Phospholipase A2 Induced Monocyte Chemotaxis to Apoptotic Cells

Kwasi Karikari
Virginia Commonwealth University

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PHOSPHOLIPASE A₂ INDUCED MONOCYTE CHEMOTAXIS TO
APOPTOTIC CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of
Science in Biochemistry at Virginia Commonwealth University.

By

KWASI KARIKARI
B.S., George Mason University 1998
Certificate, Virginia Commonwealth University 2005

Director: SUZANNE E. BARBOUR, PH.D.
ASSOCIATE PROFESSOR
DEPARTMENT OF BIOCHEMISTRY

Virginia Commonwealth University
Richmond, Virginia
June 2006
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Dedication

This manuscript is dedicated to my beloved late grandmother, “Mamaga” who tirelessly and selflessly labored to provide, nourish and comfort her family. May she rest in peace!
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>PLA₂ Family</td>
<td>13</td>
</tr>
<tr>
<td>sPLA₂ and Bioactive Lipids</td>
<td>20</td>
</tr>
<tr>
<td>sPLA₂ Receptors</td>
<td>23</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>24</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>25</td>
</tr>
<tr>
<td>Results</td>
<td>29</td>
</tr>
<tr>
<td>Discussion</td>
<td>54</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>63</td>
</tr>
<tr>
<td>Appendix-I</td>
<td>68</td>
</tr>
<tr>
<td>Vita</td>
<td>69</td>
</tr>
</tbody>
</table>
List of Table

| Table 1: Forms of Phospholipase A₂ Expressed in Mammalian Cells | 13 |
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phagocytes (including macrophage and dendritic cells) can engulf and degrade both apoptotic and necrotic cells</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Proposed model for recruitment of macrophages by “trapped” sPLA₂</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>Binding surface and mechanism of catalysis by Phospholipase A₂</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Primary structure of Pancreatic sPLA₂</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>sPLA₂ and lipid metabolism</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>Cultured supernatant containing group IIa sPLA₂ shows phospholipase activity.</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>Transition of cell population from viable to apoptotic and/or necrotic by forward and side scatter analysis</td>
<td>33</td>
</tr>
<tr>
<td>8</td>
<td>Effect of 20 ng/mL Anti-fas treatment on Jurkat cell viability</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>Propidium Iodide (PI) and Annexin-V FITC staining differentiates cell population into viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL) 4 hr time-point</td>
<td>39</td>
</tr>
<tr>
<td>10</td>
<td>Propidium Iodide (PI) and Annexin-V FITC staining differentiates cell population into viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL) 12 hr time-point</td>
<td>41</td>
</tr>
</tbody>
</table>
Figure 11: Propidium Iodide (PI) and Annexin-V FITC staining differentiates cell population into viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL) 18 hr time-point.........................................................43

Figure 12: Equivalent group IIα sPLA₂ association with control and antifas treated cells and bound enzyme remains catalytically active .................................................................46

Figure 13: “Trapped” group IIα sPLA₂ generates soluble bioactive molecules which induce THP-1 monocyte chemotaxis directly or indirectly.........................................................49

Figure 14: BPB treated sPLA₂ is no longer active.................................................................52

Figure 15: The phagocyte recognition array in the mammalian clearance of apoptotic cells .................................................................................................................................60

Figure 16: Revised model for recruitment of macrophage by “trapped” sPLA₂.................61

Figure 17: Comparison of cell viability by trypan blue exclusion method versus forward scatter by side scatter analysis using flow cytometry.......................68
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>sPLA₂</td>
<td>Secreted Phospholipase A₂</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human Acute Monocytic Leukemia Cells</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>SM</td>
<td>Sphingomyelin</td>
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<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic Acid</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lysophosphatidylserine</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan Sulfate Proteoglycan</td>
</tr>
<tr>
<td>BPB</td>
<td>para-bromophenacylbromide</td>
</tr>
<tr>
<td>CD-36</td>
<td>Scavenger Receptor</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty Acid</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
</tbody>
</table>
Abstract

PHOSPHOLIPASE A\textsubscript{2} INDUCED MONOCYTE CHEMOTAXIS TO APOPTOTIC CELLS

By
KWASI KARIKARI, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biochemistry at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

Major Director: Suzanne E. Barbour, Ph.D.
Associate Professor
Department of Biochemistry

Apoptosis is a form of programmed cell death that is essential in such processes as organ and tissue remodeling and maturation of hematopoietic cells. The clearance of apoptotic cells is essential to prevent autoimmune responses to sequestered antigens. This process is mediated by phagocytes of the monocyte lineage. Before phagocytosis can occur, macrophages must be recruited to the apoptotic cells through chemotaxis. Products of the reaction catalyzed by the phospholipases A\textsubscript{2} (PLA\textsubscript{2}) have been shown to induce
monocyte chemotaxis either directly or indirectly. Some investigators have implicated a
cytosolic calcium-independent PLA$_2$ (iPLA$_2$) in the production of these products during
apoptosis. However, a recent report suggests that the secreted group IIa (sPLA$_2$) binds to
surfaces of apoptotic cells. The “receptor” for this pool of sPLA$_2$ is the rod domain of
vimentin, an intermediate filament protein that is exposed by caspase activity when cells
undergo apoptosis. Based on these observations, we hypothesize that the exposure of
vimentin on apoptotic cells traps a pool of catalytically active sPLA$_2$ that then generates
the bioactive lipids that induce macrophage chemotaxis. In our methods, [$^3$H]-oleate
labeled E-coli is used as a substrate for sPLA$_2$ and enzyme activity is quantified by
scintillation counting of released radiolabeled oleic acid. Apoptosis is induced with anti-fas
(CD-95) on Jurkat cells and monitored through annexin-V binding and propidium iodide
(PI) staining followed by flow cytometric analyses. THP-1 monocytes are employed in
chemotaxis assay with monocyte chemotactic protein (MCP-1) as a positive control. The
preliminary data show equivalent group IIa sPLA$_2$ association with anti-fas treated and
control cells, and the enzyme remains catalytically active when bound. In line with the
hypothesis, trapped sPLA$_2$ generated soluble molecules that directly or indirectly induced
migration of THP-1 monocytes. However, the similar binding effect observed with
apoptotic or control cells is surprising and experiments are being planned to determine if
the interaction between IIa sPLA$_2$ and apoptotic cells is vimentin dependent.
Introduction

Apoptosis or programmed cell death is an active process which causes typical morphological and biochemical changes including cell shrinkage, nuclear condensation and fragmentation, and membrane alterations such as loss of sialic acid residues from membrane glycoproteins (3). Apoptosis usually affects scattered cells in a tissue and is triggered by a host of stimuli in both physiological (organ and tissue remodeling and the development and maturation of hematopoietic cells) and pathological settings. In contrast, necrosis or passive cell death is a pathological process induced by physical or chemical stimuli (11).

