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Zachary Bennett Fulkerson
University of Kentucky, zachary.fulkerson@gmail.com

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Zachary Bennett Fulkerson, Student

Dr. Susan S. Smyth, Major Professor

Dr. Bret Smith, Director of Graduate Studies

LYSOPHOSPHATIDIC ACID
PRODUCTION AND SIGNALING IN PLATELETS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Zachary Bennett Fulkerson

Lexington, Kentucky

Co-Directors: Dr. Susan S. Smyth, Professor of Internal Medicine
and Dr. Mariana Nikolova-Karakashian, Associate Professor of Physiology

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

LYSOPHOSPHATIDIC ACID
PRODUCTION AND SIGNALING IN PLATELETS

Lysophosphatidic acid (LPA) belongs to a class of extracellular lipid signaling molecules. In the vasculature, LPA may regulate platelet activation and modulate endothelial and smooth muscle cell function. LPA has therefore been proposed as a mediator of cardiovascular disease.

The bulk of circulating LPA is produced from plasma lysophosphatidylcholine (LPC) by autotaxin (ATX), a secreted lysophospholipase D (lysoPLD). Early studies suggest that some of the production of circulating LPA is platelet-dependent. ATX possesses an N-terminal somatomedin B-like domain suggesting the hypothesis that ATX interacts with platelet integrins which may localize ATX to substrate in the membrane and/or alter the catalytic activity of ATX. Using static adhesion and soluble binding assays we found that ATX does indeed bind to platelets and cultured mammalian cells in an integrin-dependent manner which is blocked by integrin function-blocking peptides and antibodies. This binding increases both the activity of ATX and localization of its product, LPA, to the platelet/cell membrane.

LPA is generally stimulatory to human platelets although platelets from a small population of donors are refractory to LPA stimulation. Likewise LPA is inhibitory to murine platelets. We previously found that LPA receptor pan-antagonists reduce agonist-induced platelet activation, and partial stimulation of LPA5 specifically increases platelet activation in humans. Since both LPA5 and LPA4 are present at significant levels in human platelets, we hypothesized that LPA4 is responsible for an inhibitory pathway and LPA5 is responsible for an inhibitory pathway. We used mice deficient in LPA4 to test this model. Isolated platelet function tests revealed no major difference between *lpa4-/-* mice

compared with WT mice although *lpa4-/-* mice were more prone to $FeCl_3$-induced thrombosis. Paradoxically, chimeric mice reconstituted with *lpa4-/-* deficient bone marrow derived cells were protected from thrombosis. These discrepancies may be explained by involvement of endothelial cells and the relative scarcity of LPA receptors in murine platelets compared with human platelets.

Taken together, these results demonstrate two critical regulators of LPA signaling and open up new avenues to further our understanding of atherothrombosis.

KEYWORDS: lysophosphatidic acid, platelet, autotaxin, signaling, integrin, G protein-coupled receptor

Zachary Fulkerson

November 23, 2011

LYSOPHOSPHATIDIC ACID
PRODUCTION AND SIGNALING IN PLATELETS

By

Zachary Bennett Fulkerson

Susan S. Smyth
Co-Director of Dissertation

Mariana Nikolova-Karakashian
Co-Director of Dissertation

Brent Smith
Director of Graduate Studies

November 23, 2011
Date

Dedicated to my father, James, who first instilled in me a love and appreciation for science and my mother, Susan, who showed me the meaning of serving others through health care.

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CHAPTER 1: INTRODUCTION

Diseases of the vascular system continue to be the most common cause of mortality in the United States with over 30% of deaths in the year 2007 being a result of heart disease or cerebrovascular disease[1]. As such, there has been much interest in characterizing vascular physiology and pathophysiology. The role of bioactive lipids in mediating vascular physiology and pathophysiology is becoming increasingly understood and appreciated. Of these, the major bioactive lipids that act on cell surface receptors are sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), both of which circulate in the blood. In the vasculature, LPA may regulate endothelial and smooth muscle cell function as well platelet function. In addition, there is evidence suggesting that platelets play a vital role in production of circulating LPA raising the question of whether locally produced platelet-derived LPA may have a role in modulating acute and chronic cardiovascular insults. To that end, the work presented herein addresses novel aspects of the role of platelets in LPA generation and the role of LPA in platelet signaling.

ROLE OF PLATELETS IN NORMAL PHYSIOLOGY

Platelets are small, anucelate cell particles that are derived from bone marrow megakaryocytes. Once activated, they function to maintain the integrity of injured vasculature by forming an initial hemostatic plug at the site of injury and subsequently aiding in the initiation of the coagulation cascade. The hallmarks of platelet activation

include adhesion, shape change, granule secretion, and cohesion (aggregation). These sequelae form the foundation of the role that platelets play both physiologically or pathologically.

Platelets form intricate and complex interactions with other blood cells and vascular cells. Most platelets are circulating in a resting state exhibiting a small discoid morphology. Even in this quiescent condition, interactions with other cells are of paramount importance. The resting state is maintained largely by platelet inhibitors such as nitric oxide (NO) and prostaglandin I2 (PGI2) which are derived from healthy endothelial cells[2, 3]. These inhibitors in general mediate increases in intraplatelet cyclic nucleoside monophosphates such as cyclic adenosine monophosphate (cAMP) which is refractory to platelet activation[2, 4]. The normal inhibition that healthy, intact endothelium exerts on platelets is perturbed during injury of the vessel.

Both collagen and von Willebrand factor (vWF) are present in the subendothelial matrix and are subseqnuently exposed when a vessel is damaged. Initial interactions of platelets with the subendothelial matrix occur through platelet receptor complex glycoprotein (GP) Ib-IX-V which binds to VW. Both vWF and GPIb-IX-V are required for adhesion of platelet under high shear conditions such as what is encountered in the arterial system[5]. Following adhesion, activation of the platelet itself occurs. Activation of platelets is elicited by many platelet agonists *in vitro*, but in the context of adhesion *in vivo*, it is mostly initiated by collagen which signals primarily through GPVI[6] and thrombin (factor IIa) which signals primarily through protease-activated receptor 1

(PAR1) and PAR4[7, 8]; thrombin itself is generated at sites of injury by the activity of tissue factor and other coagulation factors.

Collagen and thrombin, as with other platelet agonists, effect activation by signaling through a number of different pathways. Ultimately these pathways converge leading to the sequelae mentioned previously. Actin reorganization and polymerization leads to shape change which transforms the platelet from a discoid shape to their activated conformation[9] allowing them to spread to cover the injured area as well as increase contact with other platelets/cells. Exocytosis of α and dense granules result in secretion of numerous agents including adenosine diphosphate (ADP), serotonin, fibrinogen, fibronectin, *etc.*[10] Activated platelets furthermore produce the platelet agonist thromboxane A2 (TXA2)[11] as well as thrombin[12], a platelet agonist and an essential molecule in the coagulation cascade. Each of these substances promotes the growth of the hemostatic plug largely by recruiting and activating additional platelets. Fibrinogen is a multivalent ligand that can bind to αIIbβ3[13], an integrin receptor only present on platelets. Integrins exist in low-affinity and high-affinity states which may be regulated by intracellular processes; in platelets integrins transition to the high-affinity conformation during platelet activation allowing them to binding tightly with ligands such fibrinogen. Cross-linking of fibrinogen with multiple platelets allows them to cohere to each other resulting in aggregation which also allows the thrombus to grow[13]. αIIbβ3 further associates with platelet cytoskeletal elements[14] and over time contractile elements act on the cytoskeleton to allow the clot to retract[15]. Each of these hallmarks

– adhesion, shape change, granule secretion, and aggregation – allow platelets to perform their best-characterized function, hemostasis.

Outside of their traditional roles in hemostasis, it is becoming increasingly clear that platelets also play a role in other physiological processes such as angiogenesis, microbial defense, and inflammation. Platelets contain a number of substances that promote angiogenesis in their granular and membranous arsenal including a number of growth factors, CD40 ligand (CD40 is located on endothelial cells), phospholipids, matrix metalloproteases, *etc.*[16] Angiogenesis inhibitors are also found in platelet granules including angiostatin, platelet factor 4 (PF4), endostatin, and transforming growth factor (TGF) β [16]. Platelets also directly interact with the endothelium[17] suggesting this could also be a regulatory point for angiogenesis. Furthermore, both *ex vivo* and *in vivo* experimental evidence has demonstrated that platelets promote angiogenesis[18].

In the context of microbial responses, platelets possess a number of immune-related receptors including receptors for the Fc domain of immunoglobulins (FcγRII)[19] and various adhesive receptors[20]. They are furthermore able to interact directly or indirectly with bacteria[21, 22], viruses[23], protozoa[24], and fungi[25]. Many bacteria have been shown to elicit platelet activation and aggregation[21], and platelets are even capable of phagocytosis[26] or aiding in the immobilization of pathogens within platelet aggregates[22]. Aside from interaction with microbes, platelets also intricately interact with other inflammatory cells both directly and indirectly. Activated platelets interact directly with leukocytes and secrete chemokines which recruit leukocytes to sites of injury[16].

Platelets also indirectly facilitate recruitment of leukocytes as they induce increased expression of adhesive molecules and secretion of chemokines by endothelial cells[27]. Platelets are clearly an important player in the inflammatory process.

While the physiological role of platelets has largely been associated with hemostasis, platelets are more and more becoming appreciated for the integral role they play in other functions such as angiogenesis, immunity, and inflammation in both normal physiology and in the context of human disease.

ROLE OF PLATELETS IN CARDIOVASCULAR DISEASE

Historically, much of the impetus for studying platelet function in the context of human disease was in the context compromised hemostasis (*ie*, non-functional or absent platelets). The association between hemorrhagic disorders and thrombocytopenia has been observed since the late 19th century. Subsequently the role of platelets in hemostasis became clearer once it was determined that bleeding in thrombocytopenic individuals could be corrected by whole blood infusion[28]. Later platelet transfusion was found to likewise be therapeutic in treating such disorders[29]. Bleeding diatheses could additionally be caused by inherent deficiencies in platelet function even in the presence of normal platelet counts. These were largely the incentives that led to the discovery and characterization of integrin αIIbβ3 (deficient in Glanzmann Thrombasthenia), vWF (deficient in von Willebrand disease), and the GPIb-IX-V complex (deficient in Bernard-Soulier Syndrome).

While inherent or acquired deficiencies in platelet function or number are still real and relevant health concerns for a number of people, the broader health issue which concerns platelets is thrombosis rather than deficiencies in hemostasis. Compared with hemostasis, which is essentially the cessation of bleeding by the formation of a thrombus, thrombosis indicates the formation of a thrombus that occludes a blood vessel. The acute occurrence of a thrombosis, as seen in a myocardial or cerebral infarction, is a potentially catastrophic occurrence that largely follows years of chronic vascular disease, namely atherosclerosis. The central role of platelets in these disease states is apparent by the prevalence of antiplatelet therapeutics in use such as clopidogrel and aspirin. While platelets are clearly involved in these particular thrombotic endpoints, they also play central roles in the progression of such diseases.

During the past century, populations in developed parts of the world have grown progressively older, leading to increased incidence of chronic disease. Atherosclerosis, a chronic vascular disease, has reached epidemic levels in the developed world. The main risk factors are largely summed up by what is termed the metabolic syndrome and include diabetes mellitus, hypertension, dyslipidemia, obesity, *etc.* (all of which are steadily increasing in the United States). Simply stated, atherosclerosis is characterized by fatty lesions that insidiously accumulate and grow in the intima of large- and medium-sized arteries. The generally accepted view of its pathogenesis is the response-to-injury hypothesis which was first suggested by Ross and Glomset in 1973[30]. In this model, subtle and progressive endothelial damage results in an immune response and the subsequent accumulation of immune cells (*eg*, monocyte-derived macrophages),

lipids, *etc*. The response-to-injury model is supported by the predilection of portions of vessels that are subjected to turbulent blood flow to develop atheromas. Early lesions are known simply as fatty streaks and consist mostly of the pathognomonic foam cells, lipid-laden macrophages. These progress to involve infiltration of smooth muscle cells, immune cells, *etc.* Atherogenesis is therefore considered a chronic inflammatory process.

Most commonly platelets are thought to play a pathological role during acute coronary events following decades of atherogenesis. While this is true, platelets also play an important role in the injury response model of atherogenesis itself which could well be attributed to their inflammatory role described in the preceding section. Platelets are the earliest cell-types to accumulate at the site of injury. Upon their activation, they in turn can stimulate endothelial cells to express adhesion molecules and chemotactic molecules that recruit monocytes to the site of injury resulting in an inflammatory response[27, 31]. Platelets themselves secrete a host of chemokines upon activation via release of granular components and have adhesive molecules to interact directly with dendritic cells and monocytes[16]. In turn, monocyte-derived macrophages that have infiltrated the intima accumulate lipid forming foam cells whose presence are pathognomonic for atherosclerosis.

LIPIDS IN CARDIOVASCULAR DISEASE

Research in the role of lipids in atherogenesis began to accelerate in the 1970s with an observation in the Framingham study that indicated an association between early onset

heart disease and total serum cholesterol[32]. During this same period, Brown and Goldstein identified that familial hypercholesterolemia, a genetic disorder associated with accelerated atherogenesis and coronary artery disease, was caused by a disorder of the low density lipoprotein (LDL) receptor[33]. The LDL receptor normally allows for the uptake and degradation of LDL-associated cholesterol in the plasma. Paradoxically, although LDL receptor-mediated uptake of cholesterol by most cells was abolished in individuals with homozygous familial hypercholesterolemia, cholesterol could still accumulate in macrophages producing foam cells; this phenomenon led to a search for chemically modified LDL particles whose uptake into macrophages was via a mechanism independent of LDL receptor-mediated uptake[34]. It was found that cultured endothelial cells were capable of modifying native LDL such that its uptake by macrophages was greatly enhanced[35, 36], a modification that was later characterized as oxidative in nature[37]. Scavenger receptors on macrophages were identified to facilitate this uptake of modified LDL[35, 38]. In addition to their propensity to form foam cells oxidatively-modified forms of LDL were also found to be cytotoxic to a variety of cells[39-41] which made them an attractive target in the response-to-injury model of atherogenesis.

Native LDL particles themselves are heterogeneous consisting of a core of entirely hydrophobic lipids (especially cholesteryl esters) surrounded by an outer layer consisting of lipids with hydrophilic groups such as phospholipids, lysophospholipids, and unesterified cholesterol as well as a single apolipoprotein B-100 (apoB)[42]. The precise nature of *in vivo* LDL oxidation is incompletely understood, and mechanisms of oxidizing LDL *in vitro* vary widely among researchers. Not surprisingly, the term oxidized

LDL (oxLDL) does not refer to a single entity, but rather it refers a group of particles all with different lipid makeup and different degrees of oxidation. In general, LDL is considered extensively oxidized when it loses its LDL receptor recognition determinants and gains recognition determinants for scavenger receptors. Such receptor affinities are attributed to modification of the apoB protein itself[43] as well as oxidative modification of the lipid component of LDL where short-chained phosphatidylcholine derivatives also serve as scavenger receptor ligands[44]. Oxidative modification of LDL also drastically increases the relative abundance of lysophospholipids[37, 45] which will be discussed in the following section.

LYSOPHOSPHOLIPID DISTRIBUTION AND METABOLISM

While roles of lipids in cellular communication were not apparent in earlier decades, it is now recognized that there are clearly many bioactive lipids that play roles in cell signaling. These include intracellular signaling molecules such as diacylglycerol (DAG), nuclear receptor ligands such as the steroid hormones, and G protein-coupled receptor (GPCR) ligands such as the prostaglandins and lysophospholipids. Lysophospholipids are derivatives of glycerophospholipids or sphingophospholipids that contain only one radyl hydrocarbon chain (typically a fatty acyl group) (**Figure 1.1**). The major types of bioactive lysophospholipids with GPCR-mediated effects are LPA and S1P, and each of these types is composed of species with fatty acyl chains of varying lengths and degrees of saturation. Lysophosphatidylcholine (LPC) also has biologic activity in some systems,

although it is unclear if LPC acts directly through selective receptors or serves as a precursor for a primary mediator, such as LPA or a released fatty acid.

These lysophospholipids are found ubiquitously in plasma, peritoneal fluid, cerebrospinal fluid, seminal/follicular fluid, *etc*. At least 15 molecular species of LPA and both S1P and its dihydro derivative are found in the circulation of humans and mice at physiologic levels. Cumulatively, the species of LPA and S1P are present in the circulation at micromolar concentrations in humans and mice[46-49] compared with the much more abundant LPC at concentrations on the order of 100 μM[50]. These lysophospholipids largely exist associated with plasma proteins and lipoproteins[42, 51]. In regards to their metabolism, it appears that plasma lysophospholipids (especially LPA) turn over rather quickly[52] with their plasma levels being tightly regulated by their production and degradation. Both extracellular LPA and S1P are primarily removed from circulation by dephosphorylation to monoacylglycerol (MAG) and sphingosine respectively. This removal is accomplished by the broad-acting integral membrane class of enzymes known as the lipid phosphate phosphatases (LPPs), and S1P is additionally hydrolyzed by the S1P-selective phosphatases[53]. Genetic deletions of LPP3 in particular is embryonically lethal due to defects in early vascular development and patterning (*ie*, chorio-allantoic placenta and yolk sac vasculature)[54], a phenotype that is reproduced if the deletion is limited to endothelial cells (Panchatcharam *et al*, unpublished).

In contrast to their degradation, the routes of production for S1P and LPA differ as LPA is formed from hydrolysis of circulating lysophospholipids and S1P is formed by

phosphorylation of sphingosine. In the case of LPA, the primary route of production in blood plasma involves hydrolysis of LPC. The source of circulating LPC is from two general pathways. The first is lecithin cholesterol acyltransferase (LCAT) which transfers an acyl group from phosphatidylcholine to cholesterol forming LPC and cholesteryl esters. The second mostly includes secreted phospholipase A2 (PLA2) and lipoprotein-associated PLA2s (both the LCAT and PLA2 pathways are reviewed by Levitan *et al*[42]). As will be discussed in Chapter 2, secreted enzymes with PLA1 activity also generate an LPA with the fatty acyl group in the *sn*-2 position which may then undergo acyl transfer to the *sn*-1 position[55]. Hydrolysis of LPC to LPA involves the lysophospholipase D (lysoPLD) enzyme autotaxin (ATX) which is a member of the ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) family. In this family of enzymes, ATX possesses a unique ability to hydrolyze lipid substrates (specifically lysophospholipid substrates). Inactivation of the *Enpp2* gene encoding ATX in mice (*Enpp2-/-*) is embryonically lethal with severe defects in vascular maturation[56, 57]. *Enpp2+/-* mice have half-normal circulating levels of ATX and LPA[56, 57], and pharmacologic inhibition of ATX results in rapid and substantial drops in circulating LPA[52]. Transgenic overexpression of *Enpp2* using the α1-anti-trypsin inhibitor promoter to drive expression in liver increases plasma ATX/lysoPLD activity and LPA levels[49]. These findings establish that ATX is a primary regulator of LPA levels in the blood. A summary of LPC and LPA metabolism in the plasma is shown in **Figure 1.2**.

Several lines of evidence also implicate platelets as important participants in LPA production in the circulation. LPA levels in serum prepared from platelet-rich plasma

(PRP) are 5- to 10-fold higher than in platelet-poor plasma (PPP)[58-60], which suggests that activated platelets play an active role in LPA production during clotting. Experimental induction of thrombocytopenia in rats, using an anti-platelet antibody, decreases the production of LPA in serum by almost 50%[58]. Similarly, treatment of mice with a small molecule inhibitor of the platelet fibrinogen receptor, integrin αIIbβ3, causes thrombocytopenia and significantly decreases circulating LPA[61].

S1P is primarily generated by phosphorylation of sphingosine catalyzed by 2 sphingosine kinases (SPHK1 and SPHK2) and is degraded reversibly by dephosphorylation or cleaved nonreversibly by S1P lyase[62]. Combined deficiency of *Sphk1* and *Sphk2* is lethal embryonically; however, an inducible deletion of *Sphk1* in *Sphk2-/-* pups generates animals that survive to adulthood with no detectable S1P in plasma[63]. Transplant of normal bone marrow cells into these *Sphk1*- and *Sphk2*-deficient animals restores plasma S1P levels, establishing a role for marrow-derived cells in maintaining circulating S1P levels[63]. Because platelets contain Sphk but lack S1P lyase, they can synthesize and accumulate S1P[64]. Upon activation, platelets release stored S1P and until recently were assumed to be the source for the bulk of circulating S1P. However, cell reconstitution experiments indicated that red blood cells, rather than platelets, appear to be the major hematopoietic source for plasma S1P[63]. Although on an individual basis a platelet contains more S1P than a red blood cell, red blood counts (~ 5×10^{12} / L) are approximately 25-fold higher than platelet counts (~ 0.2×10^{12} / L), so that the total amount of S1P in red blood cells vastly exceeds that in platelets. In humans, S1P levels linearly correlate with hematocrit, providing further support that red blood cells are a

primary source of plasma S1P[65, 66]. In addition to platelets and red blood cells, other vascular components, including the endothelium, also contribute to circulating S1P levels[67].

LYSOPHOSPHOLIPID RECEPTORS

Even prior to the discovery of lysophospholipids receptors, the bioactivity of LPA and S1P had been indicated which included reports on the effects of lysophospholipids on vasopression and blood pressure[68, 69], contraction of smooth muscle in different organ systems[70, 71], and platelet activation[72, 73]. By this point relationships of lysophospholipids with health and disease were of interest, and the field was ripe for the discovery of lysophospholipid-specific receptors. The first of these was a GPCR discovered in 1996 that showed LPA binding and LPA-dependent activity[74]; it was eventually termed LPA1. Subsequently several other GPCRs specific to either LPA or S1P would be characterized and account for all or at least most of lysophospholipid bioactivity; there have, however, been some suggestions of LPA signaling through peroxisome proliferator-activated receptor γ (PPAR γ)[75]. The initial receptors identified were closely homologous and denoted endothelial cell differentiation gene (EDG) receptors although they were subsequently renamed to more rationally reflect their respective ligands. Of these, there are five S1P receptors (designated S1P1-5) and three LPA receptors (designated LPA1-3). At least two (and for the purposes of this dissertation, three) other LPA receptors (LPA4-6) had been identified that are divergent from the EDG-family LPA receptors showing more sequence homology to nucleotide-selective receptors than to

the earlier lysophospholipid receptors (**Figure 1.3**); LPA4 and LPA6 were formerly classified in purinergic family of receptors P2Y9 and P2Y5 respectively).

The lysophospholipid receptors are ubiquitous in tissues and signal via canonical G protein pathways (their signaling pathways have generally been identified with expression in various mammalian cell lines). Of the S1P receptors, the first to be identified was S1P1 (originally designated EDG1)[76] which couples only to Gi/o. It is broadly expressed in adult tissues[77] and critical during embryonic development as genetic deletion resulted in embryonic lethality with problems in vascular maturation[78] and neurogenesis[79]. S1P2 and S1P3 are also expressed widely, but unlike S1P1, genetic deletions are not embryonically lethal and have relatively mild phenotypes[80, 81]. They are also able to couple more promiscuously with different G protein receptors[82]. There is evidence of cooperativity among these three S1P receptors as embryonic lethality occurs earlier and with a more severe phenotype when an S1P1 deletion is coupled with an S1P2 and/or S1P3 deletion[83]. S1P4 and S1P5 have a much more narrow expression pattern with S1P4 expressed primarily in lymphocytes[84] and S1P5 in the central nervous system and spleen[85]. S1P5 knockout animals have defects in natural killer cell trafficking.[86] (S1P4 deletion has not been reported.)

As with all of the S1P receptors, the first three LPA receptors described are members of the EDG family of receptors. LPA1-3 are all widely expressed and can all couple to various G proteins. Genetic deletions of any of these receptors is not uniformly embryonically lethal[87]. The signaling in different receptors is also dependent on

individual LPA species as cellular responses vary depending on fatty acyl length and degrees of saturation. For instance unsaturated LPA species are more potent at activating most receptors[88-92]. LPA1 appears to play an important role in the developing nervous system. The most obvious phenotype of a deletion of this receptor is craniofactial defects, defects in Schwann cells, and about 50% mortality with small size (the latter are mostly attributed to suckling defects)[93]. Deletion of LPA2 yields viable animals with no overt phenotype, and double deletion of both LPA1 and LPA2 does not produce any additional defects[94]. Likewise LPA3 deletion does not produce an overt abnormality except that there are reduced litter sizes and delays in embryo implantation[95, 96].

Even prior to the discovery of LPA4, it was becoming increasingly clear that there were additional LPA signaling mechanisms as platelet aggregation and certain mitogenic responses to LPA were increasingly recognized as EDG family-independent[97-99]. As mentioned earlier, discovery of the LPA4 receptor heralded a new class of structurally divergent non-EDG family LPA receptors. Despite its structural differences, the dissociation constant for LPA4 and its ligand was comparable to that for the other receptors[100]. Deletions of LPA4 have been reported by three different groups: one genetic knockout had no obvious phenotype[101], a small hairpin ribonucleic acid (shRNA) LPA4 knockdown animal showed increased osteogenesis[102], and a separate genetic knockout showed reduced survival with hemorrhaging and vascular defects[103]. These results can be partially explained by strain differences of the knockouts and a possible redundancy of the receptors. It also suggests a role for LPA4 in vascular development

which is consistent with the phenotype of ATX knockout animals described above. Following the discovery of LPA4, two other non-EDG family LPA receptors were described, LPA5[104, 105] and LPA6[88]. Genetic deletions of these receptors have not yet been described, and their definitive functions are still speculative.

ROLE OF LYSOPHOSPHOLIPIDS IN CARDIOVASCULAR HEALTH AND DISEASE

As mentioned above, lysophospholipids are present at relevant concentrations in the plasma. They are capable of effecting cellular responses via GPCRs, and moreover, most every cell type related to the cardiovasculature has been shown to have a response to LPA and S1P.

One of the most apparent physiologic roles that both S1P and LPA play is in vasculogenesis which was demonstrated by the various receptor knockout mouse lines described above. The S1P1 receptor itself was first discovered because it was overexpressed under conditions where endothelial cells are induced to form capillary-like structures *in vitro*[76]. Likewise, deletion of the S1P1 receptor specifically in endothelial cells results in vascular development problems comparable to global knockout of the gene[106]. The role of S1P in endothelial cells in particular has thus been studied extensively. Aside from the role it plays in vascular development during embryogenesis, S1P also plays a clear role in maintaining endothelial (and also epithelial) barrier function. This has been observed *in vitro* where siRNA knockdown resulted in decreased electrical resistance of a monolayer of endothelial cells[107] and *in vivo* where pharmacological antagonism of S1P1 resulted in pulmonary edema[108]. The

effects of LPA on endothelial cells have been studied less than that of S1P. LPA does appear to have an effect on endothelial barrier function but there are inconsistencies in the literature regarding whether LPA increases[109, 110] or decreases[111, 112] barrier function. Outside of its role in barrier function, LPA plays an inflammatory role by upregulating several adhesion receptors[113] and chemokines[114] in endothelial cells. Recently, LPA has been shown to actually promote athergenosis in *Apoe-/-* mice presumably by inducing chemokine release from endothelial cells and subsequent adhesion of monocytes[115].

A further function of both S1P and LPA in endothelial cell function is that both are capable of inducing endothelial nitric oxide synthetase (eNOS) which implicates a role for these lipids in regulating vascular tone[116, 117]. Although the responses can be complex, a change in mean arterial blood pressure is observed when either S1P or LPA is administered globally in different animal models[69, 118]. In addition to modulating vascular tone via eNOS induction, smooth muscle cells themselves express receptors for both LPA and S1P[119-122]. Local administration of LPA or S1P in certain arteries (or in cultured myocytes from certain arteries) induces contraction indicating these lysophospholipids are capable of directly affecting vascular smooth muscle function[121-123]. Aside from simply causing contraction of smooth muscle cells, S1P and LPA are able to phenotypically alter smooth muscle cells, enhancing their proliferative and migratory propensity which can ultimately lead to neointima formation and vascular remodeling[89, 120].

Aside from the role that platelets play in producing LPA (see "Lysophospholipid Distribution and Metabolism" above), LPA also plays a role in platelet signaling and activation. LPA is a weak activator of platelets isolated from most human donors, although rodent platelets are refractory to stimulation by LPA. In human platelets, LPA stimulates platelet shape change[124, 125], fibronectin matrix assembly[126], and platelet-monocyte coaggregate formation[127]. LPA also synergistically enhances the actions of other platelets agonists, such as ADP and epinephrine[128, 129]. S1P may also be a weak activator of platelets although compared with LPA it is not nearly as potent requiring high concentrations (> 10 μM) to induce platelet aggregation[130]. This has led to speculation that a contaminant in S1P preparations or an S1P-derived metabolite may account for biologic activity towards platelets[131]. Likewise, variable effects of LPC on platelets have been reported. An early publication found that exposure of platelets to high dose LPC (30-100 μM) initially inhibited agonist-induced activation, but over time (30-90 minutes) could potentiate activation[132]. This latter observation of time-dependent activation of platelets by LPC suggests that activation may actually be due to LPA following lysoPLD-dependent metabolism of LPC. More on the role of LPA in platelets will be discussed in Chapter 3.

OVERVIEW

Lysophospholipids are clearly poised to play a significant role in cardiovascular health and disease. LPA and/or S1P may be involved in numerous roles in atherosclerosis as indicated by their abundant presence in lipoproteins and atheromas; their production

and metabolism by platelets and other blood cells; and their ability modulate the function of endothelial cells, smooth muscle cells, and platelets. They may indeed have a role in every stage of atherothrombosis from fatty streak formation to plaque rupture and thrombosis. To that end, the work presented herein is to delineate the role that platelets play in LPA production (Chapter 2) and to describe the mechanisms by which LPA signals in platelets (Chapter 3).

