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Neutrophil effector responses are suppressed by secretory phospholipase A_2 modified HDL

Sanja Curcic, MSc, **Michael Holzer, PhD**, **Robert Frei, MSc**, **Lisa Pasterk, MSc**, **Rudolf Schicho, PhD**, **Akos Heinemann, MD**, and **Gunther Marsche, PhD**
Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Austria

Abstract

Secretory phospholipase A_2 ($sPLA_2$) generates bioactive lysophospholipids implicated in acute and chronic inflammation, but the pathophysiologic role of $sPLA_2$ is poorly understood. Given that high-density lipoprotein (HDL) is the major substrate for $sPLA_2$ in plasma, we investigated the effects of $sPLA_2$-mediated modification of HDL ($sPLA_2$-HDL) on neutrophil function, an essential arm of the innate immune response and atherosclerosis.

Treatment of neutrophils with $sPLA_2$-HDL rapidly prevented agonist induced neutrophil activation, including shape change, neutrophil extracellular trap formation, CD11b activation, adhesion under flow and migration of neutrophils. The cholesterol-mobilizing activity of $sPLA_2$-HDL was markedly increased when compared to native HDL, promoting a significant reduction of cholesterol-rich signaling microdomains integral to cellular signaling pathways. Moreover, $sPLA_2$-HDL effectively suppressed agonist-induced rise in intracellular Ca^{2+} levels. Native HDL showed no significant effects and removing lysophospholipids from $sPLA_2$-HDL abolished all anti-inflammatory activities.

Overall, our studies suggest that the increased cholesterol-mobilizing activity of $sPLA_2$-HDL and suppression of rise in intracellular Ca^{2+} levels are likely mechanisms that counteract agonist induced-activation of neutrophils. These counterintuitive findings imply that neutrophil trafficking and effector responses are altered by $sPLA_2$-HDL during inflammatory conditions.

Keywords

HDL; secretory phospholipase A_2; lysophospholipid; neutrophil

1. Introduction

Concentrations of some secretory phospholipase A_2 ($sPLA_2$) subspecies can increase hundred-folds in plasma during acute inflammation [1]. More than one third of the mammalian PLA_2 enzymes belong to the $sPLA_2$ family, which consists of low molecular mass, Ca^{2+} dependent enzymes with a His–Asp catalytic dyad [2]. $sPLA_2$ enzymes,

Corresponding author: Gunther Marsche, PhD, Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Austria Universitätsplatz 4, 8010 Graz Tel.: +43 316 380 4513 Fax: +43 316 380 9645 gunther.marsche@medunigraz.at.

Conflict of Interest
None declared.

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including groups IIA, III, V and X, catalyze the hydrolysis of glycerophospholipids to lysophospholipids and free fatty acids at the sn-2 position. Considerable evidence implicates a potential role for groups IIA and V $sPLA_2$ in cardiovascular disease and in patients with sepsis and septic shock, and increased plasma levels of $sPLA_2$ have been observed in acute inflammatory conditions such as sepsis as well as in chronic inflammatory diseases [3-5].

Circulating levels of $sPLA_2$-IIA and increased $sPLA_2$ activities associate with cardiovascular risk in patients with sepsis and coronary artery disease [6]. Pathologic studies demonstrated the presence of $sPLA_2$ isoforms in atherosclerotic lesions and myocardial regions that have sustained ischemic injury [7]. Unexpectedly, inhibition of $sPLA_2$ in patients with acute coronary syndromes resulted in an excess rate of myocardial infarction and stroke [8] and showed no overall survival benefit in patients with severe sepsis [9]. These studies clearly suggest that the pathophysiologic roles of $sPLA_2$ are poorly understood. Most $sPLA_2$ isoforms have the unique capacity to bind and hydrolyze lipoproteins in distinct and specific manners, thereby producing various lipid mediators and modifying the lipid particles. In the acute phase of the inflammatory response $sPLA_2$ is mostly associated with high-density lipoproteins (HDL) [10], which are the major source of phospholipids in plasma [11] and are important players in the innate and adaptive immunity and cardiovascular disease [12-14]. $sPLA_2$ overexpression in apoA-I transgenic mice results in a dramatic shift of the HDL particle size toward smaller particles and virtually all plasma $sPLA_2$ is found in the HDL fraction [15]. Surprisingly, direct effects of $sPLA_2$ modified HDL on innate immune cells have not been assessed so far. Neutrophils are classically considered as short-lived phagocytes with the ability to release vast amounts of proteolytic enzymes and reactive oxygen species, both of which are important during bacterial infections. Thus, both exuberant and/or diminished responses by the innate immune system may worsen clinical outcomes in severely ill patients with acute and chronic liver diseases [16], airway diseases [17] or acute coronary syndrome [18]. Neutrophils play key roles in sepsis [19] and atherosclerosis [20] and were shown to aggravate endothelial dysfunction, to activate macrophages, promote foam cell formation and to contribute to weakening of the fibrous cap [20]. We therefore thought to assess whether $sPLA_2$-treated HDL modulates neutrophil function, an essential arm of the innate immune response.