The recognition and removal of apoptotic and necrotic cells by phagocytes (macrophage and dendritic cells) is mediated by changes in expression of membrane associated markers on the dying cell and occurs via distinct and non-competitive mechanisms. Fadok and colleagues identified a phosphatidylserine (PS) receptor on the surface of activated macrophages that selectively allows the PS dependent uptake of apoptotic cells, in addition to other receptors such as CD-36, thrombospondin and lectins (8). Binding of PS to its receptor triggers the release of anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) and inhibits production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), providing a link between the recognition of apoptotic cells and the physiological consequences of their uptake (6).

Monocyte-derived dendritic cells can engulf apoptotic cells and display the degraded peptides on their surface via a major histocompatibility complex (MHC). These
MHC-peptide complexes with the ligands for antigen receptors on T cells which can thereby regulate T cell activity (6). Uptake of necrotic cells results not only in peptide presentation on the cell surface but also, activation of the dendritic cells to express co-stimulatory molecules which are necessary for T cell activation and induction of an inflammatory response. In contrast, apoptotic cells do not trigger co-stimulatory molecules expression and without that, the MHC complexes selectively inactivate the T cell that recognize them leading to tolerance (Fig 1).
Figure 1: Phagocytes including (macrophages and dendritic cells) can engulf and degrade both apoptotic and necrotic cells leading to immune activation (a) or tolerance (b). From Green et al (33).
Infection induces apoptosis

Infection induces necrosis and cytokine production

Immunity

T cell

Tolerance

Phagocyte

MHC I

TCR

Co-stimulator

Necrotic cells

Anti-inflammatory cytokines

Apoptosis

T cell

No co-stimulation

Necrosis
Before phagocytosis can occur, monocytes must be recruited to the apoptotic cells through chemotaxis. Products of the reaction catalyzed by the phospholipases A₂ (PLA₂) family of heterogeneous enzymes whose common feature is to hydrolyze the fatty acid esterified at the sn-2 position of glycerophospholipids, have been shown to induce monocyte chemotaxis either directly or indirectly. Some investigators have implicated a cytosolic calcium-independent PLA₂ (iPLA₂) in the production of these products during apoptosis but a recent report suggests that the secreted group IIa (sPLA₂) binds to surfaces of apoptotic cells. The sPLA₂ are proteins of relatively low molecular mass (14-23 kDa), highly enriched in disulfide bonds, and require millimolar levels of Ca²⁺ for activity (16). One “receptor” for this pool of sPLA₂ is the rod domain of vimentin which is the most widely expressed type III Intermediate Filament (IF) protein.

Intermediate filament proteins associate with the nuclear envelope via the tail domain, and to the plasma membrane via the positively charged head domain. Their proposed cellular functions are to support the transport of specific population of vesicles and to maintain organelle structures (4). Consequently, this interaction between vimentin and group IIa sPLA₂ gained considerable attention following the initial observations that autoantibodies from patients with autoimmune diseases could bind apoptotic cells. Gensler et al hypothesized then that posttranslational modification or relocation of some proteins during apoptosis could be involved in autoimmunity. To test this hypothesis, they injected apoptotic Jurkat T cells into BALB/c mice and obtained monoclonal antibodies against vimentin (15). Since vimentin is a known autoantigen -- antivimentin antibodies are found in the sera of patients with Rheumatoid Arthritis (RA), Myocarditis, and Systemic Lupus
Erythematous (SLE) --these studies collectively provoked the possibility of vimentin externalization during apoptosis (4).

In the early stages of apoptosis, vimentin is partially exposed on the surface of apoptotic T cells via the action of caspases and binds to group IIa sPLA$_2$ in a Ca$^{2+}$-independent manner via its 37 kDa rod domain. Studies with IIa sPLA$_2$ mutants with charge reversal (Lys110Glu/Lys115Glu) showed that specific motifs in the interfacial binding surface are involved in the interaction with vimentin. The IIa sPLA$_2$ inhibitor LY311727, but not heparan inhibited this interaction with vimentin (4). In contrast, heparin but not LY311727 abrogated the binding of IIa sPLA$_2$ to cellular heparan sulfate proteoglycans (HSPG). Importantly, vimentin does not inhibit the catalytic activity of IIa sPLA$_2$.

Interestingly, vimentin externalization in early apoptosis occurs simultaneously with PS exposure and chromatin condensation but both are now known to be independent processes (3). An aminophospholipid translocase flips PS from the outer to the inner leaflet. Data presented by Fadok et al (1) demonstrated that loss of amino-phospholipid translocase activity alone does not result in PS appearance but more likely, results from a calcium-dependent phospholipid flip-flop that is non-specific for head group. Nevertheless, while loss of translocase activity is insufficient to result in PS appearance, loss of its function is probably a necessary event and the appearance of PS on the cell surface can be dissociated from nuclear changes during apoptosis (1). Collectively, PS exposure on the cell surface then may or may not be sufficient for the binding of IIa sPLA$_2$ to apoptotic
human T cells and suggests possibly, a role for binding sites other than anionic phospholipids on membranes of apoptotic cells.