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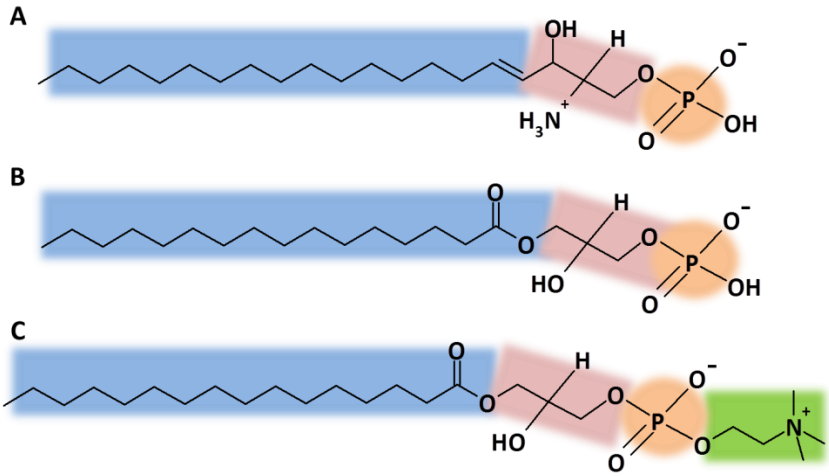
TABLE 1.1

Receptor	Knockout Phenotype	G proteins
S1P1	Embryonic Lethal (deficient vascular maturation, hemorrhaging)	Gi/o
S1P2	Hearing/balance defects	Gi/o; Gs; Gq; G12/13
S1P3	Grossly normal (infertility when deleted in conjunction with S1P2)	Gi/o; Gq; G12/13
S1P4	No knockout reported	Gs; Gq; G12/13
S1P5	Natural killer cell trafficking deficiency[86]	Gs; Gq; G12/13
LPA1	50% perinatal death and wide developmental effects; notably CNS defects among other deficiencies	Gi/o; Gq; G12/13
LPA2	Grossly normal	Gi/o; Gq; G12/13
LPA3	Male infertility; Embryo implantation delay	Gi/o; Gq
LPA4	Grossly normal*	Gs; G12/13; Gq, Gi
LPA5	No knockout reported	G12/13; Gq
LPA6	No knockout reported	G12/13; Gi

Lysophosphlipid receptors: Listed are brief overviews of the currently recognized LPA and S1P receptors, their association G proteins, and general phenotypes of knockout animals (reviewed in Chun *et al*, 2010[133])

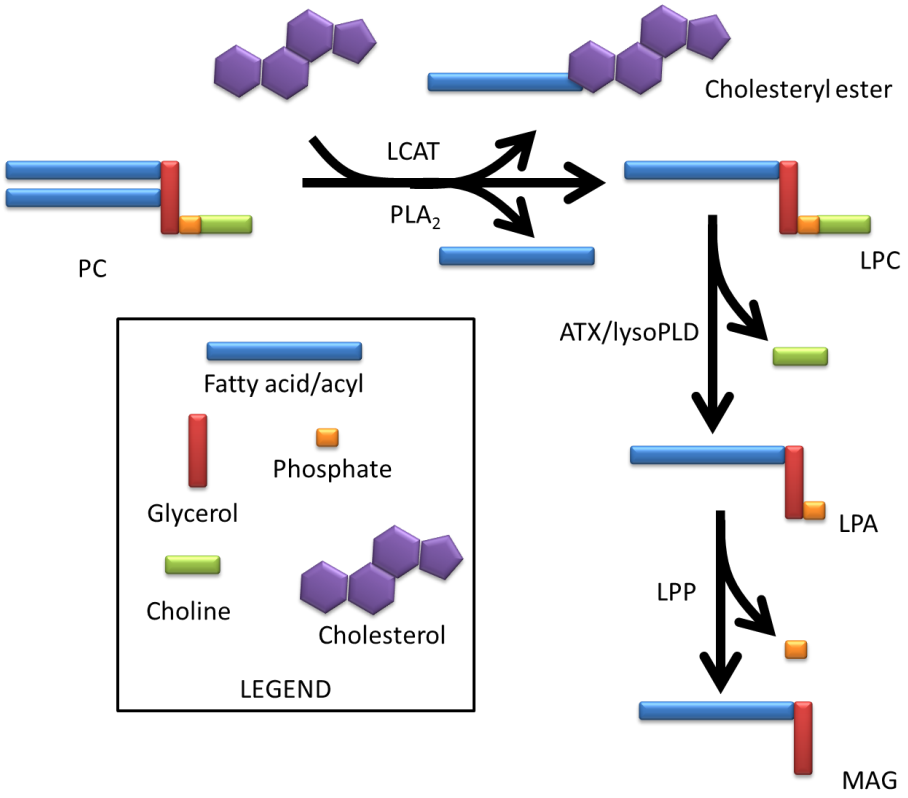
*A separate group independently created a knockout of LPA4 and found the homozygous deletion to be embryonically lethal due to vascular deficiencies and hemorrhaging[103].

FIGURE 1.1



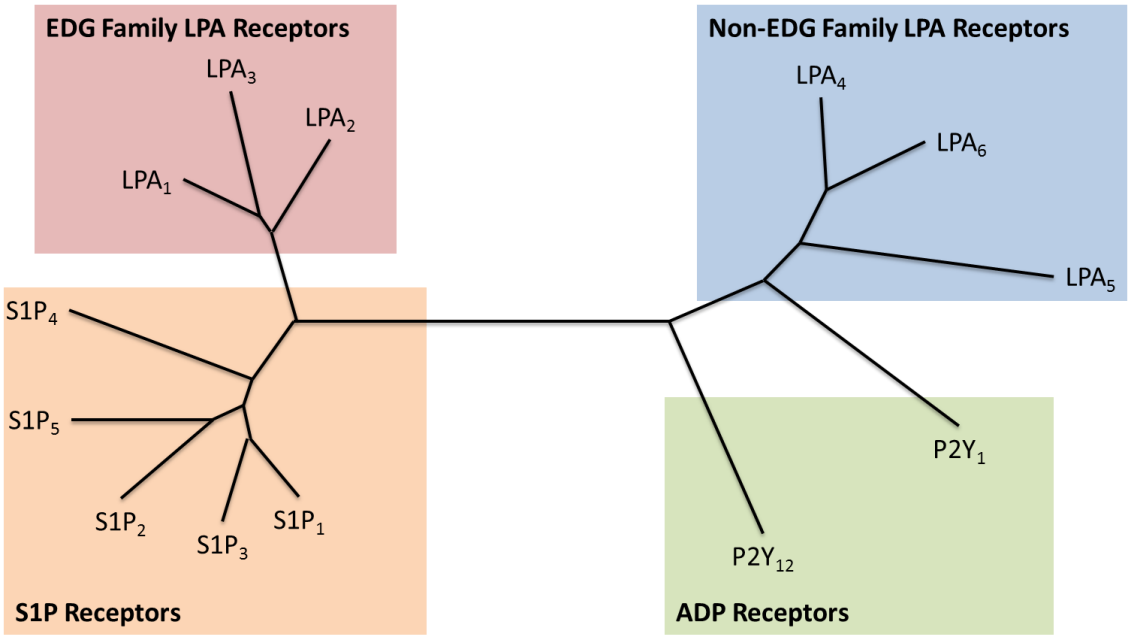
Lysophosphlipid Structure: Lysophospholipids consist of a sphingoid or glycerol backbone (highlighted in red) with a single radyl group (highlighted in blue) and a phosphate group (highlighted in orange) with or without an additional group such as choline (highlighted green). **(A)** S1P. **(B)** LPA. **(C)** LPC.

FIGURE 1.2



Schematic of Lysophospholipid Metabolism. Lysophospholipids are generated from phospholipids, typically PC, by removal of a fatty acyl by an enzyme with PLA activity by transfer of a fatty acyl to cholesterol by LCAT during cholesterol esterification. Serum phospholipids and lysophospholipids are generally associated with lipoprotein particles and are components of cellular membranes. Removal of a headgroup from a lysophospholipid (most typically choline from LPC) by ATX yields the bioactive LPA. Circulating LPA is quickly degraded to MAG by a serious of LPPs.

FIGURE 1.3



Phylogenetic Analysis of Human Lysophospholipid Receptors. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model[134]. The tree with the highest log likelihood (-7814.8060) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 289 positions in the final dataset. Evolutionary analyses were conducted in MEGA5[135]. On the left are the EDG Family of receptors while on the right are non-EDG family receptors. The ADP receptors P2Y12 and P2Y1 are shown for comparative purposes as LPA_4 and LPA_6 were originally classified as P2Y receptors.

CHAPTER 2: PLATELET INTEGRINS BIND ATX AND LOCALIZE ITS ACTIVITY

Portions of this chapter are taken, modified, and expanded upon from Fulkerson *et al*, 2011[136].

INTRODUCTION

Evidence from transgenic animals[49] and pharmacologic intervention[52] reveal the critical role of ATX in the production of circulating LPA, a finding that has only recently become appreciated. Circulating ATX levels and LPA levels correlate in human subjects as well[137]. The source of extracellular LPA has not always been clear. Intracellular LPA, which serves as an important mediator in triacylglyceride (TAG) production in most cells[138], may be produced by different mechanisms[58]. An unknown circulating lysoPLD was first suggested by Tokumura *et al* in 1986 by simple comparison of relative phospholipid species in the plasma[139]. ATX itself was later identified as a glycoprotein secreted from a line of human melanoma cells, inducing motility in an autocrine fashion[140] (hence its most common designation).

The first recognition of the enzymatic activity of ATX came when it was found to possess significant homology with an extracellular nucleotide pyrophosphatase, ENPP1[141] (ATX was subsequently given the additional designation, ENPP2). Of the ENPP family, ENPP1-3 have the greatest sequence homology. They all possess a modular structure with two amino- (N) terminal somatomedin domains (SMB1,2) followed by a phosphodiesterase domain (PDE) and finally a carboxy- (C) terminal nuclease-like domain (NUC)[142] (**Figure**

2.1A). Unlike ENPP1 and ENPP3, which are integral membrane proteins, ATX is a secreted enzyme[142]. While each of these possess activity against substrates such as nucleotides and other various phosphodiesters, ATX is unique in that it was found to also possess lysoPLD activity[143] and was indeed identified as the circulating lysoPLD found in plasma[144]. ATX itself actually has relatively poor ENPP activity compared with ENPP1 and ENPP3[145]. Although the substrates of the ENPP1-3 enzymes vary widely, the catalytic activity of each enzyme requires a critical threonine in the catalytic site of the PDE domain[143]. The actual structural determinants that account for each enzyme's diverse substrate preferences initially remained enigmatic. While the catalytic determinants are within the PDE domain, domain swapping experiments between ENPP1 and ATX have demonstrated that all three domains of ATX are necessary for lysoPLD activity.

Recently two groups have independently reported a crystal structure for ATX providing insights into the lysoPLD-specifying determinants (**Figure 2.1B**)[146, 147]. Extensive interactions exist between the PDE and NUC domains and weaker interactions between the two SMB domains and the PDE domain. An extended hydrophobic substrate binding channel leading to the active site is formed in large part by the interface between the SMB and PDE domains. This channel appears to have arisen by deletion of sequences that are present in other ENPP proteins. LysoPLD activity can be ablated while the ENPP activity remains unhindered by the introduction of mutations of hydrophobic residues that line the channel[145-147]. The pocket itself is large enough for a single acyl group explaining why ATX may use lysophospholipids as substrates but not phospholipids with

two acyl groups[146]. Taken together these observations account for the unique selectivity of ATX for lysophospholipid versus small molecule substrates.

With the evident role of ATX and LPA in human physiology and disease (see Chapter 1), there has developed an impetus for developing specific pharmacologic inhibitors to ATX. Early observations on the regulation of ATX indicated that both LPA and S1P were capable of inhibiting ATX activity[148]. This led to the identification of a number lipid-based ATX inhibitors[149-151] including the newly FDA-approved immunomodulator FTY720 (fingolimod) phosphate[152]. More recently non-lipid boronic acid-based inhibitors (*eg*, HA155) have been developed with nanomolar potency which globally inhibit ATX activity when they are administered in the blood[52, 153]. Molecules such as HA155 possess hydrophobic ring systems which allow them access to the hydrophobic pocket of ATX via van der Waals interactions[146]. This structure gives these particular inhibitors specificity to ATX compared with other ENPPs.

As described in Chapter 1, a role for platelets in production of circulating LPA has been suggested. While platelets themselves are clearly not a major source of ATX in the plasma, these observations raise the possibility that activated platelets or perhaps microparticles formed by activated platelets are a source of LPC substrate for ATX. Recent work identified acyl-protein thioesterase 1 (APT1) as being released during thrombin-activation of platelets and essential for platelet LPA production[55]. The PLA1 activity of APT1 generates *sn-2* LPC on the outer surface of the membrane bilayer, which is proposed to undergo acyl migration to *sn-1* LPC and serve as a substrate for ATX[55].

This process would necessitate that ATX localizes to the platelet surface. However, the mechanisms involved in cell localization of ATX and their importance for ATX-catalyzed production of LPA are presently unclear.

Unlike ENPP1 and ENPP3, ATX is a secreted protein although there have been suggestions that ATX may be able to accumulate at the surface of cells. One report demonstrates that ATX binds to and is cleared by liver sinusoidal scavenger receptors[154]. The modular structure of ATX itself suggests interaction with integral adhesive receptors. Its SMB domains display a degree of homology to the SMB domain of vitronectin, a protein capable of interaction with both plasma proteins and integrins via high-affinity interactions with its SMB domain[155-159]. To that end, evidence for binding of ATX to additional integrin receptors on lymphocytes[160] and oligodendrocytes[161] has been presented. Furthermore the presence of ATX has been shown in arterial thrombi of wild type mice but not mice lacking β3 integrins[49]. Integrin activation could therefore serve as a mechanism to localize the recruitment of ATX and spatially and temporally regulate LPA production. Not only would this provide a novel mechanism for localizing LPA signaling, but it would also provide a novel way for LPA production to be blocked under very specific circumstances.

In the following account, the integrin selectivity of ATX binding to platelets and mammalian cells was investigated as was the regulation of this process by cell signaling pathways. Using antibody and small molecule inhibitors of the ATX-integrin interaction and newly developed ATX mutants with impaired integrin binding it is demonstrated

that recruitment of ATX to the surface of platelets and mammalian cells is a mechanism for localized LPA production.

METHODS

Mice

All procedures conformed to the recommendations of the "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education, and Welfare publication number NIH 78-23, 1996), and were approved by the Institutional Animal Care and Use Committee. β3-deficient (*Itgβ3*$^{-/-}$) were a generous gift from Barry Coller (Rockefeller University). They were initially produced by deletion of exons 1 and 2 on the *itgb3* gene as described by Hodivala-Dilke *et al*[162]. The mice were weaned at 21 days, maintained on a 12-h light and 12-h dark cycle, and fed water and standard rodent chow (2018 Harlan Tekland Rodent Diet) *ad libitum*.

Blood Collection

Blood was collected from healthy donors with approval from the Institutional Review Board at the University of Kentucky by cubital venipuncture with a 19 gauge needle and collected into citrate (final citrate concentration 0.38%). For studies of isolated murine platelets, we collect blood by retrooribital puncture performed under anesthesia as previously described[163]. Citrated whole mouse blood (500 µl) was mixed with an equal volume of a solution of citrated saline buffer (0.38% citrate, 150 mM NaCl) and

centrifuged (300g for3 min) at room temperature to obtain a platelet-and plasma-rich buffer fraction.

Isolation of Recombinant ATX

cDNAs encoding wild type ATX, catalytically inactive ATX-T210A an N-terminal ATX fragments containing amino acids 53-143 and various site directed mutants were generated using standard methods inserted into a variant of pSecTag (Invitrogen) that was engineered for compatibility with the Gateway cloning system. Proteins were expressed by liposome mediated transfection of these constructs in suspension cultures of CHO cells. Recombinant proteins were purified from filtration concentrated CHO cell culture medium by nickel chelation chromatography as described previously[49]. Particular ATX truncations (SMB1-2) and ATX point mutations were engineered by Tao Wu, PhD (**Figure 2.2B** and **Figure 2.3B** respectively). Dr. Wu and the author additionally characterized the lysoPLD activity of the mutants using BSA-associated substrate (**Figure 2.3C**) and *bis*-paranitrophenotoluene (see "Measurement of LPA Production" for details).

Preparation of Platelets

Citrated human blood was centrifuged at 450 x g for 5 minutes to yield platelet rich plasma (PRP), to which 134 nM prostaglandin I2 (PGI2) was added. Platelets were separated from plasma by filtration of PRP using a column of Sepharose 2B (Sigma) equilibrated in HEPES-buffered modified Tyrode's buffer (138 mM NaCl, 5.6 mM dextrose, 2.7 mM KCl, 10 mM HEPES, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, pH 7.35) with

0.35% fatty acid-free bovine serum albumin (BSA; US Biological). For adhesion assays, PRP was incubated with 7 µM calcein AM for 30 minutes at 37°C prior to gel-filtration. Unless otherwise indicated, gel-filtered platelets were diluted to 200,000/µl in Tyrode's containing fatty acid free (ethanol and charcoal extracted) BSA (Sigma Aldrich).

Static Platelet/Cell Adhesion Assay

Recombinant wild type ATX, ATX variants, or fibrinogen (American Diagnostica) diluted in Tris buffer (50 mM Tris, 100 mM NaCl, pH 7.4) was incubated in wells of a black polystyrene 96 well plate (Nunc) overnight at 4°C. The wells were subsequently incubated for 1 hour at room temperature with Tyrode's containing BSA to block non-specific binding sites. Calcein-labeled platelets or CHO cells (see below) were incubated for 1 hour at 37°C; non-adherent platelets were removed by washing Tyrode's containing BSA and 2 mM Ca^{2+} and 1 mM Mg^{2+}, or Mn^{2+} , as indicated. The number of adherent platelets was determined by measuring fluorescence (abs/emi = 494/517 nm) and reference to a standard curve generated by fluorescence measurements using independently quantitated numbers of platelets. For analysis of platelet spreading and morphology, platelets were diluted to 20,000 /µl and adhesion performed on LabTek2 coverslips. Adherent platelets were fixed with 4% paraformaldehyde, permeabilized, and stained with TRITC-phalloidin.

Platelet Aggregation

Isolated platelets were prepared by gel filtration into Tyrode's with BSA as described above and diluted to 200,000 /µl. 2 mM Ca^{2+} and 1 mM Mg^{2+} was added as was

fibrinogen where indicated. 400 µl isolated platelets were aggregated using the indicated agonists at 37°C under stirring conditions. Aggregation was measured by light transmission (Platelet-Ionized Calcium Aggregometer; Model 660; Chrono-log).

Radioiodination of ATX

ATX was radiolabeled using sodium iodide-125 ($Na^{125}I$). 400-500 µg ATX was incubated with a final concentration of 0.4 M glycine, 5-10 mCi $Na^{125}I$ per mg protein, and lastly 23.1 nmol ICl / mg protein for 5 minutes on ice. ^{125}I-ATX was dialyzed overnight at 4°C against phosphate-buffered saline (PBS). Ninety-five to 99% of the ^{125}I associated with the ATX was precipitated by trichloroacetic acid. ^{125}I-ATX was combined with platelets in the absence of presence of agonists and incubated at room temperature. Platelets were centrifuged at 16,000 x g for 30 s through 30% sucrose, and the amount of radioactivity associated with the platelet pellet determined (PerkinElmer, Packard Cobar II Auto-Gamma).

CHO Cells

Chinese hamster ovary (CHO) cells stably expressing human β3 integrin were a generous gift from Zhenyu Li (University of Kentucky). For binding experiments these cells were labeled with calcein using minor modifications of the method described for platelets above. For measurements of LPA production by ATX, CHO cells were incubated with bee venom PLA2 (Sigma Chemical Co) at concentrations of 0-10 µg/ml for up to 60 minutes.

Determination of lysoPLD activity using detergent solubilized substrates. ATX activity against 18:0 LPC was determined using a spectrophotometric coupled enzymatic assay with minor adaptations for use in a microplate reader[144].

Measurement of LPA production

The lysoPLD activity of ATX was measured in the absence and presence of platelets, stably-transfected CHO cells, and purified integrins. Lipids were extracted using acidified organic solvents as described previously. When measuring changes in endogenous lipids, the unnatural lipid C17 LPA was added as an internal standard. In cases where we measured production of C17 LPA by ATX catalyzed hydrolysis of C17 LPC, we substituted C17 S1P as the recovery standard. The organic phase from these extractions was recovered, evaporated to dryness and reconstituted in 100 µl methanol. Lipids (generally 10 µl of each sample) were separated by reverse phase HPLC on an Agilent Zorbax C8 column and quantified by tandem mass spectrometry using an ABI 4000 Q-Trap hybrid linear ion trap triple quadrupole mass spectrometer. The mass spectrometer was operated in selective reaction monitoring mode to measure lipid species specific precursor product ion pairs with quantification accomplished by reference to calibration curves generated using synthetic standards obtained from Avanti Polar Lipids that were independently quantitated by phosphorous analysis as described previously[52] .

To measure real time production of ATX activity / LPA production in solution, we used fluorescent reporter systems to analyze choline liberation. Various concentrations of

LPC solubilized with Triton-X 100 were incubated with 0.1 mM amplex red (Cayman Cehmical Co.), 2 U/ml horseradish peroxidase (Sigma) and 0.2 U/ml choline oxidase (Sigma) in a black microtiter plate. Final buffer conditions were 50 mM Tris pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$. Incubations were conducted at 37°C for 1 hour and fluorescence measurements were made using an excitation wavelength of 540nm and measuring emission of 590 nm. Choline calibration curves were used to evaluate kinetics in molar quantities.

Statistics

Statistical analyses and preparation of figures were conducted using SigmaStat and SigmaPlot, respectively. Specific statistical tests are indicated in figure legends.

RESULTS

Synergistic Effect of Platelet Activation and ATX on LPA Generation

The central role of ATX in the production of circulating, bioactive LPA has been established by a combination of genetic and pharmacologic approaches. Very little is known about physiological mechanisms that may be responsible for regulating LPA production by ATX. Several observations suggest that platelets may serve as a useful model system to study regulation of ATX activity. Isolated platelets produce LPA after agonist stimulation, in a process that appears to involve platelet release of APT1 and APT1-catalyzed generation of LPC, which presumably serves as a substrate for ATX[55]. To confirm the role of ATX in platelet LPA generation, we tested the effects of a recently

described, potent, small molecule inhibitor of ATX[52]. We found that resting platelets contained significant levels of LPC, and these levels were increased ~2-fold after stimulation with maximally effective dose of thrombin (0.5 U/ml) for 30 minutes, presumably as a result of the recently described PLA1 activity of APT1[55]. This dose of thrombin increased the LPA content of isolated platelets ~6-fold after 30 minutes. The thrombin-mediated increase in platelet-derived LPA was completely attenuated in a dose-dependent manner by the ATX inhibitor HA155 (**Figure 2.4A**). Platelets contain immunologically detectable ATX[49], so these results indicate that ATX likely accounts for the previously reported ability of isolated human platelets to generate LPA in response to agonists[58, 164]. Remarkably, thrombin-stimulated LPA generation by isolated platelets was dramatically increased when recombinant ATX was added to these incubations. The increase in LPA production was dependent on the concentration of added ATX with a half maximal effect observed at concentrations of ~100 nM (**Figure 2.4B**), which is in the range of the concentration of ATX reported in human plasma[165]. When incubated with a maximally effective concentration of ATX, LPA generation by platelets was sustained at times of at least 20 minutes (**Figure 2.4C, D**). The time course of LPA in the absence of added ATX or in response to a maximally effective concentration of ATX in the absence of agonist was biphasic (**Figure 2.4C, D**), which may reflect the previously reported ability of platelets to degrade LPA[166]. The low concentration of delipidated BSA in these incubations contained trace levels of lysophospholipids including LPC that could not sustain the quantities of LPA being formed and indeed the production of LPA by ATX. Taken together, our observations of sustained, amplified LPA production by

thrombin-stimulated platelets in the presence of ATX indicates that under these conditions ATX is acting on platelet-generated LPC under conditions where the supply of substrate to the enzyme is not a rate-limiting determinant of LPA production.

Signaling Pathways Regulating Binding of Platelet Integrins to ATX

The ability of ATX to interact with integrin receptors could provide a mechanism for localizing the enzyme along the platelet surface. We therefore characterized ATX-platelet integrin interactions in more detail. LPA is a weak platelet agonist. To avoid confounding effects of ATX-generated LPA, we used a catalytically inactive mutant ATX-T210A to investigate agonist-dependent signaling pathways that regulate binding to platelets. To evaluate this interaction, we initially used a static adhesion method wherein purified recombinant ATX-T210A was immobilized followed by incubation of platelets (see Methods). We found that resting platelets alone had no measurable adhesion to ATX-T210A, but platelets stimulated with 10 µM ADP adhered to ATX-T210A maximally when it was immobilized at 5-10 µg/ml (**Figure 2.5A**; originally published in Pamuklar *et al*[49]). We further evaluated the effect of a number of platelets agonists. Agonists that act through GPCRs including ADP, PAR1 (thrombin and thrombin receptor activating peptide; TRAP) as well as agonists acting through the non-GPCR GPVI pathway (collagen), all promoted platelet adhesion to ATX-T210A (**Figure 2.5B**). In keeping with known mechanisms of integrin activation, ADP-stimulated platelet adhesion to ATX was inhibited by pretreatment of platelets with forskolin, which elevates intraplatelet cAMP, and was partially reduced by inhibitors of protein kinase A (PKI 14-22), PI3-kinase

(LY294002) or Rho kinase (Y27632) (**Figure 2.6A**). ADP-promoted platelet adhesion to ATX-T210A was also attenuated by the P2Y12 receptor antagonist cangrelor and the P2Y1 antagonist MRS-2719 (**Figure 2.6B**). Cangrelor and apyrase (an ADPase) also inhibited adhesion of TRAP-stimulated platelets to ATX (**Figure 2.6B**), identifying a role for released ADP in PAR1-stimulated platelet adhesion to ATX-T210A. In addition to classic platelet agonists, the direct integrin activator Mn^{2+} and treatment of platelets with the reducing agent dithiothreitol (DTT), which stimulates the adhesive function of several integrins, resulted in agonist- independent adhesion to ATX (**Figure 2.7**).

In agreement with the hypothesis that integrins mediate this interaction between activated platelets and immobilized ATX, we found that echistatin, an RGD-containing peptide that competitively inhibits ligand binding to β1 and β3 integrins, dose-dependently reduced platelet adhesion to ATXT210A to levels observed in unstimulated platelets (**Figure 2.8A**). To evaluate the specific integrins involved in ATX-T210A interaction with platelets we used integrin function-blocking antibodies. Monoclonal antibodies to β3 integrins (7E3) reduced adhesion by 79 ± 6% (n = 8). Monoclonal antibodies to integrin αIIbβ3 (10E5) and to β1 integrins (P4C10) also reduced adhesion of platelets to ATXT210A by 65 ± 13% (n = 4), and 87 ± 6% (n = 2), respectively (**Figure 2.8B**). The specificity of P4C10 was confirmed by its lack of an effect on fibrinogen binding to platelets which mostly involves αIIbβ3. LM609, a function blocking αVβ3 antibody, had variable effects on the adhesion of platelets from three different donors to ATX-T210A, ranging from 1 to 62% inhibition (median inhibition 22%), which may reflect low and variable levels of platelet αVβ3 expression in platelets from different

donors. Other integrin antibodies that have not previously been shown to affect binding of integrin ligands (12G10 and 23C6) had no effect on adhesion (**Figure 2.8B**). Binding to ATX-T210A was substantially, but not completely, impaired in platelets from mice lacking β3 integrin (*Itgβ3*-/-). Murine platelet adhesion was inhibited by 1B5, a hamster monoclonal antibody to murine αIIbβ3 (**Figure 2.9**). Taken together, our results indicate that the association of ATX with platelets requires integrin activation and occurs through β3 and β1 integrins.

To determine if β3 integrins were sufficient for supporting cell adhesion to ATX, we examined the effect of expressing the integrin β3 subunit alone or in combination with αIIb on CHO cell adhesion to ATX-T210A. While the parental CHO cell line did not adhere significantly to ATX-T210A, CHO cells expressing β3 integrins acquired the ability to interact with ATX-T210A in the presence of the integrin activator Mn^{2+} (**Figure 2.10**). The interaction of CHO cells with ATX-T210A was specifically mediated by β3 integrins because it was completely blocked by 7E3 (**Figure 2.10**). Because integrin β3 associates with the widely expressed vitronectin αV subunit of CHO cells (which do not express the platelet specific αIIb subunit), these observations also establish that ATX-T210A binds to αVβ3 indicating that association with this integrin may account for ATX binding to other cell types.

The preceding observations of ATX binding to integrins on platelets and mammalian cells employed microtiter plate assays with immobilized ATX. We next investigated binding of soluble ^{125}I-labeled ATX to platelets in suspension. Importantly, ^{125}I-ATX

behaved identically to unlabeled ATX in both static adhesion assays and in activity assays (data not shown). Binding of ^{125}I-ATX to activated platelets was time-dependent and saturable (**Figure 2.11A-C**). Scatchard analysis of data obtained using an excess of unlabeled ATX to define non-specific binding revealed an affinity of ~300 nM and a maximal number of ~75,000 ATX binding sites / platelet. Excess echistatin displaced bound ^{125}I-ATX (**Figure 2.11C**) and unlabeled fibrinogen dose-dependently blocked ^{125}I-ATX binding to platelets with a K_i of 220 nM (**Figure 2.11D**).

LysoPLD-Independent Effects of ATX on Platelet Aggregation and Adhesion

We sought to evaluate possible impacts integrin binding had on platelet function indepenent of its lysoPLD activity which has been reported in oligodendrocytes[167]. ATX is a monomeric protein in solution[168] and would not be expected to support platelet aggregation, which requires cross-linking of at least 2 binding sites on adjacent platelets. Under conditions where ADP-induced aggregation of washed platelets was enhanced by addition of exogenous fibrinogen, inclusion of soluble ATX-T210A did not further enhance platelet aggregation (**Figure 2.12A**). In fact, the maximal extent of aggregation of platelets was moderately lower in the presence of ATX-T210A (**Figure 2.12A** and **Figure 2.12B**) even after addition of exogenous fibrinogen (**Figure 2.12B**). The ability of ATX-T210A to reduce platelet aggregation varied between platelet preparations from different donors (**Figure 2.12B right**), but the extent of aggregation observed consistently lower in the presence of ATX-T210A. Taken together, these observations and the fact that fibrinogen competes for soluble ATX binding (**Figure 2.11D**) are

consistent with a model in which ATX and fibrinogen interact with a mutually accessible binding site(s) on platelets but only fibrinogen can support platelet aggregation.

