wide range of functions in the cell types where it is expressed and the presence of multiple promoters was partly "to achieve tissue-specific transcriptional regulation" (Sato et al., 2007). CD36 gene has been most extensively studied in Asian populations (Japan, Korea, Indonesia, Thailand and China) where 3-8% of individuals are platelet CD36 deficient (Lin et al., 1993; Santoso et al., 1993; Seo et al., 1998; Urwijitaroon et al., 1995). This deficiency was initially observed during the investigation of a Japanese patient who developed transfusion-refractory thrombocytopenia due to a platelet specific

antibody and led to discovery that 3-11% of Japanese do not express CD36 on their platelets (Tomiyama et al., 1990; Yanai et al., 2000). The antibody epitope on CD36 is called Nak[a] and the individuals lacking GPIV are called Nak[a] minus.

CD36 deficiency is divided in to two subgroups. In type I deficiency, neither platelets nor monocytes express CD36 while in type II, only platelets fail to express CD36 (Take et al., 1993; Yamamoto et al., 1990). About 10% of the Nak[a] minus group are truly null (type I deficiency) where the expression is absent in platelets, monocytes, endothelium and muscle.

Five mutations have been reported (table 1) to be associated with Type I CD36 deficiency C478T, 539delAC, 1159insA, 839-841del-->insAAAAC and 1438-1449del accompanied with or without skipping of exon 9 (nt 959-1028) in Japanese patients (Kashiwagi et al., 1993a; Kashiwagi et al., 1992; Kashiwagi et al., 1993b). The most common, C478T with an allelic frequency of 80-90% in CD36 null, produces an amino acid change from proline to serine at position 90 and a misfolded protein that appears to be targeted for intracellular degradation (Kashiwagi et al., 1993b). The nt539delAC and nt115insA mutations cause a frameshift, generating a new translation stop codon and reduce CD36 transcripts in platelets and monocytes to a great extent. The other polymorphisms create premature stop codons and proteins that are not surface expressed which are summarized in the table 1 below.

Table 1: **Common polymorphisms accounting for CD36 deficiency**

mutation	population	effect	reference
C478T	Japanese	proline90 to serine	(Kashiwagi, 1993 #66)
539-540delA	Japanese	frameshift and appearance of a premature stop codon	(Kashiwagi, 1994 #378)
1159insA	Japanese	frameshift and the appearance of a premature stop codon	(Kashiwagi, 1996 #376)
839-841del-->insAAAAC	Japanese	frameshift and appearance of a premature stop codon	(Kashiwagi, 2001 #377)
1438-1449del	Japanese	inframe 4-amino-acid deletion	(Kashiwagi, 2001 #377)
1438-1449del accompanied with skipping of exon 9 (nt 959-1028).	Japanese	frameshift and the appearance of a premature stop codon.	(Kashiwagi, 2001 #377)

Table 1.**Common polymorphisms in CD36 deficient subjects**. Table showing the 5 polymorphisms, which account for more than 90% of CD36 deficiency in Japan. These polymorphisms cause reduced transcription of CD36 or total loss of expression due to premature stop codons or proteins which cannot be processed and presented on the platelet surface.

The genetic mechanism for type II CD36 deficiency is unclear. About half of the individuals tested are heterozygous for one of the five common mutations, but many heterozygous do not have any phenotypes and this probably suggests that other mutations are involved. The Nak[a] minus phenotype is also highly prevalent in African and African-American populations with frequencies in the range of 2.4-7.8% (Curtis and Aster, 1996). Genotyping studies indicate either homozygous or heterozygous combinations of different mutations other than those reported in Asia, with a single base substitution T1264G in exon 10 being the most common (>90%). This encodes a premature stop codon and CD36 is not expressed on the cell surface.

The Nak[a] minus phenotype is however very uncommon in the Caucasian population (0-0.3%) (Yamamoto et al., 1990). This suggests "that selection pressures existed to maintain or eliminate CD36 null mutation within certain populations and these may include interaction with pathogens" (Stuart et al., 2005). One such interaction may be CD36 mediated recognition of P. falciparum in malaria. The T1264G polymorphism is reportedly associated with protection from severe malaria in Kenyans (Pain et al., 2001b), although there is a contradictory report suggesting the susceptibility of T1264G to severe malaria (Aitman et al., 2000). A study from Thailand showed that in3(TG)$_{12}$ (12 TG repeats of intron 3) was significantly associated with the reduction in risk of cerebral malaria (Omi et al., 2003).

CD36 is a highly polymorphic gene. In addition to the null mutations mentioned above, there are multiple other polymorphisms and

insertions/deletions described, including some in the promoter region that involve putative transcription factor binding site or in the 5 and 3' UTR regions. Mutations specially in the 5' and 3' UTR regions are of potential significance since translational efficiency of CD36 mRNA and CD36 expression levels have been shown to be regulated by the 5′ UTR (Griffin et al., 2001). In Asia, 9 polymorphisms with allelic frequencies of >15% are known; while in West Africa 40 common SNPs have been identified. In European population, at least 50 SNPs have been identified, including 21 with minor allele frequency of >5%, 11 of which are in putative promoter region or regulatory UTR regions. More recently, completion of the Hap Map project gave us access to a huge number of CD36 SNPs in 4 different populations, Japanese, Han from China, Yoruba from Nigeria and people with European ancestry from Utah, USA (2003). A recent study of 585 residents of eastern Italy using 21 common polymorphisms reported significant linkage disequilibrium across the entire locus with 2 blocks of preferential LD, Common haplotypes accounted for >80% of the haplotypes in each block allowing investigators to use 5 polymorphic markers as tag SNPs (33137A>G, 31118G>A, 25444G>A, 27645 del and 30294G>C) to classify the haplotype variability in the population (Ma et al., 2004).

Although the functional impact of CD36 deficiency has been well studied in different mice and rodent models, the impact of CD36 null mutations and polymorphisms in human biology is largely unknown. Our lab generated a CD36 knock-out mouse which showed hyperlipidemia (Febbraio et al., 1999) and insulin resistance (Hajri et al., 2002). In rodent models for CD36 deficiency, diets

affected phenotypic expression (Aitman et al., 1999). CD36 deficiency has been found in one strain of spontaneous hypertensive rats (SHR) expressing insulin resistance (Aitman et al., 1999; Glazier et al., 2002). Because CD36 functions as a fatty acid transporter in skeletal muscles and adipocytes and because of the probable link between CD36 null mutation and insulin resistance in the SHR rat, there has been considerable interest in human type I deficiency and heart disease and diabetes.

The relationship between CD36 deficiency and hypertrophic cardiomyopathy was reported by several groups in Japan while studying the cardiac uptake of radionuclide labeled long chain fatty acid (LCFA) analogs, such as BMIPP (β-methyl-iodophenyl-pentadecanoic-acid) (Tanaka et al., 1997a; Watanabe et al., 1997a; Watanabe et al., 1998a; Watanabe et al., 1997b; Watanabe et al., 1998b). Miyaoka et al looked for insulin resistance in genetic CD36 deficiency, by using a euglycaemic hyperinsulinaemic clamp technique and reported insulin resistance in the five CD36-deficient people tested. (Miyaoka et al., 2001). The study from Italy reported that men carrying the common AGGIG haplotype had 30% higher fasting free fatty acid levels and 20% higher triglyceride levels than non-carriers. The CAGIG haplotype was associated with lower levels of circulation free fatty acid levels in this population. The same group also reported that the overall prevalence of the AGGIG haplotype in diabetics was not different than non-carriers (Ma et al., 2004). More recently CD36 deficiency in humans was reported to be accompanied by hyperlipidemia and increased remnant lipoproteins, impaired glucose metabolism due to insulin

resistance and mild hypertension (Yamashita et al., 2007), all features suggestive of "metabolic syndrome." This may be related to defective uptake of LCFA in the heart (Nozaki et al., 1999; Tanaka et al., 1997a) and increased uptake of LCFA in the liver (Yoshizumi et al., 2000) which, in turn, may lead to increased production of VLDL and increased free fatty acid levels in plasma in these subjects. This may also contribute to insulin resistance.

CD36 on platelets:

CD36 was recognized as a major platelet glycoprotein more than three decades ago, but its role in platelet physiology has not been studied in great details. There are about 24000 CD36 molecules per platelet with at least half of those at the platelet surface (Thibert et al., 1992). Electron microscopy reveals that CD36 is located on plasma membrane and open canalicular system (OCS) in platelets (Berger et al., 1993).

A functional role of CD36 on platelets was suggested by the report of Huang et al according to which CD36 could be co precipitated from platelet membranes with the non receptor tyrosine kinases fyn, lyn, and yes (Huang et al., 1991). Interestingly, Nak[a] minus individuals show no evidence of a hemostatic defect such as bleeding diathesis. Recent reports have shown that VLDL (Englyst et al., 2003) and amyloid-like protein (Herczenik et al., 2007). increased platelet activation by the TxA2 pathway. A recent report also links platelet activation by thrombospondin-1 by inhibition of the NO signaling (Isenberg, 2007 #419) Several reports have linked CD36 autoantibodies with human thrombotic

diseases, including TTP (Tandon et al., 1994), antiphospholipid antibody syndrome (Fabris et al., 1994) and lupus (Rock et al., 1994) suggesting that this phenomenon is clinically relevant.

CD36 is unique among platelet receptors in its ability to recognize a broad variety of ligands, many of which are generated as a consequence of diseases with high thrombotic risks. The ligands include oxidized LDL (oxLDL) generated in atherosclerosis; advanced glycation end (AGE) products generated in diabetes; membranes of apoptotic cells generated in viral infections and cancer; and falciparum-infected erythrocytes. Of these, the most extensively-studied ligand is oxidized LDL (oxLDL) because of its pathogenic role in atherosclerosis.

Systemic conditions with dyslipidemic phenotype is seen in conditions like diabetes, atherosclerosis and metabolic syndrome and these can predispose to increased platelet reactivity (Carvalho et al., 1974; Davi et al., 1998). The mechanisms for increased platelet reactivity in these dyslipidemic states were largely unknown until recently when our group demonstrated that the engagement of platelet CD36 by structurally defined oxidized choline glycerophospholipid ligands (oxPC$_{CD36}$) could play an important role in the development of dyslipidemia–associated prothrombotic state. These ligands were increased in plasma of hyperlipidemic mice and human subjects, were able to bind platelet CD36 and promote platelet activation in a CD36 dependent manner. Using in vivo mouse thrombosis models, we also demonstrated that CD36 null mice were protected from "hyperlipidemia associated platelet reactivity and the accompanying prothrombotic phenotype" (Podrez et al., 2007). Another

group demonstrated that low level oxidation (0-15%) of LDL mediated increase platelet activation involving a p38 MAPK signaling (Korporaal et al., 2005; Korporaal et al., 2007) while at higher levels of oxidation (>30%) LDL could exert an opposite effect on platelet activation by interfering with integrin $\alpha_{2b}\beta_3$ (Korporaal et al., 2005).

MICROPARTICLES (MP)

Microparticles are phospholipid microvesicles that bud off normal cells during either activation or apoptosis (Combes et al., 1999; Jimenez et al., 2003a). MP were first described by Wolfe in 1967 in platelets when he referred to them as "platelet dust" (Wolf, 1967). Since their first recognition, several studies have contributed towards understanding more about MP and their role in physiology and pathology. In vitro, MP is released from endothelial cells, platelets, leukocytes, lymphocytes and erythrocytes. In vivo, some of these MP can be detected in normal or patient human plasma. MP are typically 200 nm-1000 nm in size and possess different antigenic properties depending on the type of cell (endothelium, leukocytes, platelets or cancer cells) from which they are derived or the process by which they are formed.

Formation of MP:

MP can be produced from cell **activation** by agonists. In platelets, for example, maximum MP release is induced by Ca^{2+} ionophore A23187, followed by collagen, thrombin and ADP. MP release is also stimulated by the complement complex C5b-9 or by shear stress (Barry et al., 1997; Gemmell et al., 1993; Gilbert et al., 1991; Miyazaki et al., 1996; Miyoshi et al., 1996; Siljander et al., 1996; Sims et al., 1988; Sims et al., 1989; Tans et al., 1991). In endothelial cells (EC), the process of membrane vesiculation and MP generation has been shown to follow stimulation by proinflammatory cytokines such as TNFα (Combes et al., 1999; Jimenez et al., 2003a) or interleukin (IL) 1β and by infectious agents

or their components such as lipopolysaccharide (Satta et al., 1994). Increase in membrane calcium is the most critical step for MP release particularly at the site of release (Ariyoshi and Salzman, 1996). This is further supported by the fact that chelation of extracellular calcium ions by EGTA blocks the increase in cytosolic calcium as well as the release of microparticles (Miyoshi et al., 1996). MP formation probably also requires the breakdown of the membrane skeleton at the point of release, although this has not yet been elucidated. The formation of membrane vesicles is also associated with the loss of plasma membrane asymmetry leading to the exposure of phosphatidylserine (PS) on the outer leaflet as a consequence of the calcium-dependent activation of scramblase and floppase (Daleke, 2003; Zwaal and Schroit, 1997).

MP can also be released from **apoptotic** cells. Apoptosis is characterized by "cell contraction, DNA fragmentation and dynamic membrane blebbing" (Coleman et al., 2001). Apoptotic membrane blebbing is regulated by caspase3 induced Rho-kinase I activation (Sebbagh et al., 2001).

Importance of release of MP:

There has been considerable speculation about why cells release MP. MP may be released as messengers, to transfer receptors or proteins or to initiate signaling events. The release of MP would also allow cells to escape phagocytosis by removing death signals ("eat me signals") such as phosphatidylserine (Fadok et al., 2000; Simak and Gelderman, 2006) from their surface.

Composition of microparticles:

MP are composed of mainly proteins and lipids. The composition depends on the cell they originate from and the type of stimulus involved in their formation. For example, phospholipid composition of MPs isolated from synovial fluid of patients with rheumatoid arthritis differs from that of MPs isolated from the plasma of healthy subjects (Fourcade et al., 1995). MPs also express proteins that are specific to the cell that they originate from and this characteristic can be used to determine their parental source by using antibodies directed against these specific antigens. For example, endothelial microparticles (EMP) released upon activation or apoptosis differ considerably in terms of antigenic expression (Jimenez et al., 2003b). Those expressing constitutive markers such as CD105 and CD31 are greatly increased in number in apoptosis while those expressing inducible markers such as CD62E, CD54 are increased in activation.

Function

A) Effect on blood coagulation system:

MP undergo a loss of membrane asymmetry during their formation and thus characteristically express phosphatidyl serine (PS) on their surfaces. This may contribute to thrombosis by acting as a catalytic surface for assembly of the prothrombinase complex (Sims et al., 1989). MPs are procoagulant not only because of the presence of anionic phospholipids on their surface but also because they are major carriers of blood borne tissue factor (TF). There have

been reports of monocyte derived MP that contain TF, P selectin glycoprotein ligand (PSGL-1, a protein that binds to P selectin) in human platelet poor plasma (Falati et al., 2003). P selectin and TF both play a very important role in thrombus formation. Both cell surface P selectin on activated platelets and endothelial cells and P selectin shed from these cells bind to PSGL-1 in the monocytes and this induces formation of TF-positive MP (Falati et al., 2003). P selectin on the activated platelets helps in the recruitment of these MP to the thrombus by binding to PSGL-1 on the MP (Celi et al., 2004). This ultimately leads to increased thrombin generation at the site of injury.

B) Effects of MPs on platelets, leukocytes and endothelial cells

Circulating MP have been shown to interact with platelets, leukocytes and endothelial cells (Morel et al., 2006) and may influence cell activation. Endothelial derived MP (EMP) from TNFα stimulated EC, for example, not only bound to human monocytes and monocytic cell lines via intracellular adhesion molecule (ICAM)-1 expressed on EMP, but also stimulated TF expression and procoagulant activity on the cells (Sabatier et al., 2002). Platelet MP bind, activate and aggregate neutrophils in vitro (Lo et al., 2006). In addition, another study showed that platelet MP increased the adhesion of monocytes to endothelial cells (Barry et al., 1998). A recent study showed that platelet MPs may act as carrier for arachidonic acid from pulmonary endothelial cells and this arachidonate is subsequently metabolized by MP to TxA2 (Pfister, 2004). Besides arachidonic acid, PAF, another potent mediator was documented in

platelet derived and PMN derived MPs (Iwamoto et al., 1996), potentially contributing towards platelet activation.

C) Microparticles as markers in diseases

MP have been postulated to play an important role in inflammation (Soriano et al., 2005), atherosclerosis (Falati et al., 2003) and thrombosis, and in part related to tissue factor (TF) and selectins expressed on their surface (Falati et al., 2003).

Endothelial Cell (EC)-derived MP (EMP) have been found in the blood of patients with thrombotic and inflammatory disorders. These include lupus anticoagulant (Dignat-George et al., 2004), coronary artery disease (CAD) (Bernal-Mizrachi et al., 2003; Werner et al., 2006), diabetes mellitus (Davi and Ferroni, 2005; Koga et al., 2005), thrombotic thrombocytopenic purpura (Jimenez et al., 2001), active multiple sclerosis (Minagar et al., 2001), hypertension (Gonzalez-Quintero et al., 2004), or after hematopoietic stem cell transplantation (Pihusch et al., 2006).