Hence, the presence of multiple recognition mechanisms suggests that removal of apoptotic cells is an important function and we are intrigued by the possibility that the exposure of vimentin on apoptotic cells traps a pool of catalytically active IIa sPLA₂ that then generates the bioactive lipids that induce macrophage chemotaxis to the apoptotic cell. Understanding this process which we propose to be linked in our mechanistic model (Fig 2) will require clarification of the ways in which the distinct mechanisms interact and together, these studies should illustrate a novel function of group IIa sPLA₂ and may suggest new ways to enhance the clearance of apoptotic cells and thereby prevent autoimmune responses.
Figure 2: Proposed Model for Recruitment of Macrophages by “trapped” sPLA₂.
1. A portion of vimentin is exposed on the surface of the apoptotic cell.  
2. This is a receptor for sPLA₂ that is then “trapped” on the surface of the apoptotic cell.  
3. This pool of sPLA₂ is catalytically active and generates bioactive lipids (LPC, LTC₄, PGE₂) that induce macrophage chemotaxis to apoptotic cells (either directly or indirectly).  
4. Macrophages then phagocytize and clear the apoptotic cells, thereby preventing the induction of autoimmune responses against sequestered or exposed antigens.
PLA₂ Family (sPLA₂, iPLA₂, cPLA₂)

Phospholipase A₂ enzymes can be broadly classified into three major classes on the basis of their requirements for calcium, the mechanism of their catalytic action, their molecular weight and also sequence homology (Table 1).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MW</th>
<th>Ca²⁺</th>
<th>Active Site</th>
<th>Expression</th>
<th>Location</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPLA₂ (α,β,γ)</td>
<td>85</td>
<td>uM</td>
<td>GLSGS</td>
<td>constitutive</td>
<td>cytosol</td>
<td>Ca²⁺, PO₄</td>
</tr>
<tr>
<td>iPLA₂</td>
<td>80</td>
<td>None</td>
<td>GxSxG</td>
<td>constitutive</td>
<td>cytosol</td>
<td>oligomerize</td>
</tr>
<tr>
<td>sPLA₂</td>
<td>14-18</td>
<td>mM</td>
<td>His-Asp</td>
<td>inducible</td>
<td>extracellular</td>
<td>expression</td>
</tr>
</tbody>
</table>

Table 1: Forms of Phospholipase A₂ Expressed by Mammalian Cells from Kapur (31)

The secretory enzymes, sPLA₂ groups I, II, II, II, V, X, and XII are all approximately 14 kDa and all possess a catalytic histidine residue that functions as a general base to activate a water molecule for hydrolysis of the ester bond (19). Additionally, a Ca²⁺ ion is required at the active site that functions as a Lewis acid to polarize the ester carbonyl group and facilitate attack by the water molecule. A second Ca²⁺ plays a structural role (Fig.3). The requirement for Ca²⁺ at mM concentrations for activity suggests that these enzymes do not function intracellularly where Ca²⁺ concentrations should normally be low in uM ranges (19).
Also, they possess about 7 disulfide bonds making the enzyme stable but inactive in acidic medium (Fig 4). The interface of sPLA₂s contains a collar of hydrophobic residues that surrounds the opening to the catalytic site slot and two or more cationic arginine and lysine residues. As a result of these basic residues, the interfacial binding site of sPLA₂s has a positive electrostatic potential which could explain the high affinity of these enzymes for anionic interfaces and molecules (28). The difference between the enzymes in this family is the interfacial binding surfaces that modulate their binding affinity for different kinds of phospholipids. As such, the group IIa preferentially binds anionic substrates (PS, PE) whilst the group V shows no preference.
Figure 3: Binding surface and mechanism of catalysis by Phospholipase A₂. X-ray structural model of Phospholipase A₂ showing the surface that interacts with a membrane. The interfacial binding surface contains a rim of positively charged arginine and lysine residues surrounding the catalytic active site in which a substrate lipid is bound (a). After docking on a model lipid membrane, positively charged polar groups at the interfacial binding site bind to negatively charged polar groups at the membrane surface. The binding triggers a conformational change, opening a channel lined with hydrophobic amino acids that leads from the bilayer to the catalytic site. As a phospholipid moves into the channel, an enzyme-bound Ca²⁺ ion binds to the head group positioning the ester bond to be cleaved next to the catalytic site. From Lodish et al (17).
Figure 4: Primary structure of Pancreatic sPLA₂s. From Haas et al (34).
The cytosolic PLA2 enzymes with much larger molecular weights typically 85 kDa, possess a catalytic serine residue that forms a covalent acyl ester intermediate during hydrolysis that is subsequently broken down by a water molecule. cPLA2 does not require any Ca\textsuperscript{2+} for catalytic activity, however micromolar concentrations of Ca\textsuperscript{2+} induce a translocation of the enzyme from the cytoplasm to the cell membrane where it is functionally active by phosphorylation, suggesting that cPLA2-α isoform is involved in receptor-mediated activation of cells. These serine esterases lack disulfide bonds and the three isoforms cPLA2-α, cPLA2-β, and cPLA2-γ are ubiquitously expressed. The cPLA2-γ may be involved in basal phospholipids metabolism of lower organisms such as amoeba (32). The cPLA2 enzymes do not show a preference for phospholipids with a particular head group. However, they do prefer substrates containing arachidonic acid in the sn-2 position.

The iPLA2 are calcium-independent and have molecular weight of 80-88 kDa depending on species and alternative splicing mechanisms, a catalytic serine and no requirement for Ca\textsuperscript{2+}. These serine esterases are found in the cytosolic compartment and do not have any disulfide bonds. Like the cPLA2, iPLA2 also prefers to hydrolyze the sn-2 position of phospholipids, but can also utilize the sn-1 position. Most studies suggest that iPLA2 is involved in basal lipid metabolism.
sPLA$_2$ and Bioactive Lipids

Phospholipids also contribute to cell physiology by being storehouses for signaling molecules in addition to their structural roles. The products of the PLA$_2$ reaction, lysophospholipid and the unesterified fatty acid can be converted into second messengers that then regulate cell physiology. The lysophospholipid product can be involved in the production of Platelet-Activating Factor (PAF), a pro-inflammatory lipid that binds to G-protein-coupled receptors (GPCR) on a variety of cells (35). Lysophospholipids (LPA, LPC) bind receptors and have been shown to have a variety of effects on cell physiology (32).