ATX exerts a counter-adhesive effect on oligodendrocytes that is consistent with inhibition of integrin-dependent adhesion and signaling[161]. ATX may also be capable of hydrolyzing nucleotides along the platelet surface that could impact signaling events. The inhibitory effects of ATX on platelet aggregation were independent of catalytic activity because inactive ATX-T210A also inhibited aggregation (**Figure 2.12**). Nonetheless, we sought to determine if ATX has direct inhibitory effects on platelet function by determining of ATX would alter adhesion to fibrinogen immobilized at submaximal concentrations (low density; 1 μg/ml). In the absence of agonists, platelets adhere to low-density fibrinogen and the adhesion triggers platelet activation[169, 170]. In agreement with our aggregation studies, soluble ATX blocked platelet adhesion to immobilized fibrinogen by 70% (from 9.6 ± 2.0 to 2.8 ± platelets/ high powered field). However, more platelets adhered to surfaces containing both immobilized fibrinogen and ATX than to surfaces containing fibrinogen alone (20 ± 5 versus 9.6 ± 2 platelets/field; **Figure 2.13A, B**). In addition, more platelets spread on surfaces with immobilized fibrinogen and ATX than spread on surfaces containing immobilized fibrinogen alone (**Figure 2.13A, B**). When fibrinogen was immobilized at higher density concentrations (5 μg/ml) that are known to promote maximal platelet activation and adhesion[169, 170], the inclusion of immobilized ATX did not inhibit adhesion or spreading (**Figure 2.13A, B**). Thus, rather than negatively affecting the adhesive function of platelet

integrins, as has been observed in experiments with oligodendrocytes, immobilized ATX moderately facilitates platelet adhesion to fibrinogen.

The Tandem SMB Domains are the Sole ATX Binding site for β3 Integrins

We next sought to identify the motifs in ATX responsible for interacting with platelets. ATX contains an arginine-glycine-aspartic acid (RGD) sequence motif within the second SMB-like domain (**Figure 2.2A**). Because this motif confers integrin binding to other proteins, including the structurally related SMB-like domain of vitronectin, we surmised that it would perform a similar function in ATX. Surprisingly, the ATX crystal structure (**Figure 2.1B**) revealed that the RGD motif amino acids are incompletely solvent exposed (**Figure 2.3A**). In agreement, we found that non-conservative substitutions of the RGD residues did not abolish integrin binding (Morris, unpublished observations). However, in the course of these studies, we found that mutation of a charged, surface-exposed residue at the N-terminus of the ATX SMB2 domain significantly reduced binding of ATX to platelet integrins identifying an important role for the SMB2 domain in this process[146]. To determine if the SMB2 domain is the sole integrin binding site on ATX and begin to explore interactions of ATX with integrins on other cells, we compared binding of an ATX fragment containing the tandem SMB domains to CHO cells expressing human integrin β3. Binding of these cells to the SMB domain fragment was promoted by the integrin activator Mn^{2+} and inhibited by 7E3 in an identical manner to that of WT ATX, indicating that the SMB domains (and not the PDE and NUC domains) account for ATX binding to β3 integrins (**Figure 2.14A, B**).

ATX Binding to Platelet Integrins Enables Agonist Stimulated LPA Production

Having established the properties of ATX binding to β3 integrins, we used platelets and CHO cell systems to test the hypothesis that integrin-mediated recruitment of ATX to the outer face of the plasma membrane enables localized LPA production. To investigate this possibility, we measured the rate of hydrolysis of BSA bound C17-LPC (an unnatural LPA species) in the presence of either the parental CHO cell line or αIIIbβ3 expressing CHO cells using Mn^{2+} as the integrin activator. Under conditions where, based on our measurements of the affinity of platelet αIIbβ3 for ATX, all of the added ATX would be bound to the integrin, we observed a ~2-fold increase in ATX activity in the presence of integrin-expressing cells (**Figure 2.15A**). These results indicate that integrin binding increases ATX activity against exogenous substrates. Our experiments with activated platelets indicated that integrin-bound ATX could act on endogenously generated lysophospholipid substrates. Although like resting platelets, parental and αIIbβ3 expressing CHO cells contained readily detectable LPC, we did not observe significant production of LPA when they were incubated with ATX. However, we found that preincubation of these cells with bee venom PLA2 increased cell-associated LPC levels (**Figure 2.15B**), which presumably reflects the ability of this enzyme to hydrolyze PC in the outer leaflet of the plasma membrane when added exogenously to mammalian cells. Under these conditions, ATX elicited concentration-dependent increases in cell-associated LPA, and, at lower concentrations of ATX, LPA production was increased ~4-fold by overexpression of αIIbβ3 (**Figure 2.15C**). The effect of ATX was substantially attenuated when the integrin blocking antibody 7E3 was included in these

incubations (**Figure 2.15D**). Because the parental CHO cell line failed to bind to ATX appreciably in the static adhesion assay (**Figure 2.10**), it is likely that relatively low levels of integrin binding are sufficient to sustain significant production of LPA by cell-associated ATX.

The crystal structures reported are of rodent ATX orthologs (**Figure 2.1**)[146, 147]. Tao Wu , a postdoctoral fellow in our laboratory, made variants of human ATX with nonconservative substations of charged surface exposed residues in the SMB2 domain including H117A which corresponds to H119A of the rodent sequence and was previously shown to exhibit decreased binding to platelet β3 integrins[146] (**Figure 2.2A** and also see **Figure 2.3B** for sequence details). He examined catalytic activity of these variants against mixed micelles of triton X-100 and 18:0 LPC as described in the introductory section of this chapter. The apparent K_m and V_{max} values for LPC of these SMB domain mutants were very similar to that of wild type ATX indicating that mutation of the SMB2 domain does not impair catalytic activity against detergent-solubilized substrates (**Figure 2.3C**). Activity of these variants against the small molecule substrate *bis*-paranitrophenol phosphate was also indistinguishable from wild type ATX (data not shown). We examined binding of these ATX SMB2 domain variants to β3 integrin expressing CHO cells. Binding of these cells to ATX-H117A and ATX-E109A but not ATX-R117A was significantly lower than to wild type ATX (**Figure 2.16A**). We then compared the ability of WT ATX and ATX-E109A to increase LPA production when incubated with thrombin stimulated platelets. While WT ATX produced a robust increase in LPA

production ATX E109A did not, indicating that association with β3 integrins is important for this process (**Figure 2.16B**).

DISCUSSION

LPA is produced and degraded by multiple pathways and also serves as an intermediate in the *de novo* synthesis of phospholipids. Here a mechanism is described by which the metabolic and signaling functions of LPA can be delimited by localizing LPA production to the cell surface through recruitment to activated integrin receptors. We used human platelets as a model system to dissect the regulation and specificity of integrin binding to ATX. We found that a disparate variety of platelet agonists promote ATX binding to platelets. The ability of agonists to stimulate ATX binding appears to be strongly dependent on ADP signaling through the P2Y12 purinergic receptor, as evidenced by the ability of cangrelor and apyrase to inhibit TRAP-induced adhesion to immobilized ATX. The finding that forskolin treatment abolishes platelet adhesion to ATX is also consistent with a role for P2Y12 signaling through Gi to decrease platelet cAMP levels as an important component of the signaling pathway responsible. Stimulation of platelet adhesion to ATX by Mn^{2+} in the absence of agonists implicates integrins in this process, and studies using integrin blocking antibodies and CHO cells expressing recombinant integrins indicate a role for β3 integrins (αIIbβ3 and αVβ3) and to a lesser extent β1 integrins in this process. The involvement of integrins in platelet binding to ATX is consistent with the density of ATX binding sites/platelet observed with radiolabeled soluble ATX binding experiments. These results raise the possibility that, because αVβ3

and β1 integrins are broadly expressed, cell surface recruitment of ATX through integrin binding may be a widespread phenomenon.

Consistent with this idea, binding of ATX to lymphocytes and oligodendrocytes has been reported previously[160, 161]. In the latter system ATX binding was shown to disrupt cell adhesion through a process that was postulated to involve antagonism of an unidentified adhesive receptor[161]. We found that ATX impedes fibrinogen-dependent platelet aggregation. This is likely because ATX and fibrinogen compete for binding to the same integrin receptors but as ATX is a monovalent integrin ligand it cannot support platelet aggregation. Consistent with this idea, we found that fibrinogen competitively inhibited binding of soluble radiolabeled ATX to platelets. However, when ATX and fibrinogen were immobilized together at submaximal concentrations, we found that ATX modestly enhanced, rather than inhibited, adhesion. Thus, unlike ATX binding to oligodendrocytes which appears to trigger signals that inhibit other adhesive events, our results are consistent with a model in which fibrinogen and ATX compete for the same binding sites on platelets. Our prior observation that ATX is recruited to a thrombus *in vivo* suggests that the interaction of ATX with activated platelet integrins we have characterized here *in vitro* occurs physiologically[49]. In *in vitro* assays, ATX displays ENPP activity. It is possible that localization of ATX along the platelet surface provides a mechanism for degradation of released ATP (a platelet inhibitor) and ADP (a platelet activator). To date, however, we have been unable to detect an impact of ATX on circulating nucleotides[171].

Efforts to define the physiological role of LPA signaling in platelet function have been confounded by observations of heterogeneity in human platelet responses to LPA[172] and the fact that, while LPA is a weak agonist for human platelets from most donors, it does not activate rodent platelets[49]. On the other hand, several lines of evidence implicate platelets as important participants in the production of LPA in whole blood ex vivo and in mouse models[58, 61]. We found that a recently described potent small molecule ATX inhibitor completely attenuated production of LPA by thrombin stimulated washed platelets. The delipidated BSA used in these incubations contained trace levels of LPC that were less than 1% of the quantity of LPA being generated, so clearly these results indicate that endogenous ATX acting on endogenously formed LPC is responsible for producing LPA in this system. Addition of purified ATX to washed platelets at concentrations in the range of ~100 nM, which are in line with those present in plasma, resulted in dramatically enhanced sustained production of LPA by thrombin stimulated washed platelets. We also found that ATX could hydrolyze CHO cell-associated lysophospholipids and that this activity was substantially enhanced when cells expressing integrin β3 were used as the source of substrate. We identified the N-terminal ATX tandem SMB domain as sole site of integrin binding, and generated point mutants in the SMB1 domain of ATX that exhibited parallel reductions in binding to β3 integrins and ability to generate LPA when incubated with thrombin stimulated platelets. Taken together, these results indicate that integrin binding to ATX brings the enzyme into close proximity with cell surface substrates which would generate LPA in the vicinity of its receptors (**Figure 2.17**). Intriguingly, the ATX hydrophobic substrate

binding channel through which cell surface substrates and products must enter and leave the active site is formed from the interface between the SMB and PDE domains[146, 147] raising the possibility that integrin binding alters catalytic activity of the enzyme.

While ATX can clearly generate LPA in blood plasma through hydrolysis of high concentrations of circulating LPC and this activity is important for maintaining plasma LPA levels in the ~1 µM range in the face of rapid elimination[52, 173], very little is known about the extracellular concentration of LPA in tissues. Furthermore, although a role for circulating levels of the structurally related lipid mediator S1P in control of the permeability of lymphatic and vascular endothelium is well supported by substantial data, the function of plasma LPA remains enigmatic[174]. Our results suggest that the functions of circulating and cell-associated ATX are distinct. Recruitment of circulating ATX to activated platelets or the surface of vascular endothelial and vascular smooth muscle cells could lead to cell specific LPA generation and LPA signaling with important roles in vascular inflammation and injury responses[174].

Inhibition of LPA/ATX signaling is an attractive therapeutic target in cardiovascular disease, inflammation, and cancer. To date, all of the inhibitors of ATX activity described, including several recently reported highly potent compounds target the catalytic site of the enzyme to inhibit substrate binding or catalysis[52, 175]. The ATX-integrin interaction reported here could be exploited to generate agents that selectively target ATX activity against cell-associated substrates. Indeed, antibody and small molecule inhibitors of integrin ligand interactions have already been developed as anti-

thrombotic and anti-cancer agents and one compound of this type has been reported to alter LPA signaling involved in breast cancer metastasis in a mouse model[61]. The possibility that some of the efficacy of these agents derives from inhibition of ATX recruitment to integrins warrants further examination.

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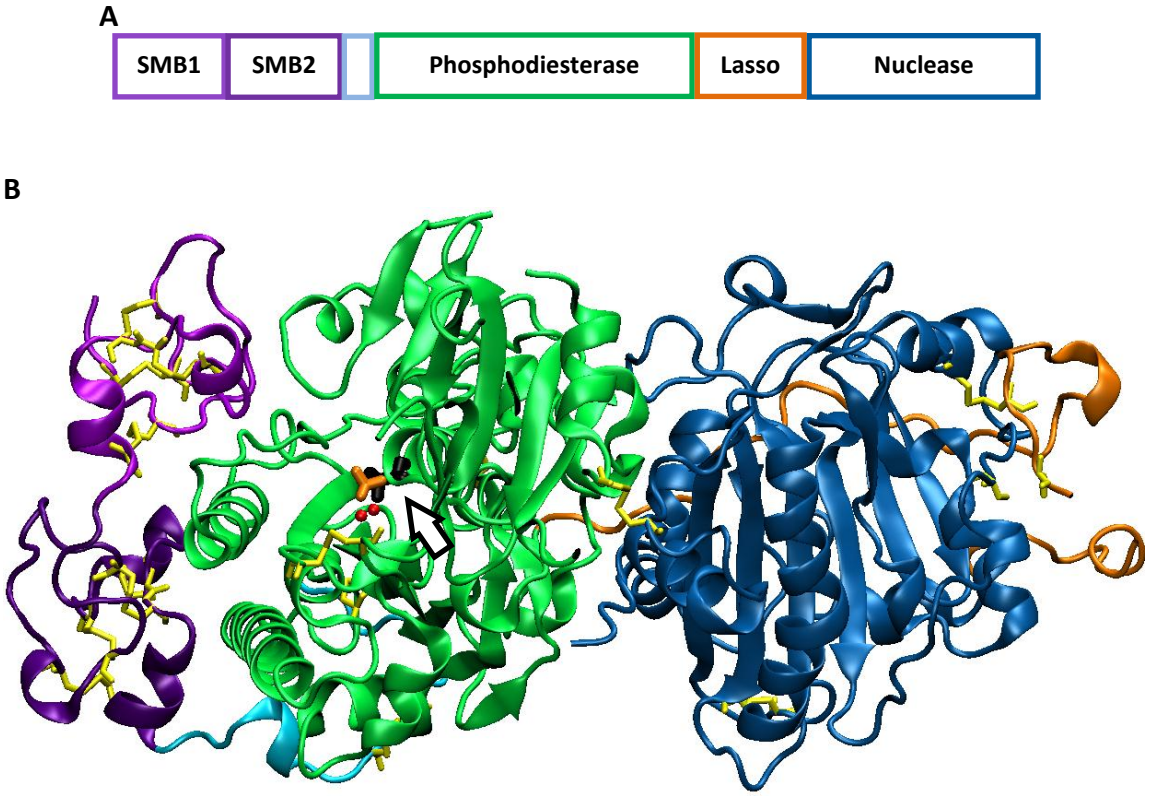
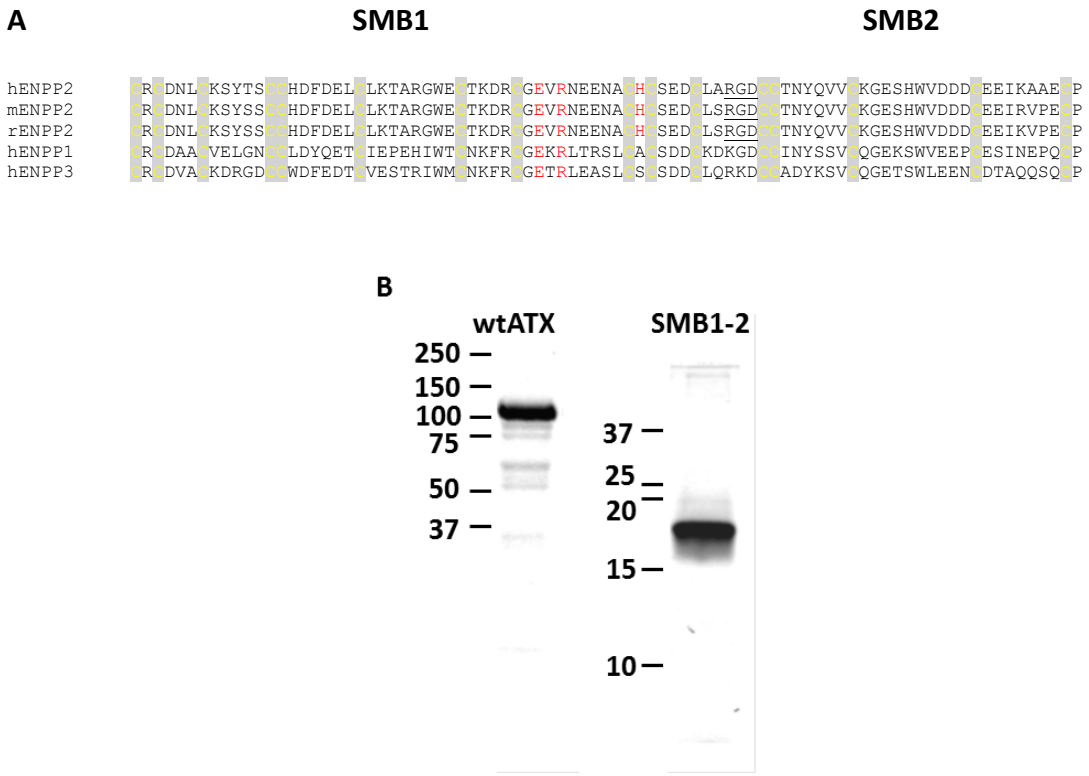
FIGURE 2.1



ATX Structure: (A) Schematic linear representation of ATX demonstrating the multimodular nature of the protein with the SMB1 domain in light magenta, the SMB2 domain in dark magenta, the phosphodiesterase domain in green, the lasso region in orange and the nuclease-like domain in blue. **(B)** Ribbon representation based upon x-ray diffraction by the crystallized ATX. As in A, the SMB1 domain is in light magenta, the SMB2 domain in dark magenta, the phosphodiesterase domain in green, the lasso region in orange and the nuclease-like domain in blue. Cysteine residues and disulfides are depicted in yellow. The active site is indicated by the arrow with bound zinc ions (red) and phosphate (orange). The critical threonine residue is black.

Figure modified from Hausman *et al*[146].

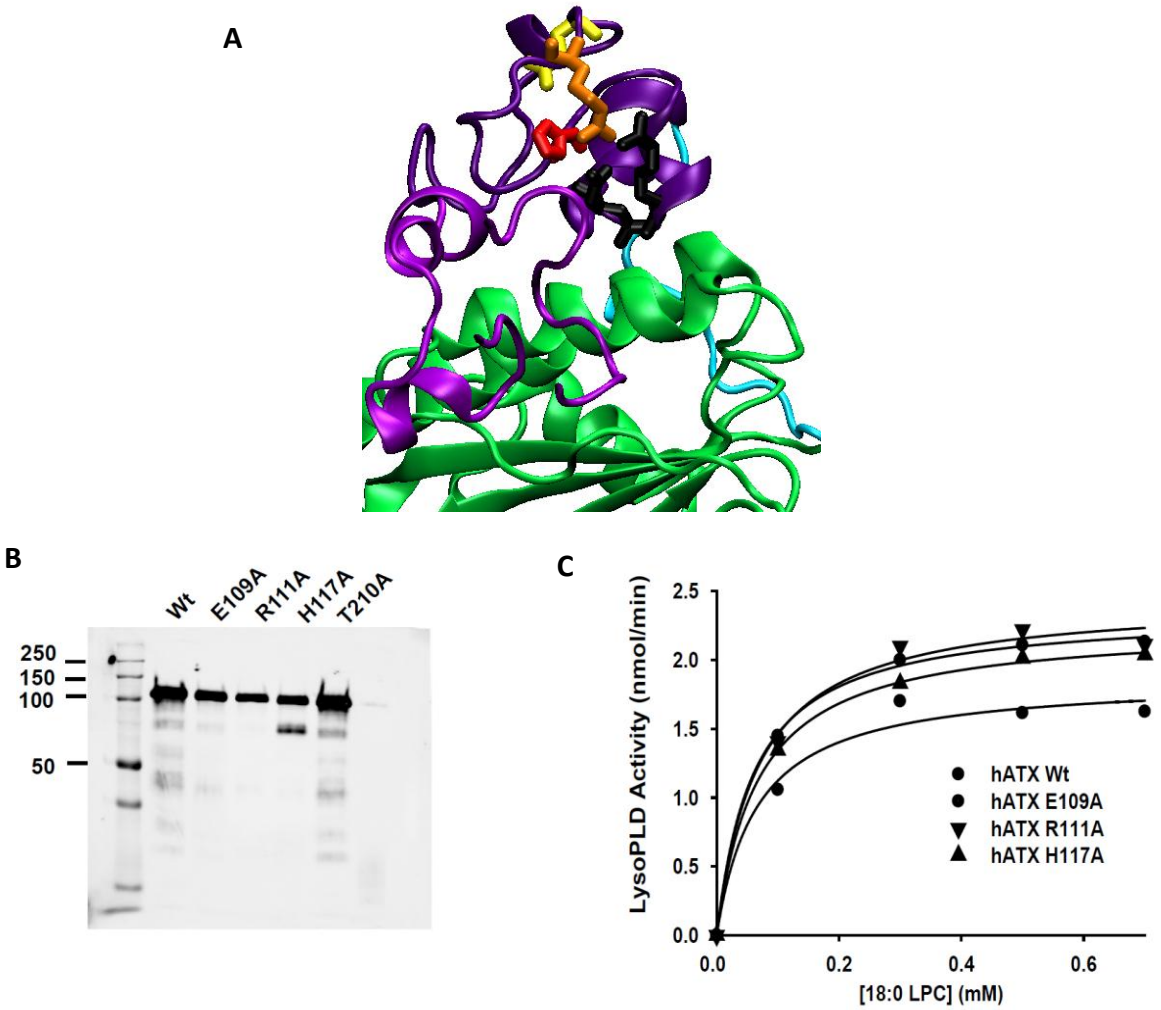
FIGURE 2.2



Production of SMB1-2 peptide: **(A)** Sequence alignment of the SMB1 and SMB2 domains of human, rat and mouse ENPP2 (ATX) and human ENPP1 and ENPP3 with the RGD sequence underlined and charged residues targeted for mutation highlighted in red. **(B)** Analysis of purified recombinant wild type ATX (wtATX) and the N-terminal tandem SMB domain fragment(SMB1-2) by SDS PAGE and staining with coomassie blue.

(B) Truncation generated by Wu[136].

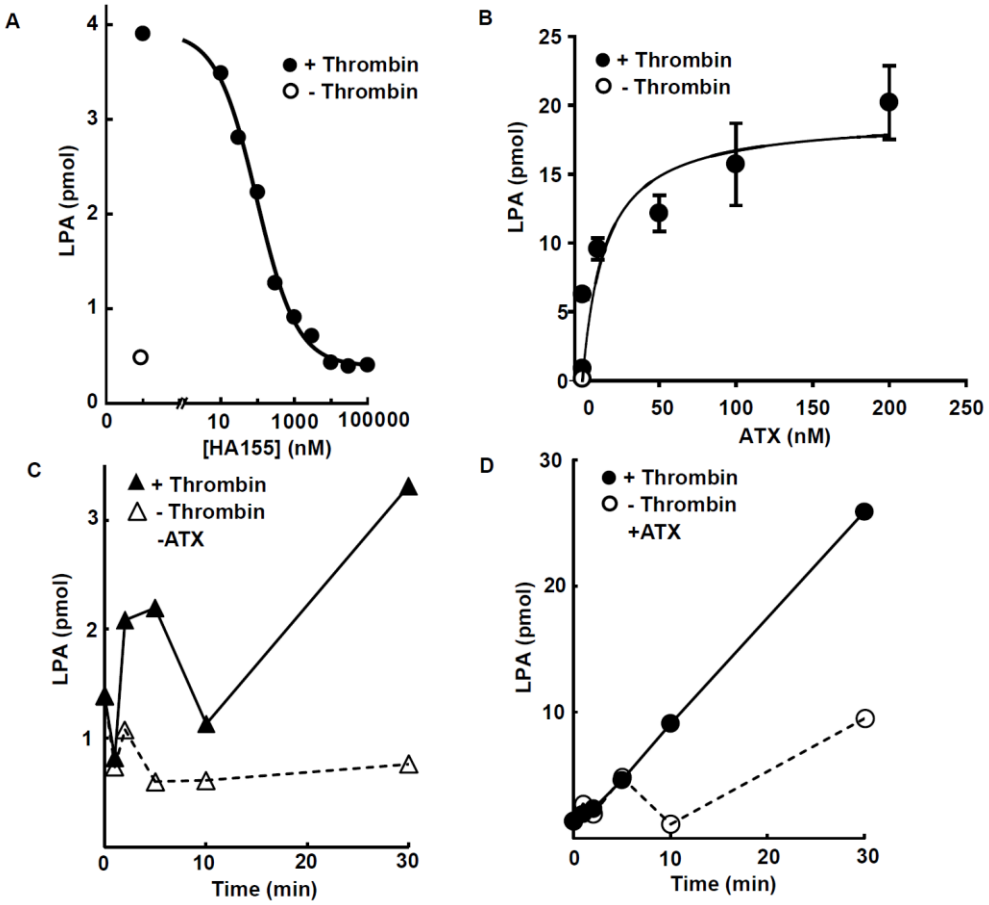
FIGURE 2.3



Preparation of SMB point mutations: (A) Ribbon diagram of the SMB1-2 domains of ATX (purple) with the surface-exposed charged residues that were targeted for mutation in SMB2 highlighted in yellow (E109), orange (R111), and red (H117). The partially exposed RGD sequence is in black. **(B)** SDS PAGE analysis of purified wild type ATX and the indicated variants. **(C)** LysoPLD of ATX mutants using BSA-associated C17-LPC substrate.

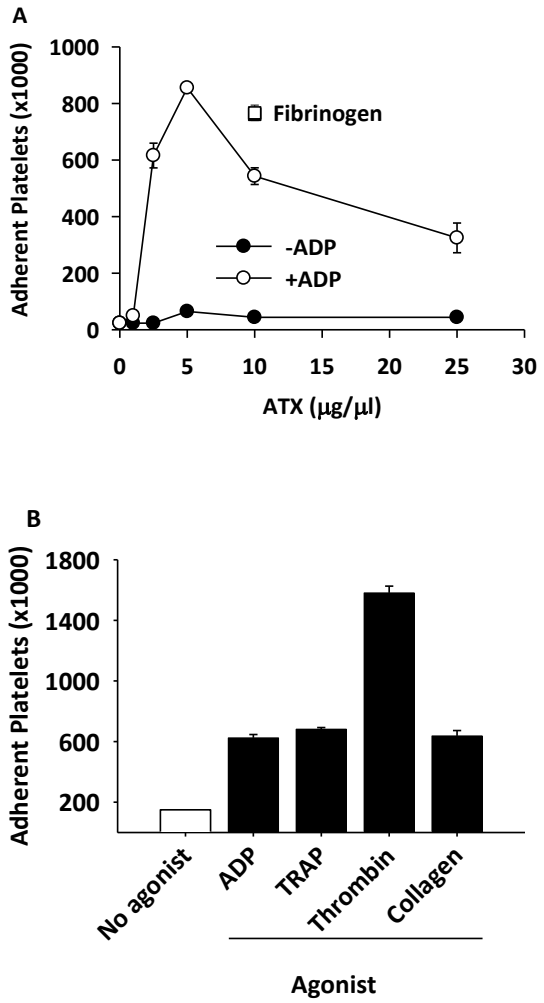
(A) Modified from Hausman *et al*[146].
(B) Mutants generated by Wu[136].

FIGURE 2.4



Synthesis of LPA by thrombin stimulated platelets is ATX-dependent and enhanced by exogenous ATX. (A) Gel-filtered platelets were treated with vehicle (○) or 0.5 U/ml thrombin (●) in the presence of the indicated concentrations of the ATX inhibitor HA155 for 30 minutes and LPA quantitated as described in the materials and methods. **(B)** LPA production by gel filtered platelets was determined in the presence of vehicle (○) or 0.5 U/ml thrombin (●) in the presence of the indicated concentrations of purified ATX and LPA quantitated as described in the materials and methods **(C, D)** LPA production by gel filtered platelets was determined at the indicated times in incubations containing no added ATX and vehicle (△) or 0.5 U/ml thrombin (▲) or 100 nM ATX and vechile (○) or 0.5 U/ml thrombin (●). Data shown are means of duplicate determinations (**A, C, D**) or mean ± SEM of triplicate determinations (**B**) from representative experiments that have been repeated using mulitple independent preparations of platelets.

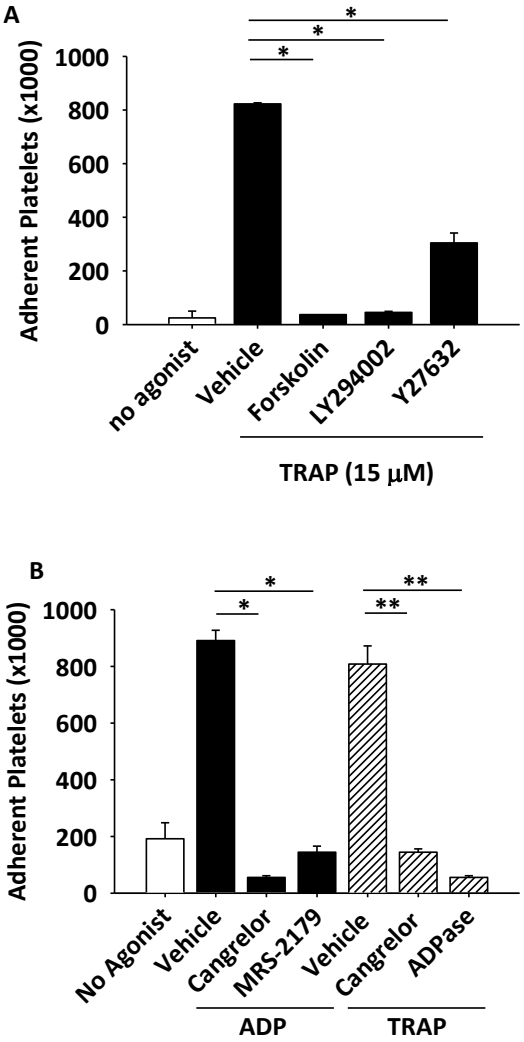
FIGURE 2.5



Activated platelets adhere to ATX: Gel-filtered, calcein-labeled platelets (200,000/μl in Tyrode's buffer with 2 mM $CaCl_2$ and 1 mM $MgCl_2$) were incubated in microtiter wells containing ATX. **(A)** Adhesion of resting platelets (closed circles) and platelets activated with 10 μM ADP (open circles) to ATX (immobilized at the indicated concentrations) and fibrinogen (immobilized at 10 μg/ml) for 1 hour at 37°C. **(B)** Adhesion of platelets to ATX (immobilized at 5 μg/ml) in the absence of agonist (open bar) or in the presence of 10 μM ADP, 15 μM thrombin receptor activating peptide (TRAP), 0.5 U/ml thrombin, or 10 μg/ml collagen for 1 hour at 37°C.