2. Materials and Methods

2.1. Materials

All laboratory reagents were from Sigma (Vienna, Austria), unless otherwise specified. Interleukin 8 (IL-8) and intracellular adhesion molecule-1 (ICAM-1) were purchased from Peprotech (London, UK). Free fatty acids (FFA), lysophosphatidylcholines (LPC) and lysophosphatydilserine 16:0 were from Avanti Polar Lipids (Birmingham, AL, USA). FFAs were dissolved in ethanol and LPCs in chloroform/methanol and stored at −20°C under argon atmosphere. Required amounts of LPC were dried under a stream of nitrogen and re-dissolved in PBS (pH 7.4). CellFix and FACS-Flow were from BD Bioscience (Vienna, Austria). Secretory phospholipase A_2 ($sPLA_2$) type III from bee venom and human recombinant secretory phospholipase A_2 type V were purchased from Cayman Europe (Tallin, Estonia). Varespladib was purchased from Eubio (Vienna, Austria). Anti-human

CD11b-FITC and CD16-PE antibodies were obtained from Biozym-Biotech (Vienna, Austria). Fluo-3-AM and SYTOX green were from Life Technologies (Vienna, Austria). Fixative solution was prepared by adding 9 ml distilled water and 30 ml FACS-Flow to 1 ml CellFix.

2.2. Isolation of HDL

HDL was isolated by density gradient ultracentrifugation as described [21-23]. Plasma density was adjusted with potassium bromide (Sigma, Vienna, Austria) to 1.24 g/ml and a two-step density gradient was generated in centrifuge tubes (16 × 76 mm, Beckman) by layering the density-adjusted plasma (1.24 g/ml) underneath a NaCl-density solution (1.006 g/ml). Tubes were sealed and centrifuged at 90.000 rpm for 4 hours in a 90Ti fixed angle rotor (Beckman Instruments, Krefeld, Germany). After centrifugation, the HDL-containing band was collected and desalted via PD10 columns (GE Healthcare, Vienna, Austria) and immediately used for experiments.

2.3. sPLA$_2$ treatment of HDL

HDL was incubated in the presence of 200 ng/ml sPLA$_2$ type III from bee venom or 400 ng/ml human recombinant type V sPLA$_2$ in PBS containing Ca^{2+} and Mg^{2+}, overnight at 37°C, in order to hydrolyse HDL-associated phospholipids.

2.4. Lysophosphatidylcholine (LPC), free-fatty acid (FFA) and lysophosphatidylserine enrichment/depletion of HDL

In order to generate LPC-, FFA-, or lysophosphatidylserine-enriched HDL, 1 mg/ml HDL was incubated with 0.6 mmol/L 16:0, 18:1, 18:2 or 20:4 FFA, with 0.6 mmol/L 16:0, 18:1, 18:2 or 20:4 LPC or with 0.6 mmol/L lysophosphatidylserine 16:0 for 2 hours at 37°C. Unbound LPCs and FFAs were removed by gel filtration and HDL-associated LPC and FFA contents were determined as described below. In some experiments, sPLA$_2$-treated HDL was incubated in the presence or absence of 50 mg/ml albumin (1 h, 37°C) and sPLA$_2$-HDL was re-isolated by density gradient ultracentrifugation in order to remove LPCs and FFAs. LPC and FFA contents were assessed as described below.