The Lipoxygenase (LOX) and Cyclooxygenase (COX) enzymes can convert unesterified polyunsaturated fatty acids (PUFA) into oxygenated derivatives (Leukotrienes and Prostaglandins, respectively), (Fig. 5). Like PAF, these molecules can exert their pro-inflammatory activities through binding to GPCR (35). Also, Prostaglandin E$_2$ (PGE$_2$) binds receptors on macrophages and dendritic cells and up-regulates the expression of CCR7, the receptor for chemokines (CCL19 and CCL21) that are essential for recruitment into secondary lymphoid tissues (36). Similarly, Leukotrienes (LTC$_4$) is necessary for optimal chemotactic responses of dendritic cells to CCL19, and LPC is a chemoattractant for monocytes (7).
Figure 5: (A) Specificity of Phospholipases. Each type of phospholipase cleaves one of the susceptible bonds represented by the arrows. The phospholipase A₂ enzyme is specific for the sn-2 position. (B) sPLA₂ and lipid metabolism. sPLA₂ esterase activity on phosphatidylcholine (PC) generates arachidonic acid (AA) from the the sn-2 position and lyso-phosphatidylcholine (LPC). Arachidonic acid is subject to downstream COX-2 and 5-LOX enzymes to yield prostaglandings (PGE₂), thromboxanes (TXA₂) and leukotrienes (LTC₄) which are pro-inflammatory lipid mediators.
sPLA$_2$ Receptors
An important connection between sPLA₂ and signal transduction pathways emerged from the discovery that some mammalian isoforms including sPLA₂ IIa bind with high affinity to receptors first associated with the toxic effect of venom-secreted PLA₂ (20). The first sPLA₂-binding protein is most abundant in brain and named N-type (neuronal type) receptor. The second type of receptor for sPLA₂ IIa was initially found in skeletal muscle and termed the M-type (muscle type) receptor (29). The M-type has been characterized as a member of a family of transmembrane proteins with similar structural organization to the C-type multilectin macrophage mannose receptor. A common function of this protein family is endocytosis and it has been proposed that the physiological role of the M-type sPLA₂ receptor is to internalize and deliver sPLA₂ IIa to specific compartments within the cell where the enzyme might exert its activity (26). Interestingly, the M-type sPLA₂ has been proposed as the main mechanism of action explaining the effects of sPLA₂ in macrophages and mast cells (18). Unlike the mouse sPLA₂ IIa, human sPLA₂ IIa has not been found to bind to the M-type receptor thus making it likely that not all of the physiological receptors for sPLA₂ have been characterized as yet (20).
Hypothesis

If exposure of vimentin on apoptotic cells traps a pool of catalytically active sPLA$_2$, then the bioactive lipids generated can induce monocyte/macrophase chemotaxis.
Materials and Methods

Materials: Jurkat clone E6-1 and THP-1 cells were obtained from ATCC (Manassas, VA). Recombinant Human CCL2/MCP-1 was obtained from R&D Systems. Vybrant Apoptosis Assay Kit used for flow cytometric analysis was obtained from Molecular Probes. Para-bromphenacylbromide (BPB) was purchased from Sigma-Aldrich.

Cell Culture: Jurkat cells were maintained in Modified RPMI-1640 medium (ATCC) supplemented with 100ug/mL penicillin and 100ug/mL streptomycin, at 7.5% CO2, 100% humidity and 37°C. THP-1 monocytes were cultured at 10^6 cells/mL in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 100ug/mL penicillin and 100ug/mL streptomycin, and also 2 mM L-glutamine at 7.5% CO2, 100% humidity and 37°C. Cell viability was determined by Trypan Blue dye exclusion with a hemacytometer. Cell viability exceeded 94% in all experiments.

Group IIa sPLA2 Plasmid Transfection: Mammalian expression vector encoding human group IIa sPLA2 was obtained from Dr. Mike Gelb (University of Washington). Human Embryonic Kidney (HEK) cells were transiently transfected with the IIa plasmid to over-express the enzyme by utilizing the FuGENE 6 Transfection protocol (Roche). After 24 hours of incubation at 7.5% CO2, 100% humidity and 37°C, harvested cultured supernatant was used as source of group IIa sPLA2.
*Induction of Apoptosis:* Fas-mediated apoptosis was induced by adding 20 ng/mL CD95 (AntiFas, BD Pharrningen) to 1x10⁶ cells/mL cultured Jurkat cells. Cells were then incubated in 7.5% CO₂, 100% humidity at 37°C for desired time point (4,12,&18 hrs), and apoptosis was monitored through annexin-V binding and PI staining followed by flow cytometric analysis.

*Acid Extraction of Group Ila Secreted PLA₂:* After apoptotic induction, Jurkat cells (10⁶ cells/mL) were resuspended in PBS. Twenty-five micro-liters (25 uL) of group Ila sPLA₂ was added on ice to equal amount of cells for both treatment and control samples. Samples were then incubated on ice for 30 mins and then centrifuged at 5000 x g for 20 mins. Cell pellets were saved and washed in 3 mL PBS. Finally, cells were re-suspended in 750 uL of 0.36N sulfuric acid and left overnight at 4°C. After 20 mins of centrifugation the next morning, the supernatant containing soluble proteins were removed for assay and stored at 4°C. Protein concentration was then determined using the Lowry BSA method. The acid extraction protocol denatures all proteins except the group Ila enzyme which remains stable but inactive in acidic medium.

*Activity Assay:* Group Ila sPLA₂ activity was measured using radiolabeled *E. coli* cells as a substrate. [³H] Oleic acid-labeled *E. coli* membranes were prepared by standard procedures (16). Equivalent amounts of acid extracted enzyme (0.4 or 1.0 ug) or cultured supernatants (50 uL) were incubated with ~50,000 cpm of *E. coli* cells in sPLA₂ assay buffer (25 mM Tris-base, pH 9.0, 10 mM calcium chloride, 5 mg/mL fatty acid-free
bovine serum albumin (FAF-BSA)) in a final volume of 500 uL for 20 mins at 37°C. The reaction was stopped by the addition of 250 uL 2N HCl and liberated fatty acids were extracted by adding 250 uL of 20 mg/mL FAF-BSA. After 20 mins incubation on ice, the reaction mixture was centrifuged at 7000 x g and 500 uL of supernatant was used for scintillation counting. The percent hydrolysis is determined by the percent of input counts released into supernatants.