Platelet derived microparticles (PMP) correlate with early phase of myocardial infarction and atherosclerosis (van der Zee et al., 2006), in patients with intermittent claudication (Tan et al., 2005), in stroke (Lee et al., 1993), deep vein thrombosis (Rectenwald et al., 2005) or sickle cell anemia (Shet et al., 2003).

The different studies mentioned above clearly demonstrate that the detection and characterization of MP is an interesting and valuable tool to

diagnose certain disease states and perhaps also function as risk calculator for certain cardiovascular conditions.

HYPOTHESES

CD36 on macrophages can initiate signals that play a significant role in fatty streak formation. The function of CD36 on platelets is incompletely characterized, but our group has recently shown that atherogenic oxidized LDL (oxLDL) binds and activates platelets in a CD36-dependent manner (Podrez et al., 2000). We also demonstrated that CD36-dependent phagocyte recognition and uptake of apoptotic cells and/or shed photoreceptor outer segments was mediated by binding of CD36 to PS and/or oxPS on their surfaces (Greenberg et al., 2006; Ryeom et al., 1996; Sun et al., 2006). Since MP express PS on their surface, **we hypothesized that they might also act as a ligand for platelet CD36 and thereby promote platelet activation.** In a clinical scenario where MP are formed, such as in inflammation, cancer, atherosclerosis or thrombosis, CD36-mediated platelet signaling might contribute to pathological thrombosis.

There has been evidence to suggest that there is significant population variance in CD36 expression. When we studied a small group of study subjects, we observed that there was considerable variation in CD36 expression (Fig17, chapter IV). We thus also hypothesized that **the variability in CD36 expression on platelets might affect platelet function and that part of this variability might be explained by genetic polymorphisms.**

In the Caucasian study reported by Ma et al (Ma et al., 2004), the phenotype (increased triglycerides and FFA) associated with the AGGIG CD36 haplotype is consistent with a decrement in CD36 function. No direct data, however, exists to support this. Since CD36 is a fatty acid transporter in heart

muscles and adipocytes, we **hypothesized that there may be a relationship between CD36 polymorphisms and its expression and function.**

In summary, we propose a novel mechanism for modulation of platelet reactivity by the surface receptor CD36 with specific ligands generated during disease states associated with increased risk of arterial thrombosis. Determination of the molecular and genetic mechanisms that control this pathway would help us to a) discover novel methods to diagnose these events and b) design novel preventative strategies to control these events.

CHAPTER II

MATERIALS AND METHODS

Preparation of platelets and platelet rich plasma:

Whole blood was collected from healthy human volunteers in 0.109 M sodium citrate, 7.4 pH (1:9 dilution) in accordance with the Cleveland Clinic Institutional Review Board. These donors had no known disease and were not taking aspirin, non steroidal anti-inflammatory drugs (NSAIDs) or any other medication. Platelet rich plasma (PRP) was obtained by centrifugation at 100g for 12min at room temperature. Platelets were counted in a Z2 particle counter (Coulter) and platelet number adjusted to 2×10^8/ml for all aggregometry experiments. For flow cytometry experiments, washed platelets were prepared by centrifuging PRP at 600g for 10min in the presence of 10 mM prostaglandin E_1 (PGE_1). PGE1 prevents any platelet activation by ADP during the process of centrifugation. The pellet was washed twice with Modified Tyrode's Buffer and then resuspended in this buffer at a concentration of 1×10^6/ml.

Detection of platelet CD36 expression:

100 µl of PRP from healthy normal donors were incubated with either PE-conjugated anti-CD36 monoclonal antibody (Santa Cruz) or isotype matched control antibody. CD36 expression was determined by flow cytometry by gating platelets with an anti-CD42b monoclonal antibody (BD Biosciences) and also on forward versus side scatter, followed by gating on PE-fluorescence vs. forward scatter for CD42b/CD36 positive events.

Generation of endothelial derived microparticles (EMP):

Human EC were isolated from umbilical veins and maintained in culture as per Jaffe et al (Jaffe et al., 1989). Cells of second passage were typically used for experiments and were incubated with 100 ng/ml TNFα (R&D Systems) and 50 ug/ml cyclohexamide (Sigma) for 24hr (Simak et al., 2002) to generate EMP. Culture supernatants were collected and nonviable cells and large cell fragments were removed by centrifugation at 4300g for 5min. The supernatants were then centrifuged at 100,000g for 90min at 10°C to pellet EMP. Pelleted EMP were resuspended in 100ul HEPES-Tyrode's buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1.0 mmol/L MgCl$_2$, 12 mmol/L NaHCO$_3$, 0.4 mmol/L Na$_2$HPO$_4$, 0.35% bovine serum albumin (BSA), 10 mmol/L HEPES, 5.5 mmol/L glucose, pH 7.4) and stored at -70°C. Freezing and thawing had no adverse effect on size or morphology of the EMP as assessed by immunofluorescence microscopy. EMP were characterized by flow cytometry (PCA Analyzer, GUAVA Technologies) and shown to bind FITC-conjugated annexin V (BD Biosciences) and phycoeryrthrin

(PE)-conjugated antibodies to CD105/endoglin (Ancell), VE-cadherin (BD Biosciences), and CD31 (BD Biosciences). In all cases, corresponding isotype matched non-immune IgGs were used as controls. CD105 positive EMP were counted by flow cytometry using 0.3 and 3 μm latex beads (Sigma) as size standards. Each confluent T75 flask of cells generated approximately 3×10^5 CD105 positive EMP.

Isolation of MP from human plasma:

Human blood was isolated from healthy normal and centrifuged at 500 g to obtain platelet poor plasma (PPP). The PPP was then centrifuged at 15000 g for 45 minutes at 20°C to pellet the MP which were resuspended in Modified Tyrode's buffer containing 0.35% BSA and stored at -70°C. Human MP were heterogenous and some expressed endothelial (CD105 and CD144), platelet (CD41), monocytic (CD14) markers. Each marker was separately counted by flow cytometry. Our colleague, Dr. McCrae, analyzed 105 subjects and a representative group of 13 was used for experiments.

Preparation of oxidized LDL (oxLDL):

OxLDL was prepared by oxidation with copper sulphate (5μm) at 37°C for 6 hours according to a previously published protocol (Febbraio et al., 2000). The amount of lipid oxidation that had taken place in each LDL sample was assessed using the TBARS (thiobarbuturic acid-reactive substances) assay (Morel and Chisolm, 1989).

Immunofluorescence flow cytometry detection of platelet-MP interactions:

Washed platelets were incubated with MP for 30min prior to incubation with the EC-specific anti-CD105 antibody. After antibody incubation, cells were pelleted, washed and analyzed by flow cytometry. Relative fluorescence intensity histograms and dot-plots were made and analyzed with Flow Jo software (Tree Star, Inc). For the MP binding studies, we used anti-CD105 antibody which is specific for endothelial cells. EMP but not platelets would bind anti-CD105. Washed platelets were pre-incubated with MP at a ratio of 1:9 for 30 minutes, the reaction mix was then centrifuged at 700g, a speed that would pellet only the platelets and washed to get rid of any free EMPs. When the resuspended pellet was incubated with anti-CD105, the platelets acquired fluorescence suggesting that CD105-positive MP were physically associated with the platelets and the change in platelet acquired anti-CD105 fluorescence was measured and statistically quantified. In some studies, platelets or MP were incubated with antibodies, oxLDL, or Annexin V (BioVision) prior to CD105 staining. Blood collection, MP incubation, antibody incubation, and data acquisition for all samples were done on the same day, using the same instrument for each individual experiment for consistency. Univariate analyses were performed using analysis of variance (ANOVA) and paired and unpaired t-tests, as appropriate. Data are presented as mean ± SD. Statistical significance was defined as $p<0.05$. Statistical analyses were performed using commercially available software (StatView 5.0; Abacus Concepts Inc, Berkley, CA).

Immunofluorescence microscopy:

For immunofluorescence microscopy, platelets were loaded with an intracellular green fluorophore (Calcein, Molecular Probes) and EMP with a red fluorophore (PKH-26, Sigma) and incubated together as above prior to imaging with a Leica DM-RXE microscope, interfaced to a PC using Q capture software (Quantitative Imaging Company).

Platelet activation studies:

Washed platelets ($1x10^6$) were incubated with EMP (1:9 ratio) or buffer control for 30min and then stimulated with ADP. In case of human MP, numbers were normalized on the basis of endothelial markers. They were then incubated for 15 mins with PE-conjugated anti-P-selectin IgG (BD Biosciences) or FITC-conjugated PAC1 (BD Biosciences), a monoclonal antibody that recognizes the activated conformation of the platelet integrin $\alpha_{2b}\beta_3$. After incubation, the platelets were centrifuged at 700 g for 10 min, resuspended in PBS, and analyzed by flow cytometry. In some studies, washed platelets were pre-incubated with anti-human CD36 antibody (clone FA6, Invitrogen) or isotype matched control IgG (Sigma).

Platelet aggregation studies:

Platelet aggregation was assessed turbidometrically with a dual channel aggregometer (Chronolog Corporation, PA) using graded doses of ADP from 1-

20 µM under constant stirring conditions. The light transmission of PPP was regarded as 100% aggregation and the light transmission of PRP before addition of agonist was regarded as 0%. Any change in light transmission after addition of agonist was recorded and expressed as a percentage of deflection.

Intravital thrombosis:

Carotid arteries in wild type and CD36 knock out mice were visualized on a Leica DM LFS microscope with water immersion objectives and recorded images with a high speed color, cooled digital camera (QImaging Retiga EXi Fast 1394) with Streampix high-speed acquisition software. We recorded the blood vessels for 30 minutes and then created a vessel- wall injury by application of a 1.5 x 1.5 mm square of Whatman filter paper soaked in saturated $FeCl_3$ solution to the surface of the vessel (we used 12.5% $FeCl_3$ for 1 minute).Then we removed the paper, covered the vessel with saline at 37˚C and recorded platelet-vessel wall interactions for 15 minutes. The thrombosed arteries were sectioned and frozen at -20˚.

Immunofluorescence staining:

Frozen thrombosed carotid sections were used for immunohistochemistry to detect endothelial derived microparticles (primary CD105; secondary, Alexa Fluor 568) and platelets (marker primary,CD61; secondary, Alexa Fluor 488) or both. Cryosections of thrombosed carotid were thawed at room temperature, post fixed

in acetone and hydrated in PBS. Tissues were blocked for 1 hour at room temperature with BSA, followed by incubation with 1:200 dilution of CD61 antibody and subsequently with 1:500 dilution of AlexaFluor 488. The same slides were also stained with CD105 antibody (1:500 dilutions) followed by Alexa Fluor 568 conjugated secondary. Confocal imaging was acquired with Leica TCS-SP2 confocal microscope (Leica Microsystems, Heidelberg) at room temperature using 63X lens. Using Adobe Photoshop software, histograms were linearly adjusted for optimal representation of the 8 bit signals, individuals channels were overlaid in RGB images and composites of panels were made for final figures.

Western Blot analysis:

Human platelets were lysed and resuspended in lysis buffer (Tris, pH 7.5 20mM, NaCl 100mM, EDTA 1mM, EGTA 1mM, Triton x 1%) containing protease inhibitors. Proteins (20 µg) were separated by SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). CD36 was identified using a monoclonal antibody (clone FA6, Invitrogen). Membranes were reprobed with an antibody with actin (Santa Cruz) or GPIX (Santa Cruz) to normalize for protein loading. Detection was performed with ECL plus Western Blotting Detection Reagents (Amersham Biosciences) and band intensity was analyzed by densitometry (Image J). Experiments were done twice in duplicate and normalized readings expressed as mean ± S.E.

OxLDL induced platelet activation:

Washed platelets (1×10^6) were incubated with oxLDL (50ug/ml) or native LDL (control) for 30min and then stimulated with or without ADP. They were then incubated for 15 minutes with PE-conjugated anti-P-selectin IgG (BD Biosciences) or FITC-conjugated PAC1 (BD Biosciences), a monoclonal antibody that recognizes the activated conformation of the platelet integrin $\alpha_{2b}\beta_3$. After incubation, the platelets were centrifuged at 700g for 10min, resuspended in PBS, and analyzed by flow cytometry. Results are representative of at least 2 independent experiments.

Study population:

The initial screen included 32 healthy human volunteers. This was followed by a larger patient population who came to Cleveland Clinic Cardiac Catheterization lab, agreed to participate in a study and the blood was collected by GeneBank. GeneBank is a large cardiovascular genetic repository in Cleveland Clinic Foundation which is involved in prospective collection of DNA, plasma and serum as well as maintenance of an extensive database of deidentified patient records. We have used 550 patients from this diverse population. Each sample was collected fresh and deidentified before use.

Tagged SNPs:

Using the haploview software offered at the international HapMap consortium website, we identified 10 SNPs with allelic frequencies >5% that "tag" major

areas of linkage disequilibrium. 10 SNPs were picked out for population CEU (population from Utah, USA with European descendency) using the algorithm-Tagger-pairwise tagging (Carlson et al., 2004; Chapman et al., 2003). The mean r^2 cut off for the tagging was 0.8.

Table 2: Identification of 10 tagged SNPs

number	#tag SNPs	Chromosome	Position	MAF
1	rs10499858	chr7	79880497	0.108
2	rs6968407	chr7	79882768	0.342
3	rs10499859	chr7	79903461	0.492
4	rs9918586	chr7	79911387	0.091
5	rs3211864	chr7	79930995	0.05
6	rs3211869	chr7	79931803	0.119
7	rs3211908	chr7	79938567	0.108
8	rs3211932	chr7	79943538	0.483
9	rs1527483	chr7	79946151	0.158
10	rs1537593	chr7	80091847	0.134

Table 2: **Tag SNPs**. Table showing the 10SNPs with minor allelic frequency (MAF) of 5%.Also shows the position of each SNP. The numbers were allocated according to the position of the SNPs. In haplotype analyses, these numbers were used rather than the SNP designation.

SNP genotyping:

Genotyping of SNPs was carried out by the 5'allelic discrimination assay (the TaqMan assay) as described previously (Shen et al., 2007). SNP assay probes, Assay-on-Demand or Assay-by-Design, were from Applied Biosystems (ABI, Foster City, CA, USA). PCR for SNP genotyping was performed in a 5 μl volume containing 20ng of genomic DNA sample and 2 μl TaqMan Universal PCR Master Mix a GeneAmp PCR System 9700. The PCR products were scanned by an ABI PRISM 7900HT Sequence Detection System. Alleles of SNPs were called analyzed with software version 2.1. For quality control, SNP genotyping results were verified for eight random selected DNA samples by direct DNA sequence analysis as described (Shen et al., 2007). DNA sequence analysis was performed by Big Dye Terminator Cycle Sequencing v 1.1 Mix (ABI, Foster City, CA, USA) using ABI 3100 Genetic Analyzer.

Genetic Data analysis:

A) SNP analysis:

The mean fluorescence intensity of CD36 expression on platelets was categorized into 2 groups by their median (< 3.75 or > 3.75). We generally considered phenotypes as outcomes and SNPs as predictors. If outcome was binary, we used fisher exact test of the association and calculated OR (Odds Ratio) by logistic regression. If the outcome was multilevels, we used trend test

or the linear-by-linear association tests (Agretsi, 2002; Horthorn, 2006) R coin package: independence test) to test the association between the SNPs and traits. We fitted proportional odds ordinal logistic regression models, which apply maximum likelihood estimation or penalized maximum likelihood estimation (Agretsi, 2002; Harrel, 1998; R) to estimate the ratio of the cumulative odds of the upper quintiles and lower quintiles of the traits. Since we were testing the associations of 10 SNPs simultaneously, we considered the multiple comparisons adjustment, where we implemented a correction introduced by Benjamini & Hochberg (Benjamini and Hochberg, 1995) controlling the false discovery rate (FDR), the expected proportion of false discoveries amongst the rejected hypotheses. The FDR is a less stringent condition than the family wise error rate (FWER), so it is more powerful than those controlling FWER. Only those with P values < 0.05 and FDR values < 0.2 were reported as statistically significant findings.

B) Haplotype analyses:

We applied the 'haplo.stats' package in R (Schaid et al., 2002) (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html) to identify the association between a trait and haplotypes. A suite of R routines, referred to as "haplo.score", was used to compute score statistics to test associations between haplotypes and traits. "haplo.score.slide" was used to identify sub-haplotypes from a group of loci. It is useful for a series of loci where little is known of the association between a trait and haplotypes.

We first ran "haplo.score.slide" on all contiguous SNP subsets of size, *n.slide*. Using a range of *n.slide* values (=*2,3,4,5*), the region with the strongest association consistently have low p-values for locus subsets containing the associated haplotypes.

For each phenotype, we considered different SNPs subsets with a range of *n.slide* values, which meant that we were testing the associations of multiple subsets (30 subsets) simultaneously. Therefore, we need to consider the multiple comparisons adjustment. We considered two error measures: the false discovery rate (FDR) and the tail probability of the proportion of false positives (TPPFP). We used Bonferroni procedure to get adjusted p-values, then applied augmentation approach to these p-values to get generalized TPPFP adjusted p-values (van der Laan, 2004, 2). Also we considered a multiple comparisons adjustment implemented by Benjamini & Hochberg correction to control FDR. We report the SNP subsets with TPPFP adjusted p-values less than 0.2 have statistically significant association with the trait.

For each of these significant SNP subsets, we ran "haplo.score" to identify the haplotypes which are significantly associated with the trait. We considered those haplotypes with p values of score tests less than 0.05 as potential significant ones.