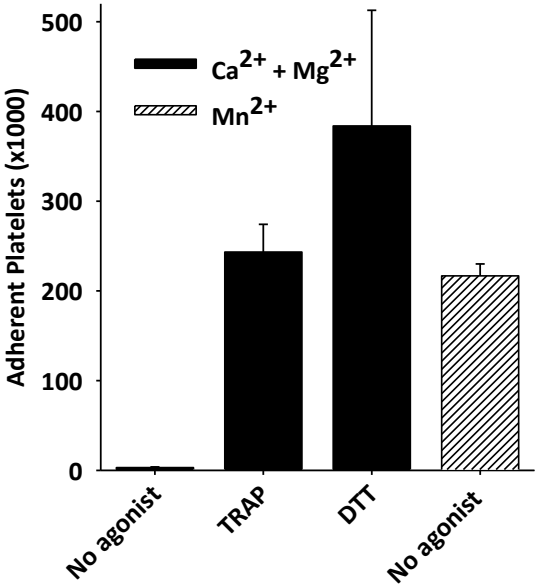
(A) Modified from Pamuklar *et al*[49].

FIGURE 2.6



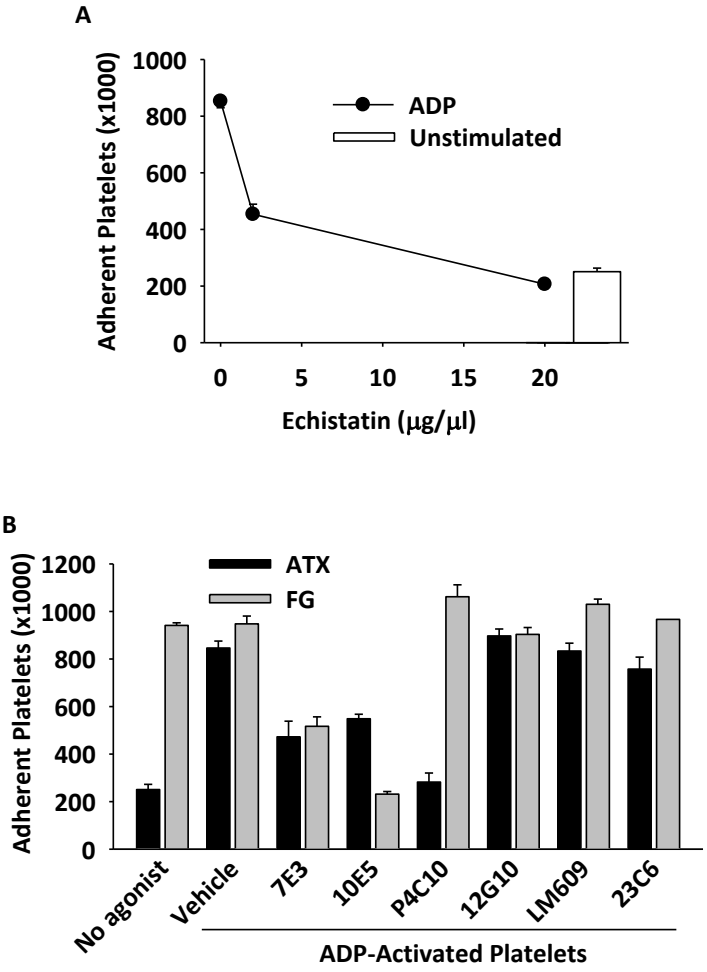
Platelet inhibitors block binding to ATX: (A) Platelets were incubated in wells with immobilized ATX in the absence of agonist or presence of TRAP and with either 100 μM forskolin, 20 μM LY294002 (PI3-kinase inhibitor) or 2.5 μM Y27632 (Rho kinase inhibitor). (*P<0.05 versus vehicle, ANOVA). **(B)** Where indicated, 0.5 μM cangrelor (P2Y12 antagonist), 10 μM MRS-2179 (P2Y1 antagonist), or 1 U/ml apyrase (ADPase) was included in the assay. (*P<0.05 versus ADP/vehicle, ANOVA; **P<0.05 versus TRAP/vehicle, ANOVA).

FIGURE 2.7



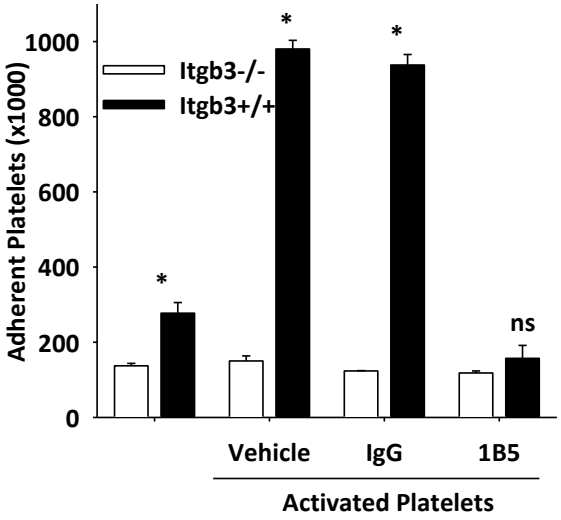
Mn^{2+} induces adhesion of resting platelets: Platelets (in Tyrode's buffer with the indicated divalent cations) were incubated in microtiter wells containing ATX (immobilized at 5 µg/ml) with vehicle (no agonist), 15 µM TRAP, 2.5 µM DTT, or 50 µM Mn^{2+}.

FIGURE 2.8



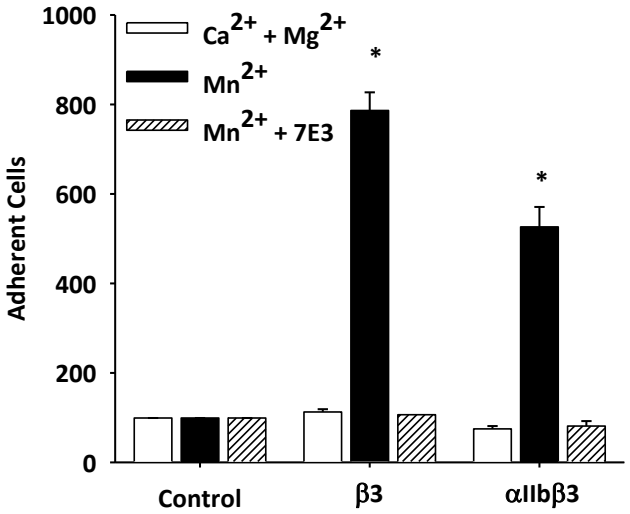
Human platelet adhesion is inhibited by blocking integrins: (A) Platelet adhesion to microtiter wells with immobilized ATX without (open bar) or with 10 μM ADP (closed circles) and the indicated concentrations of echistatin, an RGD-containing disintegrin. **(B)** Results obtained with platelets from one donor incubated with wells containing immobilized ATX (dark bars) or fibrinogen (gray bars; immobilized at 10 μg/ml) in the presence of 10 μM ADP and 20 μg/ml of a β3-blocking antibody (7E3), an αIIbβ3-blocking antibody (10E5), a β1-blocking antibody (P4C10), a β1 antibody that has no effect on ligand binding (12G10), an αVβ3-blocking antibody (LM609), and an αVβ3 antibody that has no effect on ligand binding (23C6).

FIGURE 2.9



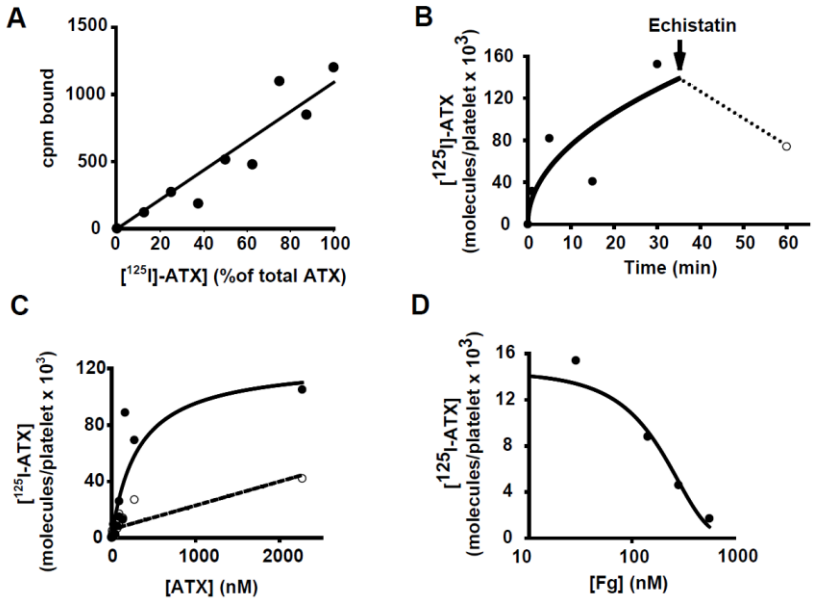
Activated murine platelets adhere to ATX in β3-dependent manner: Platelets were isolated from wild-type (Itgβ+/+) or Itgβ3-/- mice and incubated (200,000/µl) with wells containing immobilized ATX or fibrinogen in the presence of 150 µM PAR4 activating peptide. Results are representative of those obtained in 3 separate experiments. (*P<0.05 versus Itgβ+/+ platelets, 2-way ANOVA, ns = not significant).

FIGURE 2.10



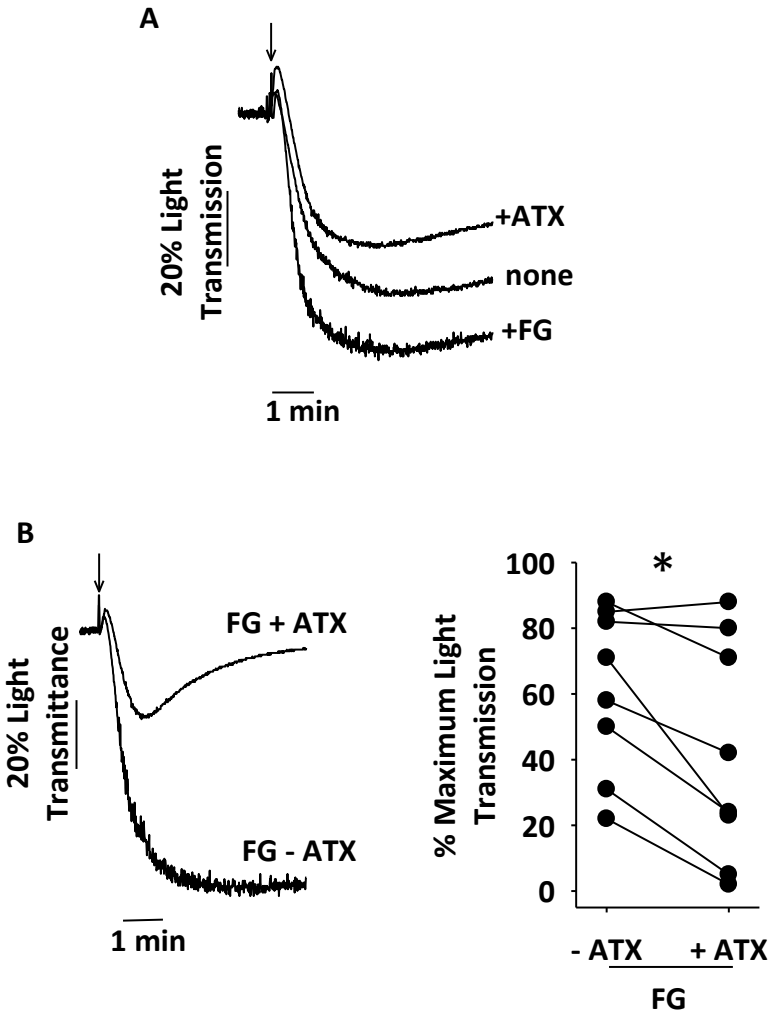
CHO cells transfected with β3 integrin adhere to ATX: Calcein-labeled CHO cells (300 cells/µl) (control) or CHO cells that were stably expressing β3 or αIIbβ3 were incubated with immobilized ATX in the presence of 2mM $CaCl_2$ and 1mM $MgCl_2$ (open bars), 50 µM Mn^{2+} (black bars) or 50 µM Mn_2 and 20 µg/ml of the function-blocking integrin β3 antibody 7E3 (hatched bars). (*P<0.05 versus control CHO cells, ANOVA). Results are presented as the number of adherent platelets or CHO cells (mean ± SD) from triplicate determinations.

FIGURE 2.11



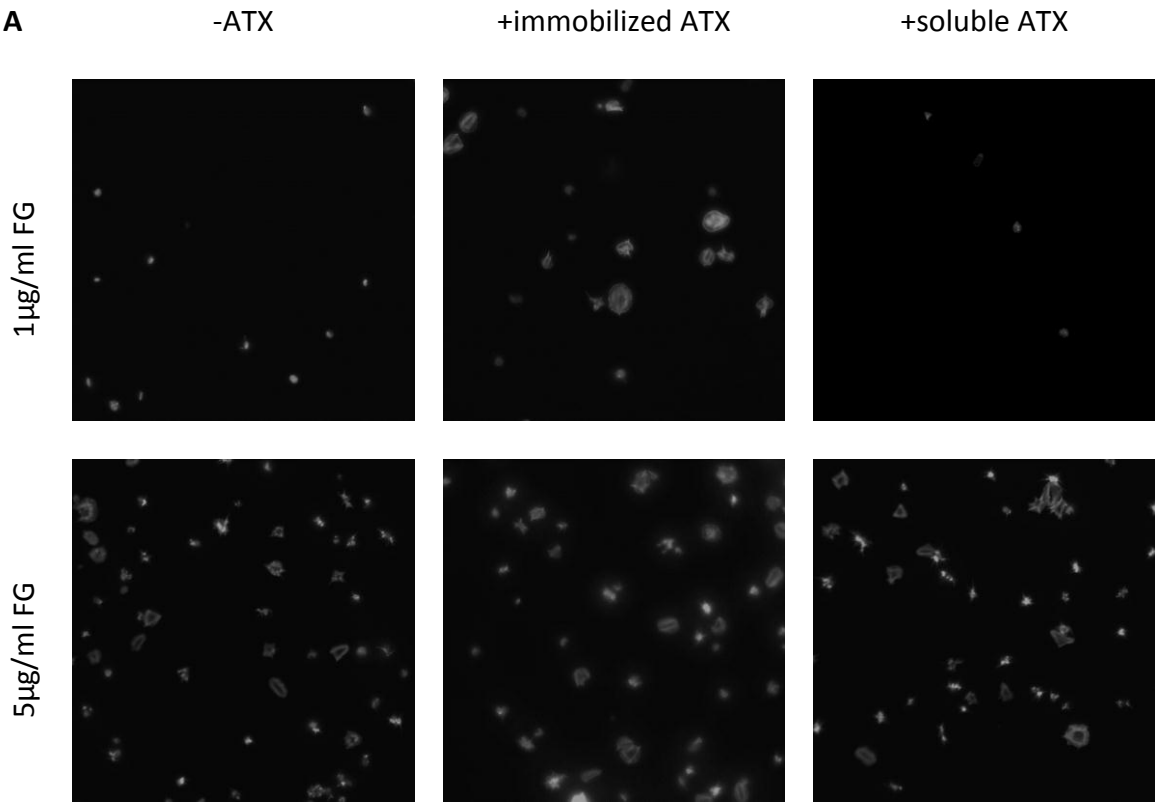
Affinity and stochiometry of ^{125}I-ATX binding to activated platelets. (A) 15 μM TRAP-activated platelets (200,000/μl) were incubated with a total of 73 nM ATX, composed of varying amounts of ^{125}I-labeled ATX and unlabeled ATX and platelet bound ATX determined as described in the materials and methods. **(B)** TRAP-activated platelets were incubated with ^{125}I-ATX for the indicated times. At 30 min, echistatin (20 μg/ml) was added and incubations continued for an additional 30 min to determine reversibility. **(C)** TRAP-activated platelets (●) or resting platelets (○) were incubated with the indicated concentrations of ATX for 30 min. **(D)** TRAP-activated platelets were incubated with [^{125}I]-ATX in the presence of the indicated concentrations of fibrinogen. Data are means of duplicate determinations.

FIGURE 2.12



Non-lysoPLD effects of ATX on platelet aggregation: **(A)** Gel-filtered platelets (200,000/µl) were stirred in the absence or presence of 200 µg/ml fibrinogen (+FG) or 50 µg/ml ATXT210A (+ATX). Platelet aggregation was stimulated by the addition of 15 µM TRAP at the time indicated by the arrow. **(B)** Gel-filtered platelets (200,000/µl) stirred in the presence of 200 µg/ml fibrinogen without ATX (FG – ATX) or with 50 µg/ml ATX (FG + ATX). 15 µM TRAP was added at the time indicated by the arrow. Left, representative light transmission tracing from single donor; right, cumulative maximal percent aggregation from different donors (*P <0.05, paired t-test).

FIGURE 2.13


A
-ATX
+immobilized ATX
+soluble ATX
1µg/ml FG
5µg/ml FG

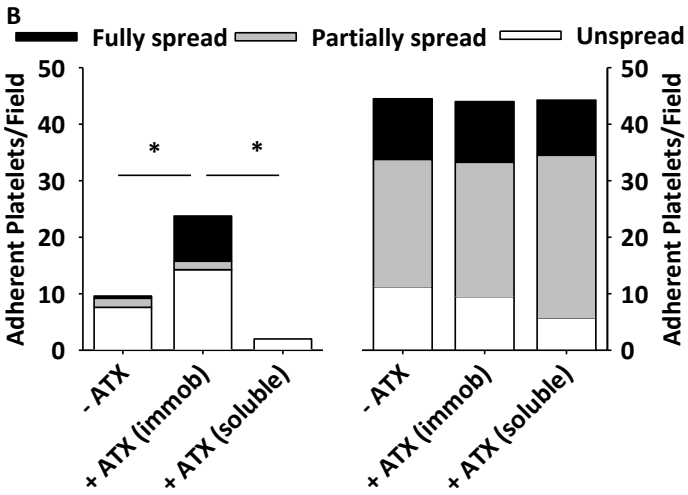
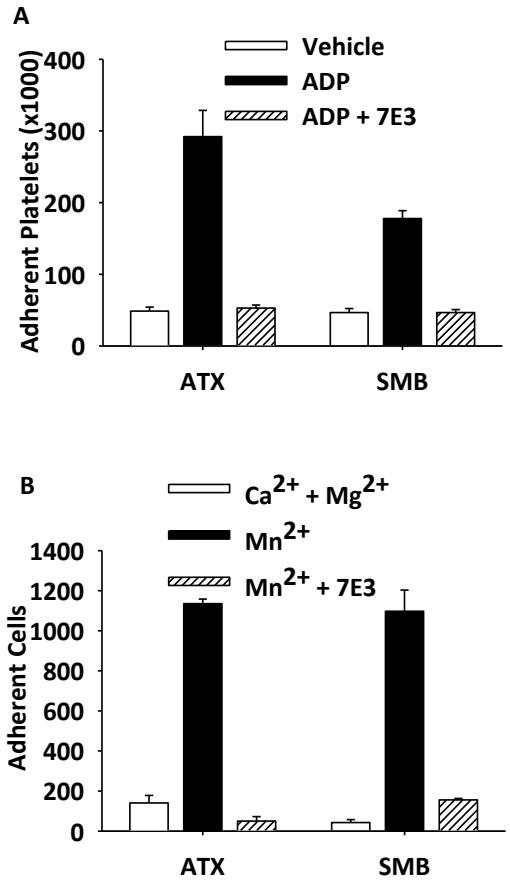

B
Fully spread
Partially spread
Unspread
Adherent Platelets/Field
50
40
30
20
10
0
*
*
- ATX
+ ATX (immob)
+ ATX (soluble)
- ATX
+ ATX (immob)
+ ATX (soluble)

FIGURE 2.13 (CAPTION)

Non-lysoPLD effects of ATX on platelet adhesion. Platelets (20,000/µl) were incubated with wells pre-coated with either 1 µg/ml or 5 µg/ml. **(A)** Where indicated plates were coated with ATX or FG and adherent platelets were stained with TRITC-phallodoin. Platelets were visualized by widefield fluorescent microscopy. **(B)** The total number of platelets/field in 4-5 fields were counted. Where indicated, the number of platelets adherent in the presence of soluble ATX was also determined. The platelets in each field were scored as fully spread (defined by the presence of cortical actin reorganization), partially spread (presence of filopodia) or unspread (neither cortical actin or filopodia). (*$P < 0.05$, One way ANOVA on total number of adherent platelets).

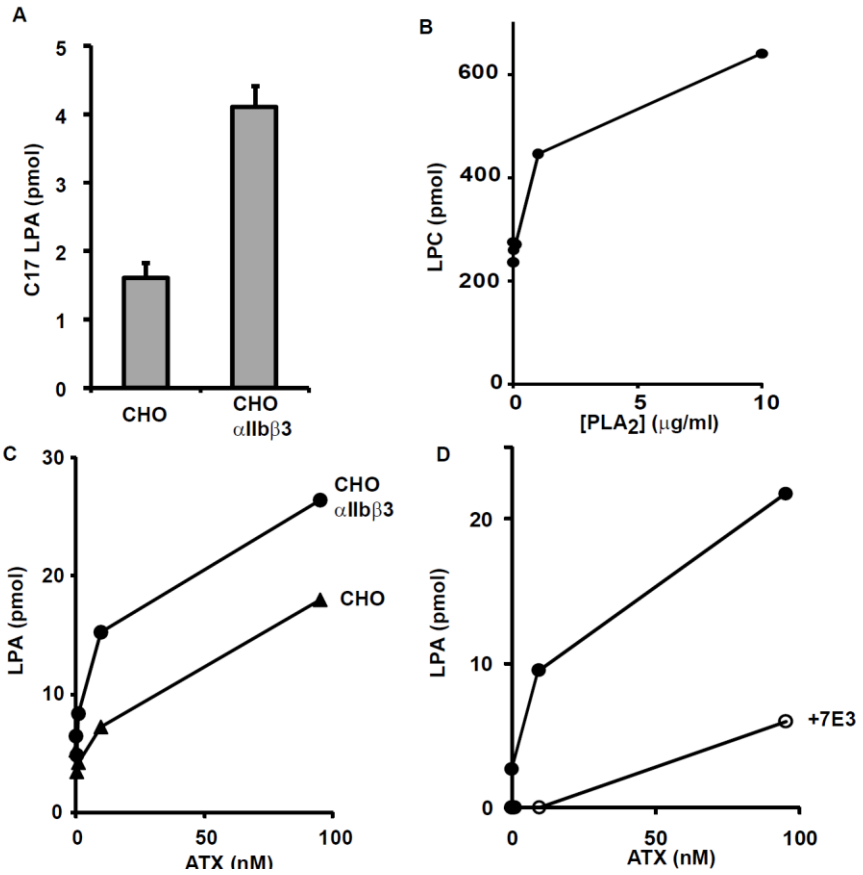
FIGURE 2.14



The SMB domain of ATX possesses integrin-binding capacity: (A) Human platelets were incubated with immobilized ATX or the N-terminal SMB1-2 fragment in the presence of 2 mM $CaCl_2$ and 1 mM $MgCl_2$ alone (open bars), with 10 µM ADP (black bars), or with 10 µM ADP and 20 µg/ml of the function blocking integrin β3 antibody 7E3 (hatched bars). **(B)** CHO cells stably expressing the human β3 integrin subunit were incubated with immobilized ATX or the N-terminal SMB1-2 fragment in the presence of 2mM $CaCl_2$ and 1mM $MgCl_2$ (open bars), 50 µM $MnCl_2$ (black bars) or 50 µM $MnCl_2$ and 20 µg/ml 7E3 (hatched bars). Data shown are means ± SD of triplicate determinations.

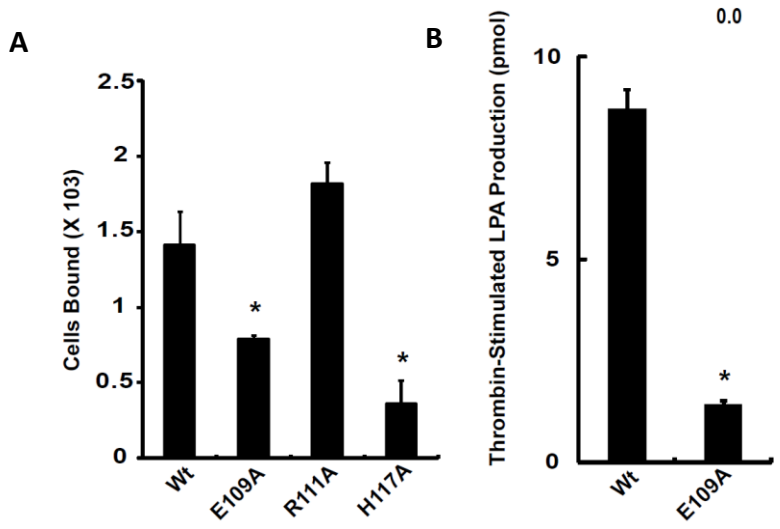
(A) modified from Hausman *et al*[146].

FIGURE 2.15



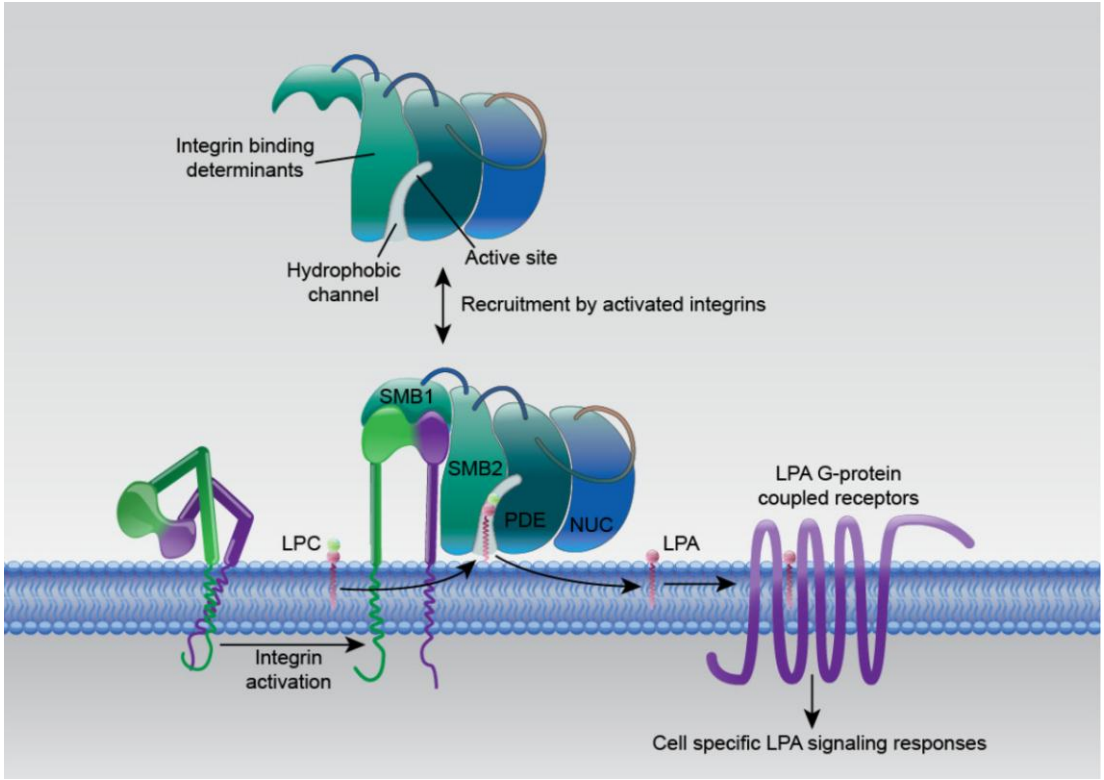
ATX activity against exogenously provided and cell-associated substrates is increased by integrin binding. **(A)** Control or αIIbβ3 expressing CHO cells were incubated with 100 nM ATX in Tyrode's buffer containing 1 mM $MgCl_2$, 50 μM $MnCl_2$,, 100 μM C17 LPC, and 0.35% BSA. Production of C17 LPA was measured after a 1 hour incubation at 37°C. **(B,C)** Control or αIIbβ3 expressing CHO cells were preincubated with bee venom PLA2 to increase LPC production **(B)** and then incubated with the indicated concentrations of ATX in the Tyrode's buffer used for the experiment shown in panel (A) omitting the C17 LPC and production of LPA determined as described in the materials and methods. **(D)** αIIbβ3 expressing CHO cells were preincubated with bee venom PLA2 and then incubated with the indicated concentrations of ATX in the Tyrode's buffer in the presence or absence of 10 μg/ml of the integrin blocking antibody 7E3 and production of LPA determined as described in the materials and methods. The data shown are means ± SD of triplicate determinations (A) or duplicate determinations (B,C).

FIGURE 2.16



ATX SMB2 mutants with impaired binding to β3 integrins display reduced LPA generating capacity. (A) Binding of β3 integrin expressing CHO cells to wild type ATX and the indicated ATX variants was determined as described in the materials and methods (mean ± SD of triplicate determinants). **(B)** The effect of wild type ATX and ATX E019A on LPA production by thrombin stimulated platelets was determined as described in the materials and methods (mean ± SD of triplicate determinations).

FIGURE 2.17



Current Model. Recruitment of ATX to the cell surface by activated integrins enables localized production of LPA by hydrolysis of cell surface lysophospholipid substrates.

CHAPTER 3: LPA4 SIGNALING IN MURINE PLATELETS

INTRODUCTION

Among the effects LPA has on vascular and blood cells described in Chapter 1, LPA acts as an agonist to human platelets. Activation of human platelets by LPA likely involves G12/13 stimulation of the GTPase Rho, which regulates myosin light chain kinase and actin reorganization leading to shape change[125, 127]. Reports vary on the extent to which LPA elicits intracellular Ca^{2+} release in platelets[129, 176]. LPA can also activate tyrosine kinases SRC and SYK in platelets[176]. Higher concentrations of LPA are required to activate platelets in whole blood than those effective at activating isolated platelets, which may be due in part to sequestrations of LPA by plasma albumin or gelsolin or by the metabolism of LPA by blood cell-associated LPPs[177]. Additionally, ADP receptor antagonists block LPA-induced platelet aggregation in whole blood, indicating that released ADP potentiates platelet LPA responses[127].

The identity of the stimulatory human platelet LPA receptor(s) has not been established. Human platelets contain mRNAs for LPA1-5[20, 130, 172]. Interestingly LPA species with alkyl-linked and highly unsaturated radyl chains are uniquely potent platelet activators[98, 99, 178]. This unusual pharmacology is not observed with recombinant LPA1-4 expressed in heterologous expression systems, indicating that these receptors are unlikely to be primary mediators of the major stimulator effects of LPA on human platelets. LPA has also been proposed as an endogenous activator of PPARγ and intravascular infusion of

LPA appears to affect smooth muscle cell function through PPARγ[179]. In platelets, activation of PPARγ has been associated with inhibition of platelet function[180].