2.5. Determination of FFAs and LPCs

HDL-associated lipids were extracted according to Bligh and Dyer [24] and dried under a stream of nitrogen. Dried lipid extracts were resuspended in 200 µl $CHCl_3$/MeOH (1:1, v/v) containing 1 pmol/µl of LPC 17:1 serving as internal standard. Chromatographic separation of lipids was performed by an Accela HPLC (Thermo Scientific) on a Thermo Hypersil GOLD C18, 100 × 1 mm, 1.9 µm column. Solvent A was a water solution of 1% ammonium acetate (v/v) and 0.1% formic acid (v/v) and solvent B was acetonitrile/2-propanol (5:2, v/v) supplemented with 1% ammonium acetate (v/v) and 0.1% formic acid (v/v), respectively. The gradient was run from 35% to 70% B for 4 min, then to 100% B in additional 16 min with subsequent hold at 100% for 10 min. The flow rate was 250 µl/min. Phospholipid species were determined by a TSQ Quantum ultra (Thermo Scientific) triple quadrupole instrument in positive ESI mode. The spray voltage was set to 4500 V and capillary voltage to 35 V. The LPC species were detected in a precursor ion scan on m/z 184 at 34 eV. Peak

areas were calculated by QuanBrowser for all lipid species and the calculated peak areas for each species were expressed as a % of internal standard. Results are expressed as nmol/mg HDL protein. FFA content was assessed using a non-esterified fatty acids kit (Diasys, Holzheim, Germany). In some experiments the Azwell LPC Assay Kit (Hölzl Diagnostika) was used to assess the LPC content of $sPLA_2$-HDL.

2.6. Neutrophil isolation

Human polymorphonuclear leukocytes (PMNL) were isolated as previously described [25;26], from peripheral blood of healthy volunteers according to a protocol approved by the Ethics Committee of the Medical University of Graz. Prior to blood sampling from healthy volunteers, all donors signed an informed consent form. Platelet-rich plasma was removed by centrifugation of citrated whole blood. Erythrocytes were removed by dextran sedimentation. PMNL were isolated by Histopaque gradient centrifugation. Any erythrocyte contamination of the PMNL pellet was removed by hypotonic shock lysis. The purity and viability of neutrophil preparation was greater than 95%. All functional assays of neutrophils were performed in assay buffer (PBS with Ca^{2+} and Mg^{2+}, HEPES 10 mmol/L, glucose 10 mmol/L, bovine serum albumin 0.1%, pH 7.4).

2.7. Neutrophil shape change assay

Neutrophil shape change was measured as previously described [27;28]. Isolated PMNL were resuspended in assay buffer and aliquots of cells (about 3×10^5 cells per sample) were preincubated with HDL samples and then stimulated in 37°C shaking water bath with interleukin-8 (IL-8), N-formyl-methionyl-leucyl-phenylalanin (fMLP) or complement component 5a (C5a) for 4 min, with lipopolysaccharide (LPS) in the presence of 2 % serum for 90 min or with *E.coli* bacteria for 60 min at a final volume of 100 μl. Cells were transferred to ice and 150 μl of ice-cold fixative solution was added to terminate the reaction and maintain the change in cell shape until analysis. The samples were then analyzed on a FACScalibur flow cytometer (BD Biosciences). Eosinophils were distinguished from neutrophils according to granularity (side scatter) and by their autofluorescence in the FL-2 channel. Shape change was determined as the increase in the forward scatter property of the cell compared with vehicle stimulation.

2.8. CD11b activation

Isolated PMNL were preincubated with HDL samples and stimulated with IL-8 (3 nmol/L), fMLP (5 nmol/L) or C5a (30 nmol/L) for 4 min at 37°C in shaking water bath in the presence of FITC-conjugated Ab to the active epitope of CD11b. Cells were fixed and then analyzed by flow cytometry [29].

2.9. Neutrophil adhesion under flow conditions

Vena8 biochips (Cellix Ltd, Dublin, Ireland) were coated with 10 μg/ml intracellular adhesion molecule-1 (ICAM-1) at 4°C overnight in a humidified box. On the next day, the chips were washed twice with distilled water, blocked with 0.1 % bovine serum albumin for 30 minutes and rinsed with distilled water. Isolated PMNL were resuspended in assay buffer (containing Ca^{2+} and Mg^{2+}) and treated with vehicle, HDL, or $sPLA_2$-HDL for 15 min at

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37°C. Cells (3×10^6/ml) were then perfused over the ICAM-1 coated channels at constant shear stress of 0.5 dyne cm^{-2} for 5 minutes using the Mirus nanopump (Cellix). Neutrophil adhesion was recorded on an Olympus IX70 fluorescence microscope and an Olympus UPIanFI-X20/0.40 lens, using a Hamamatsu ORCA-ER digital camera and the Olympus CellP software. Cell images were taken 5 minutes after the start of perfusion and adherent neutrophils were analyzed using ImageJ software (National Institutes of Health) as described before [30].