**Flow Cytometric Analysis of Cell Size and Viability:** Cells were pelleted, washed 2X in PBS and resuspended in 1X Binding Buffer (1XBB: 0.01 M Hepes/NaOH (pH 7.4) 0.14 M NaCl, 2.5 mM CaCl₂) at a concentration of 10⁶ cells/mL. Five micro-liters (5 uL) each of 10 ug/mL Propidium Iodide (PI, Sigma) and Annexin-FITC were added to 100 uL of cells in 1X Binding Buffer, and incubated at room temperature for 15 mins. Samples were then transferred to ice, volume brought to 0.5 mL with 1X Binding Buffer and analyzed on a BD FACSCAN flow cytometer. The distribution of cell size and density is represented by forward scatter versus side scatter dot plots respectively and gates were set based on control samples to distinguish normal and shrunken cells. Annexin-FITC positive cells were determined as described in the Vybrant Apoptosis Assay kit by setting quadrants to separate viable cells from PI permeant cells, and non-apoptotic cells from those staining highly for the Annexin probe. Percent quadrant cell population was determined by statistical analysis of gated region using CellQuest and WinMDI 2.8 softwares.
Transmigration Assay and Production of Candidate Chemoattractant Cell Supernatants:
After induction of apoptosis, cells were resuspended in 100 uL of PBS (9.4 uL of 1M CaCl₂/3 mL PBS) per 100,000 cells on ice. Twenty-five micro-liters (25 uL) of group IIa sPLA₂ was added and incubated for 30 mins on ice, followed by 20 mins in a 37°C water bath with the shaker at moderate speed. Cells were then centrifuged at 10,000 x g for 5 mins and supernatants used in chemotaxis assay. Transmigration assays were performed using 8um pore size Transwell plates (Fisher). One hundred micro-liters (100 uL) of chemoattractant or supernatants from treated or non-treated cells described above were added to 500 uL of 2% FCS medium and placed into the lower chamber. Also, 10⁵ THP-1 monocytes were added to the upper chamber and the assay was incubated for 2 hrs at 37°C. Migrated and non-migrated monocytes were determined utilizing a BD Coulter Counter and transmigration was assessed as percent of total cells recovered in lower chamber.

Para-bromophenacylbromide (BPB) Inactivation of IIa sPLA₂: Group IIa sPLA₂ was dialyzed with PBS for 16 hours using 3.5K dialysis tubing. Four hundred microliters of dialyzed enzyme was incubated with 10 uL BPB (200 mM in Methanol) on ice for 30 minutes and tested for catalytic activity.

Statistical Analysis: Data shown are mean and standard deviation of a single representative experiment done in triplicate. Statistical significance was determined by using the student’s t-test with p< 0.05.
Results

* Cultured supernatant containing group IIa sPLA$_2$ shows phospholipase activity: The source of IIa sPLA$_2$ for these studies was a cultured supernatant from transiently transfected HEK cells in serum free medium as described in methods. Quantification of IIa sPLA$_2$ activity shows a remarkable seventeen fold hydrolysis compared to control (Fig 6).
Figure 6: Cultured supernatant containing group IIa sPLA₂ shows phospholipase activity. HEK cells were transiently transfected with group IIa sPLA₂ vector plasmids or control in serum-free DME medium. Cultured supernatants were harvested after 24 hrs and activity quantified as percent hydrolysis by scintillation counting of released radiolabeled oleic acid.
Forward scatter (FSC), Side scatter (SSC), AnnexinV-FITC, and Propidium Iodide (PI) staining indicates cells are undergoing apoptosis induced by anti-fas: To induce apoptosis, Jurkat cells (10⁶/mL) were added to 20 ng/mL anti-fas and incubated for desired time-point. Cells were then stained and analyzed by flow cytometry. The forward-angle light scatter (FSC) relates to the cell diameter and the side-angle light scatter reflects the density of inner cellular structures (10). During the initial stages of apoptosis, the cell shrinks while the membrane remains intact. During necrosis, cell swelling occurs as a result of the early failure of the membrane integrity. As a consequence of these cellular changes, the FSC decreases during the initial phases of apoptosis while SSC increases or remains unchanged (6). After some hours, decrease in both FSC and SSC becomes evident (Fig 7). Taking into consideration that trypan blue positive cells are rather dead cells that have passed the early apoptotic stage, this observation nevertheless correlated with the percent cell viability calculations as assessed by trypan blue exclusion for the 24 hr time course with 20 ng/mL anti-fas treatment (Fig 8). At 0 hr, trypan blue dye exclusion showed cells were about 95% viable. Control cells fluctuated in that range to 92% viability at 30 hrs. In contrast, the anti-fas treated cells gradually decreased to 41.3% viability at 30 hrs.
Figure 7: Transition of Cell Population from Viable to Apoptotic and/or Necrotic by Forward and Side Scatter Analysis. Flow cytometry for 24hr time course of 20 ng/mL AntiFas treated cells (fas-bottom row) and non-treated cells (ctr-top row). The distribution of cell size is represented by forward scatter (vital cells in R1) and side scatter (apoptotic/necrotic cells in R2) dot plots. The percentage of control R2 cell population was 4.9, 11.8, 10.0, and 11.1 for the 4, 8, 10, and 12 hour time-point respectively. The percentage of anti-fas treated R2 cell population was 10.4, 19.1, 19.3, and 17.5 for the 4, 8, 10, and 12 hour time-point respectively. For the second experimental batch, the percentage of control R2 cell population was 3.7, 6.2, 6.5, and 10.0 for the 14, 16, 20, and 24 hour time-point respectively. For the second experimental batch, the percentage of anti-fas treated R2 cell population was 8.5, 22.0, 24.2, and 31.1 for the 14, 16, 20, and 24 hour time-point respectively.
Figure 8: Effect of 20 ng/mL Anti-Fas treatment on Jurkat cells Viability.
Jurkat cells (1x10⁶/mL) were induced to undergo apoptosis by addition of 20 ng/mL AntiFas. Cell viability at each time point was determined by Trypan Blue dye exclusion method.
In Figures 9, 10, and 11, we used annexinV-FITC staining to discriminate between apoptotic and non-apoptotic cells for the different time-points. In a Ca\textsuperscript{2+}-dependent manner, annexinV-FITC binds preferentially to anionic lipid species such as phosphatidylserine (PS) which is normally absent in the outer leaflet of the plasma membrane, and shows minimal binding to other phospholipids species such as phosphatidylcholine (PC) which is constitutively present in the outer leaflet of the plasma membrane. When apoptosis occurs, PS is translocated to the outer leaflet of the membrane and this is observed in the early phase of apoptosis.