To calculate the odds ratio of the significant haplotypes for the specific phenotype, we applied the "haplo.glm". The base haplotype is the most frequent one.

CHAPTER III

ENDOTHELIAL DERIVED MICROPARTICLES BIND AND ACTIVATE PLATELETS IN A CD36 DEPENDENT MANNER

Characterization of EMP:

Human EC were isolated from umbilical veins were incubated with 100 ng/ml TNFα and 50 ug/ml cyclohexamide overnight to generate EMP. EMP were characterized by flow cytometry and shown to bind FITC-conjugated annexin V (a marker for surface PS) and phycoeryrthrin (PE)-conjugated antibodies to the EC specific proteins CD105/endoglin, VE-cadherin, and CD31 (BD Biosciences) (Fig 5). In all cases, corresponding isotype matched non-immune IgGs were used as controls. As CD105 gave most robust fluorescence, it was used for the subsequent assays.
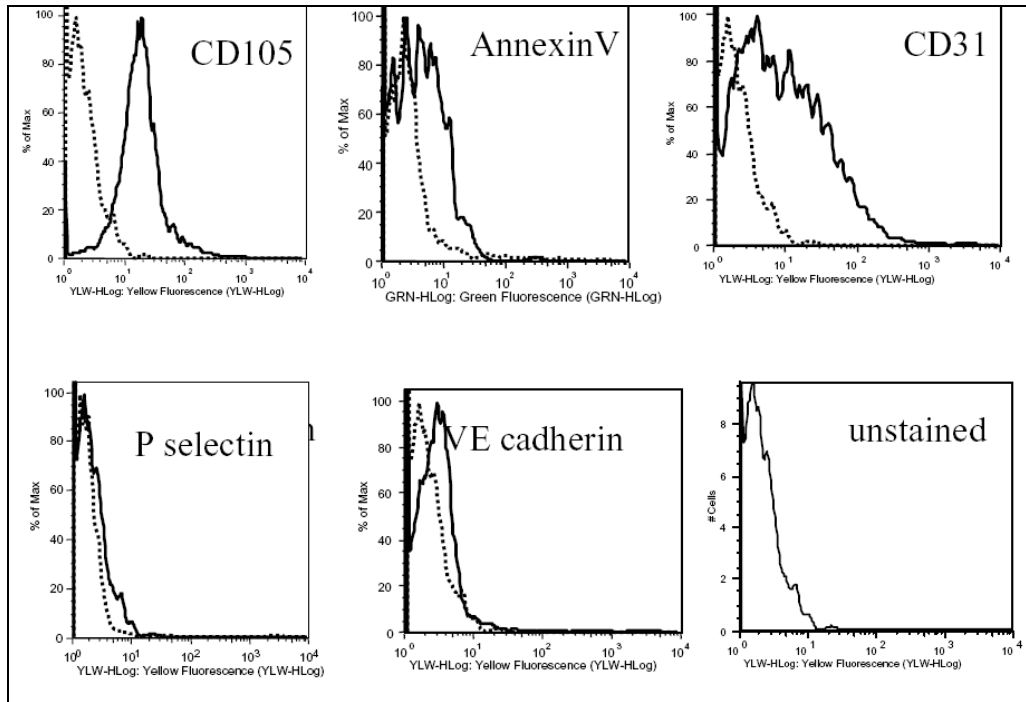
Fig 6. **Characterization of EMP.** HUVECs were treated with TNFα and Cyclohexamide and EMP were obtained from the supernatant. Flow cytometry histograms of EMP shown to bind FITC-conjugated annexin V and phycoeryrthrin (PE)-conjugated antibodies to CD105/endoglin, VE-cadherin, and CD31 (BD Biosciences).

EMP bind to platelets:

As shown in Fig 7A, EMP but not platelets (Fig 7B) bound anti-CD105. When

washed platelets were pre-incubated with EMP prior to incubation with anti-

CD105, the platelets acquired fluorescence (Fig 7B), suggesting that CD105-

positive EMP were physically associated with the platelets.   This physical

association was confirmed using 2-color fluorescence microscopy (Fig 8): EMP were labeled with a red fluorophore (Fig 8A, bottom panel) and platelets were labeled with a green fluorophore (Fig 8A, top panel). As shown in Fig. 8B, the red EMP formed rosettes with the green platelets.
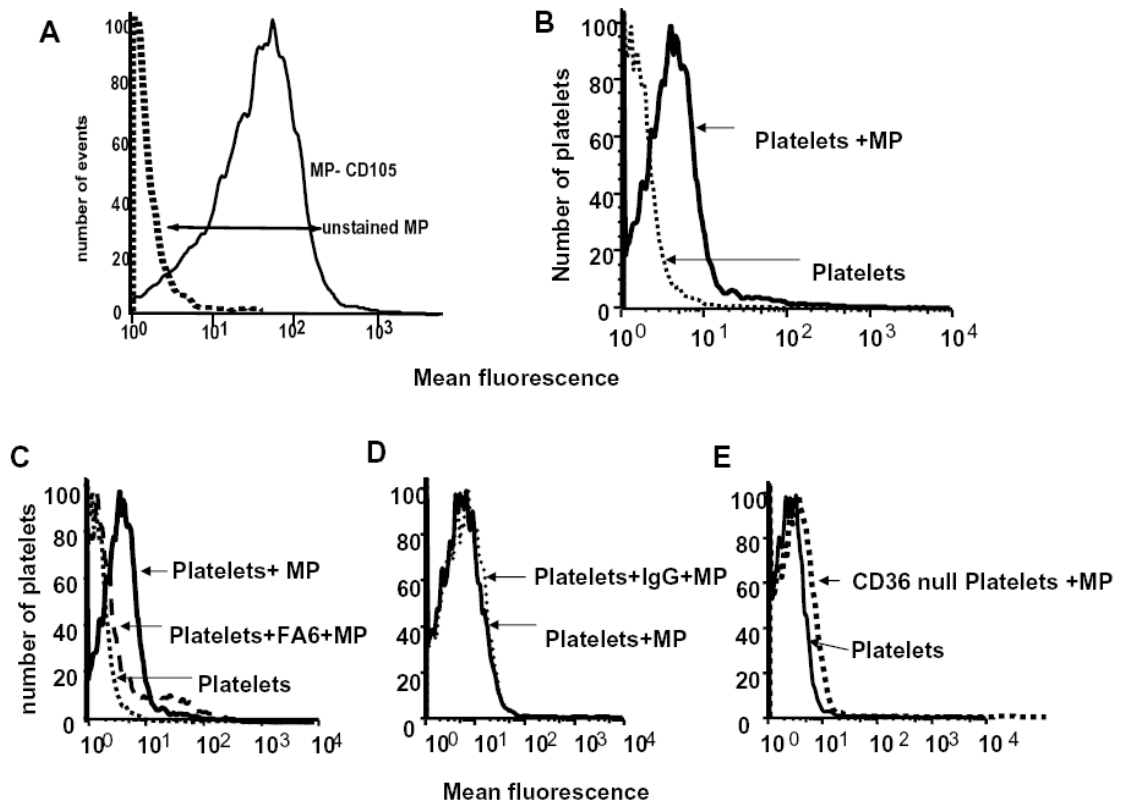
Fig 7. **CD36-dependent binding of EMP to platelets detected by immunofluorescence flow cytometry.** EMP were generated and purified from HUVEC cultures treated with TNFα (100ng/ml) and cyclohexamide (50µg/ml) for 24hrs. (A) Flow cytometry histogram showing that EMP stain with a PE-conjugated anti-CD105 IgG (solid line) but not an isotype matched control IgG (dotted line). (B) Flow cytometry histogram showing that platelets did not react with the anti-CD105 IgG (dotted line) but when incubated with EMP at a ratio of 1:9, the platelets acquired PE fluorescence (solid line). (C) Platelets and EMP were mixed together and stained with anti-CD105 IgG as in panel B, except the platelets were first pre-incubated with anti-CD36 IgG FA6 (dashed line); or (Panel D) isotype matched control IgG (dotted line). (E) Platelets and EMP were mixed

together and stained with anti-CD105 IgG as in panel B, except platelets were from a donor shown to be CD36 null. Platelet-associated PE fluorescence was reduced by more than 95%. In all cases histograms represent one of at least 3 separate experiments.

EMP binding to platelets is CD36 dependent:

We used two independent methods to define the role of CD36 in platelet-EMP interactions. As shown in Fig. 7C, pre-incubation of washed platelets with a monoclonal anti-CD36 antibody inhibited the acquisition of anti-CD105 positivity in a flow cytometry assay; control IgG had no effect (Fig 7D). Similarly, using immunofluorescence microscopy, oxLDL, an alternative ligand for CD36 (Fig. 8C) or antibody to CD36 (Fig. 8E), inhibited platelet-EMP interaction whereas control IgG (Fig 8F) and native LDL (Fig 8D) had no effect. Furthermore, platelets isolated from a CD36 null donor did not bind EMP (Fig 7E).
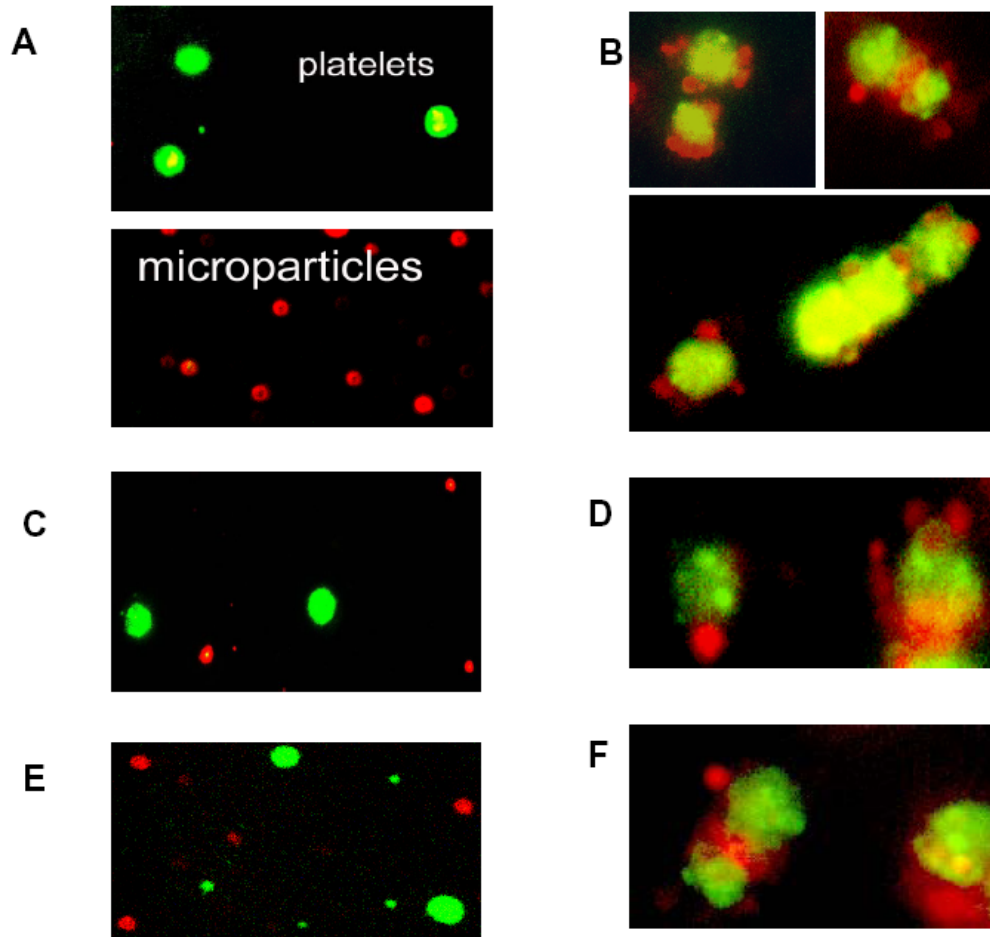
Fig 8. **CD36-dependent binding of EMP to platelets detected by immunofluorescence microscopy.** (A) Washed platelets (top panel) were loaded with calcein and visualized by green fluourescence. EMP (bottom panel) were loaded with PKH26 and visualized by red fluorescence. (B) Platelet-EMP rosettes seen when calcein-loaded platelets and PKH26-loaded EMP were incubated together at a 1:9 ratio for 30min prior to visualization. (C) Fluorescence tagged platelets and EMP were mixed together as in panel B except that platelets were first incubated with oxLDL (50µg/ml) or (D) nLDL (50ug/ml) as control or (E) an inhibitory anti-CD36 IgG or (F) its IgG control prior to visualization.

Blockade of MP-PS inhibited platelet-MP interaction:

Incubation of MP with annexin V to block exposed PS dramatically reduced platelet acquired CD105 fluorescence (Figure 9A). A control protein, thioredoxin, had no effect. In addition, incubation of MP with a monoclonal anti-PS antibody significantly reduced the platelet-MP interaction whereas a non-immune control IgM did not (Figure 9B). These data suggest that the ligand for CD36 on the MP surface is phosphatidylserine.
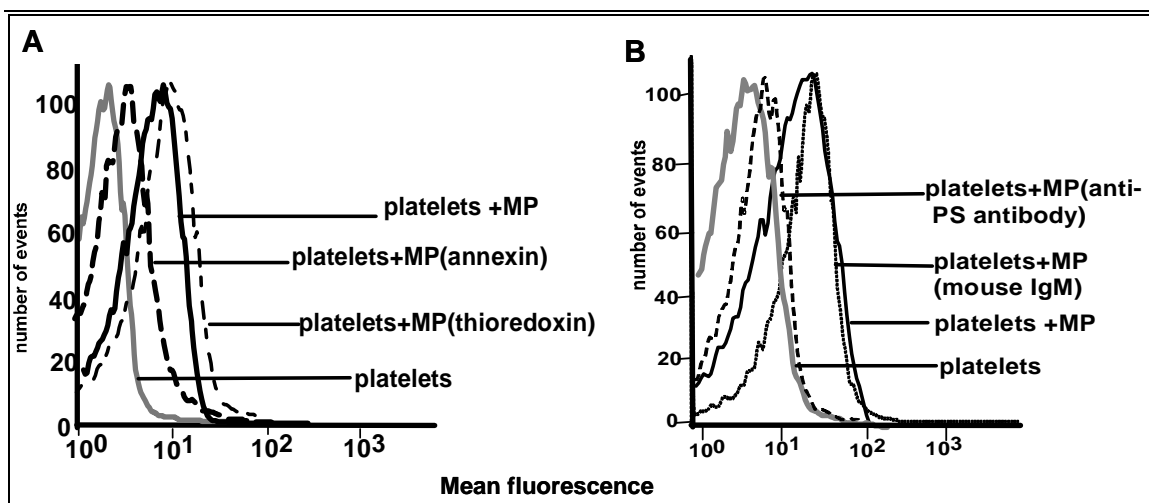
Fig 9. **Binding of EMP to platelets is PS dependent.** Platelets and EMP were mixed together and stained with anti-CD105 IgG as in Figure 1B, except the EMP were first pre-incubated with annexin V (150nmol) to block exposed PS. Platelet associated anti-CD105 fluorescence was reduced by >95% (Figure 9A). Pre-incubation of EMPs with thioredoxin, a control protein had no effect. Similarly, EMP preincubated with anti PS antibody (25µg/ml) were mixed with platelets and stained with CD105. The platelet associated fluorescence was significantly reduced (Figure 9B, dashed line) whereas the control IgM had no effect (Figure 9B, dotted line) .The histograms are representative of at least 2 separate experiments.