2.10. Migration assay

Migration of freshly isolated human neutrophils (1.5×10^5 cells per well) was assessed using 96-well transwell plates with a pore size of 8 μm (Corning) as described [31]. Neutrophils were preincubated with vehicle, $sPLA_2$-HDL, HDL or PLA_2 for 15 minutes at 37°C and seeded into the upper wells. Cells were allowed to migrate towards IL-8 (10 nmol/L) for 1 hour at 37°C. Cells that had migrated to the lower compartment were enumerated by flow cytometric counting for 30 s. Spontaneous migration was determined in wells containing only assay buffer. To calculate the chemotactic index, the number of cells migrated in response to IL-8 was divided by the number of spontaneously migrated cells.

2.11. Ca^{2+} Flux

Changes in intracellular Ca^{2+} levels in neutrophils were analyzed by flow cytometry as described previously [32]. PMNL were loaded with the cell membrane permeable Ca^{2+}-sensitive dye Fluo-3-AM (2 μmol/L) in the presence of 0.02 % F-127 pluronic acid for 60 min at room temperature. Cells were then washed, stained with anti-CD16 (PE) and resuspended in assay buffer. Neutrophils were identified as CD16-positive cells.

2.12. Neutrophil extracellular traps (NET)

The kinetics of NET formation was measured as previously described [33]. Isolated human neutrophils (5×10^4 cells per well) were treated in 96-well black plates in a final volume of 200 μl in the presence of SYTOX green (5 μmol/L), a cell-impermeable nucleic acid dye. 10 nmol/L PMA was used to induce NET formation. NET formation was observed by measuring mean fluorescence (Ex 488 nm, Em 523 nm) every 10 min for 5 hours at 37°C (FlexStation II; Molecular Devices).

2.13. Cholesterol-rich microdomain (lipid raft) assessment

Isolated human neutrophils were incubated with different HDL preparations for 5 to 120 min at 37°C; subsequently cells were washed with PBS and incubated with 1 μg/ml FITC-cholera toxin B for 1 hour at room temperature. Neutrophils were fixed and lipid raft abundance was measured by flow cytometry. For fluorescence microscopy, cells were spun on glass coverslips using a Cytospin 3 centrifuge (Shandon). The cells were mounted with Vectashield mounting medium including DAPI (Szabo Scandic, Vienna, Austria) and analyzed using OLYMPUS fluorescence microscope equipped with a Hamamatsu ORCA CCD camera.

2.14. Cholesterol efflux measurement

Isolated neutrophils were labeled for 3 hours with [^{3}H]-cholesterol (1 μCi/ml) at 37°C in the presence of 2 μg/ml Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz 58-035. The cells were then washed three times with PBS containing 1 mg/ml bovine serum albumin by centrifugation. Efflux to the respective cholesterol acceptors was allowed to occur for 1 hour at 37°C in a total volume of 400 μl. After the efflux period, cells were pelleted and the radioactivity in cells and supernatant was measured.

2.15. Filipin staining of unesterified cholesterol

Neutrophils were incubated with HDL samples, cells were then washed with ice cold PBS, fixed on ice for 20 min, washed with PBS and stained for 1 h with filipin (100 μg/ml). Cells were washed before quantification by flow cytometry (LSR II, BD Biosciences).

2.16. Statistical analysis

Data are shown as mean ± SD for n observations. Experiments were repeated three to six times with neutrophils from different donors. Statistical analyses were performed using GraphPad Prism Version 4.03. Student's t-test was used for experiments comparing two groups and one-way ANOVA with Tukey multiple-comparison post hoc test was used for experiments comparing three or more groups. Significance was accepted at $p<0.05$.