We and others have reported that platelets from a subset of normal donors (~20%) fail to aggregate in response to LPA[98, 172]. Lack of LPA-induced aggregation in LPA-nonresponsive individuals is associated with the presence of an LPA-promoted inhibitory signaling response in platelets, and interestingly individuals with coronary artery disease are more likely to have LPA-responsive platelets[172]. The presence of LPA-mediated inhibitory signals may explain the variability among different human donors observed in platelet responses to LPA. Among the LPA receptors expressed in human platelets, LPA4 and LPA5 are the most abundant and are indeed among the most abundantly expressed of all platelet GPCRs[20, 172, 181]. We have found that the subset of donors who fail to aggregate in response to LPA have higher expression levels of LPA4[172]. Finally, in contrast to the majority of human platelets, LPA inhibits agonist-activation of mouse platelets[49].

We predict that differential expression levels of LPA4 and LPA5 explains the difference in LPA responsive between murine platelets and platelets derived from most human donors as well as the difference noted between human responders and non-responders. Since LPA4 expression levels are higher in human non-responders and LPA4 is capable of coupling to Gs increasing cAMP levels in cultured cell systems[100], we hypothesized that LPA4-dependent signaling is responsible for inhibitory signals of LPA. We likewise predicted that the most significant LPA-dependent platelet-activating pathway is via

LPA5. This is because LPA5 is the second most abundant transcript found in platelets[181] and preferentially binds to alkyl-LPAs which are more potent platelet agonists than acyl-LPAs[99, 182].

Led by Julie Oestreich, PharmD, PhD, we began to investigate these signaling pathways using complementary methods. One of these methods involved investigation of the role of LPA5 in human platelet aggregation using bromophosphonate-based LPA mimetics (BrP-LPA). These compounds have a chiral center and exist to one of two enantiomeric forms. The *anti* form of the compound is a pan-LPA receptor antagonist (for LPA1-5). The *syn* form of the compound also shows antagonism for LPA1-4 but partial agonism for LPA5[183]. We have previously conducted studies to evaluate the effects of these compounds on human platelet aggregation and found that *syn*-BrP-LPA, the partial LPA5 agonist, induces promotes platelet aggregation (**Figure 3.1**) while *anti*-BrP-LPA inhibits agonist-depndent and specifically LPA-dependent aggregation (**Figure 3.2**) (Julie Oestreich and Susan Smyth, unpublished data). This supported the hypothesis that LPA5 plays a stimulatory role in platelet signaling. To further investigate the role of these receptors in platelet signaling, we also investigated platelet activation and thrombosis is LPA4-deficient mice (*lpa4-/-*). In whole blood aggregation assays of *lpa4-/-* platelets were slightly more prone to aggregate in the presence of moderate doses of a PAR4 agonist peptide (AYPGKF) at moderate concentrations (**Figure 3.3A**). The *lpa4-/-* platelets also exhibited slightly more P-selectin expression (**Figure 3.3B**) although in this case the results were not statistically significant (Smyth, unpublished). LPA had only moderate effects on isolated murine platelets, but we nonetheless sought to evaluate

whether there was an *in vivo* thrombotic phenotype. While blood counts were identical in wild type (WT) vs *lpa4-/-* animals (**Table 3.1**), *lpa4-/-* mice displayed a pro-thrombotic phenotype during a ferric chloride ($FeCl_3$)-induced carotid artery thrombosis (**Figure 3.4**) (Smyth, unpublished).

In light of these findings, I continued to evaluate our hypothesis that platelet LPA4 mediates a predominately inhibitory pathway.

METHODS

Mice

All procedures conformed to the recommendations of the "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education, and Welfare publication no. NIH 78-23, 1996) and were approved by the Institutional Animal Care and Use Committee. Mice were weaned at 21 days, maintained on a 12-h light and 12-h dark cycle, and fed water and standard rodent chow (2018 Harlan Tekland Rodent Diet) *ad libitum*. Mice were originally received as a generous gift from Xianjun Fang (Virginia Commonwealth University). The null mice were produced by deletion of the 3' portion of intron 2 and the 5' fragment of exon 3 of the *lpa4* gene as described in Lee *et al.*[101] We maintained the mice on a mixed *129/Sv* and *C57BL/6* background.

Blood Collection

Blood was collected from healthy donors with approval from the Institutional Review Board at the University of Kentucky by cubital venipuncture with a 19 gauge needle and

collected into citrate (final citrate concentration was 0.38%). For studies of isolated murine platelets, we collect blood by retrooribital puncture performed under anesthesia as previously described[163].

Preparation of Platelets

Citrated human blood was centrifuged at 450 x g for 5 minutes to yield platelet rich plasma (PRP), to which 134 nM prostaglandin I2 (PGI2) was added. Platelets were separated from plasma by filtration of PRP using a column of Sepharose 2B (Sigma) equilibrated in HEPES-buffered modified Tyrode's buffer (138 mM NaCl, 5.6 mM dextrose, 2.7 mM KCl, 10 mM HEPES, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, pH 7.35) with 0.35% fatty acid-free BSA (US Biological) . For adhesion assays, PRP was incubated with 7 µM calcein AM for 30 minutes at 37°C prior to gel-filtration. Unless otherwise indicated, gel-filtered platelets were diluted to 200,000 /µl in Tyrode's containing fatty acid free (ethanol and charcoal extracted) BSA (Sigma Aldrich).

Bone marrow transplantation

Recipient mice (C57BL/6 background) were γ-irradiated with cesium-137 as the radiation source (Mark I Irradiator). The first radiation dose was 7 Gy (700 rad). Donor mice were then anesthetized and euthanized with isoflurane followed by cervical dislocation. They were sterilized by submersion in 70% ethanol and the femurs and tibia were isolated into sterile PBS. Following isolation, the ends of the long bones were cut off and a 27.5 gauge needle was used to flush isolation media (RPMI media supplemented with 10mM HEPES, 25 U/mL heparin and 5% FBS) until bone marrow was

completely removed. The bone marrow components were pelleted using an Eppendorff 5810 R centrifuge at 1500 RPM at 4°C for 10 minutes. The pellet was resuspended in 3 ml 0.2% saline to lyse the erythrocytes for 45 seconds followed by addition of 7 ml 1.2% saline to stop the lysis. The cells were pelleted once more and resuspended to ~50 x 10^6 cells/mL in PBS supplemented with 2% heat-inactivated FBS. The recipient mice were then given a second radiation dose of 5 Gy (500 rad) which occurred at least 3 hours after the first dose. Following the second radiation dose, the recipient mice were injected with the prepared bone marrow-derived cells from the donor mice (~5 x 10^6 cells/recipient).

Fibrinogen Binding

Mice were anesthetized with isoflurane, and blood was collected by puncture of the retrobulbar venous plexus into tubes containing 1/10th the volume of 3.2% sodium citrate (final concentration of 0.32% citrate). Citrated blood was then diluted 1:40 into Tyrode's buffer (138 mM NaCl, 5.6 mM dextrose, 2.7 mM KCl, 10 mM HEPES, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, pH 7.35) with 0.35% fatty acid-free BSA (US Biological). Prior to use 1 mM $MgCl_2$ was added to the diluted blood. 18 µl of blood was added to 9 µl of FITC-anti-FG (Dako) which had been diluted 1:10 in Tyrode's + BSA + $MgCl_2$ and 3 µl of 10x agonist. Following a10-minute incubation, reactions were stopped by adding 500 µl Tyrode's + BSA + $MgCl_2$ and 500 µl 4% paraformaldehyde in PBS. Fibrinogen binding was determined by fluorocytometric analysis (BD FACSCalibur Flow Cytometer; 342975). In experiments which involved evaluating the effects of LPA, LPA stocks (in chloroform)

were dried and then reconstituted in Tyrode's + BSA by successive vortexing and sonicating.

Platelet Aggregation

For light transmission aggregometry, isolated platelets were prepared by gel filtration into Tyrode's with BSA as described above and diluted to 200,000 /µl. 2 mM Ca^{2+} and 1 mM Mg^{2+} was added as was fibrinogen where indicated. 400 µl isolated platelets were aggregated using the indicated agonists at 37°C under stirring conditions. Aggregation was measured by light transmission (Platelet-Ionized Calcium Aggregometer; Model 660; Chrono-log). For whole blood aggregation, blood was collected into hirudin and diluted 1:1 with normal saline. 300 µl diluted blood were added to test wells, and aggregation was determined by recording electrical impedence (Multiplate; Model MP0010, Dynabyte Medical).

Static Platelet Adhesion

Recombinant type I collagen (from rat tail; BD) or fibrinogen (American Diagnostica) diluted in Tris buffer (50 mM Tris, 100 mM NaCl, pH 7.4) was incubated in wells of an 8-well coverslip chamber (LabTek2) overnight at 4°C. The wells were subsequently incubated for 1 hour at room temperature with Tyrode's containing BSA to block non-specific binding sites. Platelets were diluted to 20,000 /µl in Tyrode's containing BSA and 1 mM Mg^{2+} and allowed to adhere to wells for one hour at 37°C. Non-adherent platelets were removed by successive washings with Tyrode's containing BSA and 1 mM

Mg^{2+} and adherent platelets were fixed with 4% paraformaldehyde, permeabilized, and stained with TRITC-phalloidin.

Carotid Artery Thrombosis

4% $FeCl_3$ (247 mM) solution was prepared in normal saline on the day of use. Mice were anesthetized with inhaled isoflurane (1-2.5%), and their body temperature was maintained at 37°C by varying the output of an EPZ type halogen heat lamp. A flow probe (Model 0.5 PSB, Trnasomic System, Inc.) was placed on the left carotid artery. Thrombosis was initiated by the application of two 1 – 2-mm pieces of filter paper (Whatman no. 3) on opposite sides of the carotid artery that had been submerged in the $FeCl_3$ solution for 5 seconds just prior to application. The filter paper was removed after 3 minutes. The flow probe was used to record the time thrombotic occlusion, which was defined as the time to complete cessation of blood flow for at least 30 seconds.

Cytokine Assays

Membranes impregnated with cytokine antibodies (RayBiotech) were blocked in PBS + 0.1% casein at 4°C overnight. The membranes were then incubated with mouse plasma for 2 hours at room temperature and washed thrice with PBS + 0.1% tween. The membranes were then incubated with biotin-conjugated cytokine antibodies (RayBiotech) for 2 hours at room and washed 4 times with PBS + tween. Finally the membranes were incubated in the dark with IRDye 800CW-conjugated streptavidin (LI-COR) for 2 hours at room temperature and washed thrice with PBS + tween and then twice with PBS.

Statistics

Statistical analyses and preparation of figures were conducted using SigmaStat and SigmaPlot, respectively. Specific statistical tests are indicated in figure legends.

RESULTS

Binding of Fibrinogen is Not Altered in *lpa4-/-* Platelets

LPA typically acts as an agonist in human platelets while LPA is refractory to activation in murine platelets. The agonistic or antagonistic activity may be due to relative levels of LPA4 expressed by platelets. In our preliminary studies (described in the previous section) we found only mild to no differences in activation of *lpa4-/-* platelets compared to WT platelets using a whole blood aggregation assay and PAR4-stimulating peptide or collagen as an agonist. We sought to find if there were more subtle differences in platelet activation as specifically measured by fibrinogen binding. Histograms based upon fibrinogen binding (**Figure 3.5A**) showed no clear difference between *lpa4-/-* platelets or WT platelets whether evaluated by the percentage of platelets to have positive fibrinogen binding as indicated by fluorescence above a pre-set threshold (**Figure 3.5B left**), evaluated by the geometric mean (**Figure 3.5B right**), or evaluated by any other measure of central tendency (not shown). Similarly, no difference between *lpa4-/-* platelets and WT platelets was noted when platelets were exposed to 10 µM oleyl LPA and agonist (**Figure 3.6A, B**).

lpa$_4$-/- Platelets Adhere More Strongly to Collagen than WT Platelets

We further sought to evaluate the ability of *lpa4-/-* platelets to adhere to different adhesive proteins. We were particularly interested in this since our earlier studies suggested that *lpa4-/-* mice were prone to thrombosis with $FeCl_3$. Platelets derived from *lpa4-/-* mice adhered to fibrinogen in a comparable manner as did platelets derived from WT mice (**Figure 3.7A, B**). Interestingly, *lpa4-/-* platelets from older mice (~2 years) adhered to collagen a greater extent than platelets from age-matched WT littermate mice (**Figure 3.7B, C**). However, platelets derived from younger *lpa4-/-* mice (~3 months) did not show increased absolute numbers of adherent platelets to collagen compared with age-matched WT controls. The amount of platelet spreading did increase in *lpa4-/-* mice compared with WT mice (although not to a point that was statistically significant) (**Figure 3.8C**). We therefore hypothesized that any differences we saw in the ability of *lpa4-/-* platelets and WT platelets to adhere to collagen was due to differences in the platelet levels of GPVI, the major collagen receptor which facilitates signaling. To evaluate this, we used a FITC-labeled anti-GPVI monoclonal antibody to evaluate levels of GPVI on *lpa4-/-* and WT platelets by fluorocytometry and found no difference in the levels of GPVI (**Figure 3.8A, B**)

Mice with *lpa4-/-* Derived Bone Marrow Are Protected from Thrombosis

Our earlier data suggested that *lpa4-/-* mice had a thrombotic tendency. Likewise *lpa4-/-* platelets seem to adhere more efficiently to immobilized collagen although aggregation and fibrinogen binding assays showed little to know difference in activation

response of *lpa4-/-* platelets when compared with WT mice. This raised the distinct possibility that the observed pro-thrombotic phenotype could be due to LPA4 receptors in cells aside from platelets. Expression of *lpa4* has been reported in endothelial cells[103]. We therefore generated chimera mice by lethally irradiating recipient mice and rescuing them with bone marrow derived from *lpa4-/-* mice or WT mice. In general, complete blood counts were similar between mice reconstituted with *lpa4-/-* bone marrow and WT bone marrow (**Table 3.2**). Not surprisingly, all irradiated mice developed a relative pancytopenia when compared with non-irradiated mice (**Table 3.1** and **Table 3.2**). After conducting thrombosis experiments on these chimeras, we found that animals transplanted with WT platelets were more prone to thrombosis than animals transplanted with platelets from *lpa4-/-* donor mice with non-transplanted mice exhibiting an intermediate phenotype (**Figure 3.9**). This was in stark contrast to the thrombosis experiments conducted previously in global *lpa4-/-* mice which exhibited a pro-thrombotic phenotype (**Figure 3.4**).

Despite this discrepancy, there is a clear effect that LPA4 plays on thrombosis. It is not currently clear whether LPA4 plays a protective role or deleterious role. In any case, the effect is not likely to be due to platelet LPA4 in these animals since isolated platelets have little to no appreciable phenotype in aggregation, flow cytometry, or adhesion. We therefore evaluated cytokine levels in these mice using antibodies against a variety cytokines related to atherosclerosis. No differences were appreciated between the two genotypes (**Table 3.3**).

DISCUSSION

LPA has typically been characterized as a weak platelet agonist although we have found previously that in some humans, LPA is refractory to platelet activation[172] as it is in murine platelets[49]. This stimulatory activity of LPA on human platelets seems to involve LPA5 because LPA stimulatory effects are elicited by an LPA mimetic that has partial agonist activity at LPA5 but is antagonistic to other LPA receptors[183] (**Figure 3.1**). This observation is consistent with earlier reports that alkyl-LPA, which acts more potently at LPA5, is a stronger platelet agonist than acyl LPAs[98, 182]. Because LPA4 expression is abundant in human platelets[20], has higher expression in humans whose platelets have refractory responses to LPA[172], and can couple to Gs[100], we surmised that this is likely the receptor responsible for LPA-dependent platelet inhibition. Because LPA acts as an inhibitor to murine platelets, we used an *lpa4-/-* mouse model to test the hypothesis that the inhibitory LPA signaling pathway is mediated by LPA4. We evaluated their platelet responses in *in vitro* and *in vivo* settings.

Earlier findings in our laboratory suggested that there was perhaps a moderate pro-stimulatory phenotype in isolated *lpa4-/-* platelets (**Figure 3.3**), but the finding that was especially compelling was the *in vivo* thrombosis data using a $FeCl_3$-injury model, a model that is collagen-dependent[184]. *Lpa4-/-* mice exhibited a substantially pro-thrombotic phenotype compared with WT mice (**Figure 3.4**). As I began evaluating these pathways, again using isolated platelets, I could not find any differences in stimulatory signaling in platelets derived from *lpa4-/-* mice compared with those

derived from WT mice (**Figure 3.5** and **Figure 3.6**). I sought also to look at inhibitory pathways by measuring the phosphorylation of vasodilator-stimulated protein, a marker of platelet inhibition, via Western blotting and flow cytometry although sufficient signal strength was never achieved to draw any conclusive results (data not shown). In short, very few of the studies I conducted on isolated platelets yielded significant results. Indeed one of the only assays I conducted that displayed a difference between *lpa4-/-* platelet and WT platelets was adhesion to collagen (**Figure 3.7**), and even then the results were age-dependent and only suggestive. Since there was a modest effect of the LPA4 receptor on collagen adhesion, I decided to go back to the $FeCl_3$ (collagen-dependent model). This time instead of using global knockouts, I decided to look at the effect of LPA4 receptors specifically in bone marrow-derived cells. This, I hoped, would yield more-platelet specific information. As described in the previous section, the thrombosis data in these chimeras were paradoxically the exact opposite of our laboratory's earlier observations in global knockouts. Chimeras that were transplanted with *lpa4-/-* bone marrow were actually protected from thrombosis rather than being prone to thrombosis (**Figure 3.9**).

It is difficult to reconcile the difference in these findings if one were to use a platelet-centric view point. Interestingly, although LPA receptors and LPA responses have clearly been identified in human platelets, the same cannot be said for mouse platelets with the exception of our laboratory's earlier findings that LPA inhibits platelet activation[49]. Recently, a non-biased evaluation of the platelet transcriptome of both humans and mice revealed that most LPA receptor transcripts are present in human platelets[20]. In

stark contrast to this there were no LPA receptor transcripts expressed in murine platelets with the exception of exceedingly small levels of *lpa4* which itself was at the threshold of detectability[20]. Over the course of these studies, we attempted to directly evaluate LPA4 protein levels, although like many membrane-bound proteins, it proved difficult to evaluate. There is a clear presence of a phenotype using *in vivo* thrombosis and a lack of a phenotype in isolated platelet activity assays. This indicates that the role that LPA4 plays in thrombosis is actually in a cell type different than platelets. LPA4 expression has been reported in endothelial cells[103] making this a likely candidate for being responsible for some of the thrombosis phenotypes seen when comparing *lpa4-/-* thrombosis with WT thrombosis. Even in the chimeric animals, it is certainly plausible that some of the endothelial cells were actually devoid of LPA4 in the animals transplanted with *lpa4-/-* bone marrow. The chimeras were produced by lethal irradiation and undoubtedly had endothelial damage which may have been replaced by LPA4-deficient cells as there have been reports that circulating endothelial progenitor cells are bone marrow-derived[185]. In the chimeras, injury itself promoted thrombosis as chimeras reconstituted with WT bone marrow were more prone to exhibit full occlusion than non-irradiated mice (**Figure 3.9**). If anything, LPA4-depletion seemed to protect animals from this radiation-dependent injury and subsequent propensity toward thrombosis. This suggests that a number of different mechanisms involving inflammatory responses, fibrosis, *etc.* may be dependent on LPA4 in one or more yet-to-be-identified cells. An attempt to elucidate such factors by evaluating soluble cytokines did not yield any striking findings (**Table 3.3**).

In conclusion, while the LPA4 receptor plays an important, if still ambiguous role, in thrombosis, this may or may not be due specifically to platelet LPA4. The assays presented here involving isolated platelets did not identify a specific role for LPA4 in contrast to our earlier paper that identified an inhibitory role of LPA in murine platelets[49]. When these observations are viewed in light of the extremely small levels of LPA4 transcript identified in murine platelets[20], the most reasonable explanation is that very slight changes in LPA4 expression in murine platelets could result in vastly different responses of murine platelets to LPA and other stimulator and/or inhibitory agents. These could all be dependent upon mouse strain, sex, age, and environmental variations. Indeed phenotypes as severe as embryonic mortality are starkly different in LPA4-deficient mice derived in different laboratories[101, 103], so differences in platelet function are certainly plausible. It is still very likely that in humans, LPA4 is responsible for a platelet-inhibitory pathway while LPA5 promotes platelet activation. Murine models may simply not be a good model to investigate these given the extreme scarcity of these receptors when compared with human platelets.

Future work in this area would necessarily include understanding the relative role of vascular cells and blood cells using a number of different chimeras (WT to *lpa4-/-*, *lpa4-/-* to WT, *etc.*). To further understand the relative roles of LPA4 and LPA5 in humans, an additional investigatory tool would be knock-in mice that express significant levels of human LPA4 and/or human LPA5 (either specifically in bone marrow-derived cells using a viral vector or a global genomic knock-in).

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TABLE 3.1

Genotype	n	WBC (K/ml)	Hct (%)	Hgb (g/dL)	Platelet (K/ml)
Wild-type	9	6.5 ± 0.9	46 ± 2	12 ± 0.4	695 ± 78
Lpa4-/-	9	7.7 ± 0.7	44 ± 1	12 ± 0.3	656 ± 42

Complete blood counts of wild-type and *lpa4-/-* mice.

TABLE 3.2

Genotype	n	WBC (K/ml)	Hct (%)	Hgb (g/dL)	Platelet (K/ml)
Wild type	6	12.0 ± 1.8	26.4±	5.6±1.6	476± 119
Lpa4-/-	6	11.1 ± 2.0	27.4±	6.1±1.7	645 ± 80

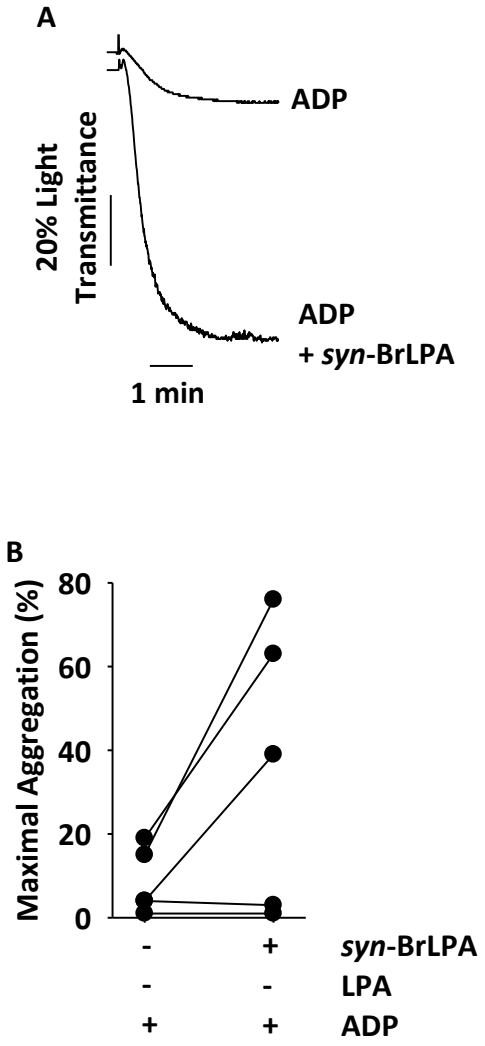
Complete blood counts of chimera mice transplanted with bone marrow from wild-type and *lpa4-/-* mice.

TABLE 3.3

	Lpa4-/-			WT		
GCSF	0.230	±	0.006	0.225	±	0.006
IL-5	0.072	±	0.032	0.035	±	0.000
RANTES	0.083	±	0.004	0.077	±	0.004
GM-CSF	0.110	±	0.000	0.105	±	0.003
IL-6	0.042	±	0.003	0.042	±	0.003
TNFα	0.127	±	0.004	0.113	±	0.008
IFNγ	0.177	±	0.122	0.052	±	0.002
IL-13	0.098	±	0.004	0.088	±	0.002
VEGF	0.018	±	0.003	0.018	±	0.002
IL-1α	0.048	±	0.002	0.052	±	0.003
L-Selectin	0.558	±	0.016	0.515	±	0.020
IL-1β	0.097	±	0.007	0.088	±	0.009
MCP1	0.093	±	0.003	0.078	±	0.006
FGF basic	0.225	±	0.110	0.107	±	0.009
IL-2	0.025	±	0.003	0.022	±	0.002
M-CSF	0.093	±	0.003	0.085	±	0.006
CD40	0.068	±	0.004	0.062	±	0.003
IL-3	0.070	±	0.003	0.065	±	0.006
MIP-3α	0.113	±	0.003	0.097	±	0.010
Eotaxin	0.017	±	0.002	0.015	±	0.005
IL-4	0.102	±	0.006	0.098	±	0.012
P-Selectin	0.273	±	0.019	0.218	±	0.028

Atherogenic Cytokines Unaffected in *lpa4-/-* Mice: Levels of select atherogenic cytokines were ascertained in platelet-poor plasma derived from *lpa4-/-* mice (left) and WT littermates (right). Values indicate mean intensity and standard deviation for each group. N = 3 for each group. No cytokines showed statistically significant differences using a Student's t-test.

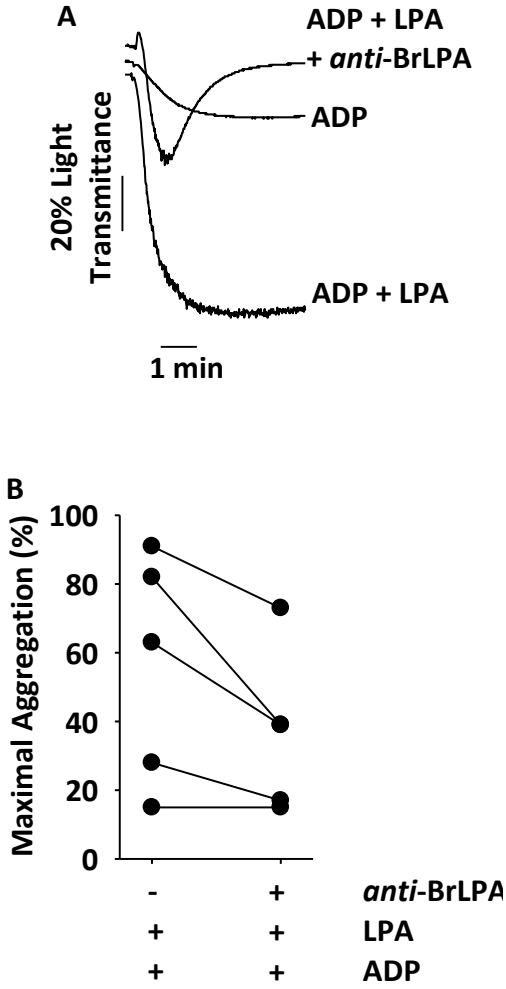
FIGURE 3.1



Agonism of LPA_5 Promotes Aggregation. Light transmission aggregation of human platelets after isolation by gel-filtration. Platelets were treated with vehicle or 10 µN *syn*-BrLPA analog immediately prior to the addition of 20 µM ADP. **(A)** Representative tracing from a single donor. **(B)** Cumulative maximal percent transmission from multiple donors. P = 0.08 (Paired t test).

Oestreich and Smyth, unpublished.

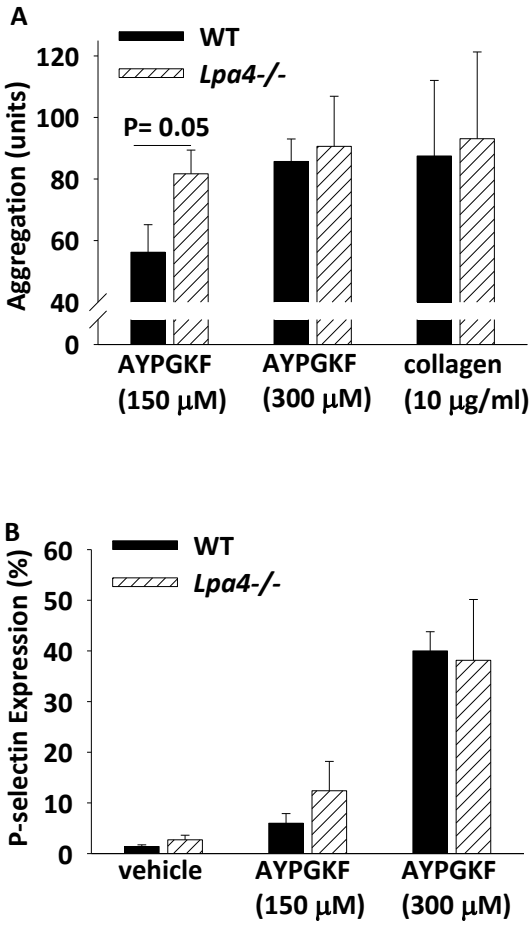
FIGURE 3.2



LPA Pan-antagonism Inhibits Aggregation. Light transmission aggregation of human platelets after isolation by gel-filtration. Platelets were treated with vehicle or 10 µN *syn*-BrLPA analog immediately prior to the addition of 20 µM ADP. **(A)** Representative tracing from a single donor. **(B)** Cumulative maximal percent transmission from multiple donors. P = 0.06 (Paired t test).

Oestreich and Smyth, unpublished.

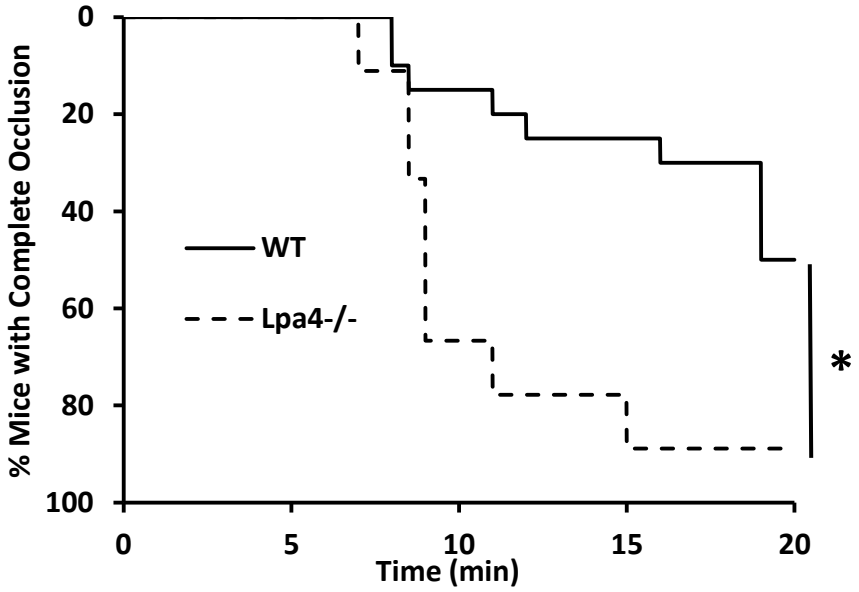
FIGURE 3.3



Platelet Activation in *lpa4-/-* mice. (A) Whole blood aggregation of mice treated with a peptide agonist to the PAR4 receptor (AYPGKF) at the indicated concentrations or with 10 μg/ml collagen. AT 150 μM AYPGKF p = 0.05 (Student t-test). **(B)** P-selectin expression measured by flow cytometry in ploatelets treated with AYPGKF at the indicated concentrations.