EMP enhance platelet activation and aggregation:

To assess the functional consequences of EMP-platelet CD36 interactions, we incubated PRP with EMP prior to assessment of activation and aggregation responses to graded concentrations of the agonist ADP. EMP at a ratio of 1:9 (9 EMP per platelet) did not induce platelet aggregation (not shown). In contrast, in combination with low doses of ADP, EMP induced a significant increase in the rate and extent of the aggregation response (Fig 10A). This effect was not observed in platelets from CD36 null donors (Fig 10B) demonstrating CD36 dependence. Fig.10C shows that EMP significantly enhanced the extent of aggregation of CD36 expressing platelets induced by ADP concentrations of 1-4 µM but not 20 µm (white and black bars). No EMP effect was observed at any ADP concentration in CD36 null platelets (hatched and checkerboard bars). We also observed that responses of CD36 expressing platelets to graded doses of ADP (1-20µM) were significantly higher than those of CD36 null platelets (white and hatched bars).
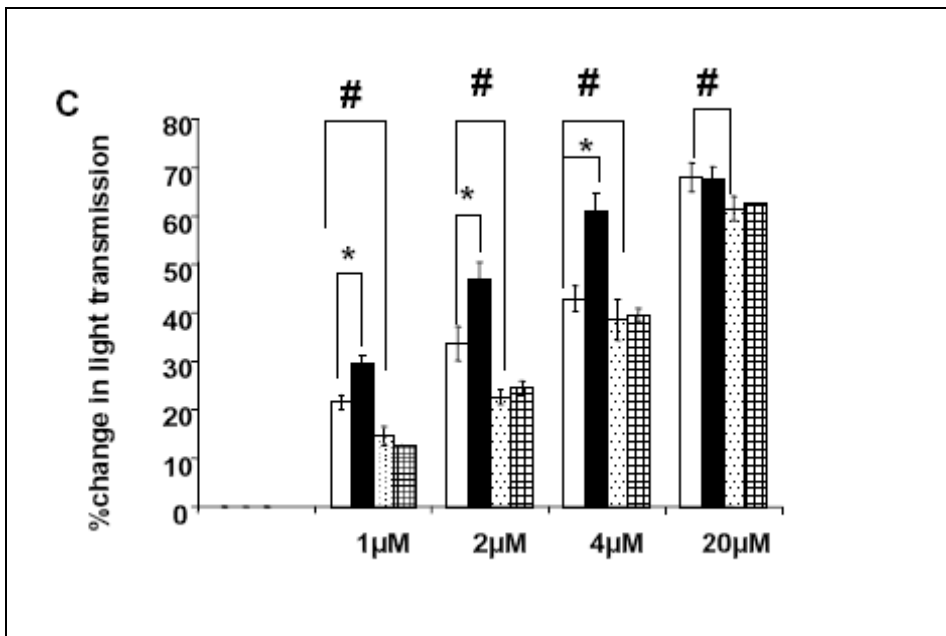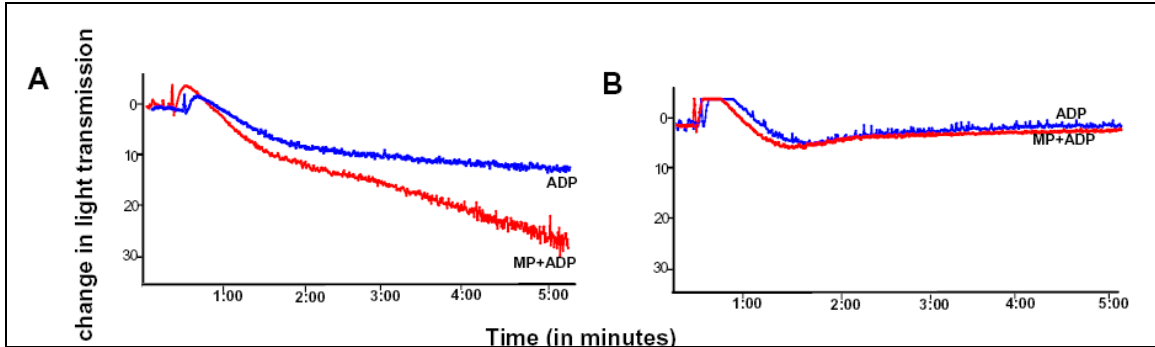
Fig 10. **CD36-dependent enhancement of platelet aggregation by EMP in response to low doses of ADP.** EMP were added to PRP obtained from healthy CD36 expressing (A) or CD36 null (B) donors and then stimulated with 2µM ADP. Aggregration was assessed turbidometrically with a dual channel aggregometer. Tracings show representative aggregometry curves from n=4. (C) Maximum aggregation response of CD36 expressing platelets (open and closed bars) and CD36 null platelets (hatched and checkerboard bars) incubated with EMP (closed and checkerboard bars) or with buffer control (open and hatched bars) in response to graded doses of ADP. Values are the means of measurements done in triplicate from CD36 expressing or null donors and are expressed as +/- SE. Significant differences between CD36 expressing platelets with(open bar) or without(closed bar) prior incubation with EMP are shown by(*) sign. Significant differences between ADP dose responses between CD36

expressing (hatched bar) and CD36 null platelets (checkerboard bar) are shown by (#) sign.

EMP enhance platelet activation and secretion:

To determine the effect of EMP on platelet activation and secretion, we assessed platelet P-selectin expression, a marker of α-granule secretion (Fig 11A) and PAC1 binding, a marker of integrin $\alpha_{2b}\beta_3$ activation (Fig 11B) in response to 2 μM ADP. In both cases we observed that EMP induced a marked increase in the expression of these markers. These effects were significantly diminished by pre-incubation of platelets with anti-CD36 IgG (Fig 11A and 11B). Furthermore, washed platelets from a CD36 null donor showed neither increased P-selectin expression nor PAC1 binding in the presence of EMP and 2 μM of ADP (Fig 11C and 11D).
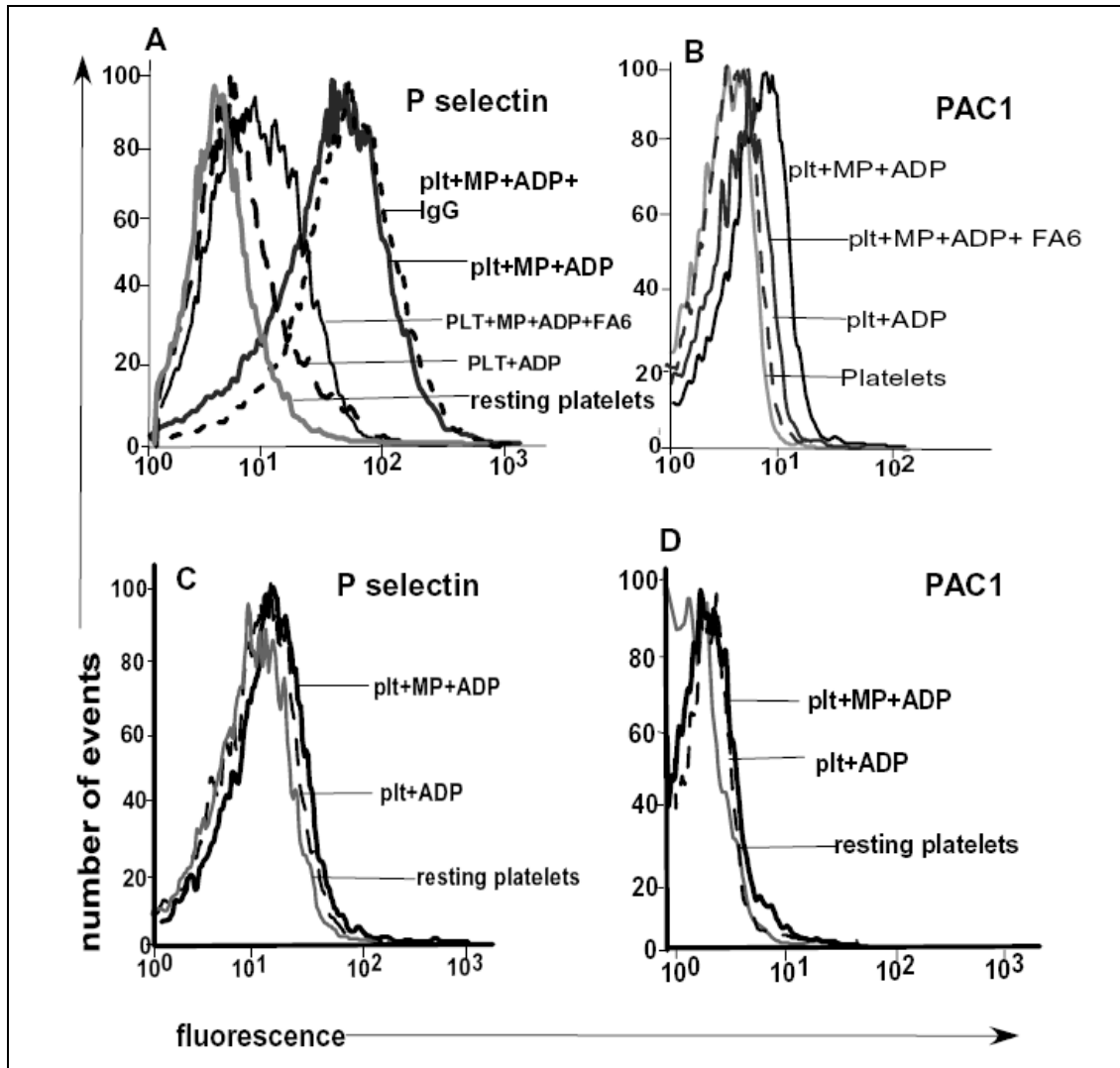
Fig 11. **CD36-dependent enhancement of platelet activation by EMP in response to low dose ADP.** Washed platelets from CD36 expressing donors (A) and (B) or CD36 null donors (C) and (D) were incubated with EMP (1:9) or buffer control for 30min and then stimulated with 1µM ADP. They were then incubated with either PE-conjugated anti-P-selectin (A) and (C) or FITC-conjugated PAC1 (B) and (D) and analyzed by flow cytometry. (A and B) Histograms showing increased anti-P-selectin (A) and PAC-1 (B) binding to CD36-expressing platelets when pre-incubated with EMP. Binding was significantly decreased when platelets were pre-incubated with anti-CD36 IgG, but not an isotype matched control IgG. (C and D) Histograms showing lack of increased anti-P-selectin (C) or PAC-1 (D) binding to CD36 null platelets when pre-incubated with EMP. Histograms are representative of three independent experiments.

Isolation and characterization human blood-derived MP:

Human MP (MP) were characterized by endothelial (CD105 and CD144), platelet (CD41), monocytic (CD14) markers and each marker was separately counted by flow cytometry. Our colleague, Dr. McCrae analyzed 105 subjects and a representative group of 13 subjects was used for experiments.

Table 3: **Characteristics of MP isolated from normal human plasma**

donor	Annexin V APC	CD 105 PE	CD 14 PE	CD 41 PE-Cy5
donor 1	14730	6030	1980	900
donor 2	180	270	90	450
donor 3	8190	3960	1125	17440
donor 4	9450	4865	225	23940
donor 5	9630	2655	720	2250
donor 6	13950	1485	585	1680
donor 7	360	180	135	2295
donor 8	5040	4500	315	2520
donor 9	540	3420	135	1710
donor 10	5490	2025	90	1350
donor 11	NA	8963	12	13249
donor 12	8820	2025	675	20644
donor 13	10170	1710	720	22470
Mean ± SD	7213±4988	3238±2453	524±352	8531± 9412

Table 3:   Characteristics of MP isolated from blood of normal human subjects.   MP were isolated from platelet poor plasma of normal human subjects. Human MP were analyzed by flow cytometry and were quantified by light scatter and annexin V staining. MP were also characterized by cell of origin using antibodies to endothelial: (CD105), platelet (CD41) and monocytic (CD14) markers. Each marker was separately counted by flow cytometry. Also shown here are the average count per ml ± SD for each MP marker. We analyzed 105 subjects and a representative group of 13 was used for experiments. NA: not available

MP isolated from normal healthy subjects bind and activate platelets in a CD36-dependent manner:

As noted by others (Berckmans et al., 2001; Jimenez et al., 2001; Simak et al., 2002), MP can be isolated from healthy subjects with no thrombosis history or risk. MP isolated from 13 healthy human subjects were used for the studies discussed below. As shown in Figure 6A, MP isolated from normal healthy volunteers stained positively for CD105. When washed platelets were pre-incubated with human plasma derived MP at a ratio of 9 MP per platelet prior to incubation with anti-CD105, the platelets acquired fluorescence (Figure 12B), suggesting that CD105-positive MP were physically associated with the platelets. To determine the effect of blood borne MP on platelet activation, we assessed platelet PAC1 binding, a marker of integrin $\alpha_{2b}\beta_3$ activation (Figure 12C) in response to 2 µM ADP. Human plasma-derived MP induced a marked increase in the expression of PAC1. This effect was significantly diminished by pre-incubation of platelets with anti-CD36 IgG (Figure 12C). These studies show that MP isolated from normal human plasma similar to those generated in vitro from cultured EC could bind and activate platelets in a CD36 dependent manner.
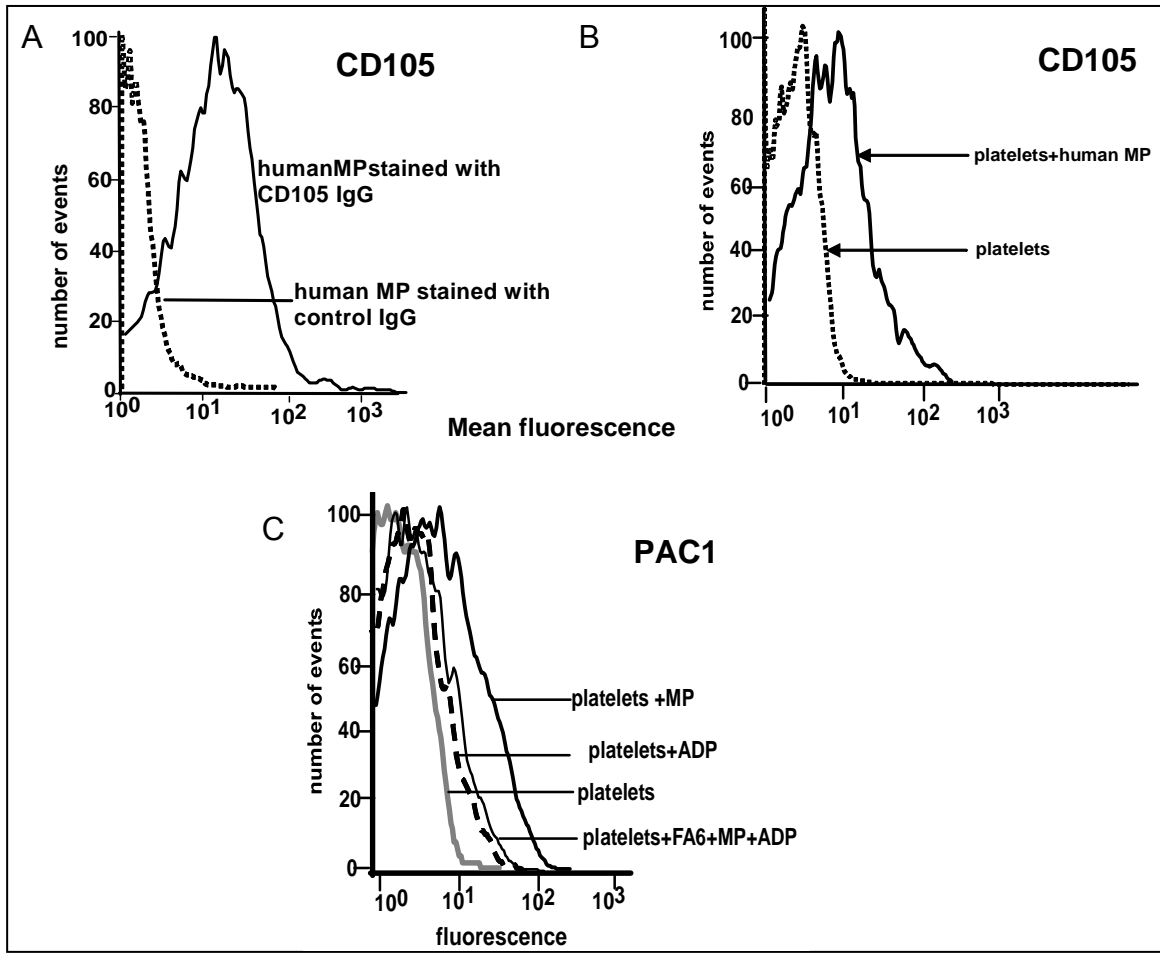
Figure 12. MP isolated from normal healthy subjects bind and activate platelets in a CD36 dependent manner. MP were isolated from normal healthy volunteers and characterized and counted. (A) Flow cytometry histogram showing that human blood-derived MP stain with a PE-conjugated anti-CD105 IgG (solid line). (B) Flow cytometry histogram showing that platelets did not react with the anti-CD105 IgG (dotted line) but when incubated with human (CD105 positive) MP at a ratio of 1:9, the platelets acquired PE fluorescence (solid line). Washed platelets from CD36 expressing donors were incubated with CD105 positive human blood-derived MP (1:9) or buffer control for 30min and then stimulated with 1μM ADP. They were then incubated with FITC-conjugated PAC1 and analyzed by flow cytometry. (C) Histogram showing increased PAC-1 binding to CD36-expressing platelets when pre-incubated with human blood-derived MP (solid bold line). Binding was significantly decreased when platelets were pre-incubated with anti-CD36 IgG (solid thin line). Histograms are representative of at least three different experiments.