Consequently, the annexinV-FITC binding assay was combined with propidium iodide (PI), a vital dye exclusion test in order to collect information about membrane integrity which fails early during necrosis whilst remaining intact during the initial phase of apoptosis (9). Compared to control, the 12 hr time point sample showed about a 3.4 fold in early apoptotic (LR) cell population (13% vs 4%). We concentrated on the 12 hr time point because our data consistently showed a higher ratio of early apoptotic cell population (annexin-V (+), PI (-)) compared to control cells from other time points (Figs 9,10,11). Moreover, the 12 hour data showed the least ratio of late apoptotic or dead cells (UR:19%) per early apoptotic cell population (LR:13%). Additionally, it is noteworthy that the minimal percentage of non-apoptotic dead cells in UL (1.14%) indicates that the dead cells seen in UR (19.02%) were cells that transitioned through the apoptotic process since they showed binding to annexinV-FITC. Quantitatively, the data show a decline in viable cell population in anti-fas treated group from 78% (4hr) to 67% (12hr) to 56% (18hr). In
contrast, viable cell population in control group remained unchanged at 91% (4hr), 91% (12hr), and 92% (18hr).
Figure 9: Propidium Iodide (PI) and Annexin-V FITC staining differentiates cell population into viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL). UL: PI (+), AnnexinV-FITC (-); UR: PI (+), AnnexinV-FITC (+) LL: PI (-), Annexin V-FITC (-); LR: PI (-), Annexin V-FITC (+). Data represents 4 hr time-point.
### 4 hr Control

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### Annexin FITC vs PI(FL3-H)

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Figure 10: Propidium Iodide (PI) and Annexin-V FITC staining differentiates cell population into viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL). UL : PI (+), AnnexinV-FITC (-); UR : PI (+), AnnexinV-FITC (+); LL : PI (-), Annexin V-FITC (-); LR : PI (-), Annexin V-FITC (+). Data represents 12 hr time-point.
Flow Cytometry

### 12 hr Control

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### Annexin FITC vs PI (FL3-H)

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### Annexin FITC vs PI (FL3-H)

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Figure 11: Propidium Iodide (PI) and Annexin-V FITC staining differentiates cell population into viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic (UL). UL: PI (+), AnnexinV-FITC (-); UR: PI (+), AnnexinV-FITC (+)
LL: PI (-), Annexin V-FITC (-); LR: PI (-), Annexin V-FITC (+). Data represents 18 hr time-point.
Group IIa sPLA₂ associates with apoptotic cells and bound enzyme remains catalytically active: Having established conditions to generate apoptotic cells, we next determined the association of group IIa sPLA₂ with Jurkat cells. Surprisingly, our results in Fig 12 are not in accord with other findings that anti-fas stimulation results in an increase binding of IIa sPLA₂ to apoptotic T cells (2). In the first part of this assay, we allowed sufficient interaction between group IIa enzyme exposed to equal populations of anti-fas treated or control cells without activating its phospholipase activity by keeping samples on ice, without any source of calcium in the preparation. The 12 hr flow cytometry data (Fig 10) indicated a 3-fold increase (13% vs 4%) in the number of apoptotic cells in antifas-treated compared to control populations. Additionally, total annexin-FITC positive cell population showed a time-dependent increase from 21%, to 32%, to 43% for the 4 hr, 12 hr, and 18 hr time-points respectively. On the contrary, group IIa sPLA₂- cell bound extracts from all three time-points showed similar activity between anti-fas treated and control groups that was not statistically different. We expected the anti-fas treated cells to bind more to the enzyme based on the observation by Boilard et al that exposed rod domain of vimentin was a docking site for group IIa enzyme (4). However, the use of primary human cells in Boilard’s experiment stands in contrast to our Jurkat cell line. Yet, the results provoke the idea that group IIa interaction with apoptotic cells may be vimentin-independent since viable cells do not expose the intermediate filament protein.
Figure 12: Equivalent group IIa sPLA₂ association with control and anti-fas treated cells and bound enzyme remains catalytically active. Control and anti-fas-treated Jurkat cells harvested at 4, 12, and 18 hrs were added to 25 uL of cultured IIa sPLA₂ and processed for acid extraction as described in methods. Following protein concentration determination by the Lowry BSA method, 1.0 or 0.4 ug of soluble protein was then used to quantify the activity of the enzyme. (C+) = control+IIa, (C-) = control, (F-) = anti-fas, and (F+) = anti-fas+IIa.
Group IIa sPLA₂ generates soluble molecules that induce THP-1 Monocyte chemotaxis directly or indirectly to apoptotic cells: Monocyte Chemotactic Protein-1 (MCP-1) is a potent βchemokine that recruits monocytes and promotes cell adhesion and transmigration across an endothelia layer into tissues via a PKC signaling pathway (13). In Fig 13, we used the MCP-1 as a positive control to compare potency of the group IIa sPLA₂ products as chemoattractants. Supernatants of anti-fas (F+) and expectedly, of control (C+) cells treated with IIa sPLA₂ contained a chemotactic factor which was equally as effective as MCP-1 in recruiting THP-1 monocytes. On the other hand, supernatant of anti-fas treated cells without group IIa sPLA₂ treatment (F-) minimally affected transmigration of monocytes and was about 50% less compared to MCP-1. Also, group IIa sPLA₂ (IIa only) had no effect on chemotaxis.
Figure 13: Group IIa sPLA₂ generates soluble bioactive molecules which induce THP-1 monocyte chemotaxis directly or indirectly. Control and antifas-treated Jurkat cells were harvested at 12hrs and processed to obtain cell supernatants for chemotaxis as described in methods. Six hundred microliters of 10 nM MCP-1 prepared in 2% FCS RPMI 1640 medium, and 100 uL of possible chemoattractant for samples IIa only, F-, F+, and C+ was added to 500 uL of 2% FCS RPMI 1640 medium and placed in lower chamber of transwells. All samples received 100,000 THP-1 monocytes in upper chamber. (+)= with IIa, (-)= without IIa.
BPB treated sPLA₂ is no longer active: Group IIa sPLA₂ can generate chemotactic lipids directly through catalysis or indirectly through binding to receptors followed by activation of other phospholipases. To determine whether group IIa sPLA₂ catalytic activity is necessary to augment monocyte chemotaxis, the enzyme was treated with para-bromophenacylbromide (BPB), an irreversible inhibitor of IIa sPLA₂ that does not affect other forms of PLA₂ (34). The activity assay of BPB-treated sPLA₂ showed the enzyme was inhibited (Fig 14). Consequently, the next step in our experimental design is to test the effect of the catalytically inhibited group IIa sPLA₂ on monocyte chemotaxis.
Figure 14: BPB treated sPLA2 is no longer active. Dialyzed group IIa supernatants (400 uL) is treated with 10 uL of BPB (200 mM para-bromophenacylbromide dissolved in methanol). After 30 minutes incubation on ice, inhibitor treated (BPB+IIa) and active IIa enzymes were tested for catalytic activity in a radiolabeled assay as described in methods.
Discussion