Smyth, unpublished.

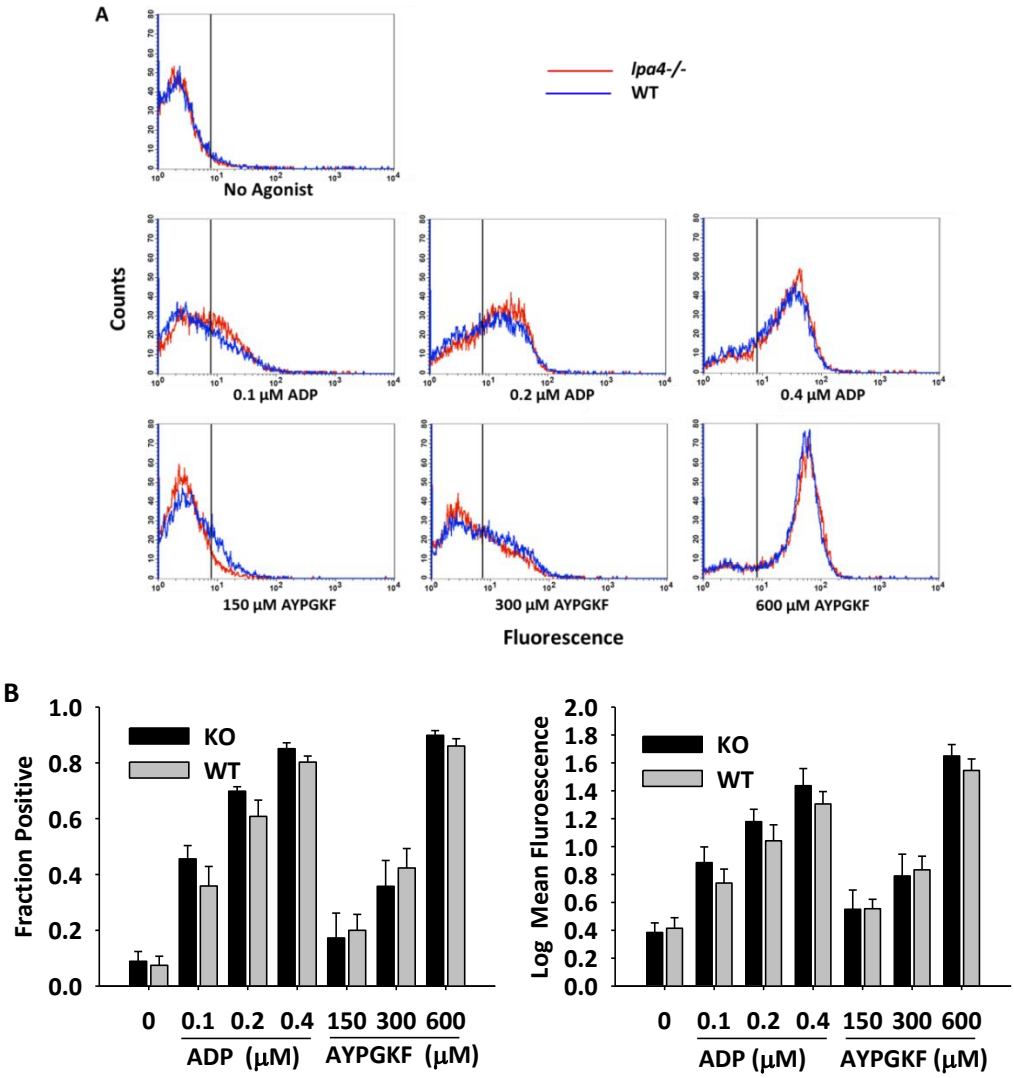
FIGURE 3.4



***lpa4*-/- mice have a prothrombotic phenotype:** $FeCl_3$-induced thrombosis of the left carotid artery in animals with a global deletion of the LPA4 receptor (*Lpa4-/-*; dashed line) and WT controls (solid line). * p = 0.003 Log-rank test.

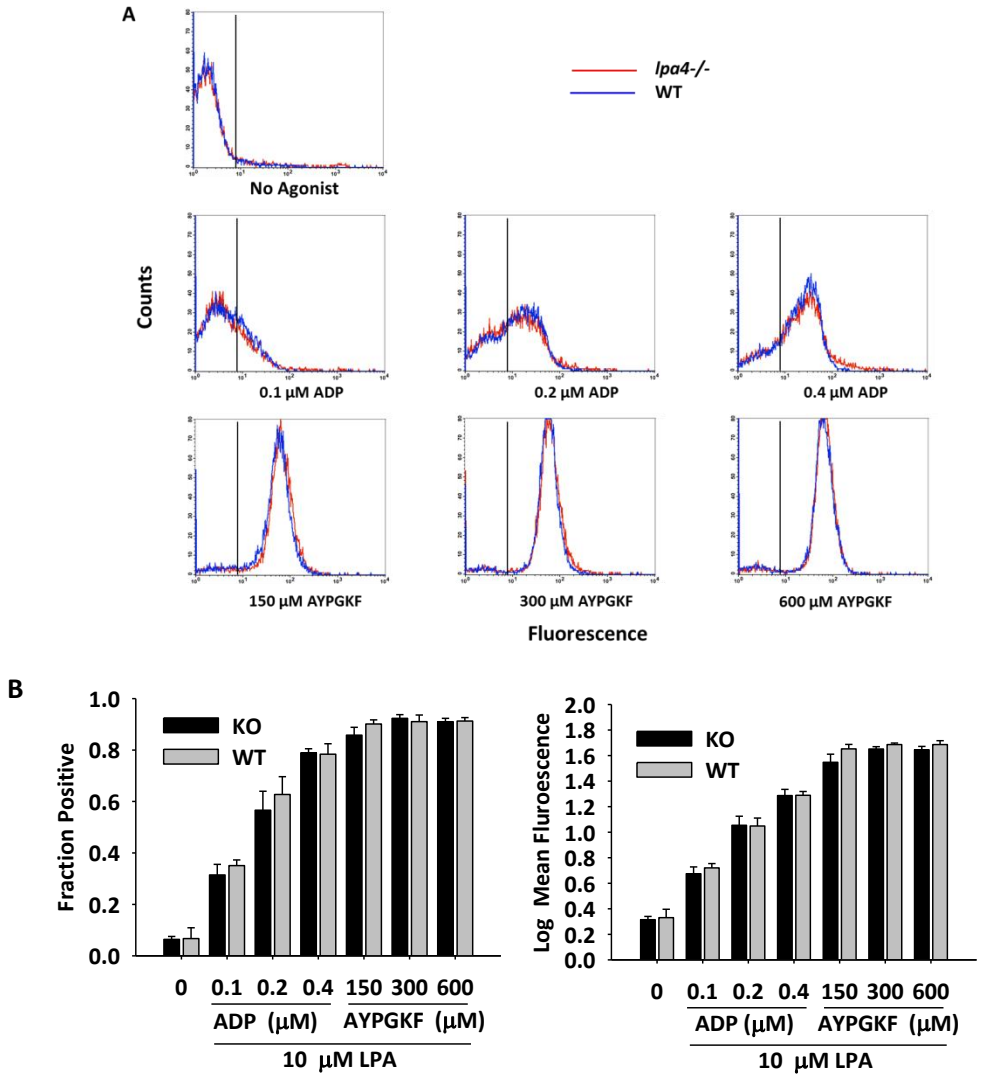
Smyth, unpublished.

FIGURE 3.5



***lpa4-/-* Platelets Have Normal Fibrinogen Binding Response to Agonists:** A FITC-conjugated anti-fibrinogen antibody was used to evaluate fibrinogen binding to platelets. **(A)** Representative histograms of *lpa4-/-* (red) and WT (blue) platelets incubated with ADP or a PAR4 agonist peptide (AYPGKF) at the indicated concentrations. The vertical line indicates the threshold for labeling a platelet as having positive fluorescence. **(B)** Cumulative data of fibrinogen binding for platelets from *lpa4-/-* mice (black bars; n = 3) and WT platelets (gray bars; n = 4) after stimulation. The left panel indicates the fraction of platelets having positive fluorescence. The right panel shows the geometric mean fluorescence. Any differences *lpa4-/-* and WT fibrinogen binding (fluorescence) were not statistically significant.

FIGURE 3.6



***lpa4-/-* Platelets Have Normal Fibrinogen Binding Response to Agonists with LPA:** A FITC-conjugated anti-fibrinogen antibody was used to evaluate fibrinogen binding to platelets. **(A)** Representative histograms of *lpa4-/-* (red) and WT (blue) platelets incubated with 10 µM oleyl LPA and/or ADP or a PAR4 agonist peptide (AYPGKF) at the indicated concentrations. The vertical line indicates the threshold for labeling a platelet as having positive fluorescence. **(B)** Cumulative data of fibrinogen binding for platelets from *lpa4-/-* mice (black bars; n = 3) and WT platelets (gray bars; n = 4) after stimulation. The left panel indicates the fraction of platelets having positive fluorescence. The right panel shows the geometric mean fluorescence. Any differences *lpa4-/-* and WT fibrinogen binding (fluorescence) were not statistically significant.

FIGURE 3.7

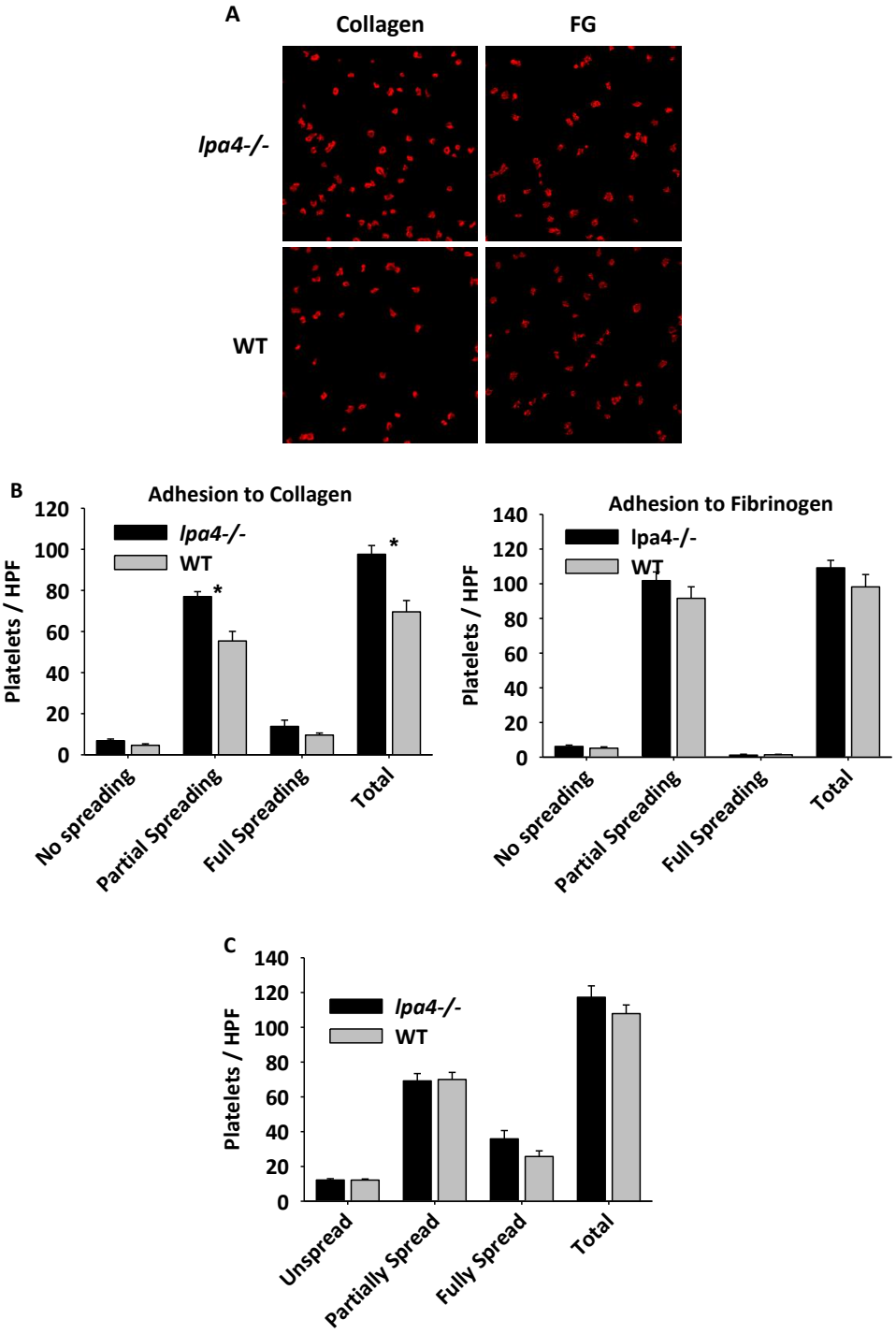
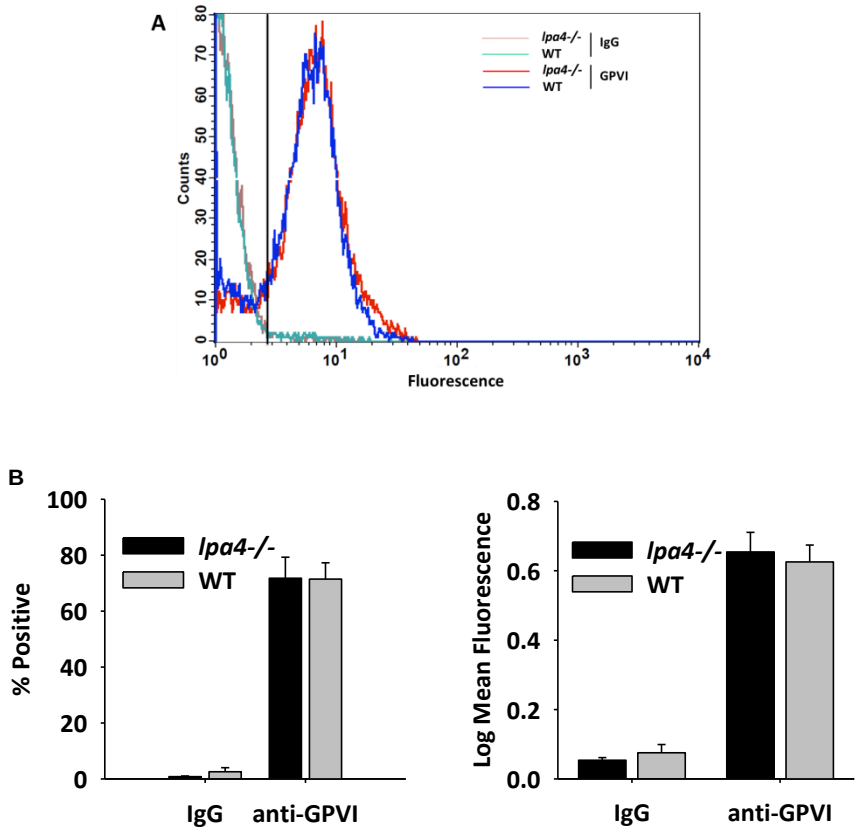

A
Collagen
FG
lpa4-/-
WT
B
Adhesion to Collagen
lpa4-/-
WT
Platelets / HPF
No spreading
Partial Spreading
Full Spreading
Total
Adhesion to Fibrinogen
lpa4-/-
WT
Platelets / HPF
No spreading
Partial Spreading
Full Spreading
Total
C
lpa4-/-
WT
Platelets / HPF
Unspread
Partially Spread
Fully Spread
Total

FIGURE 3.7 (CAPTION)

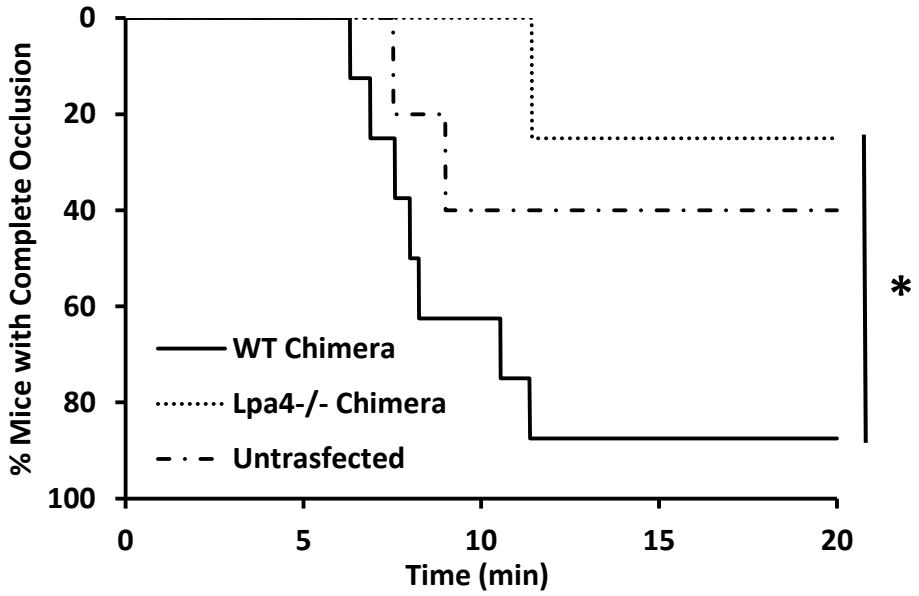
***lpa4-/-* Platelets Have Increased Binding to Collagen:** Adhesion of murine platelets to platelets coated with either 10 µg/ml collagen or 5 µg/ml fibrinogen (FG). **(A)** Light micrographs showing adhesion of platelets stained with TRITC-phalloidin. Platelets were acquired from mice approximately 2 years old. **(B)** Quantification of the number of platelets per high-powered field (HPF) shown in panel (A). Scoring was also conducted based on the degree of spreading. Left: adhesion to collagen. Right: adhesion to fibrinogen. **(C)** Quantification and scoring of platelets derived from mice approximately 3 months old adhering to collagen. * $p < 0.05$ Student t test.

FIGURE 3.8



***lpa4-/-* Platelets Have Normal Levels of GPVI:** A FITC-conjugated anti-GPVI antibody was used to evaluate GPVI in platelets. **(A)** Representative histograms of *lpa4-/-* (red) and WT (blue) platelets incubated with control IgG (lighter color) or anti-GPVI (darker color). The vertical line indicates the threshold for labeling a platelet as having positive fluorescence. **(B)** Cumulative data of GPVI for platelets from *lpa4-/-* mice (black bars; n = 4) and WT platelets (gray bars; n = 4) after. The left panel indicates the fraction of platelets having positive fluorescence. The right panel shows the geometric mean fluorescence. Any differences *lpa4-/-* and WT fluorescence were not statistically significant.

FIGURE 3.9



Bone marrow-derived cells lacking LPA4 are protected from thrombosis: $FeCl_3$-induced thrombosis of the left carotid artery in lethally irradiated mice rescued with *lpa4-/-* bone marrow (long dash), lethally irradiated mice rescued with WT bone marrow (solid line), or non-irradiated mice (short dash). *P = 0.002 Log-rank test.

CHAPTER 4: DISCUSSION

In this dissertation, I have discussed two aspects of LPA as they relate to platelets: the role of platelets in LPA production (Chapter 2), and the role of LPA in platelet signaling (Chapter 3). In Chapter 2, interpretation of the results was largely offered in context of the specific molecular interactions (*ie*, ATX-integrin interactions) which govern the lysoPLD activity of ATX and localization of its product. In Chapter 3, I specifically discussed the roles of LPA5 and LPA4 (especially the latter) in platelet LPA signaling. In this particular section, a slightly broader perspective will be taken as we consider the overarching implications of these findings.

ATX AND LPA IN PHYSIOLOGY AND PATHOPHYSIOLOGY

Before beginning a discussion on a potential role of ATX or LPA in health and disease, it is important to consider where these signaling functions have physiological and pathophysiological effects, particularly in the vasculature. In the plasma, LPA is present at micromolar concentrations[46, 48] although interestingly it is unknown what the role of this global circulating LPA is in a normal physiological setting. The plasma levels of LPA are tightly dependent on its relative production by ATX and degradation by the LPPs[49, 52]. However, whether LPA is serving a specific signaling purpose in the mature vascular system (*eg*, maintaining vascular tone) or is simply an intermediate step in the degradation of LPC is still not understood. There have been reports that global intravenous administration of LPA has effects on vascular tone[69] and even effects on the

progression of atherosclerosis in animal models[115]. These findings do raise some interesting mechanistic oddities since the rapid turnover of LPA would result in near-immediate removal of LPA added as a bolus[52]. Nonetheless, the findings suggest that the large pool of circulating LPA may have an important role in the vasculature. An additional peculiarity in human disease is that the venom of the brown recluse spider (*Loxosceles reclusa*) is a lysoPLD which produces measurable LPA[186, 187], suggesting that there are serious implications if there are perturbations in LPA levels.

Although the specific physiological role of LPA is not entirely understood in adults, the balance of LPA production and degradation seems to be critical for embryonic development as deletions of both ATX and LPP3 results in embryonic lethality with vascular defects[54, 56, 57]. This implies that LPA has some effect in physiological or pathological vascular development in adults as well. Of course, the ability of LPA to effect specific cellular processes would imply its ability either (1) to be generated in a precisely controlled manner, (2) to signal via receptors that are located on very specific cell types, or (3) to have complimentary actions with other specific cell-signaling entities. A preliminary (and superficial) glance would suggest that the first scenario is unlikely to be the case since LPC and ATX are found at biologically significant levels in the plasma at all times. The second scenario is also unlikely the case since LPA receptors are found ubiquitously on blood cells and vascular cells. Finally the third option that LPA requires additional factors is unlikely since in *in vitro* settings, LPA is capable of eliciting cellular responses on its own.

IMPLICATIONS OF LOCALIZED LYSOPLD ACTIVITY

Our own data, presented in Chapter 2, convincingly demonstrates the ability of ATX to bind to the surface of platelets, a process that is clearly mediated by the SMB-domain of ATX and integrins on the platelet surface. This certainly forces reexamination of the first scenario mentioned above, that the production of LPA may be controlled spatially and temporally. The PDE domain and the SMB domain form a portion of the proposed hydrophobic channel of ATX, by which LPC enters the active site and LPA leaves. This indicates that integrin binding plays a role in regulating the catalytic activity of the enzyme itself. This proposal is supported by our finding that BSA-associated substrate in the media is hydrolyzed more rapidly when platelet integrins are present and active (**Figure 2.15A**). More than this, even when there is no (or negligible) BSA-bound substrate available for ATX to hydrolyze, ATX can not only access membrane substrates (**Figure 2.4B, D**) but it does so in an integrin-dependent manner producing membrane-associated LPA (**Figure 2.15C, D**). Once again, the question that arises is the importance of this membrane-associated LPA that is generated in a seemingly very controlled manner.

It is reasonable to propose that the location of LPA dictates its signaling function – that there are actually different functional pools of LPA. Perhaps the global plasma pool of LPA is nothing more of an intermediate in the metabolism of LPC. Alternatively, plasma LPA could act globally on vascular tone or some other broad-reaching function. In either case, membrane-associated LPA could certainly be poised to play a more selective

signaling role. In platelets, it may be that ATX only gets recruited to the membrane surface when there is a certain level of integrin activation which would typically happen if there is underlying activation of the platelets themselves. The effect of generating membrane-associated LPA could elicit robust and specific effects because (1) the LPA production is controlled spatially and temporally, (2) the local concentration of LPA could increase dramatically, and (3) being already located in the membrane, this pool of LPA may have much easier access to membrane LPA receptors (ligands for LPA receptors incorporate into the membrane-spanning hydrophobic region of the receptor[188]).

Given a mechanism for how membrane-associated LPA is generated and how it may facilitate cell-specific (or platelet-specific) LPA signaling, the question again arises of what the ultimate functional consequences of this is. In the context of platelets, there are a few possible scenarios which are not at all mutually exclusive. Increased local production of LPA may facilitate changes in platelet responses to stress during the process of activation (*ie*, promotion or attenuation of platelet activation). The data presented in Chapter 3 do indicate that in human platelets, LPA5 mediates stimulatory pathways. The role of LPA4 is much more ambiguous as platelets isolated from *lpa4-/-* mice do not show a robust phenotype in various platelet function assays, and *in vivo* thrombosis oddly produces conflicting results if thrombosis is conducted in an animal with a global deletion of LPA4 or if the animal only has bone marrow-derived cells that lack LPA4. Since LPA4 expression has been reported in the endothelium, it is likely that LPA-dependent interactions between platelets and endothelial cells plays an important role although the exact function still remains enigmatic.

In addition to facilitating LPA signaling in platelets specifically, the interaction of ATX with integrins may serve slightly different roles. Since platelets are among the first blood components to localize to a site of injury, it is certainly feasible that LPA is delivered to these sites by activated platelets rather than exclusively acting on platelet LPA receptors. In our own studies we found that LPA formed in isolated platelets tended to remain platelet-associated (data not shown). However, these studies occurred in physiologic solutions that were devoid of lipoproteins and at most contained a maximum of 0.35% BSA. Lipoproteins and BSA are both present at significant levels in the plasma, both are capable of associating with lysophospholipids. It is therefore very possible that *in vivo* LPA could be sequestered from platelets and added to the global circulating pool or even to other cells. This would explain the earlier findings in the literature that animals with thrombocytopenia have decreased circulating LPA[58, 61].

In addition to the possibility that platelets facilitate LPA delivery, it is further possible that ATX itself may be delivered to sites of injury. Platelets are capable of not only binding the integrin ligand fibrinogen but also capable of internalizing fibrinogen upon binding[189-191] which allows platelets to store up fibrinogen in granules and release it upon activation[192]. Since ATX binds platelet αIIβ3 as fibrinogen, it is quite possible that platelets internalize, store, and secrete ATX in a manner identical to fibrinogen. Our own data supports this hypothesis. In earlier publications we found that platelets derived from WT mice had associated ATX (as measured by Western blot) while platelets derived from mice deficient in the β3 integrin subunit did not[49]. While this in of itself does not indicate whether the ATX is externally associated with the platelet or

internalized, our lipid measurements in platelets (presented here in Chapter 2) uniformly show increased LPA production when the platelets were activated with thrombin even in the absence of exogenously added ATX. We ultimately found that this thrombin-induced LPA production was indeed ATX-dependent (**Figure 2.4**).

Again, the notion of ATX internalization and release does not exclude the role of integrin binding in localizing LPA production to the platelet itself and may indeed be a manner by which the effects of LPA are made even more cell-specific. As platelets exocytose the contents of their granules, the local concentration of ATX could be dramatically increased and brought into proximity of the membrane and membrane-derived substrate when it binds to integrins. At sites of injury, the integrins available with which ATX could interact would not merely be limited to platelet αIIbβ3. The data presented in Chapter 2 indicate that ATX is capable of interacting with other αVβ3 as well as at least some integrins that incorporate a β1 subunit. Integrin αVβ3 itself is fairly ubiquitously expressed on many cells including many cells in the blood and vasculature, so there is no reason that this phenomenon would be exclusive to platelets. Moreover, the binding of ATX to the surface of cells may not even be limited to integrins. One particular group has suggested that ATX may be able to interact with certain scavenger receptors[154] which could provide a link between ATX and lysophospholipids found within lipoproteins which also possess ligands for several scavenger receptors. Given all of this, ATX and LPA could play a number of signaling roles in multiple cell types in the vasculature and particularly at sites of injury.

OVERVIEW OF FINDINGS AND HYPOTHESIZED ROLES IN ATHEROSCLEROSIS

In Chapter 1, we discussed both the role of platelets and LPA in cardiovascular physiology and pathophysiology. Let us now review progression of atherosclerosis and the role that ATX-integrin interaction and cell-specific LPA production might play. This overview is succinctly illustrated in **Figure 4.1**. As a resting platelet circulates in healthy, uninjured vessels it accumulates certain plasma proteins such as fibrinogen through integrin-mediated endocytosis[189, 191] and likely accumulates ATX via a similar mechanism. As the platelet approaches an area of vascular damage, interactions with the subendothelial matrix induce platelet activation which induces granule secretion of a number of proteins including fibrinogen and probably ATX. Activation also induces an increase in PLA activity increasing the amount of lysophospholipids in the extracellular leaflet of the lipid bilayer and providing substrate for ATX[55]. As integrins on platelets and endothelial cells activate, ATX from the plasma and ATX secreted from platelets are drawn to the membrane of the integrin-bearing cells thus increasing LPA production and localization. LPA itself acts chemotactically drawing in other cell types such as circulating monocytes[115]. LPA further facilitates cell recruitment by inducing endothelial cells to secrete more chemokines and by increasing the adhesiveness of endothelial cells[114]. LPA may also facilitate migration of monocytes/macrophages into the subendothelium and induce dedifferentiation and proliferation of smooth muscle cells. As lipids, particularly LPC, begin to accumulate at the site of injury due to interactions of oxLDL with monocyte/macrophage scavenger receptors, this may provide an additional concentrated reservoir of substrate for ATX to make use of in addition to what is found

in cell membranes. There are therefore many possible sources of LPA generation during a typical response to vascular injury. Indeed, during a particular injury response, not all of the newly produced LPA may even be used for signaling. Rather, much of the LPA could actually be stored in high concentrations in the lipid core of an atheroma (which indeed is the most thrombogenic component)[45]. During plaque rupture, this atherosclerotic LPA pool is likely extruded into the plasma and depending on relative LPA signaling pathways in platelets may be among the agents that promotes acute thrombosis (particularly when coupled with *de novo* LPA production in platelets and other blood cells).