MP isolated from normal subjects augmented platelet aggregation in a CD36-dependent manner:

Human plasma derived MP were incubated with PRP from healthy human volunteers prior to stimulation with 2 µM ADP. Since we observed a subtle decrement in CD36 null platelet responses to low doses of ADP (Figure 10C), ADP concentrations were adjusted to produce the same aggregation response in both the CD36 null and CD36 expressing platelets. As shown in Figure 13 left panel, when human plasma derived MP were incubated with CD36 expressing platelets, there was a significant enhancement in the aggregation response to ADP. This was not observed when human plasma derived MP were incubated with CD36 null platelets (Figure 13, right panel). These studies show that blood-borne MP have similar effects as culture derived EMP on platelet function.
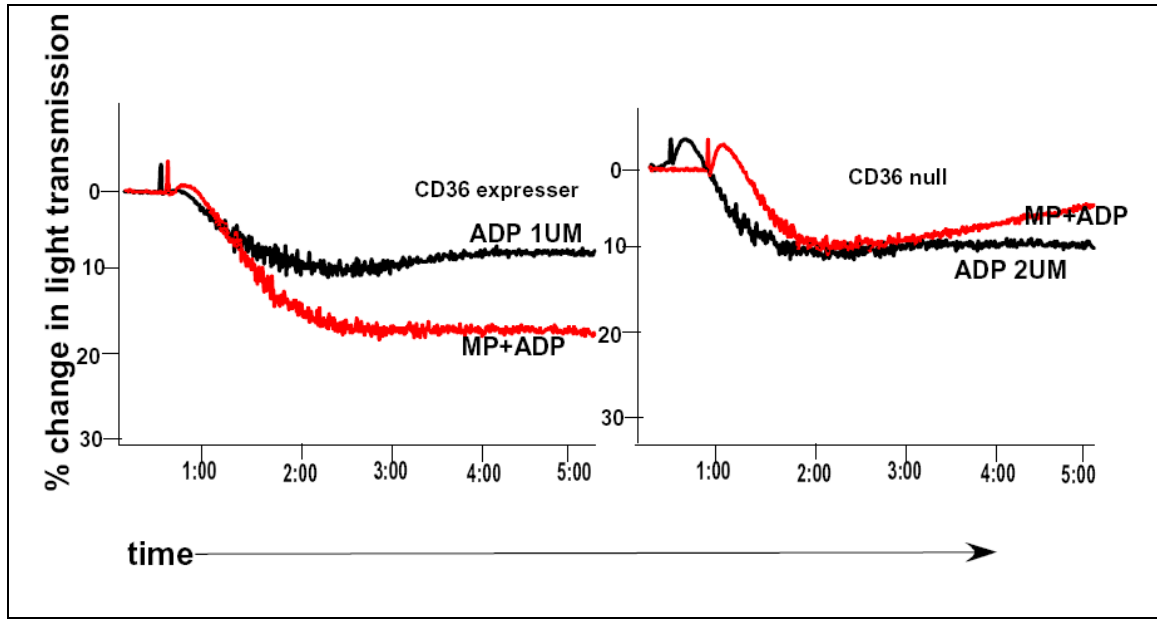
Figure 13. MP isolated from normal healthy subjects augment platelet aggregation in a CD36 dependent manner. PRP from healthy CD36 expressing (A) or CD36 null (B) human subjects was mixed with MP isolated from plasma of 13 normal healthy subjects and then stimulated with 1µM ADP (A) or 2µM ADP (B). Aggregation was assessed turbidometrically as in Figure 10. Tracing is representative of findings from at least 5 different normal subjects.

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CD105 staining in thrombi from CD36 null mice was decreased:

Ongoing studies in the lab by a colleague had shown that the carotid artery occlusion times in CD36 wild type was significantly longer compared to knock out mice. We hypothesized that one of the contributing factors to this could be EMP-platelet interaction in CD36 wild type mice.To determine if MP and platelets interact in vivo, carotid arteries were injured by $FeCl_3$ in wild type and CD36 knock out mice. The thrombosed arteries were sectioned and immunostained with an endothelial cell specific antibody to CD105 and a platelet specific antibody to CD61, followed by detection with secondary antibodies conjugated with different color fluorophores. Nuclei were stained with DAPI. We reasoned that CD105 staining of thrombi would reflect incorporation of EC-derived MP into the thrombi. Images (Fig. 14) were acquired by confocal microscope from wild type (A) and CD36 null mice (B). Each image has four panels showing blue nuclei (DAPI), red CD105 suggestive of EMP, green platelets (CD61) and a merged image. When we compared these two images, we observed significantly more CD105 staining within the thrombi from wild type mice (A) and also more colocalizing areas between the red EMP and the green platelets (yellow) in the merge imaged compared to CD36 null mice (B).
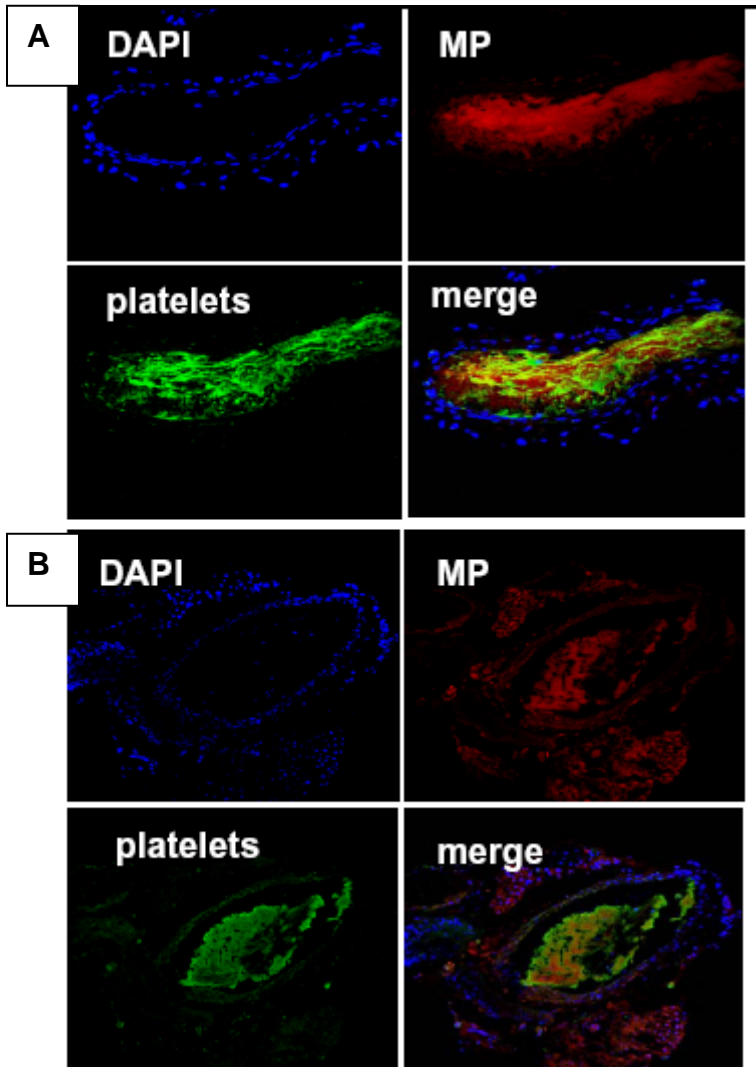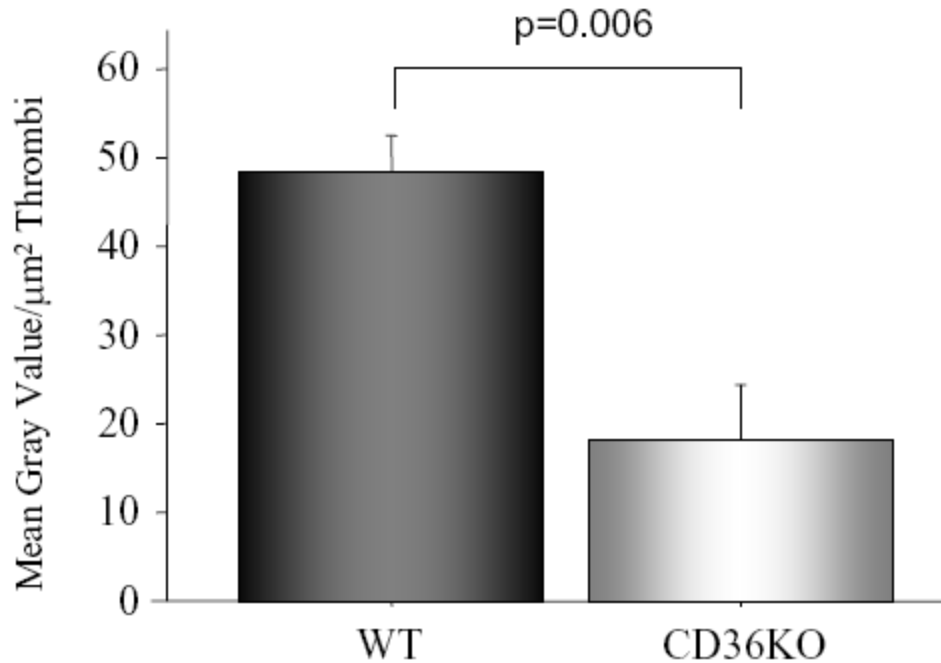
Fig 14: **Detection of EMP in thrombi of CD36 wild type and knock out mice.**
Immunohistochemistry of cryo preserved carotid artery sections following FeCl3
injury in A) wild type and B) CD36 knock out mice. Section were stained with
DAPI (blue), CD105 (red), platelets (green) and images merged. The merged
section when compared show less CD105 in the CD36 null mice compared to the
wildtype. (C) Quantification of CD105 fluorescenc in thrombi showed a significant
difference (pvalue=0.006)between WT and null mice.

LEVELS OF CD36 EXPRESSION ON PLATELETS MODULATE PLATELET FUNCTION

Development of an assay to quantify platelet CD36:

Platelet rich plasma from healthy human volunteers was stained with anti CD36 antibody or its isotype control to quantify CD36 expression levels on platelet surface. Mean fluorescence intensity was quantified by a standard curve generated with the PE-Quantibrite Beads (BD Biosciences) using a mixture of beads tagged with defined amounts of PE ranging from 200 to 70000 molecules per bead (Fig 15). Knowing the molar ratio of PE to anti-CD36 immunoglobulin (supplied by the manufacturer) allowed us to calculate the number of bound antibody molecules on each platelet by comparing mean fluorescence intensity to the standard curve, and hence, the number of CD36 molecules per platelet. Fig 16 shows typical anti-CD36 fluorescence histograms of platelets from a high (top panel), low (bottom left) and null (bottom right) donor.
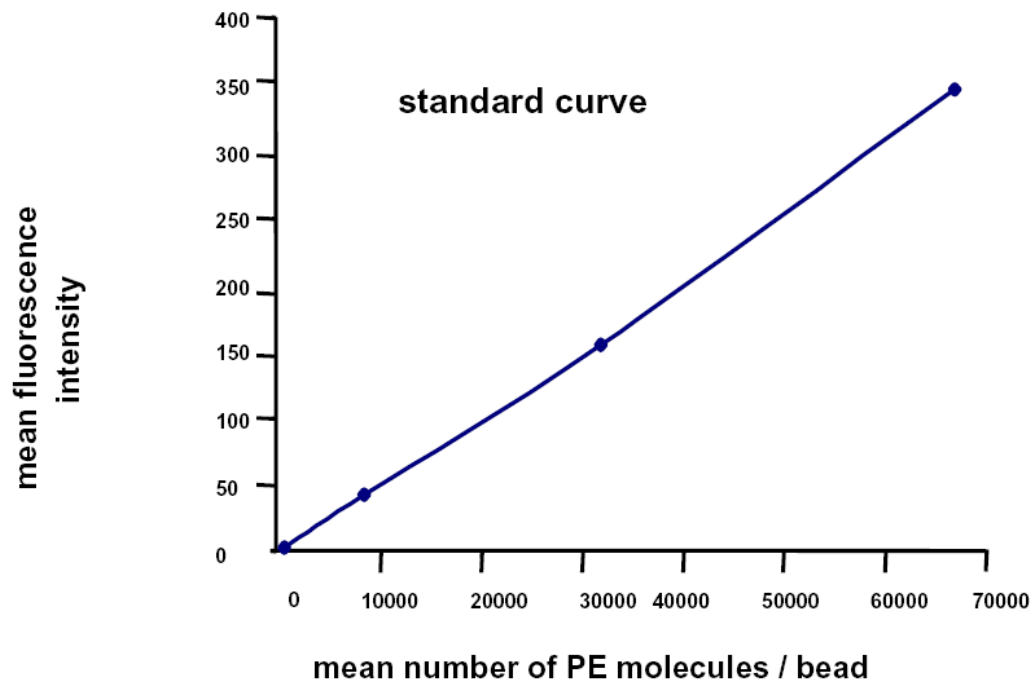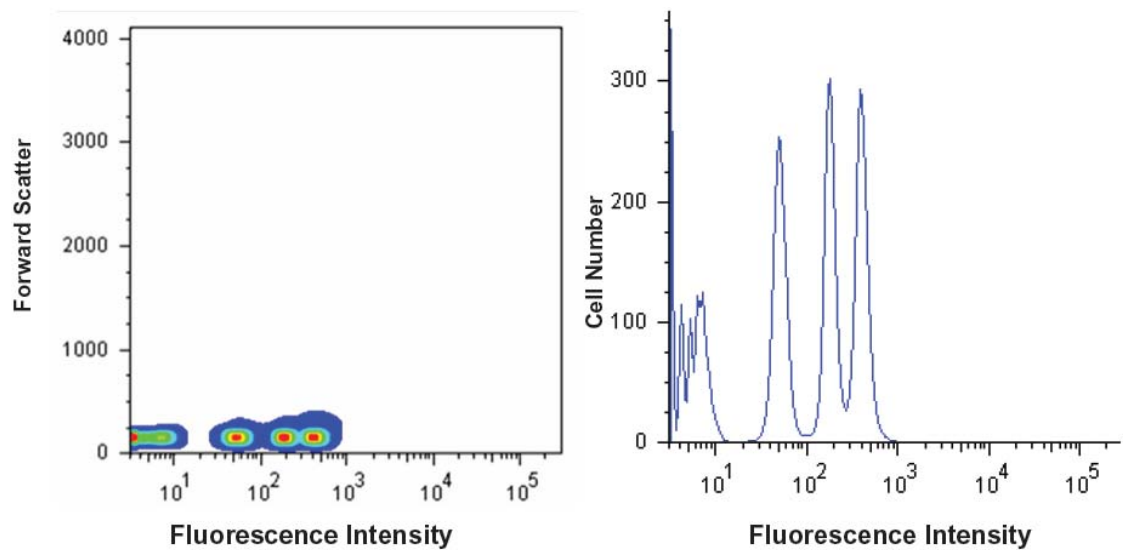
Fig 15. **Quantification of platelet CD36 expression:** Flow cytometry of PE-Quantibrite beads on forward scatter vs fluorescence intensity (upper left panel) and histogram (upper right panel) showing four ranges of fluorescent beads and when plotted against the number of PE molecules per bead as per manufacturer's instructions, a graph standard curve (bottom panel) was obtained.
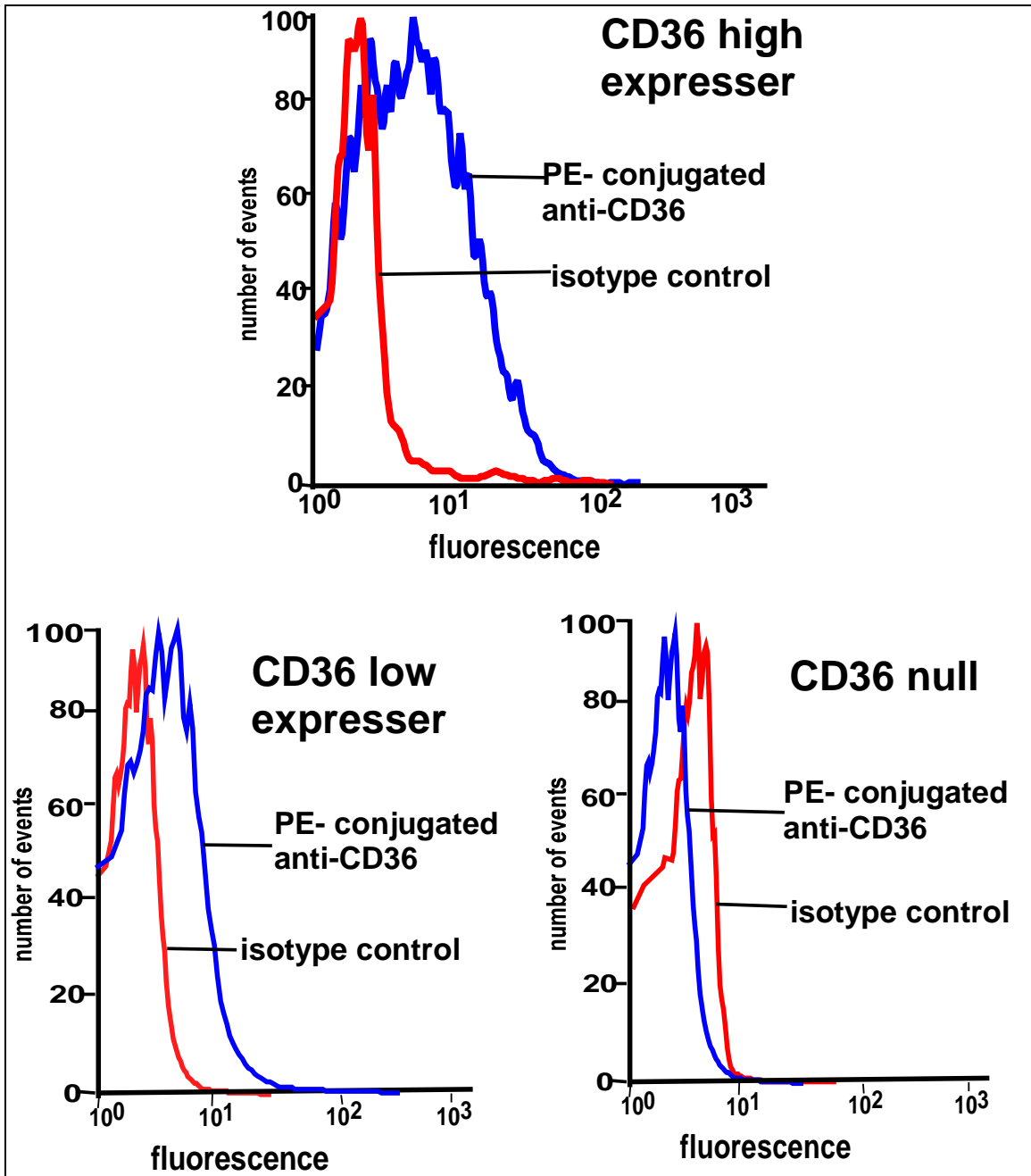
Fig 16. **Detection of CD36 expression in human subjects**: Platelet Rich Plasma (PRP) from human subjects were incubated with either PE-conjugated CD36 antibody (blue) or its isotype control (red) and then analyzed by flow cytometry. This helped to identify CD36 expresser (Top panel), CD36 low expresser (left, bottom panel) and CD36 null donors (right, bottom panel).