3. Results

3.1. sPLA$_2$ modification of HDL generates particles with potent shape change inhibitory activity

Stimulation of PMNL with chemoattractants results in rapid reorganization of the cytoskeleton and changes of their shape, which can be assessed by flow cytometry (Supplementary Figure IA to ID) [34]. These responses are important prerequisites for granulocyte adhesion, polarization, locomotion, and degranulation. To elucidate the role of sPLA$_2$ in chemoattractant-induced shape change responses, neutrophils were added to 20 % serum pretreated for 20 hours in the absence or presence of sPLA$_2$ type III. Cells were subsequently stimulated with interleukin 8 (IL-8) and increase in the forward scatter (shape change response) was assessed by means of flow cytometry. Surprisingly, sPLA$_2$-treated serum almost completely reversed IL-8 induced shape change (Figure 1A). Given that HDL is the major carrier of phospholipids in plasma and the major substrate for sPLA$_2$ [10;15], we isolated HDL from sPLA$_2$-treated plasma by ultracentrifugation and assessed whether the HDL containing fraction shows similar effects. Indeed, HDL isolated from sPLA$_2$ treated plasma effectively suppressed neutrophil shape change, whereas HDL isolated from control plasma showed no effect (Figure 1B). We next assessed whether direct modification of HDL with sPLA$_2$ (sPLA$_2$-HDL) generates particles with shape change inhibitory activity. The lysophosphatidylcholine (LPC) content of sPLA$_2$-HDL and control HDL is shown in Supplementary Table I. We observed that sPLA$_2$-HDL effectively inhibited the activation of neutrophils induced by various agonists (IL-8, formy-methionyl-leucyl-phenylanaline (fMLP), lipopolysaccharide (LPS), complement 5a (C5a) and *E. coli* bacteria) (Figure 2A to 2E and Supplementary Figure ID), whereas native HDL or addition of sPLA$_2$ alone showed

no effects. Importantly, $sPLA_2$-HDL-induced inhibition of shape change was seen in neutrophils from all donors. Several HDL preparations from different donors showed comparable inhibitory effects after $sPLA_2$ treatment. Notably, also low-density lipoprotein treated with $sPLA_2$ suppressed agonist induced shape change (Supplementary Figure II). $sPLA_2$-HDL did not impact neutrophil viability (Supplementary Figure III). These results suggest that $sPLA_2$ induced hydrolysis of HDL-phospholipids is required for the observed effects. The inhibitory activity of $sPLA_2$-HDL on neutrophils was dose dependent (Figure 2F).

Notably, addition of $sPLA_2$-HDL after stimulation of neutrophils (with LPS or IL-8) rapidly reversed shape change (Supplementary Figure IV).

3.2. $sPLA_2$-generated lysophosphatidylcholines are the active moiety in HDL

Next, we assessed whether the potent effects of $sPLA_2$-HDL on neutrophils result from phospholipid hydrolysis products that are formed and enriched in $sPLA_2$-HDL. Therefore, we added an excess of fatty-acid free albumin to $sPLA_2$-HDL to remove lysophospholipids and free fatty acids and re-isolated HDL using density gradient ultracentrifugation. The lysophospholipid and FFA content following albumin incubation was effectively reduced as shown in Supplementary Figure V. We observed that the activity of $sPLA_2$-HDL was not due to reduced particle size or other structural alterations, but rather depended on the presence of lysophospholipids and/or free fatty acids in HDL (Figure 3A and 3B). In order to investigate whether specific free fatty acids and/or lysophospholipids mediate the effects on neutrophils, we enriched HDL with different free fatty acids and lysophospholipids. We observed that the major $sPLA_2$-HDL associated lysophospholipid LPC 16:0 was the most effective, whereas other LPCs and free fatty acids showed weak or no inhibitory effects (Figure 3C and 3D). Interestingly, besides LPC 16:0 also lysophosphatidylserine 16:0 showed potent inhibitory activity (Supplementary Figure VI).

3.3. $sPLA_2$-HDL suppresses CD11b activation, adhesion, migration and extracellular trap (NET) formation of neutrophils

When stimulated, neutrophils display an increased activation of CD11b, which is an important integrin for leukocyte adhesion and migration. In order to further investigate the effects of $sPLA_2$-HDL on neutrophil function, we measured CD11b activation. $sPLA_2$-HDL potently inhibited CD11b activation induced by IL-8, fMLP and C5a (Figure 4A to 4C).

Interestingly, LPS induced IL-8 production of neutrophils was not inhibited by $sPLA_2$-HDL (Supplementary Figure VII). Neutrophils, along with other inflammatory cells, infiltrate atherosclerotic plaques. An intriguing role has recently been suggested for neutrophil extracellular trap (NET) formation in this process [35;36]. NETs are pro-inflammatory, antimicrobial structures consisting of extracellular chromatin decorated with granular and cytoplasmic proteins, such as myeloperoxidase and neutrophil elastase. Of particular interest, stimulation of NET formation with the protein kinase C activator phorbol 12-myristate 13-acetate was effectively suppressed by $sPLA_2$-HDL (Figure 4D).