ROLE OF LPA, ATX, AND INTEGRINS IN OTHER DISEASE STATES

As stated, the interaction between ATX and integrins would by no means be limited to platelets. Also while this interaction has mostly been discussed in the context of cardiovascular disease, it should be noted that disregulation of ATX and integrins likely contribute to other disease states. ATX itself was originally discovered as a glycoprotein secreted from a line of human melanoma cells[140] and has long been an attractive target for anti-cancer therapeutics. Apart from its ability to promote angiogenesis, LPA can also directly induce migration of different cancer cells indicating an additional role in regulating cancer motility[193-195]. Furthermore LPA levels are elevated in ascites and blood of individuals with ovarian cancer, and ATX expression is even enhanced in certain cancer cell types[196-199]. The role of integrins in cancer has been well established as well. Many integrins, particularly αVβ3, show increased expression in a variety of tumors, and

their function has been mostly attributed to their role in angiogenesis, their role in promoting adhesion-dependent tissue invasion, or their association with growth factor receptors[200]. Clearly, the facilitation of local LPA production in tumor cells may be an additional role that integrins play in cancer progression (especially given overexpression of ATX in different cancer types). Furthermore, platelets themselves have been shown to produce LPA in the tumor microenvironment consequently promoting metastasis[61]. Platelets even support the survival of circulating tumor cells and metastasis[201], while many cancers are associated with a hypercoagulable state[202]. The factors involved in such interactions are likely multivariable, and could potentially involve ATX/LPA. In any case, the role of an ATX-integrin interaction is an attractive target for future study in a number of cancer fields and may even provide novel targets for cancer therapeutics.

FINAL COMMENTS

In conclusion, the work described here clearly describes the role that platelets have in LPA production and how the interaction of ATX with integrins not only enhances its activity but also localizes LPA production in close proximity to LPA receptors. We mostly investigated this in the context of platelets and further evaluated LPA signaling in platelets via the LPA4 and LPA5 receptors. While the most obvious implications of this work relate to cardiovascular health and disease, it is important to recognize that these mechanisms of LPA metabolism would not be limited to cells in the cardiovasculature. Furthermore, with the increasing appreciation of the role of platelets in other processes

aside from hemostasis and thrombosis, platelet LPA may have implications that reach into a number of different disease states as well.

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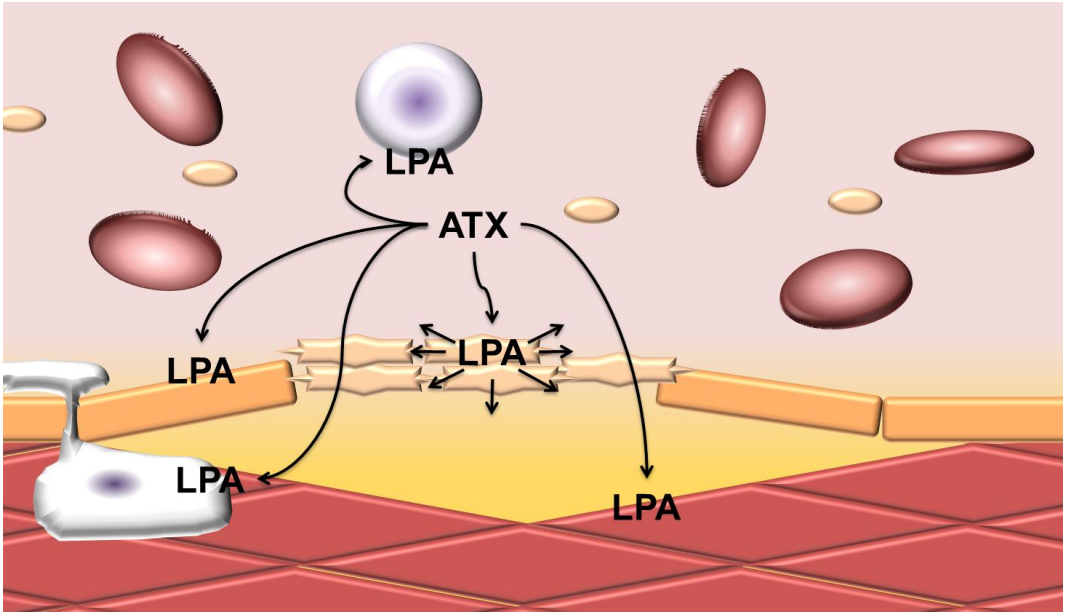
FIGURE 4.1



Hypothetical Role of ATX-Integrin Interaction in LPA Localization and Atherogenesis. ATX is recruited to the surface of platelets and likely other integrin-possessing cells including endothelial cells, smooth muscle cells, and leukocytes. This localization could facilitate many of the hallmarks of LPA signaling and indeed the hallmarks of atherogenesis including platelet activation, smooth muscle dedifferentiation and proliferation, endothelial cell activation, monocyte migration, *et cetera*. While we hypothesize that the main function of the interaction between ATX and integrin is to localize LPA to a specific cell, it is also feasible that LPA could act in a paracrine manner on several cell types in close proximity. Local production of LPA may also be responsible for the high levels of this lipid found in the lipid-rich core of atherosclerotic plaques.

APPENDIX: ABBREVIATIONS

Abbreviation	Description
10E5	Monoclonal antibody that blocks integrin αIIbβ3
^{125}I	Iodine-125 (radioactive isotope
^{125}I-ATX	^{125}I-labeled ATX
12G10	Nonblocking monoclonal antibody against β1 integrins
23C6	Nonblocking monoclonal antibody against integrin αVβ3
7E3	Monoclonal antibody that blocks β3 integrins
ADP	adenosine diphosphate
***anti*-BrP-LPA**	*Anti* enantiomer of the bromophosphonate LPA analog
apoB	Apolipoprotein B-100
APT1	acyl-protein thioesterase 1
ATX	Autotaxin
ATX-E109A	Point mutation of the SMB_1 domain of ATX
ATX-H117A	Point mutation of the SMB_1 domain of ATX
ATX-R117A	Point mutation of the SMB_1 domain of ATX
ATX-T210A	Catalytically inactive point mutation of ATX (threonine in catalytic site mutated to alanine)
AYPGKF	Peptide sequence for a PAR_4 receptor agonist
BrP-LPA	Racemic mixture of the bromophosphonate LPA analog
C17 LPA	Unnatural LPA species with an acyl chain of 17 carbons
C17 LPC	Unnatural LPC species with an acyl chain of 17 carbons
C17 S1P	Unnatural S1P species with radyl chain of 17 carbons
cAMP	Cyclic adenosine monophosphate
CHO	Chinease hmaster ovary
DAG	Diacylglycerol
DTT	Dithiothreitol
EDGx	Endothelial cell differentiation gene receptor (x denotes numerical identity)
eNOS	Endothelial nitric oxide synthetase
ENPP	Ectonucleotide pyrophosphatase/phosphodiesterase
ENPPx	Enzymes of the ectonucleotide pyrophosphatase phosphodiesterase family (x denotes numerical identity; ATX is further denoted $ENPP_2$)
F2Y720	S1P mimetic drug (fingolimod)
FcγRII	Fc gamma receptor
FITC	Fluorescein isothiocyanate
GP	Glycoprotein
GPCR	G protein-coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

LCAT	Lecithin cholesterol acyltransferase
LDL	Low-density lipoprotein
LM609	Monoclonal antibody that blocks integrin αVβ3
LPA	Lysphosphatidic acid
LPAx	Lysophosphatidic acid receptor (x denotes numerical identity)
LPC	Lysophosphatidylcholine
LPPx	Lipid phosphate phosphatase (x denotes numerical identity)
LY204002	PI3-kinase inhibitor
lysoPLD	Lysophospholipase D
MAG	Monoacylglycerol
MRS-2719	$P2Y_1$ receptor inhibitor
NO	Nitric oxide
NUC	Nuclease-like domain of ATX
oxLDL	Oxidized low-density lipoprotein
P2Yx	Purinergic receptors type 2Y (x deontes numerical identity)
P4C10	Monoclonal antibody that blocks β1 integrins
PAR	Protease-activated receptor
PBS	Phosphate-buffered saline
PC	Phosphoatidylcholine
PDE	Phosphodiesterase domain of ATX
PF4	Platelet factor 4
PGI2	Prostaglandin I2
PKI 14-22	Protein kinase A inhibitor
PLAx	Phospholipase A (X = 1 or 2 and indicates activity at acyl at the *sn-1* position or *sn-2 position)*
PPAR	Peroxisome proliferator-activated receptor
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
RGD	Integrin binding peptide sequence (arginine-glycine-aspartic acid)
S1P	Sphingosine 1-phosphate
S1Px	Sphingosine 1-phosphate receptor (x denotes numerical identity)
shRNA	Small hairpin ribonucleic acid
SMBx	Somatomedin B-like domain of ATX (x = 1 or 2)
Sphkx	Sphingosine kinase (x denotes numerical identity)
***syn*-BrP-LPA**	*Syn* enantiomer of the bromophosphonate LPA analog
TAG	Triacylglyceride
TGF-β	Transforming growth factor β
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
TXA2	Thromboxane A2
vWF	von Willebrand Factor
Y27632	Rho kinase inhibitor

REFERENCES

1. Xu J, Kochanek KD, Murphy SL, Tejada-Vera B. Deaths: Final data for 2007. *Natl Vital Stat Rep*. 2010;58:1-135
2. Radomski MW, Palmer RM, Moncada S. The role of nitric oxide and cgmp in platelet adhesion to vascular endothelium. *Biochem Biophys Res Commun*. 1987;148:1482-1489
3. de Graaf JC, Banga JD, Moncada S, Palmer RM, de Groot PG, Sixma JJ. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation*. 1992;85:2284-2290
4. Noe L, Peeters K, Izzi B, Van Geet C, Freson K. Regulators of platelet camp levels: Clinical and therapeutic implications. *Curr Med Chem*. 2010;17:2897-2905
5. Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell*. 1998;94:657-666
6. Clemetson JM, Polgar J, Magnenat E, Wells TN, Clemetson KJ. The platelet collagen receptor glycoprotein vi is a member of the immunoglobulin superfamily closely related to fcalphar and the natural killer receptors. *J Biol Chem*. 1999;274:29019-29024
7. Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV, Jr., Tam C, Coughlin SR. A dual thrombin receptor system for platelet activation. *Nature*. 1998;394:690-694
8. Kim S, Foster C, Lecchi A, Quinton TM, Prosser DM, Jin J, Cattaneo M, Kunapuli SP. Protease-activated receptors 1 and 4 do not stimulate g(i) signaling pathways in the absence of secreted adp and cause human platelet aggregation independently of g(i) signaling. *Blood*. 2002;99:3629-3636
9. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol*. 1992;118:1421-1442
10. Reed GL, Fitzgerald ML, Polgar J. Molecular mechanisms of platelet exocytosis: Insights into the "secrete" life of thrombocytes. *Blood*. 2000;96:3334-3342
11. Szczeklik A, Gryglewski RJ, Musial J, Grodzinska L, Serwonska M, Wojcik-Switek L, Marcinkiewicz E. Arachidonic acid-induced platelet aggregation and thromboxane a2 generation in patients with coronary heart disease. *Acta Biol Med Ger*. 1978;37:741-742
12. Ahmad SS, London FS, Walsh PN. The assembly of the factor x-activating complex on activated human platelets. *J Thromb Haemost*. 2003;1:48-59
13. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins iib and/or iiia. *J Clin Invest*. 1983;72:325-338
14. Knezevic I, Leisner TM, Lam SC. Direct binding of the platelet integrin alphaiibbeta3 (gpiib-iiia) to talin. Evidence that interaction is mediated through

the cytoplasmic domains of both alphaiib and beta3. *J Biol Chem*. 1996;271:16416-16421

15. Schoenwaelder SM, Jackson SP, Yuan Y, Teasdale MS, Salem HH, Mitchell CA. Tyrosine kinases regulate the cytoskeletal attachment of integrin alpha iib beta 3 (platelet glycoprotein iib/iiia) and the cellular retraction of fibrin polymers. *J Biol Chem*. 1994;269:32479-32487
16. Smyth SS, McEver RP, Weyrich AS, Morrell CN, Hoffman MR, Arepally GM, French PA, Dauerman HL, Becker RC. Platelet functions beyond hemostasis. *J Thromb Haemost*. 2009;7:1759-1766
17. Massberg S, Enders G, Leiderer R, Eisenmenger S, Vestweber D, Krombach F, Messmer K. Platelet-endothelial cell interactions during ischemia/reperfusion: The role of p-selectin. *Blood*. 1998;92:507-515
18. Rhee JS, Black M, Schubert U, Fischer S, Morgenstern E, Hammes HP, Preissner KT. The functional role of blood platelet components in angiogenesis. *Thromb Haemost*. 2004;92:394-402
19. Chong BH, Pilgrim RL, Cooley MA, Chesterman CN. Increased expression of platelet igg fc receptors in immune heparin-induced thrombocytopenia. *Blood*. 1993;81:988-993
20. Rowley JW, Oler A, Tolley ND, Hunter B, Low EN, Nix DA, Yost CC, Zimmerman GA, Weyrich AS. Genome wide rna-seq analysis of human and mouse platelet transcriptomes. *Blood*. 2011
21. Clawson CC, White JG. Platelet interaction with bacteria. I. Reaction phases and effects of inhibitors. *Am J Pathol*. 1971;65:367-380
22. Clawson CC, White JG. Platelet interaction with bacteria. Ii. Fate of the bacteria. *Am J Pathol*. 1971;65:381-397
23. Bik T, Sarov I, Livne A. Interaction between vaccinia virus and human blood platelets. *Blood*. 1982;59:482-487
24. Cox D, McConkey S. The role of platelets in the pathogenesis of cerebral malaria. *Cell Mol Life Sci*. 2010;67:557-568
25. Maisch PA, Calderone RA. Role of surface mannan in the adherence of candida albicans to fibrin-platelet clots formed in vitro. *Infect Immun*. 1981;32:92-97
26. Youssefian T, Drouin A, Massé J-M, Guichard J, Cramer EM. Host defense role of platelets: Engulfment of hiv andstaphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation. *Blood*. 2002;99:4021-4029
27. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. Cd40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature*. 1998;391:591-594
28. Duke WW. The relation of blood platelets to hemorrhagic disease. *Journal of the American Medical Association*. 1910;55:1185-1192
29. Gardner FH, Cohen P. The value of platelet transfusions. *Med Clin North Am*. 1960;44:1425-1439
30. Ross R, Glomset JA. The pathogenesis of atherosclerosis. *New England Journal of Medicine*. 1976;295:369-377

31. Gawaz M, Neumann F-J, Dickfeld T, Koch W, Laugwitz K-L, Adelsberger H, Langenbrink K, Page S, Neumeier D, Schömig A, Brand K. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. *Circulation*. 1998;98:1164-1171
32. Kannel WB, Castelli WP, Gordon T. Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the framingham study. *Ann Intern Med*. 1979;90:85-91
33. Brown MS, Goldstein JL. Expression of the familial hypercholesterolemia gene in heterozygotes: Mechanism for a dominant disorder in man. *Science*. 1974;185:61-63
34. Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci U S A*. 1979;76:333-337
35. Henriksen T, Mahoney EM, Steinberg D. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. *Proc Natl Acad Sci U S A*. 1981;78:6499-6503
36. Henriksen T, Mahoney EM, Steinberg D. Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis*. 1983;3:149-159
37. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A*. 1984;81:3883-3887
38. Kodama T, Reddy P, Kishimoto C, Krieger M. Purification and characterization of a bovine acetyl low density lipoprotein receptor. *Proc Natl Acad Sci U S A*. 1988;85:9238-9242
39. Hessler JR, Morel DW, Lewis LJ, Chisolm GM. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Arteriosclerosis*. 1983;3:215-222
40. Hessler JR, Robertson AL, Jr., Chisolm GM, 3rd. Ldl-induced cytotoxicity and its inhibition by hdl in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis*. 1979;32:213-229
41. Morel DW, Hessler JR, Chisolm GM. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J Lipid Res*. 1983;24:1070-1076
42. Levitan I, Volkov S, Subbaiah PV. Oxidized ldl: Diversity, patterns of recognition, and pathophysiology. *Antioxid Redox Signal*. 2010;13:39-75
43. Haberland ME, Olch CL, Folgelman AM. Role of lysines in mediating interaction of modified low-density lipoproteins with the scavenger receptor of human monocyte macrophages. *Journal of Biological Chemistry*. 1984;259:1305-1311
44. Podrez EA, Poliakov E, Shen Z, Zhang R, Deng Y, Sun M, Finton PJ, Shan L, Gugiu B, Fox PL, Hoff HF, Salomon RG, Hazen SL. Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor cd36. *J Biol Chem*. 2002;277:38503-38516

45. Siess W, Zangl KJ, Essler M, Bauer M, Brandl R, Corrinth C, Bittman R, Tigyi G, Aepfelbacher M. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc Natl Acad Sci U S A*. 1999;96:6931-6936
46. Baker DL, Desiderio DM, Miller DD, Tolley B, Tigyi GJ. Direct quantitative analysis of lysophosphatidic acid molecular species by stable isotope dilution electrospray ionization liquid chromatography-mass spectrometry. *Analytical Biochemistry*. 2001;292:287-295
47. Ohkawa R, Nakamura K, Okubo S, Hosogaya S, Ozaki Y, Tozuka M, Osima N, Yokota H, Ikeda H, Yatomi Y. Plasma sphingosine-1-phosphate measurement in healthy subjects: Close correlation with red blood cell parameters. *Ann Clin Biochem*. 2008;45:356-363
48. Hosogaya S, Yatomi Y, Nakamura K, Ohkawa R, Okubo S, Yokota H, Ohta M, Yamazaki H, Koike T, Ozaki Y. Measurement of plasma lysophosphatidic acid concentration in healthy subjects: Strong correlation with lysophospholipase d activity. *Ann Clin Biochem*. 2008;45:364-368
49. Pamuklar Z, Federico L, Liu S, Umezu-Goto M, Dong A, Panchatcharam M, Fulkerson Z, Berdyshev E, Natarajan V, Fang X, van Meeteren LA, Moolenaar WH, Mills GB, Morris AJ, Smyth SS. Autotaxin/lysopholipase d and lysophosphatidic acid regulate murine hemostasis and thrombosis. *J Biol Chem*. 2009;284:7385-7394
50. Zhao Z, Xiao Y, Elson P, Tan H, Plummer SJ, Berk M, Aung PP, Lavery IC, Achkar JP, Li L, Casey G, Xu Y. Plasma lysophosphatidylcholine levels: Potential biomarkers for colorectal cancer. *J Clin Oncol*. 2007;25:2696-2701
51. Okajima F. Plasma lipoproteins behave as carriers of extracellular sphingosine 1-phosphate: Is this an atherogenic mediator or an anti-atherogenic mediator? *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2002;1582:132-137
52. Albers HM, Dong A, van Meeteren LA, Egan DA, Sunkara M, van Tilburg EW, Schuurman K, van Tellingen O, Morris AJ, Smyth SS, Moolenaar WH, Ovaa H. Boronic acid-based inhibitor of autotaxin reveals rapid turnover of lpa in the circulation. *Proc Natl Acad Sci U S A*. 2010;107:7257-7262
53. Brindley DN, Pilquil C, Sariahmetoglu M, Reue K. Phosphatidate degradation: Phosphatidate phosphatases (lipins) and lipid phosphate phosphatases. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2009;1791:956-961
54. Escalante-Alcalde D, Hernandez L, Le Stunff H, Maeda R, Lee HS, Jr Gang C, Sciorra VA, Daar I, Spiegel S, Morris AJ, Stewart CL. The lipid phosphatase lpp3 regulates extra-embryonic vasculogenesis and axis patterning. *Development*. 2003;130:4623-4637
55. Bolen AL, Naren AP, Yarlagadda S, Beranova-Giorgianni S, Chen L, Norman D, Baker DL, Rowland MM, Best MD, Sano T, Tsukahara T, Liliom K, Igarashi Y, Tigyi G. The phospholipase a1 activity of lysophospholipase a-i links platelet activation to lpa production during blood coagulation. *J Lipid Res*. 2011;52:958-970

56. Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, Noji S, Yatomi Y, Aoki J, Arai H. Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. *J Biol Chem*. 2006;281:25822-25830
57. van Meeteren LA, Ruurs P, Stortelers C, Bouwman P, van Rooijen MA, Pradere JP, Pettit TR, Wakelam MJ, Saulnier-Blache JS, Mummery CL, Moolenaar WH, Jonkers J. Autotaxin, a secreted lysophospholipase d, is essential for blood vessel formation during development. *Mol Cell Biol*. 2006;26:5015-5022
58. Aoki J, Taira A, Takanezawa Y, Kishi Y, Hama K, Kishimoto T, Mizuno K, Saku K, Taguchi R, Arai H. Serum lysophosphatidic acid is produced through diverse phospholipase pathways. *J Biol Chem*. 2002;277:48737-48744
59. Tigyi G, Miledi R. Lysophosphatidates bound to serum albumin activate membrane currents in xenopus oocytes and neurite retraction in pc12 pheochromocytoma cells. *J Biol Chem*. 1992;267:21360-21367
60. Eichholtz T, Jalink K, Fahrenfort I, Moolenaar WH. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J*. 1993;291 (Pt 3):677-680
61. Boucharaba A, Serre CM, Gres S, Saulnier-Blache JS, Bordet JC, Guglielmi J, Clezardin P, Peyruchaud O. Platelet-derived lysophosphatidic acid supports the progression of osteolytic bone metastases in breast cancer. *J Clin Invest*. 2004;114:1714-1725
62. Yatomi Y. Plasma sphingosine 1-phosphate metabolism and analysis. *Biochim Biophys Acta*. 2008;1780:606-611
63. Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, Camerer E, Zheng YW, Huang Y, Cyster JG, Coughlin SR. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science*. 2007;316:295-298
64. Yatomi Y, Yamamura S, Ruan F, Igarashi Y. Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J Biol Chem*. 1997;272:5291-5297
65. Hanel P, Andreani P, Graler MH. Erythrocytes store and release sphingosine 1-phosphate in blood. *FASEB J*. 2007;21:1202-1209
66. Ito K, Anada Y, Tani M, Ikeda M, Sano T, Kihara A, Igarashi Y. Lack of sphingosine 1-phosphate-degrading enzymes in erythrocytes. *Biochem Biophys Res Commun*. 2007;357:212-217
67. Venkataraman K, Lee YM, Michaud J, Thangada S, Ai Y, Bonkovsky HL, Parikh NS, Habrukowich C, Hla T. Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. *Circ Res*. 2008;102:669-676
68. Tokumura A, Fukuzawa K, Akamatsu Y, Yamada S, Suzuki T, Tsukatani H. Identification of vasopressor phospholipid in crude soybean lecithin. *Lipids*. 1978;13:468-472
69. Tokumura A, Fukuzawa K, Tsukatani H. Effects of synthetic and natural lysophosphatidic acids on the arterial blood pressure of different animal species. *Lipids*. 1978;13:572-574

70. Tokumura A, Fukuzawa K, Tsukatani H. Contractile actions of lysophosphatidic acids with a chemically-defined fatty acyl group on longitudinal muscle from guinea-pig ileum. *J Pharm Pharmacol*. 1982;34:514-516
71. Tokumura A, Fukuzawa K, Yamada S, Tsukatani H. Stimulatory effect of lysophosphatidic acids on uterine smooth muscles of non-pregant rats. *Arch Int Pharmacodyn Ther*. 1980;245:74-83
72. Tokumura A, Fukuzawa K, Isobe J, Tsukatani H. Lysophosphatidic acid-induced aggregation of human and feline platelets: Structure-activity relationship. *Biochem Biophys Res Commun*. 1981;99:391-398
73. Gerrard JM, Kindom SE, Peterson DA, Peller J, Krantz KE, White JG. Lysophosphatidic acids. Influence on platelet aggregation and intracellular calcium flux. *Am J Pathol*. 1979;96:423-438
74. Hecht JH, Weiner JA, Post SR, Chun J. Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *The Journal of Cell Biology*. 1996;135:1071-1083
75. Zhang C, Baker DL, Yasuda S, Makarova N, Balazs L, Johnson LR, Marathe GK, McIntyre TM, Xu Y, Prestwich GD, Byun H-S, Bittman R, Tigyi G. Lysophosphatidic acid induces neointima formation through pparγ activation. *The Journal of Experimental Medicine*. 2004;199:763-774
76. Hla T, Maciag T. An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to g-protein-coupled receptors. *J Biol Chem*. 1990;265:9308-9313
77. Chae SS, Proia RL, Hla T. Constitutive expression of the s1p1 receptor in adult tissues. *Prostaglandins Other Lipid Mediat*. 2004;73:141-150
78. Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL. Edg-1, the g protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest*. 2000;106:951-961
79. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol*. 2005;25:11113-11121
80. Kono M, Belyantseva IA, Skoura A, Frolenkov GI, Starost MF, Dreier JL, Lidington D, Bolz SS, Friedman TB, Hla T, Proia RL. Deafness and stria vascularis defects in s1p2 receptor-null mice. *J Biol Chem*. 2007;282:10690-10696
81. Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ, Kingsbury MA, Zhang G, Brown JH, Chun J. Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its g protein-coupled receptor, lp(b3)/edg-3. *J Biol Chem*. 2001;276:33697-33704
82. Windh RT, Lee MJ, Hla T, An S, Barr AJ, Manning DR. Differential coupling of the sphingosine 1-phosphate receptors edg-1, edg-3, and h218/edg-5 to the g(i), g(q), and g(12) families of heterotrimeric g proteins. *J Biol Chem*. 1999;274:27351-27358

83. Kono M, Mi Y, Liu Y, Sasaki T, Allende ML, Wu YP, Yamashita T, Proia RL. The sphingosine-1-phosphate receptors s1p1, s1p2, and s1p3 function coordinately during embryonic angiogenesis. *J Biol Chem*. 2004;279:29367-29373
84. Graeler M, Goetzl EJ. Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic t cells. *FASEB J*. 2002;16:1874-1878
85. Im DS, Heise CE, Ancellin N, O'Dowd BF, Shei GJ, Heavens RP, Rigby MR, Hla T, Mandala S, McAllister G, George SR, Lynch KR. Characterization of a novel sphingosine 1-phosphate receptor, edg-8. *J Biol Chem*. 2000;275:14281-14286
86. Walzer T, Chiossone L, Chaix J, Calver A, Carozzo C, Garrigue-Antar L, Jacques Y, Baratin M, Tomasello E, Vivier E. Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. *Nat Immunol*. 2007;8:1337-1344
87. Ishii I, Fukushima N, Ye X, Chun J. Lysophospholipid receptors: Signaling and biology. *Annu Rev Biochem*. 2004;73:321-354
88. Yanagida K, Masago K, Nakanishi H, Kihara Y, Hamano F, Tajima Y, Taguchi R, Shimizu T, Ishii S. Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/lpa6. *J Biol Chem*. 2009;284:17731-17741
89. Yoshida K, Nishida W, Hayashi K, Ohkawa Y, Ogawa A, Aoki J, Arai H, Sobue K. Vascular remodeling induced by naturally occurring unsaturated lysophosphatidic acid in vivo. *Circulation*. 2003;108:1746-1752
90. Hayashi K, Takahashi M, Nishida W, Yoshida K, Ohkawa Y, Kitabatake A, Aoki J, Arai H, Sobue K. Phenotypic modulation of vascular smooth muscle cells induced by unsaturated lysophosphatidic acids. *Circ Res*. 2001;89:251-258
91. Tokumura A, Iimori M, Nishioka Y, Kitahara M, Sakashita M, Tanaka S. Lysophosphatidic acids induce proliferation of cultured vascular smooth muscle cells from rat aorta. *Am J Physiol*. 1994;267:C204-210
92. Jalink K, Hengeveld T, Mulder S, Postma FR, Simon MF, Chap H, van der Marel GA, van Boom JH, van Blitterswijk WJ, Moolenaar WH. Lysophosphatidic acid-induced ca2+ mobilization in human a431 cells: Structure-activity analysis. *Biochem J*. 1995;307 (Pt 2):609-616
93. Contos JJ, Fukushima N, Weiner JA, Kaushal D, Chun J. Requirement for the lpa1 lysophosphatidic acid receptor gene in normal suckling behavior. *Proc Natl Acad Sci U S A*. 2000;97:13384-13389
94. Contos JJ, Ishii I, Fukushima N, Kingsbury MA, Ye X, Kawamura S, Brown JH, Chun J. Characterization of lpa(2) (edg4) and lpa(1)/lpa(2) (edg2/edg4) lysophosphatidic acid receptor knockout mice: Signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol Cell Biol*. 2002;22:6921-6929
95. Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, Suzuki H, Amano T, Kennedy G, Arai H, Aoki J, Chun J. Lpa3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature*. 2005;435:104-108
96. Hama K, Aoki J, Inoue A, Endo T, Amano T, Motoki R, Kanai M, Ye X, Chun J, Matsuki N, Suzuki H, Shibasaki M, Arai H. Embryo spacing and implantation timing are differentially regulated by lpa3-mediated lysophosphatidic acid signaling in mice. *Biol Reprod*. 2007;77:954-959

97. Hooks SB, Santos WL, Im DS, Heise CE, Macdonald TL, Lynch KR. Lysophosphatidic acid-induced mitogenesis is regulated by lipid phosphate phosphatases and is edg-receptor independent. *J Biol Chem*. 2001;276:4611-4621
98. Tokumura A, Sinomiya J, Kishimoto S, Tanaka T, Kogure K, Sugiura T, Satouchi K, Waku K, Fukuzawa K. Human platelets respond differentially to lysophosphatidic acids having a highly unsaturated fatty acyl group and alkyl ether-linked lysophosphatidic acids. *Biochem. J.* 2002;365:617-628
99. Gueguen G, Gaigé B, Grévy J-M, Rogalle P, Bellan J, Wilson M, Klaébé A, Pont F, Simon M-F, Chap H. Structure−activity analysis of the effects of lysophosphatidic acid on platelet aggregation†. *Biochemistry*. 1999;38:8440-8450
100. Noguchi K, Ishii S, Shimizu T. Identification of p2y9/gpr23 as a novel g protein-coupled receptor for lysophosphatidic acid, structurally distant from the edg family. *Journal of Biological Chemistry*. 2003;278:25600-25606
101. Lee Z, Cheng CT, Zhang H, Subler MA, Wu J, Mukherjee A, Windle JJ, Chen CK, Fang X. Role of lpa4/p2y9/gpr23 in negative regulation of cell motility. *Mol Biol Cell*. 2008;19:5435-5445
102. Liu YB, Kharode Y, Bodine PV, Yaworsky PJ, Robinson JA, Billiard J. Lpa induces osteoblast differentiation through interplay of two receptors: Lpa1 and lpa4. *J Cell Biochem*. 2010;109:794-800
103. Sumida H, Noguchi K, Kihara Y, Abe M, Yanagida K, Hamano F, Sato S, Tamaki K, Morishita Y, Kano MR, Iwata C, Miyazono K, Sakimura K, Shimizu T, Ishii S. Lpa4 regulates blood and lymphatic vessel formation during mouse embryogenesis. *Blood*. 2010;116:5060-5070
104. Kotarsky K, Boketoft A, Bristulf J, Nilsson NE, Norberg A, Hansson S, Owman C, Sillard R, Leeb-Lundberg LM, Olde B. Lysophosphatidic acid binds to and activates gpr92, a g protein-coupled receptor highly expressed in gastrointestinal lymphocytes. *J Pharmacol Exp Ther*. 2006;318:619-628
105. Lee CW, Rivera R, Gardell S, Dubin AE, Chun J. Gpr92 as a new g12/13- and gq-coupled lysophosphatidic acid receptor that increases camp, lpa5. *J Biol Chem*. 2006;281:23589-23597
106. Allende ML, Yamashita T, Proia RL. G-protein-coupled receptor s1p1 acts within endothelial cells to regulate vascular maturation. *Blood*. 2003;102:3665-3667
107. Singleton PA, Dudek SM, Ma SF, Garcia JG. Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation. Novel role for hyaluronan and cd44 receptor family. *J Biol Chem*. 2006;281:34381-34393
108. Sanna MG, Wang SK, Gonzalez-Cabrera PJ, Don A, Marsolais D, Matheu MP, Wei SH, Parker I, Jo E, Cheng WC, Cahalan MD, Wong CH, Rosen H. Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral s1p1 antagonist in vivo. *Nat Chem Biol*. 2006;2:434-441
109. English D, Kovala AT, Welch Z, Harvey KA, Siddiqui RA, Brindley DN, Garcia JG. Induction of endothelial cell chemotaxis by sphingosine 1-phosphate and stabilization of endothelial monolayer barrier function by lysophosphatidic acid,

potential mediators of hematopoietic angiogenesis. *J Hematother Stem Cell Res*. 1999;8:627-634