In this work, we have studied the involvement of secretory type IIa phospholipase in the signaling cascade that leads to the recruitment of monocytes to apoptotic cells. Induction of apoptosis in Jurkat cells with 20 ng/mL antifas and subsequent analyses with trypan blue dye showed the gradual decline of treated cells from 95% to about 41% viability at 30 hours post-treatment. This observation correlated with the forward by side scatter dot plot analysis (Fig 7), and we were convinced that cells were undergoing apoptosis. To differentiate between early and late apoptotic, as well as necrotic cells populations, we utilized annexinV-FITC and propidium iodide staining technique. Consequently, we identified a more than three-fold increase in the ratio of early apoptotic cells in treatment group compared to control at the 12 hour time point (Fig 10).

Using transiently transfected HEK cells to overexpress the IIa sPLA$_2$, we determined a robust activity (seventeen fold hydrolysis) of the cultured supernatant with [³H]-oleate $E. coli$ as a substrate. Upon addition of group IIa sPLA$_2$ to treated and control cells, the IIa sPLA$_2$ activity assays showed that the enzyme associates equivalently with both cell populations for the different time points (4,12,&18 hrs). This was unexpected because the flow data showed a time-dependent increase in total annexin-FITC binding to treated but not control cells. Since the annexin-FITC probe is specific for phosphatidylserine binding, we were unequivocally convinced that treated cells were transitioning through stages of apoptosis and the early part of that process results in the exposure of vimentin to the cell surface. Further analysis of chemotactic activity using supernatants again showed that the IIa sPLA$_2$ generated similar bioactive chemoattractant
molecules when bound to control or treated cells. On the other hand, neither supernatants from non-IIa sPLA2 treated apoptotic cells nor cultured IIa sPLA2 alone could induce any significant level of THP-1 monocyte migration compared to the potent chemokine, MCP-1. Upon treatment with para-bromophenacyl bromide, the catalytic activity of sPLA2 IIa was completely abolished.

Collectively, our results challenges previous data about the involvement of vimentin in group IIa sPLA2 interactions with apoptotic cells. Boilard et al had reported that vimentin, an intermediate filament and part of the cell’s cytoskeleton, is exposed to the extra-cellular surface by caspase activity early in apoptosis to which the group IIa sPLA2 then binds. Our observation that the exogeneous IIa sPLA2 enzyme bound equivalently to control and treated (apoptotic) cells, even though the early apoptotic cell population in treated sample was three-fold more, suggests that the enzyme may be binding through other means. Also, it is worth mentioning that Boilard’s experiments involved human primary cells whilst we used a cultured cell line.

Data presented by Murakami et al (18) and others suggest an alternative mechanism for the IIa sPLA2 association with cells through Heparan Sulfate Proteoglycans (HSPG). The cell surface HSPGs fall into two families of molecules that differ in their core protein domain structures, the integral syndecans and GPI-anchored glypican (23). The syndecans have core proteins with a transmembrane and cytoplasmic domain and they possess heparan sulfate chains near the N-terminus distal to the plasma membrane (27). By contrast, the glypicans lack a membrane spanning domain, are anchored to the external surface of the plasma membrane via glycosylphosphatidylinositol (GPI) and have three
heparan sulfate chains near the C-terminus which are close to the plasma membrane (30). These sulfated glycosaminoglycans contain fixed anionic sites that confer an overall electronegative charge to their structures depending on the cell type (36). Practically, the group IIa sPLA₂ (with clusters of positively charged lysines and arginines, Fig 4) binds to HSPG via the anionic glycosaminoglycan moiety (21).

Structurally, GPI-anchored proteins generally occur in plasma membrane micro-domains like caveolae and lipid rafts that contain intermediate filament proteins including vimentin (14,22). Previously, glypican 1 has been shown to recruit IIa sPLA₂ into caveolae-like compartments of cells. Dynamic changes occur in the subcellular distribution of glypican which moves to the nucleus and punctate caveolae-like domain, depending upon the activation state of the cell (25). Caveolae form a unique endocytic compartment at the surface of most cells, are capable of importing molecules and delivering them to specific locations within the cell as well as compartmentalizing a variety of signaling activities (24). By means of this caveolae-mediated endocytic event, bound sPLA₂ IIa can be translocated to the perinuclear compartments in proximity to COX-2 (22). Also, since caveolae are a site of Ca²⁺ storage and entry into the cell, sPLA₂ present inside caveolae signalsomes may retain enzyme activity even after internalization and translocation to the perinuclear domain. Although it is not known whether vimentin and HSPGs co-localize in these membrane micro-domains, these proteins may play a role in the sequestering and distribution of IIa sPLA₂ in apoptotic cells.