CD36 expression on platelets is variable:

Quantification of the flow cytometry data allowed us to assess the range of CD36 expression in the healthy human population. In a sample size of 32 normal healthy volunteers, the CD36 expression on platelets ranged from as low as zero to as high as 14000 molecules per platelet. The mean was 6005±1484, the median was 5766 per platelet.
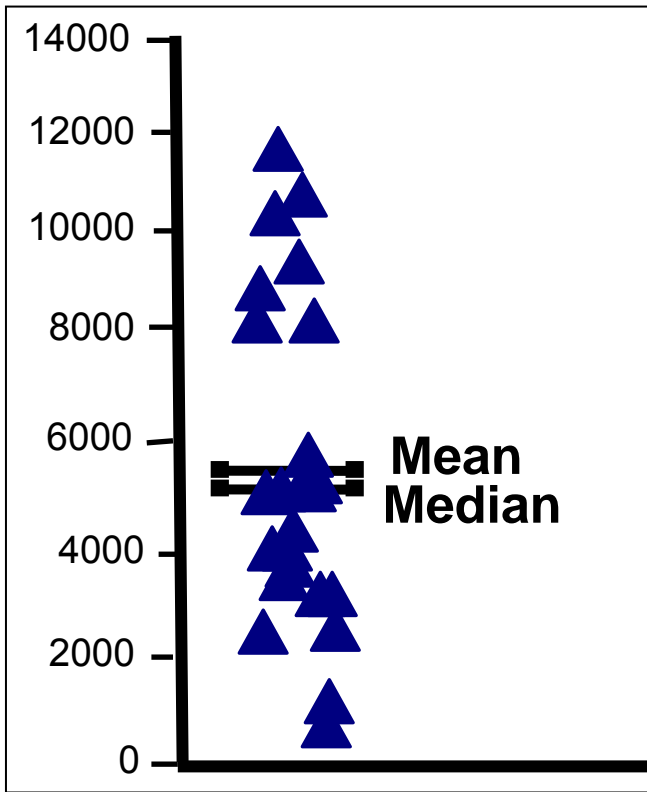
Fig 17: **CD36 distribution in the population is variable.** CD36 expression on platelets in a small group of healthy human volunteers was quantified as described above. The range of CD36 expression was as low as 2000/platelet to as high as 14000/platelet. Also shown are the mean (6005) and median (5766) values.

CD36 expression levels on platelets from individual donors were consistent over time:

Of the 32 normal healthy volunteers tested, a representative group of five individual donors showed consistent levels of CD36 expression on at least five repeated analyses. Analyses done over three years at different times in the day were also consistent (Fig 18).

Donor12 on 8.26.05 **Donor12 on 2.12.06** **Donor12 on 8.22.07**
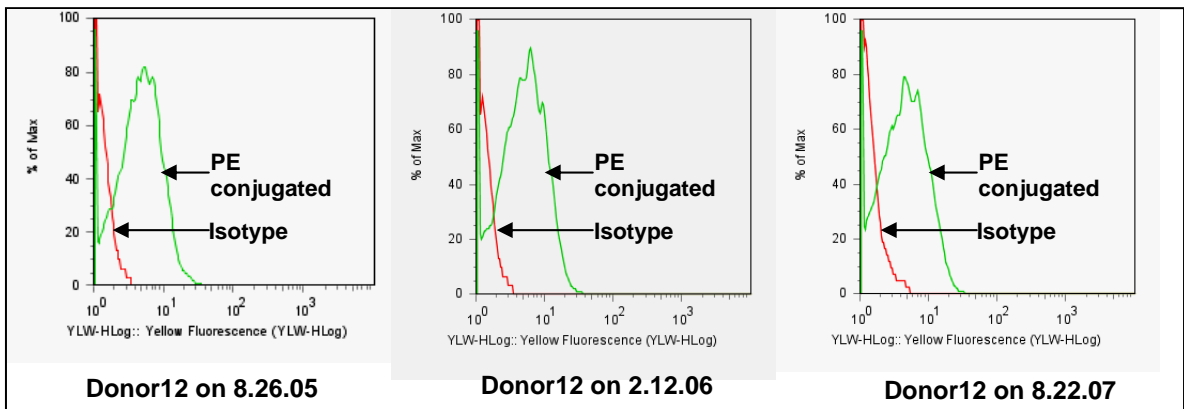
Fig 18. **CD36 expression on platelets were consistent over time**. 3 representative histograms from the same subject over three years (2005, 2006 and 2007) at different times of the day. CD36 expression is shown by green and its isotype control by red.

Table 4: Summary of CD36 analysis in different donors on different days

Donor	Mean±SD	Coefficient of variation
12	2.35± 1	4.32%
10	4.59±1.3	6.54%
9	1.28±0.5	8.35%
31	1.19±0.7	6.16%

Table 4 shows the mean, SD and coefficient of variance for each reading for 4 donors on 4 different time points.

Change of CD36 expression with platelet activation does not show significant difference among donors:

As reported by others (Kestin et al., 1993; Murasaki et al., 2007; Rinder et al., 1991), we found that when activated, platelet CD36 levels increases by about 20%. Importantly, there was no significant difference in the % increase in CD36 expression among donors (high expresser and medium expresser), regardless of resting levels of expression (Fig 19). This suggests that the wide variability in platelet CD36 expression cannot be accounted by differences in degrees of in vivo or ex vivo platelet activation among donors.
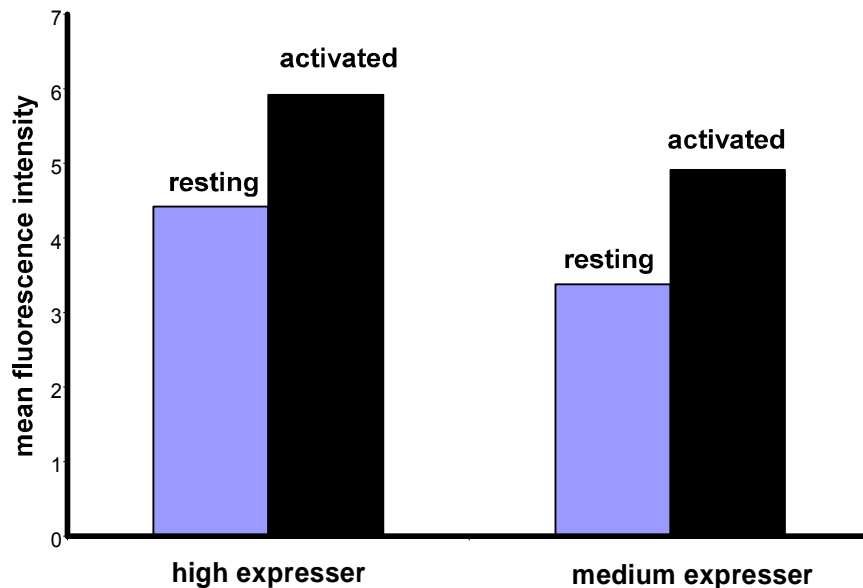
Fig 19. **Difference in platelet CD36 expression between resting and activated platelets.** Platelets from high and medium expresser were stained with either a PE- conjugated anti CD36 antibody or its isotype control. After activation by ADP10uM, platelets from high or medium donors (black bars) showed the same difference in expression when compared to resting (blue bars) platelets.

CD36 surface expression correlates with total CD36 protein:

Variable CD36 surface expression on platelets could be due to differences in cellular localization or distribution rather than total expression. To test this, platelet protein lysates from high, medium and low expessers were subjected to Western Blot analyses to detect total CD36 protein (Fig 20A). Platelet GPIX or actin were used as internal controls (Fig 20B). Platelet CD36 was expressed as a ratio to GPIX and showed considerable variance. We saw a good association (r^2=0.99) between the number of CD36 surface molecules   and the normalized CD36 protein (Fig 20C) suggesting that the variance in CD36 expression was due to differences in megakaryocyte CD36 synthesis and not intracellular distribution.
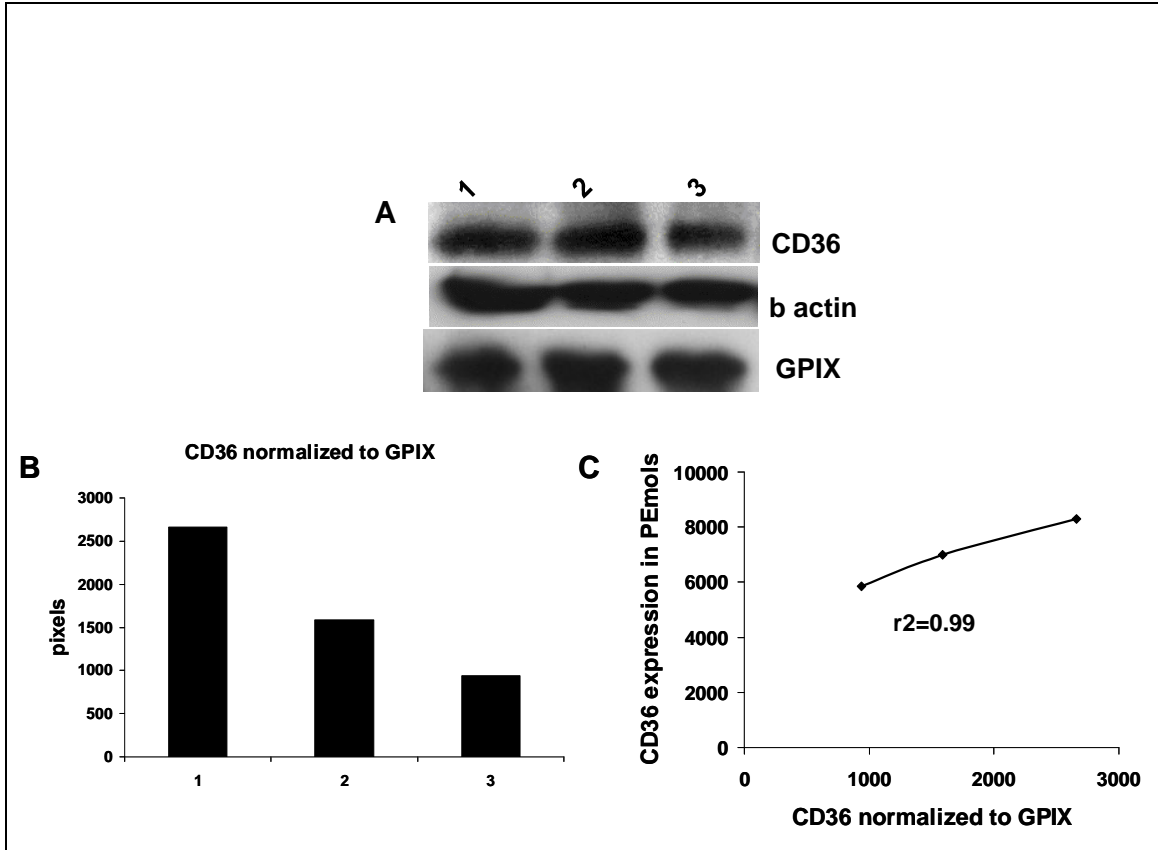
Fig 20. **Platelet CD36 surface expression correlates with total CD36 protein:**
Platelet lysates were immunoblotted (A) with an anti CD36 antibody (clone FA6,
Invitrogen). A specific CD36 band was observed at 88kDa in all samples but of
varying band density. The blot was stripped and reprobed with either a polyclonal
antibody to actin or a monoclonal antibody to platelet GPIX to normalize CD36
expression. Plotting the normalized CD36 expression against the flow based
surface expression of the samples (C), suggested a possible association
between the two (r^2=0.99).

Variability in CD36 expression modulates platelet function:

To assess the functional consequences of this variability in CD36 expression we studied the effect of oxLDL on platelets from donors with high, medium and low levels of expression (Fig 21B). It is known that oxLDL can activate platelets in a CD36 dependent manner (Podrez et al., 2007). To assess platelet activation and aggregation, we incubated PRP or washed platelets with oxLDL. oxLDL induced a significant increase in the rate and extent of aggregation response (Fig 21A, left panel). This effect was not observed in null donors (Fig 21A right panel) demonstrating CD36 dependence.

To determine the effect of oxLDL on platelet activation and secretion, we assessed platelet P-selectin expression, a marker of α-granule secretion. We observed that oxLDL could induce a marked increase in the expression of P-selectin (not shown). We also found a good correlation (correlation coefficient=0.98) between platelet activation by oxLDL and level of CD36 expression (Fig 21C).
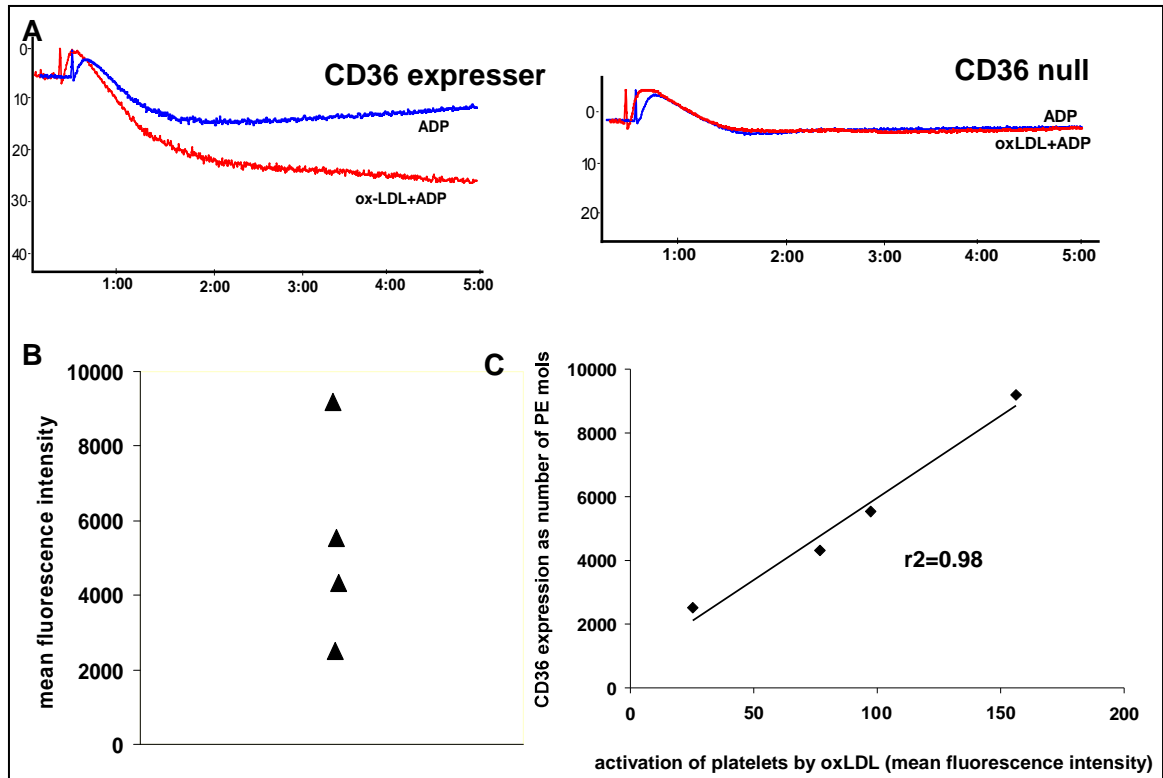
Fig 21. **CD36 expression and oxLDL mediated platelet activation**. PRP from healthy CD36 expressing (A, left panel) or CD36 null (A, right panel) donors were incubated with oxLDL for 30 minutes and then stimulated with ADP 2µM. Aggregation was assessed turbidometrically with a dual chamber aggregometer. Tracings show representative aggregometry curves from n=3. (B) Selection of four normal healthy subjects with their CD36 expression ranging from null to high. (C) Washed platelets from the selected subjects were incubated with oxLDL for 30 minutes and the lipid mediated platelet activation was detected by anti P-selectin antibody. Activation data plotted against surface expression of CD36 suggested a good correlation (0.98).