110. Minnear FL, Patil S, Bell D, Gainor JP, Morton CA. Platelet lipid(s) bound to albumin increases endothelial electrical resistance: Mimicked by lpa. *Am J Physiol Lung Cell Mol Physiol*. 2001;281:L1337-1344
111. Neidlinger NA, Larkin SK, Bhagat A, Victorino GP, Kuypers FA. Hydrolysis of phosphatidylserine-exposing red blood cells by secretory phospholipase a2 generates lysophosphatidic acid and results in vascular dysfunction. *J Biol Chem*. 2006;281:775-781
112. van Nieuw Amerongen GP, Vermeer MA, van Hinsbergh VW. Role of rhoa and rho kinase in lysophosphatidic acid-induced endothelial barrier dysfunction. *Arterioscler Thromb Vasc Biol*. 2000;20:E127-133
113. Rizza C, Leitinger N, Yue J, Fischer DJ, Wang DA, Shih PT, Lee H, Tigyi G, Berliner JA. Lysophosphatidic acid as a regulator of endothelial/leukocyte interaction. *Lab Invest*. 1999;79:1227-1235
114. Lin CI, Chen CN, Chen JH, Lee H. Lysophospholipids increase il-8 and mcp-1 expressions in human umbilical cord vein endothelial cells through an il-1-dependent mechanism. *J Cell Biochem*. 2006;99:1216-1232
115. Zhou Z, Subramanian P, Sevilmis G, Globke B, Soehnlein O, Karshovska E, Megens R, Heyll K, Chun J, Saulnier-Blache JS, Reinholz M, van Zandvoort M, Weber C, Schober A. Lipoprotein-derived lysophosphatidic acid promotes atherosclerosis by releasing cxcl1 from the endothelium. *Cell Metab*. 2011;13:592-600
116. Igarashi J, Michel T. Agonist-modulated targeting of the edg-1 receptor to plasmalemmal caveolae. Enos activation by sphingosine 1-phosphate and the role of caveolin-1 in sphingolipid signal transduction. *J Biol Chem*. 2000;275:32363-32370
117. Kou R, Igarashi J, Michel T. Lysophosphatidic acid and receptor-mediated activation of endothelial nitric-oxide synthase. *Biochemistry*. 2002;41:4982-4988
118. Forrest M, Sun SY, Hajdu R, Bergstrom J, Card D, Doherty G, Hale J, Keohane C, Meyers C, Milligan J, Mills S, Nomura N, Rosen H, Rosenbach M, Shei GJ, Singer, II, Tian M, West S, White V, Xie J, Proia RL, Mandala S. Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J Pharmacol Exp Ther*. 2004;309:758-768
119. Subramanian P, Karshovska E, Reinhard P, Megens RT, Zhou Z, Akhtar S, Schumann U, Li X, van Zandvoort M, Ludin C, Weber C, Schober A. Lysophosphatidic acid receptors lpa1 and lpa3 promote cxcl12-mediated smooth muscle progenitor cell recruitment in neointima formation. *Circ Res*. 2010;107:96-105
120. Kluk MJ, Hla T. Role of the sphingosine 1-phosphate receptor edg-1 in vascular smooth muscle cell proliferation and migration. *Circ Res*. 2001;89:496-502
121. Ohmori T, Yatomi Y, Osada M, Kazama F, Takafuta T, Ikeda H, Ozaki Y. Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via s1p2. *Cardiovasc Res*. 2003;58:170-177

122. Coussin F, Scott RH, Wise A, Nixon GF. Comparison of sphingosine 1-phosphate-induced intracellular signaling pathways in vascular smooth muscles: Differential role in vasoconstriction. *Circ Res*. 2002;91:151-157
123. Tigyi G, Hong L, Yakubu M, Parfenova H, Shibata M, Leffler CW. Lysophosphatidic acid alters cerebrovascular reactivity in piglets. *Am J Physiol*. 1995;268:H2048-2055
124. Gerrard JM, Robinson P. Lysophosphatidic acid can activate platelets without increasing 32p-labelling of phosphatidic acid. *Biochim Biophys Acta*. 1984;795:487-492
125. Retzer M, Siess W, Essler M. Mildly oxidised low density lipoprotein induces platelet shape change via rho-kinase-dependent phosphorylation of myosin light chain and moesin. *FEBS Lett*. 2000;466:70-74
126. Olorundare OE, Peyruchaud O, Albrecht RM, Mosher DF. Assembly of a fibronectin matrix by adherent platelets stimulated by lysophosphatidic acid and other agonists. *Blood*. 2001;98:117-124
127. Haseruck N, Erl W, Pandey D, Tigyi G, Ohlmann P, Ravanat C, Gachet C, Siess W. The plaque lipid lysophosphatidic acid stimulates platelet activation and platelet-monocyte aggregate formation in whole blood: Involvement of p2y1 and p2y12 receptors. *Blood*. 2004;103:2585-2592
128. Eriksson AC, Whiss PA, Nilsson UK. Adhesion of human platelets to albumin is synergistically increased by lysophosphatidic acid and adrenaline in a donor-dependent fashion. *Blood Coagul Fibrinolysis*. 2006;17:359-368
129. Nilsson UK, Svensson SP, Grenegard M. Synergistic activation of human platelets by lysophosphatidic acid and adrenaline. *Haematologica*. 2002;87:730-739; discussion 739
130. Motohashi K, Shibata S, Ozaki Y, Yatomi Y, Igarashi Y. Identification of lysophospholipid receptors in human platelets: The relation of two agonists, lysophosphatidic acid and sphingosine 1-phosphate. *FEBS Letters*. 2000;468:189-193
131. Siess W. Athero- and thrombogenic actions of lysophosphatidic acid and sphingosine-1-phosphate. *Biochim Biophys Acta*. 2002;1582:204-215
132. Joist JH, Dolezel G, Cucuianu MP, Nishizawa EE, Mustard JF. Inhibition and potentiation of platelet function by lysolecithin. *Blood*. 1977;49:101-112
133. Chun J, Hla T, Lynch KR, Spiegel S, Moolenaar WH. International union of basic and clinical pharmacology. Lxxviii. Lysophospholipid receptor nomenclature. *Pharmacological Reviews*. 2010;62:579-587
134. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci*. 1992;8:275-282
135. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. Mega5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*. 2011
136. Fulkerson Z, Wu T, Sunakura M, Vander Kooi C, Morris AJ, Smyth SS. Binding of autotaxin to integrins localizes lysophosphatidic acid production to platelets and mammalian cells. *J Biol Chem*. 2011

137. Watanabe N, Ikeda H, Nakamura K, Ohkawa R, Kume Y, Aoki J, Hama K, Okudaira S, Tanaka M, Tomiya T, Yanase M, Tejima K, Nishikawa T, Arai M, Arai H, Omata M, Fujiwara K, Yatomi Y. Both plasma lysophosphatidic acid and serum autotaxin levels are increased in chronic hepatitis c. *Journal of Clinical Gastroenterology*. 2007;41:616-623
138. Takeuchi K, Reue K. Biochemistry, physiology, and genetics of gpat, agpat, and lipin enzymes in triglyceride synthesis. *American Journal of Physiology - Endocrinology And Metabolism*. 2009;296:E1195-E1209
139. Tokumura A, Harada K, Fukuzawa K, Tsukatani H. Involvement of lysophospholipase d in the production of lysophosphatidic acid in rat plasma. *Biochim Biophys Acta*. 1986;875:31-38
140. Stracke ML, Krutzsch HC, Unsworth EJ, Arestad A, Cioce V, Schiffmann E, Liotta LA. Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. *J Biol Chem*. 1992;267:2524-2529
141. Murata J, Lee HY, Clair T, Krutzsch HC, Arestad AA, Sobel ME, Liotta LA, Stracke ML. Cdna cloning of the human tumor motility-stimulating protein, autotaxin, reveals a homology with phosphodiesterases. *Journal of Biological Chemistry*. 1994;269:30479-30484
142. Stefan C, Jansen S, Bollen M. Npp-type ectophosphodiesterases: Unity in diversity. *Trends in Biochemical Sciences*. 2005;30:542-550
143. Gijsbers R, Aoki J, Arai H, Bollen M. The hydrolysis of lysophospholipids and nucleotides by autotaxin (npp2) involves a single catalytic site. *FEBS Lett*. 2003;538:60-64
144. Umezu-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J, Arai H. Autotaxin has lysophospholipase d activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J Cell Biol*. 2002;158:227-233
145. Cimpean A, Stefan C, Gijsbers R, Stalmans W, Bollen M. Substrate-specifying determinants of the nucleotide pyrophosphatases/phosphodiesterases npp1 and npp2. *Biochem J*. 2004;381:71-77
146. Hausmann J, Kamtekar S, Christodoulou E, Day JE, Wu T, Fulkerson Z, Albers HMHG, van Meeteren LA, Houben AJS, van Zeijl L, Jansen S, Andries M, Hall T, Pegg LE, Benson TE, Kasiem M, Harlos K, Kooi CWV, Smyth SS, Ovaa H, Bollen M, Morris AJ, Moolenaar WH, Perrakis A. Structural basis of substrate discrimination and integrin binding by autotaxin. *Nat Struct Mol Biol*. 2011;18:198-204
147. Nishimasu H, Okudaira S, Hama K, Mihara E, Dohmae N, Inoue A, Ishitani R, Takagi J, Aoki J, Nureki O. Crystal structure of autotaxin and insight into gpcr activation by lipid mediators. *Nat Struct Mol Biol*. 2011;18:205-212
148. van Meeteren LA, Ruurs P, Christodoulou E, Goding JW, Takakusa H, Kikuchi K, Perrakis A, Nagano T, Moolenaar WH. Inhibition of autotaxin by lysophosphatidic acid and sphingosine 1-phosphate. *J Biol Chem*. 2005;280:21155-21161
149. Baker DL, Fujiwara Y, Pigg KR, Tsukahara R, Kobayashi S, Murofushi H, Uchiyama A, Murakami-Murofushi K, Koh E, Bandle RW, Byun H-S, Bittman R, Fan D, Murph M, Mills GB, Tigyi G. Carba analogs of cyclic phosphatidic acid are selective

inhibitors of autotaxin and cancer cell invasion and metastasis. *Journal of Biological Chemistry*. 2006;281:22786-22793

150. Cui P, McCalmont WF, Tomsig JL, Lynch KR, Macdonald TL. A- and β-substituted phosphonate analogs of lpa as autotaxin inhibitors. *Bioorganic & Medicinal Chemistry*. 2008;16:2212-2225
151. Ferry G, Moulharat N, Pradère J-P, Desos P, Try A, Genton A, Giganti A, Beucher-Gaudin M, Lonchampt M, Bertrand M, Saulnier-Blache J-S, Tucker GC, Cordi A, Boutin JA. S32826, a nanomolar inhibitor of autotaxin: Discovery, synthesis and applications as a pharmacological tool. *Journal of Pharmacology and Experimental Therapeutics*. 2008;327:809-819
152. van Meeteren LA, Brinkmann V, Saulnier-Blache JS, Lynch KR, Moolenaar WH. Anticancer activity of fty720: Phosphorylated fty720 inhibits autotaxin, a metastasis-enhancing and angiogenic lysophospholipase d. *Cancer Letters*. 2008;266:203-208
153. Albers HMHG, van Meeteren LA, Egan DA, van Tilburg EW, Moolenaar WH, Ovaa H. Discovery and optimization of boronic acid based inhibitors of autotaxin. *Journal of Medicinal Chemistry*. 2010;53:4958-4967
154. Jansen S, Andries M, Vekemans K, Vanbilloen H, Verbruggen A, Bollen M. Rapid clearance of the circulating metastatic factor autotaxin by the scavenger receptors of liver sinusoidal endothelial cells. *Cancer Letters*. 2009;284:216-221
155. Sigurdardottir O, Wiman B. Identification of a pai-1 binding site in vitronectin. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*. 1994;1208:104-110
156. Preissner KT. Structure and biological role of vitronectin. *Annual Review of Cell Biology*. 1991;7:275-310
157. Stefansson S, Su EJ, Ishigami S, Cale JM, Gao Y, Gorlatova N, Lawrence DA. The contributions of integrin affinity and integrin-cytoskeletal engagement in endothelial and smooth muscle cell adhesion to vitronectin. *J Biol Chem*. 2007;282:15679-15689
158. Seiffert D, Loskutoff DJ. Evidence that type 1 plasminogen activator inhibitor binds to the somatomedin b domain of vitronectin. *Journal of Biological Chemistry*. 1991;266:2824-2830
159. Schar CR, Blouse GE, Minor KH, Peterson CB. A deletion mutant of vitronectin lacking the somatomedin b domain exhibits residual plasminogen activator inhibitor-1-binding activity. *Journal of Biological Chemistry*. 2008;283:10297-10309
160. Kanda H, Newton R, Klein R, Morita Y, Gunn MD, Rosen SD. Autotaxin, an ectoenzyme that produces lysophosphatidic acid, promotes the entry of lymphocytes into secondary lymphoid organs. *Nat Immunol*. 2008;9:415-423
161. Fox MA, Colello RJ, Macklin WB, Fuss B. Phosphodiesterase-ialpha/autotaxin: A counteradhesive protein expressed by oligodendrocytes during onset of myelination. *Mol Cell Neurosci*. 2003;23:507-519
162. Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Coller BS, Teitelbaum S, Hynes RO. Beta3-integrin-deficient

mice are a model for glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest*. 1999;103:229-238

163. Jirouskova M, Shet AS, Johnson GJ. A guide to murine platelet structure, function, assays, and genetic alterations. *J Thromb Haemost*. 2007;5:661-669
164. Sano T, Baker D, Virag T, Wada A, Yatomi Y, Kobayashi T, Igarashi Y, Tigyi G. Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine 1-phosphate generation in blood. *Journal of Biological Chemistry*. 2002;277:21197-21206
165. Nakamura K, Igarashi K, Ide K, Ohkawa R, Okubo S, Yokota H, Masuda A, Oshima N, Takeuchi T, Nangaku M, Okudaira S, Arai H, Ikeda H, Aoki J, Yatomi Y. Validation of an autotaxin enzyme immunoassay in human serum samples and its application to hypoalbuminemia differentiation. *Clinica Chimica Acta*. 2008;388:51-58
166. Smyth SS, Sciorra VA, Sigal YJ, Pamuklar Z, Wang Z, Xu Y, Prestwich GD, Morris AJ. Lipid phosphate phosphatases regulate lysophosphatidic acid production and signaling in platelets. *Journal of Biological Chemistry*. 2003;278:43214-43223
167. Fox MA, Alexander JK, Afshari FS, Colello RJ, Fuss B. Phosphodiesterase-i alpha/autotaxin controls cytoskeletal organization and fak phosphorylation during myelination. *Mol Cell Neurosci*. 2004;27:140-150
168. Tokumura A, Majima E, Kariya Y, Tominaga K, Kogure K, Yasuda K, Fukuzawa K. Identification of human plasma lysophospholipase d, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J Biol Chem*. 2002;277:39436-39442
169. Coller BS, Kutok JL, Scudder LE, Galanakis DK, West SM, Rudomen GS, Springer KT. Studies of activated gpiib/iiia receptors on the luminal surface of adherent platelets. Paradoxical loss of luminal receptors when platelets adhere to high density fibrinogen. *The Journal of Clinical Investigation*. 1993;92:2796-2806
170. Jiroušková M, Jaiswal JK, Coller BS. Ligand density dramatically affects integrin αiibβ3-mediated platelet signaling and spreading. *Blood*. 2007;109:5260-5269
171. Federico L, Pamuklar Z, Smyth SS, Morris AJ. Therapeutic potential of autotaxin/lysophospholipase d inhibitors. *Curr Drug Targets*. 2008;9:698-708
172. Pamuklar Z, Lee JS, Cheng HY, Panchatcharam M, Steinhubl S, Morris AJ, Charnigo R, Smyth SS. Individual heterogeneity in platelet response to lysophosphatidic acid: Evidence for a novel inhibitory pathway. *Arterioscler Thromb Vasc Biol*. 2008;28:555-561
173. Tomsig JL, Snyder AH, Berdyshev EV, Skobeleva A, Mataya C, Natarajan V, Brindley DN, Lynch KR. Lipid phosphate phosphohydrolase type 1 (lpp1) degrades extracellular lysophosphatidic acid in vivo. *Biochem J*. 2009;419:611-618
174. Smyth SS, Cheng HY, Miriyala S, Panchatcharam M, Morris AJ. Roles of lysophosphatidic acid in cardiovascular physiology and disease. *Biochim Biophys Acta*. 2008;1781:563-570
175. Gierse J, Thorarensen A, Beltey K, Bradshaw-Pierce E, Cortes-Burgos L, Hall T, Johnston A, Murphy M, Nemirovskiy O, Ogawa S, Pegg L, Pelc M, Prinsen M,

Schnute M, Wendling J, Wene S, Weinberg R, Wittwer A, Zweifel B, Masferrer J. A novel autotaxin inhibitor reduces lysophosphatidic acid levels in plasma and the site of inflammation. *J Pharmacol Exp Ther*. 2010;334:310-317

176. Maschberger P, Bauer M, Baumann-Siemons J, Zangl KJ, Negrescu EV, Reininger AJ, Siess W. Mildly oxidized low density lipoprotein rapidly stimulates via activation of the lysophosphatidic acid receptor src family and syk tyrosine kinases and ca2+ influx in human platelets. *J Biol Chem*. 2000;275:19159-19166
177. Smyth SS, Sciorra VA, Sigal YJ, Pamuklar Z, Wang Z, Xu Y, Prestwich GD, Morris AJ. Lipid phosphate phosphatases regulate lysophosphatidic acid production and signaling in platelets: Studies using chemical inhibitors of lipid phosphate phosphatase activity. *J Biol Chem*. 2003;278:43214-43223
178. Simon M-F, Chap H, Douste-Blazy L. Human platelet aggregation induced by 1-alkyl-lysophosphatidic acid and its analogs: A new group of phospholipid mediators? *Biochemical and Biophysical Research Communications*. 1982;108:1743-1750
179. Zhang C, Baker DL, Yasuda S, Makarova N, Balazs L, Johnson LR, Marathe GK, McIntyre TM, Xu Y, Prestwich GD, Byun HS, Bittman R, Tigyi G. Lysophosphatidic acid induces neointima formation through ppargamma activation. *J Exp Med*. 2004;199:763-774
180. Chou TC, Shih CY, Chen YT. Inhibitory effect of alpha-lipoic acid on platelet aggregation is mediated by ppars. *J Agric Food Chem*. 2011;59:3050-3059
181. Amisten S, Braun OO, Bengtsson A, Erlinge D. Gene expression profiling for the identification of g-protein coupled receptors in human platelets. *Thromb Res*. 2008;122:47-57
182. Williams JR, Khandoga AL, Goyal P, Fells JI, Perygin DH, Siess W, Parrill AL, Tigyi G, Fujiwara Y. Unique ligand selectivity of the gpr92/lpa5 lysophosphatidate receptor indicates role in human platelet activation. *J Biol Chem*. 2009;284:17304-17319
183. Zhang H, Xu X, Gajewiak J, Tsukahara R, Fujiwara Y, Liu J, Fells JI, Perygin D, Parrill AL, Tigyi G, Prestwich GD. Dual activity lysophosphatidic acid receptor pan-antagonist/autotaxin inhibitor reduces breast cancer cell migration in vitro and causes tumor regression in vivo. *Cancer Res*. 2009;69:5441-5449
184. Du H, Zawaski JA, Gaber MW, Chiang TM. A recombinant protein and a chemically synthesized peptide containing the active peptides of the platelet collagen receptors inhibit ferric chloride-induced thrombosis in a rat model. *Thromb Res*. 2007;121:419-426
185. Murayama T, Tepper OM, Silver M, Ma H, Losordo DW, Isner JM, Asahara T, Kalka C. Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. *Exp Hematol*. 2002;30:967-972
186. Lee S, Lynch KR. Brown recluse spider (loxosceles reclusa) venom phospholipase d (pld) generates lysophosphatidic acid (lpa). *Biochem J*. 2005;391:317-323
187. van Meeteren LA, Frederiks F, Giepmans BN, Pedrosa MF, Billington SJ, Jost BH, Tambourgi DV, Moolenaar WH. Spider and bacterial sphingomyelinases d target

cellular lysophosphatidic acid receptors by hydrolyzing lysophosphatidylcholine. *J Biol Chem*. 2004;279:10833-10836

188. Sardar VM, Bautista DL, Fischer DJ, Yokoyama K, Nusser N, Virag T, Wang DA, Baker DL, Tigyi G, Parrill AL. Molecular basis for lysophosphatidic acid receptor antagonist selectivity. *Biochim Biophys Acta*. 2002;1582:309-317
189. Harrison P, Wilbourn B, Debili N, Vainchenker W, Breton-Gorius J, Lawrie AS, Masse JM, Savidge GF, Cramer EM. Uptake of plasma fibrinogen into the alpha granules of human megakaryocytes and platelets. *The Journal of Clinical Investigation*. 1989;84:1320-1324
190. Wencel-Drake JD, Boudignon-Proudhon C, Dieter MG, Criss AB, Parise LV. Internalization of bound fibrinogen modulates platelet aggregation. *Blood*. 1996;87:602-612
191. Handagama P, Bainton DF, Jacques Y, Conn MT, Lazarus RA, Shuman MA. Kistrin, an integrin antagonist, blocks endocytosis of fibrinogen into guinea pig megakaryocyte and platelet alpha-granules. *J Clin Invest*. 1993;91:193-200
192. Kaplan KL, Dauzier MJ, Rose S. Adp and epinephrine-induced release of platelet fibrinogen. *Blood*. 1981;58:797-802
193. Bian D, Su S, Mahanivong C, Cheng RK, Han Q, Pan ZK, Sun P, Huang S. Lysophosphatidic acid stimulates ovarian cancer cell migration via a ras-mek kinase 1 pathway. *Cancer Res*. 2004;64:4209-4217
194. Shida D, Kitayama J, Yamaguchi H, Okaji Y, Tsuno NH, Watanabe T, Takuwa Y, Nagawa H. Lysophosphatidic acid (lpa) enhances the metastatic potential of human colon carcinoma dld1 cells through lpa1. *Cancer Res*. 2003;63:1706-1711
195. Hama K, Aoki J, Fukaya M, Kishi Y, Sakai T, Suzuki R, Ohta H, Yamori T, Watanabe M, Chun J, Arai H. Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through lpa1. *J Biol Chem*. 2004;279:17634-17639
196. Yang SY, Lee J, Park CG, Kim S, Hong S, Chung HC, Min SK, Han JW, Lee HW, Lee HY. Expression of autotaxin (npp-2) is closely linked to invasiveness of breast cancer cells. *Clin Exp Metastasis*. 2002;19:603-608
197. Baumforth KR, Flavell JR, Reynolds GM, Davies G, Pettit TR, Wei W, Morgan S, Stankovic T, Kishi Y, Arai H, Nowakova M, Pratt G, Aoki J, Wakelam MJ, Young LS, Murray PG. Induction of autotaxin by the epstein-barr virus promotes the growth and survival of hodgkin lymphoma cells. *Blood*. 2005;106:2138-2146
198. Zhang G, Zhao Z, Xu S, Ni L, Wang X. Expression of autotaxin mrna in human hepatocellular carcinoma. *Chin Med J (Engl)*. 1999;112:330-332
199. Kishi Y, Okudaira S, Tanaka M, Hama K, Shida D, Kitayama J, Yamori T, Aoki J, Fujimaki T, Arai H. Autotaxin is overexpressed in glioblastoma multiforme and contributes to cell motility of glioblastoma by converting lysophosphatidylcholine to lysophosphatidic acid. *J Biol Chem*. 2006;281:17492-17500
200. Desgrosellier JS, Cheresh DA. Integrins in cancer: Biological implications and therapeutic opportunities. *Nat Rev Cancer*. 2010;10:9-22

201. Karpatkin S, Pearlstein E, Ambrogio C, Coller BS. Role of adhesive proteins in platelet tumor interaction in vitro and metastasis formation in vivo. *J Clin Invest*. 1988;81:1012-1019
202. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nat Rev Cancer*. 2011;11:123-134

VITA

Author Name

Zachary Bennett Fulkerson

Date of Birth

February 20, 1984

Place of Birth

Owensboro, Kentucky

Education

University of Kentucky College of Arts and Sciences, Lexington, KY
BA, Chemistry (minor Classics), *Summa Cum Laude*, May 2006
BS, Biology (minor Music Performance), *Summa Cum Laude*, May 2006

Professional and Scholastic Positions

Vice-Chair, American Physician Scientists Association Annual Meeting Committee, 2011

Co-Chair, Gordon Research Semianr on Hemostasis, 2010

Institutional Representative, American Physician Scientists Association University of Kentucky Chapter, 2009-2010

Awards

Travel Award, Southeastern Regional Lipid Conference, 2010

Travel Award, Southeastern Regional Lipid Conference, 2009

Funding

1F30HL099272-01, Regulation of Lysolipid Signaling by Autotaxin-Integrin Interactions on Platelets, Awarded 2010

5T32HL0727-43-06, Interdisciplinary Cardiovascular Training Grant, Awarded 2008

Publications

Fulkerson Z, Wu T, Sunkara M, Vander Kooi, C, Morris AJ, Smyth S.Binding of autotaxin to integrins localizes lysophosphatidic acid production to platelets and mammalian cells. (2011) *J Biol Chem*, 286: 34654 - 34663.

Hausmann J, Kamtekar S, Christodoulou E, Day JE, Wu T, **Fulkerson Z**, Albers HMHG, van Meeteren LA, Houben AJS, van Zeijl L, Jansen S, Andries M, Hall T, Pegg LE, Benson TE, Kasiem M, Harlos K, Kooi CWV, Smyth SS, Ovaa H, Bollen M, Morris AJ, Moolenaar WH, Perrakis A. Structural basis of substrate discrimination and integrin binding by autotaxin. (2011) *Nat Struct Mol Biol*, 18:198 - 204.

Morris AJ, Panchatcharam M, Cheng HY, Federico L, **Fulkerson Z**, Selim S, Escalante-Alcalde D, and Smyth SS. Regulation of blood and vascular cell function by bioactive lysophospholipids. (2009) *J Thromb Haemostasis*, 7:39 - 43.

Pamuklar Z, Federico L, Liu S, Goto M, Dong A, Panchatcharam M, **Fulkerson Z**, Berdyshev E, Natarajan V, Fang F, Mills GB, Morris AJ, and Smyth SS. Autotaxin/lysophospholipase D and lysophosphatidic acid regulate thrombosis and hemostasis in mice. (2009) *J Biol Chem*, 284:7385 - 7394

Fulkerson Z, Morris AJ, Smyth SS. 2008. Interactions of lysophospholipids with platelets. *International Review of Thrombosis*

Abstracts (Poster and Oral Presentations)

American Society of Clinical Investigation and Association of American Physicians Joint Meeting 2011, Chicago, IL

Binding of autotaxin to integrins localizes lysophosphatidic acid production to the plasma membrane of platelets and mammalian cells (Poster Presentation)

Midwest Platelet Conference 2010, Chapel Hill, NC

Regulation of LPA Production by ATX-Integrin Interaction (Oral Presentation)

Southeastern Regional Lipid Conference 2010, Cashiers, NC

The N-terminal tandem somatomedin b-like domain regulates the autotaxin/lysophospholipase D activity by interdomain interactions and integrin binding (Oral Presentation)

Gordon Research Conference in Hemostasis 2010, Waterville Valley, NH

Generation of LPA is Enhanced by the Interaction of the Lysophosholipase D Autotaxin with Integrins on Activated Platelets (Poster Presentation)

Gordon Graduate Research Seminar in Hemostasis 2010, Waterville Valley, NH

Generation of LPA is Enhanced by the Interaction of the Lysophosholipase D Autotaxin with Integrins on Activated Platelets (Poster Presentation)

American Society of Clinical Investigation and Association of American Physicians Joint Meeting 2010, Chicago, IL

Autotaxin/lysophospholipase D is localized to the platelet surface by interaction with platelet integrins (Poster Presentation)

Southeastern Regional Lipid Conferece 2009, Cashiers, NC

Generation of the bioactive lipid mediator lysophosphatidic acid by Autotaxin/Lysophospholipase D through interaction with platelet integrins (Oral Presentation)

Federation of American Societies for Experimental Biology Summer Research Conference Lysolipid Mediators in Health and Disease 2009, Carefree, AZ

Interaction of the lysospholipase D autotaxin with integrins (Poster Presentation)

Arteriosclerosis, Thrombosis, and Vascular Biology National Meeting, Washington, DC

Generation of Distinct Molecular Species of the Bioactive Lipid Mediator Lysophosphatidic Acid by Autotaxin/Lysophospholipase D Through Interaction with Platelet Integrins (Oral Presentation)

American Society of Clinical Investigation and Association of American Physicians Joint Meeting 2009, Chicago, IL

Generation of Distinct Molecular Species of the Bioactive Lipid Mediator Lysophosphatidic Acid by Autotaxin/Lysophospholipase D through Interaction with Platelet Integrins (Poster Presentation)

Society Memberships

American Heart Association
American Medical Association
American Student Medical Association
American College of Physicians
Southern Medical Association

Zachary B. Fulkerson