Additionally, the binding of group IIa sPLA₂ to heparan sulfate chains of glypican facilitates its presentation to the putative sPLA₂ IIa receptor, which transduces signals
leading to increased COX-2 expression (18). In accord with this hypothesis, COX-2 induction by sPLA₂ IIa in rat serosal mast cells appears to involve a receptor-mediator pathway (18,23). This type of receptor system utilizing both HSPG and a signal transducing receptor subunit has been shown for the FGF receptor system in which the ligand, its tyrosine kinase receptor and HSPG form a stable complex on the cell surface (18) On the other hand, in hematopoetic cells such as platelets and mast cells, sPLA₂ IIa is stored in secretory granules rather than binding to cell surface HSPG (18). Hence, the implication is that subcellular distribution of sPLA₂ IIa varies according to cell type and HSPG molecular species.

Furthermore, results from the chemotaxis assay suggest that the group IIa enzyme generates soluble bioactive molecules that induce monocyte chemotaxis directly or indirectly. Since the mechanism by which group IIa sPLA₂ specifically generates chemoattractant molecules from cellular phospholipids after interacting with HSPG is not clear, it is possible instead that the cytosolic calcium-independent PLA₂ (iPLA₂-VIA), can be partly responsible for generating the chemoattractant. Evidence presented by Lauber et al (7) that apoptotic cells generate chemotactic factors due to caspase-3 mediated activation of iPLA₂ that stimulate the attraction of monocytes support this idea. However, iPLA₂ activity was not determined and the evidence remains inconclusive. Hence, to determine if catalytic activity of group IIa sPLA₂ is required for monocyte recruitment, we used the para-bromophenacyl bromide (BPB) to inhibit the enzyme. BPB irreversibly inhibits IIa sPLA₂ by alkylating the imidazole side chain on histidine-53 at the active site (34). The results show a complete abolishment of activity (fig 15). Consequently, the next step in our
experimental design is to test the effect of the catalytically inhibited group IIa sPLA₂ on monocyte chemotaxis.

Additionally, Atsumi et al (14) provided convincing evidence that the perturbed membrane asymmetry of cells undergoing apoptosis enables the access of sPLA₂. This validates our speculation that group IIa sPLA₂ might contribute to the generation of LPC via the hydrolysis of PC from the outer leaflet of apoptotic cells (Fig 13). Moreover, our model Jurkat cells are known to contain about 40% PC (31) in the outer leaflet of their plasma membrane and we are almost certain that in future experiments, LPC would be identified as the major chemo-attractant in the supernatants. In this experiment, we provide evidence to support the observation that group IIa sPLA₂ might also contribute to the generation of LPC via hydrolysis of phosphatidylcholine (PC) from the outer leaflet of apoptotic Jurkat cells. This would confirm the results in Lauber et al which concluded that only LPC was able to attract THP-1 cells and maximal chemotactic activity of LPC was observed at a concentration of 20 to 30 uM, whereas higher concentrations reduced the migration activity of THP-1 cells (7).

In summary, we have provided experimental evidence from this study that recruitment of macrophage to sPLA₂ -bound control and apoptotic cells is an indiscriminatory process. This is an important step before the timely removal of apoptotic cells to prevent the release of toxic and immunogenic intracellular contents into the surrounding tissue (37). The mechanism involved in recognition and removal has been studied extensively over the past decade and a number of receptors that mediate the process have been identified (Fig 15). Based on previous studies (1,8,9) and data from this
experiment, we also conclude that PS exposure is a separate and unrelated event to IIa sPLA₂ binding to apoptotic cells. Hence, once the macrophages are indiscriminately recruited to the source of the chemotactic molecules, then the decision is made as to which cells are to be phagocytosed based on the diverse and many apoptotic cell recognition mechanisms such as the PS receptor, mannose receptor or the lectin receptors expressed on the macrophage (Fig 15).

Figure 15: The phagocyte recognition array in the mammalian clearance of apoptotic cells. From Fadok et al (6).
Finally, in our future studies, we will establish vimentin-negative Jurkat cells using siRNA techniques and determine whether vimentin directly impacts on group IIa sPLA_2 hydrolysis of cellular phospholipids as well as the ability to generate chemotactic factors. Also, based on our revised hypothesis (Fig 16) that group IIa sPLA_2 binds to cells indiscriminately by interacting with glypican 1 or other HSPGs, we will elucidate the effect of heparin, a competitive inhibitor of group IIa sPLA_2 on enzyme binding and ability to generate chemotactic factors.
Secreted phospholipase A$_2$ is captured by the heparan sulfate chains of a HSPG and thus accumulates on the plasma membrane from which the enzyme liberates arachidonic acid. Downstream COX and LOX enzymes utilize the arachidonic acid to generate several pro-inflammatory lipid mediators, some of which are potential chemoattractants that induces monocyte chemotaxis to the site of chemoattractant. Once macrophages reach the source of chemotactic molecules, the decision is made as to which cells are to be phagocytosed based on the diverse and many apoptotic cell recognition mechanisms.
Literature Cited
Literature Cited


Figure 17: Comparison of cell viability by trypan blue exclusion method versus forward scatter by side scatter analysis using flow cytometry.
Kwasi KariKari was born on April 26, 1970 in Accra, Ghana. He immigrated to the United States as a teenager and graduated from Erasmus Hall High, Brooklyn, NY in 1989. He became a naturalized U.S. Citizen in 1996 while living in Virginia and received a Bachelor of Science in Chemistry from George Mason University, Fairfax, VA in 1998. Subsequently, he obtained a Collegiate Professional Teachers License from the State of Virginia and taught chemistry and physics in Prince William County Schools before enrolling in graduate school at Virginia Commonwealth University (VCU) in 2004. As a graduate student, he was inducted into the Phi Kappa Phi Honor Society and also recognized with an award for “Excellence in Biochemistry” by the Biochemistry Department. Additionally, he was initially awarded a “Pre-Medical Basic Health Science Certificate” in Biochemistry and then joined the laboratory of Dr. Suzanne Barbour in May 2005. He completed his research thesis for the Masters of Science in Biochemistry degree in the summer of 2006 and continued on to Michigan State University College of Human Medicine for medical studies.