CHAPTER V

PLATELET CD36 EXPRESSION LEVELS ARE ASSOCIATED WITH GENETIC

POLYMORPHISMS

Platelet CD36 expression is variable: replicability in a larger population:

Using the quantitative immunofluorescence flow cytometric technique to assess

the level of platelet CD36 expression described in Chapter IV, we studied a large

sample size of 567 successive subjects recruited through the Cleveland Clinic

Cardiac Catheterization lab.   As with the normal volunteers, this group also

showed a wide variability in platelet CD36 expression (Fig 22). The mean was

7876±1924 CD36 molecules per platelet and the median was 7611.
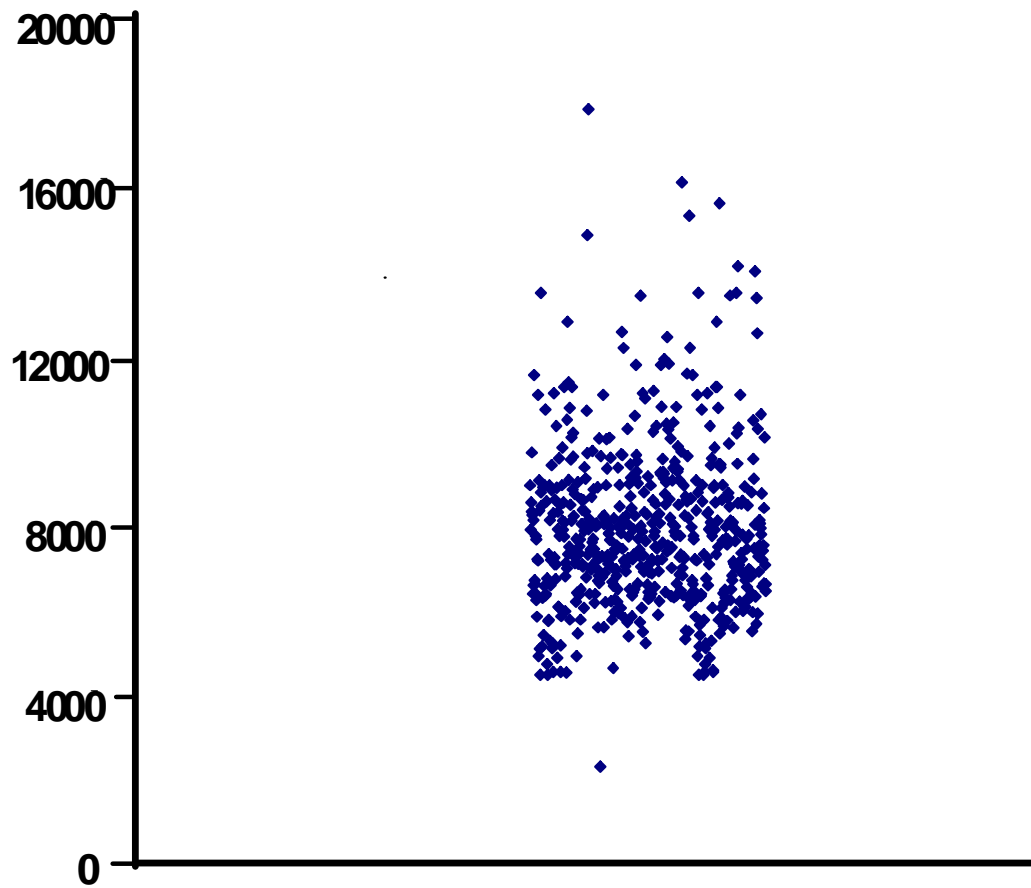
Fig 22: **CD36 distribution in a large patient population:** The variability of CD36 expression in a small group of normal healthy population was replicated in a large (567) patient population. The mean is 7876±1924, median is 7611 CD36 molecules per platelet.

Patient profile:

Table 5 shows the anthropometric and biochemical data for the study subjects focusing on those variables that relate to CD36 function. The reference values for each if the phenotypes were blood cholesterol (CHOL) 200mg/dl; HDL 60mg/dl (in female) and 50mg/dl (in male); serum triglycerides (TG) 200mg/dl; blood glucose (GLU) 120mg/dl; platelet count 150,000/ml and Body mass index (BMI) 30. Most patients had a past history of coronary artery disease (CAD) which was consistent with the fact that they all presented at the Cardiac Catheterization Lab. Almost 70% patients showed history of smoking in the past and more than 80% were hypertensive (criteria, 120/90) and more than 50% reported a history of cardiac disease.

Variable	All Patients (%)	Caucasian Patients(%)
HxCurrentSmoking	68(12.5%)	63(12.7%)
Hx PastSmoking	371(68.2%)	337(67.7%)
Hx CAD	315(56.1%)	295(57.5%)
Hx MI	290(52.6%)	273(54%)
Hx Stroke	43(10%)	38(9.7%)
Hx HTN >120/90	466(85.2%)	427(85.1%)
Hx Diabetes	163(30%)	144(28.9%)
Cholesterol ≥200	117(23.2%)	112(24.2%)
LDL ≥120	100(20.4%)	96(21.3%)
HDL ≥50(M) or ≥60(F)	180(35.8%)	162(35.1%)
Triglyceride(TG) ≥200	90(17.9%)	87(18.8%)
Glucose(GLU) ≥120	126(24.2%)	115(24.1%)
Platelet count ≥150	475(91.5%)	437(91.6%)
BMI >30	260(45.9%)	232(44.7%)
Hx.CABG/PCI	256(45.3%)	245(47.4%)
Stenosis		
Normal	147(26.1%)	128(24.9%)
Mild	104(18.5%)	94(18.3%)
Moderate	27(4.8%)	26(5%)
Severe	285(50.6%)	267(51.8%)

Table 5: **Phenotypic traits of the study subjects.** CHOL=cholesterol; HDL=high density lipopoprotein; LDL=low density lipopoprotein; TG=triglycerides, GLU=glucose; PLT=platelet count; BMI=body mass index; hx=history of; CABG=coronary artery by-pass graft surgery; PCI= per cutaneous intervention. All numbers are total counts and percentages (%).

SNP analysis:

10 tagged SNPs for CD36 were identified from the HapMap website as described in the "Materials and Methods" section (Table 2). Table 5 shows the major findings of our SNP analysis in 567 patients. All the SNPs were in Hardy-Weinberg equilibrium. When the MFI was considered as a continuous variable, the none of the SNPs were found to be associated with CD36 expression levels, although one SNP seemed to have an important effect in females (rs1537593, p value= 0.019). When the MFI was considered as a binary variant (based on median values), three SNPs (rs3211864, p value0.023, OR=0.55; rs3211932, p value=0.02, OR=0.617 and rs1537593, p value=0.03, OR=1.067) were significantly associated with CD36 expression level. In 2 of 3 SNP analyses, owing to small number of subjects and the low frequency of the minor allele, homozygotes or heterozygotes for the rare allele were grouped together. In case of SNP rs 3211864, the presence of the minor allele (AG or GG) was associated with lower CD36 expression (OR=0.55). Similarly in case of rs1537593, the minor allele (TT or CT) was associated with lower CD36 levels. In rs3211932, a common polymorphism, both the T and the C allele were almost equally distributed and only the heterozygous (CT) was significantly associated with levels of CD36.

As Caucasians represent the majority of this population, we reanalyzed the data excluding the African-American subjects (48 of 567). The same effect was observed for the SNPs rs3211864 and rs3211932 in the Caucasian population (519 of 567) (table 6), where the minor alleles were associated with lower levels

of CD36. One interesting observation in the Caucasians was that one of the above three SNPs in Table 5 was not significantly associated with the CD36 expression levels (rs 1537593).

Table 6A: SNP analysis in all patients using MFI as a continuous variable

SNP	Mean±SD(1)	Mean±SD(2)	Mean±SD(3)	ANOVA/t test	r^2	model
rs10499859	3.39±1.4	3.92±1.7	4.1±1.6	0.11	<0.01	codom
rs6968407	4.25±1.7	3.96±1.6	4.22±1.7	0.61	<0.01	codom
rs10499858	3.96±1.6	3.96±1.7		0.75	<0.01	codom
rs9918586	3.95±1.6	3.95±1.6		0.95	<0.01	codom
rs3211864	3.9±1.6	3.6±1.3	2.8±1.9	0.21	<0.01	codom
rs3211869	3.95±1.65	3.88±1.65	2.69	0.51	<0.01	codom
rs3211908	3.9±1.6	3.8±1.8		0.2	<0.01	codom
rs3211932	3.99±1.5	3.8±1.6	3.9±1.7	0.88	<0.01	heterosis
rs1527483	4.08±1.6	4.14±2		0.66	<0.01	codom
rs1537593	3.9±1.7	4±1.5	2.26±0.2	0.12	<0.01	1 dom.

B: SNP analysis in all patients using MFI as a binary variant (median)

MFI	Median < 3.725	Median > 3.725	P-value (FDR)	Odds ratio (95% C.I.)
rs3211864			A/G+G/G vs.A/A	A/G+G/G vs.A/A
A/A	236(84%)	255(90.4%)	vs.A/A	0.555(0.331,0.924)
A/G	43(15.3%)	27(9.6%)	0.023	
G/G	2(0.7%)	0(0%)	(0.234)	
rs3211932				C/T vs. T/T
T/T	78(28%)	100(35.7%)	0.022	0.617(0.422,0.902)
C/T	153(54.8%)	121(43.2%)	(0.125)	C/C vs. T/T
C/C	48(17.2%)	59(21.1%)		0.959(0.592,1.553)
rs1537593				TT+CT vs CC
C/C	225(79.5%)	228(80.9%)	0.038	0.108(0.014, 0.861)
C/T	49(17.3%)	53(18.8%)	(0.127)	
T/T	9(3.2%)	1(0.4%)		

Table 6 **SNP analysis in all patients**. A) SNP analysis in all subjects using MFI as a continuous variable. The genotypes are designated as 1, 2 or 3 and are compared by either ANOVA or T test. A linear correlation coefficient is designated by r^2. For each SNP, the dominant genotype is designated by the model column. None of the SNPs were associated with CD36 expression levels in the in the total population. B) SNP analysis using binary va.lues for MFI. The frequency of each genotype is denoted in percentage (%). P value was corrected with FDR. Only those with P values < 0.05 and FDR values < 0.2 were reported as statistically significant findings. When calculating Odds Ratio, owing to smaller number of subjects, homozygotes for the rare allele (GG in case of SNP rs3211864) and heterozygotes (AG for rs3211864) were grouped as carriers of the G allele.

Table 7A: SNP analysis in 519 Caucasian patients using MFI as a continuous variable

SNP	Mean±SD(1)	Mean±SD(2)	Mean±SD(3)	ANOVA/t test	r^2	model
rs10499859	3.39±1.4	3.92±1.7	4.1±1.6	0.11	<0.01	codom
rs6968407	4.25±1.7	3.96±1.6	4.22±1.7	0.61	<0.01	codom
rs10499858	3.96±1.6	3.96±1.7		0.75	<0.01	codom
rs9918586	3.95±1.6	3.95±1.6		0.95	<0.01	codom
rs3211864	3.9±1.6	3.6±1.3	2.8±1.9	0.21	<0.01	codom
rs3211869	3.95±1.65	3.88±1.65	2.69	0.51	<0.01	codom
rs3211908	3.9±1.6	3.8±1.8		0.2	<0.01	codom
rs3211932	3.99±1.5	3.8±1.6	3.9±1.7	0.88	<0.01	heterosis
rs1527483	4.08±1.6	4.14±2		0.66	<0.01	codom
rs1537593	3.9±1.7	4±1.5	2.26±0.2	0.12	<0.01	1 dom.

7B: SNP analysis in males

SNP	Mean±SD(1)	Mean±SD(2)	Mean±SD(3)	ANOVA/ttest	r^2	model
rs10499859	3.88±1.3	3.92±1.7	3.84±1.5	0.9	<0.01	codomt
rs6968407	3.89±1.6	3.91±1.6	3.94±1.4	0.55	<0.01	codom
rs10499858	3.91±1.5	3.83±1.8		0.62	<0.01	codom
rs9918586	3.9±1.5	3.89±1.6		0.88	<0.01	codom
rs3211864	3.9±1.5	3.66±1.3	3	0.27	<0.01	2 dom
rs3211869	3.898±1.5	3.86±1.6		0.78	<0.01	codom
rs3211908	3.92±1.6	3.79±1.7		0.37	<0.01	codom
rs3211932	3.95±1.6	3.81±1.5	4±1.7	0.6	<0.01	codom
rs1527483	3.85±1.56	4.07±1.8		0.33	<0.01	codom
rs1537593	3.859±1.5	3.97±1.5		0.7	<0.01	codom.

C: SNP analysis in females

SNP	Mean±SD(1)	Mean±SD(2)	Mean±SD(3)	ANOVA/t test	r^2	model
rs10499859	4.01±1.5	3.92±1.7	4.31±1.7	0.16	<0.01	1 dom
rs6968407	4.25±1.7	3.96±1.6	4.22±1.7	0.61	<0.01	heterosis
rs10499858	4.04±1.6	4.47±2		0.39	<0.01	codom
rs9918586	4.04±1.5	4.11±1.6		0.8	<0.01	codom
rs3211864	4.09±1.7	3.7±1.3	2.72	0.52	<0.01	codom
rs3211869	4.07±1.7	3.98±1.7	2.69	0.64	<0.01	codom
rs3211908	4±1.6	3.9±2.2		0.39	<0.01	codom
rs3211932	4.2±1.2	4±1.9	3.9±1.7	0.69	<0.01	heterosis
rs1527483	4.049±1.56	4.434±2.5		0.46	<0.01	codom
rs1537593	4.06±1.7	4.2±1.5	2.26±0.2	0.018	<0.01	1 dom.

D: SNP analysis in all Caucasians: using MFI as a binary (median) variable

SNP	MFI ≤ 3.74	MFI ≥ 3.74	P-value (FDR)	Odds ratio (95% C.I.)
rs3211864*				A/G +G/G vs. A/A
A/A	215	233	A/G +G/G vs. A/A	0.515
A/G	41	24	0.019	(0.302,0.877)
G/G	2	0		
rs3211932				C/T vs. TT
T/T	65	92	0.005	0.529 (0.353,0.792)
C/T	143	107		C/C vs. T/T
C/C	48	57		0.839 (0.510,1.381)

Table 7: **SNP analysis in Caucasian patients**. A: SNP analysis in all Caucasian subjects using MFI as a continuous variable. The genotypes are designated as 1, 2 or 3 and are compared by either ANOVA or T test. A linear correlation coefficient is designated by r^2. For each SNP, the dominant genotype is designated by the model column. None of the SNPs were associated with CD36 expression levels in the in the total (A) or the male (B) Caucasian population. However, in the female population (C) one SNP rs1537593 was significantly associated (p=0.018) with CD36 expression levels. (D) When the CD36 expression was divided into two groups their by median values, analysis revealed two SNPs to be significantly associated with median CD36 expression levels (3.74); rs3211864 and rs3211932. P value was corrected with FDR. Only those with P values < 0.05 and FDR values < 0.2 were reported as statistically significant findings. Odds ratio (OR) for each SNP was calculated with 95%CI.

Haplotype analysis:

Haplotype analyses in all patients revealed (Table 8) that a number of haplotypes were associated with CD36 expression. In all instances, the p values were corrected by TPPFP which is midway between the most conservative bon-Ferroni and the least conservative False Discovery Rate (FDR) statistic. Considering TPPFP at a significance level of 0.2, 4 haplotypes were found to be significantly associated with CD36 expression levels. These are CA, GTGT, TGTC, and GTGTC. Of these, the most significant haplotype was GTGTC (3,4,5,6,7), p value= 0.0044, TPPFP= 0.13. G allele of SNP5 (rs3211864) seemed to have an important effect in the outcome of these haplotypes as seen with the SNP analysis.

Table 8: Haplotype analysis of CD36 expression in all patients

Trait	SNPs	P value	TPPFP	OR(95%CI)	significant
MFI	4,5	0.0068	0.2042	0.61 (0.39,0.96)	CA
				0.46 (0.27,0.78)	TG
	5,6	0.0141	0.4218	0.48 (0.28,0.81)	GT
	3,4,5	0.0101	0.3028	0.59 (0.37,0.94)	ACA
				0.45 (0.26,0.77)	GTG
	4,5,6	0.0092	0.2772	0.47 (0.28,0.81)	TGT
	5,6,7	0.0283	0.4545	0.53 (0.32,0.87)	GTC
	3,4,5,6	0.0064	0.1909	0.47 (0.28,0.78)	GTGT
	4,5,6,7	0.0067	0.2013	0.48 (0.28,0.82)	TGTC
	2,3,4,5,6	0.0095	0.2844	0.47 (0.28,0.8)	CGTGT
	3,4,5,6,7	0.0044	0.1321	0.46 (0.28,0.78)	GTGTC
	4,5,6,7,8	0.0152	0.4545	0.42 (0.24,0.73)	TGTCT

Table 8. **Haplotypes associated with MFI**. showing the haplotypes associated with CD36 expression. P value, adjusted P value (TPPFP) and OR (Odd's ratio) of the significant base is also shown. GTGTC was the haplotype associated most significantly with levels of CD36 (p= 0.0044, TPPFP= 0.42). The Odds of this haplotype of being associated with the CD36 levels was 0.42.

CHAPTER V

DISCUSSION

Endothelial derived microparticles bind and activate platelets in a CD36 dependent manner

In the present study we demonstrated using inhibitory antibodies, a competitive ligand (oxLDL), and platelets from CD36 null donors, that EMP bound to resting platelets in a CD36-dependent manner. Binding was assessed by both flow cytometry and immunofluorescence microscopy. Our studies using annexin V or more specifically a monoclonal antibody to PS to mask surface PS/oxPS suggest that it is PS exposed on EMP surfaces during their formation that serves as a ligand for platelet CD36. This is consistent with previous studies from our lab and others that showed that PS and/or oxPS serve as a ligand on apoptotic cells and shed photoreceptor outer segments facilitating CD36-

dependent recognition and internalization by phagocytic cells (Greenberg et al., 2006; Ryeom et al., 1996; Sun et al., 2006). In these previous studies, CD36 was shown to initiate a signal in the phagocyte that resulted in internalization of the bound particle (Greenberg et al., 2006; Ryeom et al., 1996; Sun et al., 2006).

We and others have also defined CD36-mediated signals in macrophages that are required for oxLDL uptake and foam cell formation and for clearance of bacterial products (Stuart et al., 2005). Since platelets are probably not phagocytic cells, we hypothesized that CD36 signaling induced by MP might modulate platelet activation. To test this hypothesis, we demonstrated using both turbidometric platelet aggregation studies and flow cytometry assays that EMP augment platelet aggregation at low doses of the weak agonist ADP (1-4 µM). This effect was abrogated by CD36 inhibitory antibodies and was not observed in platelets from CD36 null donors.

The classic platelet activation model suggests that agonists, such as collagen, thrombin and epinephrine, interact with platelet surface receptors, most of which are G protein coupled receptors (GPCR), to initiate intracellular signaling events that lead to activation of integrins, reversible platelet aggregation, and secretion of platelet granule contents. "Outside-in" signals mediated by integrin $\alpha_{2b}\beta_3$ and additional GPCR activation via secretion of ADP and thromboxane result in enhancement of the signal and ultimately stable platelet aggregation and thrombus formation. Recent studies have significantly advanced and refined this model. A large number of signaling molecules (receptors and ligands) have been identified that appear to act within the platelet-

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platelet contact zone after the aggregation event. These include ephrins and their receptors, eph kinases (Prevost et al., 2005; Prevost et al., 2004), gas6 (Angelillo-Scherrer et al., 2005; Angelillo-Scherrer et al., 2001), and its tyrosine kinase receptors, mer, tyro3, and axl, PECAM-1 (Falati et al., 2006), CD40 and CD40L (Henn et al., 1998; Hermann et al., 2001) and semaphorin 4D (Zhu et al., 2007). It has been suggested that after aggregation, platelets form a "synapse" (Brass et al., 2006) facilitating signaling by membrane tethered receptor/ligand pairs and localizing secreted and shed ligands. This promotes growth and stability of the thrombus (Jackson, 2007).

Our studies suggest that CD36 can also function as a signaling receptor on platelets capable of modulating platelet activation and aggregation. Unlike the case of most of the receptor-ligand pairs described above, CD36 may function on resting platelets to signal and sensitize platelets to activation by other agonists. Importantly, the ligands that we have shown to bind CD36 on platelets (oxidized lipids and EMP) are generated as a consequence of diseases known to be associated with increased thrombotic risk. We thus propose a model whereby CD36 ligands presented to platelets renders them "hyperreactive", predisposing patients to pathological thrombosis. In support of this model, we demonstrated that both EMP isolated from HUVEC culture and MP isolated from the blood of healthy human subjects augmented platelet aggregation in a CD36-dependent manner. In times of pathological stress, the number of circulating MP presenting to platelets increases, leading to binding and activation and this might account, at least in part, for the higher risk of thrombosis in these stressful conditions. Our

model does not preclude a role for CD36 in the platelet synapse. It is also possible that ligands such as EMP that are generated during an acute thrombotic event could interact with CD36 in the platelet contact zone to increase responses to other signals and thereby provide a positive feedback loop during normal hemostasis.
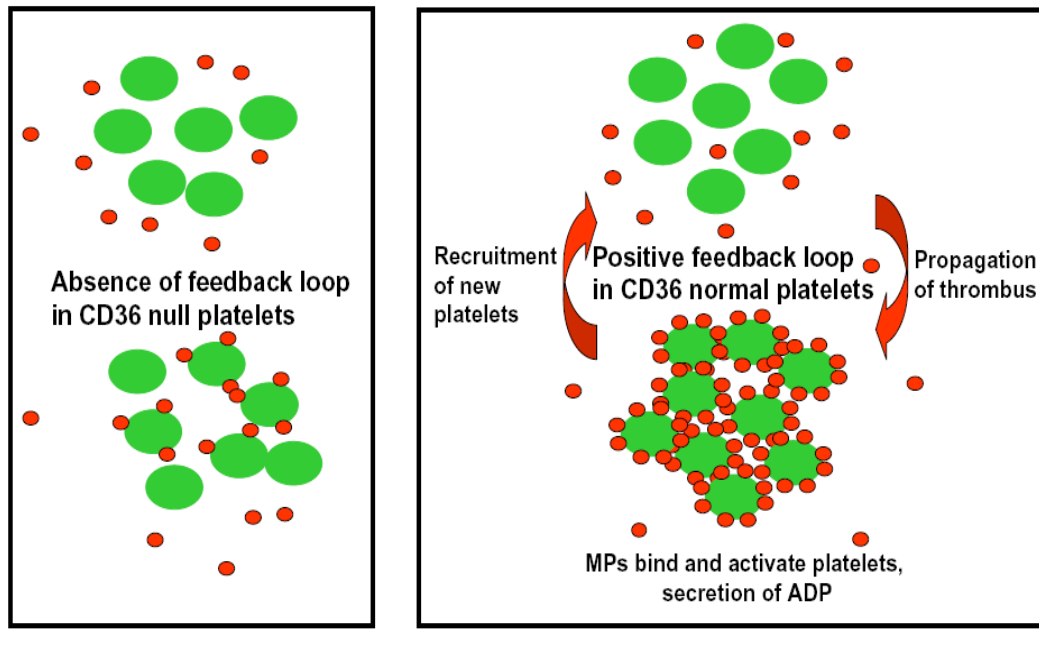
Fig 23. **Model showing platelet CD36-MP interaction**. EMP bind platelet CD6 and in turn can activate platelets, thus leading to recruitment of more platelets in a thrombi of CD36 expressing platelets(right panel) forming a feed forward loop promoting thrombus formation; whereas in the CD36 null platelets, this loop is absent

In fact our data supports this; ADP dose response in CD36 null donors was blunted compared with CD36 expressing donors. The mechanism of this effect is unclear but our in-vivo study showed decreased EMP (CD105) staining in the thrombi from CD36 null mice suggesting that MP generated during normal hemostasis might serve as a ligand to facilitate platelet activation. Thus CD36 could potentially modulate platelet function in both resting state as well as after platelet activation in the platelet synapse.

Levels of CD36 expression on platelets modulate platelet function

In the first part of our studies we established the hypothesis that CD36-ligand interactions could "prime" or sensitize platelets to activation by low doses of agonists. We had seen that the absence of CD36 could significantly alter platelet function. We thus hypothesized that levels of CD36 expression in healthy individuals could modulate platelet function.

We reported here using a quantitative flow cytometric method that CD36 expression on resting platelets among individual donors is highly variable with expressions ranging from 200 to 14000 molecules/ platelet. A recent study in this lab, in collaboration with others, has characterized the role of oxLDL as a ligand for platelet CD36 and shown that oxLDL can mediate platelet activation (Podrez et al., 2007). We showed here by flow cytometry and platelet aggregation studies that oxLDL could modulate platelet function in a CD36 dependent manner.

When we selected four donors who were high, middle or low expressers and assessed platelet activation after pre-incubation with oxLDL, we observed that the extent of oxLDL induced platelet activation correlated closely with CD36 levels on the platelets (r^2=0.98) (Fig 21). These data suggest that individuals with high expression of CD36 on platelets might be more susceptible to platelet activation and, in turn, more prone to acute thrombotic events, while those with low levels of expression might have lower risk.

The mechanisms responsible for variability of platelet CD36 expression are not known. We propose here that a component of the variability might be

genetic, but it is also likely that non-genetic e.g. environmental factors contribute. CD36 expression in monocytes and macrophages has been shown to be regulated by a number of conditions and agents. For example, in monocytes and macrophages PPARγ agonists, statins, vitamin E, HIV protease inhibitors, diet (fat), hyperglycemia, oxLDL, cytokines (IL4, TGFb, M-CSF) have been shown to influence CD36 expression (Feingold et al., 2004; Feng et al., 2000; Han et al., 2000; Han et al., 1999; Huh et al., 1995; Huh et al., 1996; Kwok et al., 2007; Tontonoz et al., 1998), but it is not known whether any of these agents could affect platelet CD36 expression at the megakaryocytic level.

CD36 (GPIV) expression on platelets has also seen to vary in certain disease conditions. For example, Rinder et al had showed that cardiopulmonary bypass produced significant increase in platelet Glycoprotein IV expression after 2-4 hours of the bypass (Rinder et al., 1991). Changes in platelet surface CD36 expression were also observed by Kestin et al who reported an increase in expression of GPIV detected by the monoclonal antibody OKM5 in a group of sedentary subjects after strenuous exercise (Kestin et al., 1993). In another report, CD36 expression detected by two antibodies, OKM5 and GS95 in subjects who underwent coronary stenting surgery reflected the level of platelet mediated restenosis (Murasaki et al., 2007). In all of these studies increase in CD36 expression was associated with evidence of platelet activation. This is consistent with our study where we showed that platelet CD36 expression increased by 20% after activation.

Platelet CD36 expression levels are associated with genetic

polymorphisms

CD36 is a highly polymorphic gene, yet systematic studies related to its genetic polymorphisms and their functional consequences are limited. In one of the few studies reported, 5 polymorphisms in Caucasians were used to define two specific LD blocks in the CD36 gene and thereby classify the population into

several haplotypes (Ma et al., 2004). There was a correlation between one of these haplotypes and circulating free fatty acid levels. Since CD36 is a fatty acid transporter, the results suggested an association with CD36 expression on fat and/or muscle, but there was no experimental evidence to support this.

Generally, a haplotype is a collection of SNPs that are inherited together. This coinheritance suggests that some of these SNPs may be strongly linked to each other; this is known as linkage disequilibrium (LD). This strong association also implies that analysis of any one of these SNPs within a region of LD would provide us information on all the other SNPs in that region. These "informative" SNPs are known as Tagged SNPs (Project, 2003). In our study, we identified 10 regions of LD based on data available through the Human Genome project and then chose 10 tagged SNPs for analysis from each region based on the criterion of at least 5% frequency of the minor allele.

We then used these ten tagged SNPs to genotype 567 unrelated subjects who presented to the Cleveland Clinic Cardiac Catheterization lab for cardiovascular complaints. SNP analysis revealed that 3 SNPs were significantly associated with CD36 levels. Interestingly, both the minor alleles of these 3 SNPs were associated with low CD36 expression.

Polymorphisms can affect the protein expression levels by several mechanisms. An unstable protein can result if the polymorphism is within the coding region of the gene. These mutant proteins are often mistargeted and degraded, for example, CD36 null mutation C478T. Such polymorphisms could result in single amino acid changes or could cause a frame shift, generating a

missense and /or translation stop codon and reduce transcripts (as seen in some CD36 null mutations). Polymorphisms can also occur in non-coding exons that can affect the mRNA stability or mRNA translational efficacy. SNPs located at the intron-exon splice site could alter protein structure or expression by altering splicing. SNPs in the gene promoter/ enhancer regions could affect transcription factor binding and thus influence mRNA transcription. Recently, mutations/ polymorphisms in regulatory micro RNA binding sites have been described and related to transcriptional activity.

SNPs can be either synonymous (silent) or non-synonymous (a change in the protein). While non-synonymous SNPs are associated with most single gene diseases, most SNPs are non-coding and most SNPs identified in genetic association studies of complex multigenic diseases are non-coding. Most CD36 SNPs identified to date are in the non-coding region. In our study, all 10 tag SNPs were non-coding. They were mostly intronic, although one of them was in the 5' untranslated region of the mRNA coding region (rs9918586). All the 3 SNPs significantly associated with low CD36 expression levels in our study were intronic. Although we have shown an association between these 3 SNPs and CD36 levels, it does not imply that these SNPs are responsible for reduced CD36 protein expression. These tag SNPs probably predict the presence of other SNPs which are in LD and these are equally or more likely to be responsible for the change in CD36 protein expression.

There have been several studies in the past on polymorphisms of platelet receptors and their functional impact. The most clinically relevant platelet

polymorphisms involve surface glycoprotein molecules that play a key role in platelet adhesion, activation and aggregation. These include, α_{3a}, α_{2b}, GPIbα, GPIbβ and integrin $\alpha2$. For example the PIA^2 polymorphism (leu/pro 33, 1565T/C), first shown by Kunicki and Aster (Kunicki and Aster, 1978) is a T→C substitution at position1565 in Exon 2 of $\alpha_{2b}\beta_3$ gene (Newman et al., 1989). There is conflicting evidence, however about whether this mutation could affect platelet function. The same group also identified a Sra polymorphism in $\alpha3a$ (Arg636→cys) (Santoso et al., 1994) but this was not associated with any change in platelet expression of $\alpha_{2b}\beta_3$ on the platelet nor with any platelet adhesive function. Afshar-Kharghan identified a Kozak-sequence polymorphism in GP1bα (-5C→T) where the -5C allele is associated with increased surface expression of GP1bα on platelets or cell lines expressing these mutants (Afshar-Kharghan et al., 1999). Kozak sequence consensus states that translation is most efficient with C at -5 position of the start codon ATG (Kozak, 1984) In the same lines, the Br^a polymorphism (Br 1648G→A) was associated with lower platelet expression levels of integrin $\alpha2\beta1$ (Kunicki et al., 1997), and in the -807C→T polymorphism, the T allele was associated with higher levels of $\alpha_2\beta_1$ (Corral et al., 1999a; Corral et al., 1999b).

The present study structure does not give us enough information about CD36 inheritance in general. This draws our attention to the fact that the population under discussion is a mixed population in terms of health and disease including hypertension, obesity, smoking and atherosclerosis. Also, there has been considerable evidence that hydroxylmethyl-glutaryl CoA inhibitors or statins

can actually reduce the platelet surface expression of CD36 and thus reduce platelet reactivity to oxLDL, thereby contributing towards anti-thrombotic risk (Bruni et al., 2005; Puccetti et al., 2002; Puccetti et al., 2003; Puccetti et al., 2005). Our sample group is heterogenous, some of patients may or may not have been on statins, and this may mask some of the genetic effects. We therefore need to repeat the study in a large normal healthy population who are not on any medication and without any risk factors for atherosclerotic diseases to address this issue.

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APPENDIX

<u>Association of CD36 tagged SNPs with other phenotypes:</u>

Although not included in the initial proposal, accessibility to other phenotypic data (Table 4, Chapter V) for the patient population through Gene Bank allowed us to do association analyses between these phenotypes and CD36 genotypes. Pilot studies revealed that CD36 SNPs were associated with BMI (body mass index). Considering all patients, G allele in the SNP rs3211864 was significantly associated with lower BMI (body mass index) (P value= 0.001). Interestingly, the minor allele of the same SNP was also associated with lower levels of CD36 expression (Chapter IV). Similar results were obtained with analysis of the data in Caucasian patients (data not shown). Haplotype analyses also revealed an association of CD36 with BMI (Table 2). This association is probably relevant because our group in collaboration with others have shown that body weight of CD36 null male (less so in female) mice were lower than their age matched wild type controls (Goudriaan et al., 2002). We also showed that the daily food intake was also lower in CD36 null mice compared to the wild types. Interestingly, Laugerette et al demonstrated that CD36 was present at the apex of taste buds and acted as "oral sensor" of fat. CD36 null mice were less attracted by fatty diet (Laugerette et al., 2005) suggesting that this could contribute towards less body weight and BMI. Recently, a study of 219 Korean patients with severe coronary artery disease revealed that homozygosity of the polymorphism (TG repeat in intron 3) was significantly associated with higher BMI in men compared to control male subjects (Min Yun et al., 2007). We show here that CD36 SNPs could

associate with BMI, however further analysis with a large number of normal control subjects is required to conclude anything definitive.

Table 1: SNP analysis for BMI in all patients

Phenotype			Pvalue (FDR)	Odds ratio (95% CI)
BMI	≤30	>30		
rs3211864			A/G+G/G vs. A/A	
A/A	279	213	0.001	2.317
A/G	26	44	(0.014)	(1.388,3.870)
G/G	0	2		
rs1527483			A/G vs. G/G	
G/G	266	213	0.041	1.641
A/G	35	46	(0.207)	(1.020,2.639)

Table 1: **SNPs associated with BMI.** Two SNPs were significantly associated with BMI. The p value is supplemented with (FDR). Odds Ratio with 95%CI for each SNP was calculated.

Table 2: Haplotype analysis in all patients: BMI

Trait	SNPs	Pvalue	TPPFP	OR(95%CI)	significant
BMI	4,5	0.0036	0.1087	2.27 (1.36,3.79)	TG
	5,6	0.0036	0.1066	2.31 (1.39,3.86)	GT
	3,4,5	0.0033	0.0981	2.5 (1.49,4.22)	GTG
	4,5,6	0.0132	0.3951	2.34 (1.41,3.89)	TGT
	5,6,7	0.0065	0.1964	2.32 (1.39,3.88)	GTC
	2,3,4,5	0.0133	0.3951	2.5 (1.49,4.2)	CGTG
	3,4,5,6	0.0093	0.2803	2.53 (1.51,4.24)	GTGT
	4,5,6,7	0.0183	0.4128	2.29 (1.37,3.84)	TGTC
	5,6,7,8	0.0124	0.3733	2.42 (1.02,5.75)	AACT
				2.17 (1.25,3.75)	GTCT
	2,3,4,5,6	0.0113	0.339	2.47 (1.47,4.15)	CGTGT
	3,4,5,6,7	0.0138	0.3996	2.54 (1.52,4.27)	GTGTC
	5,6,7,8,9	0.0026	0.0771	2.59 (1.07,6.27)	AACTG
				2.36 (1.1,5.05)	ATCCA
				2.21 (1.27,3.86)	GTCTG

Table 2: **Haplotypes associated with BMI.** Haplotype analysis revealed several haplotypes to be associated with BMI. P value and TPPFP for each haplotype combination are shown. OR with 95% CI are also shown.