To show that the inhibition of neutrophil activation was dependent on the enzymatic activity of $sPLA_2$, varespladib (an inhibitor of $sPLA_2$ types IIA, V and X but not $sPLA_2$ type III)

was added during incubation of $sPLA_2$ with HDL. In agreement with results above, varespladib prevented the effects of $sPLA_2$ type V-treated HDL on neutrophil shape change and CD11b activation induced with IL-8 (Figure 5) and other agonists (Supplementary Figure VIII). To assess whether the $sPLA_2$-HDL-induced inhibition of CD11b activation is associated with functional alterations, we examined neutrophil adhesion to intercellular adhesion molecule 1 coated surfaces under shear stress. $sPLA_2$-HDL significantly reduced neutrophil adhesion under flow conditions (Figure 6A and 6B). Moreover, in accordance with these data, incubation of neutrophils with $sPLA_2$-HDL potently inhibited neutrophil migratory response towards IL-8 (Figure 6C).

3.4. $sPLA_2$-HDL suppresses agonist induced Ca^{2+} flux and reduces lipid raft assembly

Cytoplasmic Ca^{2+} changes in neutrophils play a key role in a series of pathways leading to activation. Stimulation with IL-8 resulted in a marked rise in the intracellular Ca^{2+}, which was effectively suppressed by $sPLA_2$-HDL, whereas control HDL showed no effect (Figure 7A). Interestingly, we observed that $sPLA_2$-HDL on its own moderately induced Ca^{2+} flux in unstimulated (resting) cells. To gain insights into the potential mechanism(s) involved in $sPLA_2$-HDL effects on neutrophils, we next examined the involvement of cAMP, as previous investigations showed that lysophospholipids increase intracellular cAMP in neutrophils. However, we did not observe altered cAMP levels in neutrophils treated with $sPLA_2$-HDL (Supplementary Figure IX). Native HDL differs from $sPLA_2$-HDL in its lysophospholipid content, raising the possibility that the variant effects of these particles on neutrophil function may be related to their cholesterol efflux activity. Interestingly, cholesterol efflux capability of $sPLA_2$-HDL was increased when compared to control HDL (Figure 7B), reflected by a significantly decreased membrane free cholesterol content in neutrophils as shown by filipin fluorescence staining (Figure 7C).

Given that cholesterol-rich lipid rafts are known to play an important role in leukocyte activation, we investigated whether increased cholesterol efflux capability of $sPLA_2$-HDL alters lipid raft abundance in neutrophils. For that purpose, FITC-labeled cholera toxin B, which binds to the raft constituent ganglioside GM1, was used to visualize lipid rafts. Indeed, we observed that $sPLA_2$-HDL caused rapid depletion of cholera-toxin positive lipid rafts in neutrophils (Figure 7D, 7E). ABCA1 and SR-BI have been reported to localize selectively in lipid rafts or caveolae [37;38] and to be involved in cholesterol efflux. However, neither the ABCA1-inhibitor probucol [39] nor the SR-BI inhibitor BLT-1 [40] diminished $sPLA_2$-HDL and native HDL mediated efflux from neutrophils, suggesting that the increased cholesterol efflux capability of $sPLA_2$-HDL is independent of these receptors (Supplementary Figure X). Moreover, when cholesterol efflux experiments were performed with J774 macrophages, cholesterol efflux capability of $sPLA_2$-HDL was not different compared with native HDL, suggesting a cell type specific effect (Supplementary Figure XI).

4. Discussion

The studies presented here demonstrate that $sPLA_2$ modification of HDL generates an anti-inflammatory particle that has the ability to effectively alter neutrophil function. Of

particular interest is the observation that $sPLA_2$-HDL, but not control HDL, effectively depleted the free cholesterol content of neutrophils and suppressed agonist-induced rise in intracellular Ca^{2+} levels. It is now well established that alterations in membrane cholesterol content cause disruption of cholesterol-rich signaling microdomains integral to cellular signaling pathways known as lipid rafts [41]. Several lines of evidence suggest that HDL regulates the plasma membrane cholesterol content through the ability to remove cholesterol and other lipid species from cells, thereby dampening plasma membrane receptor signaling [42]. Our studies suggest that the increased cholesterol-mobilizing activity of $sPLA_2$-HDL as well as the suppression of agonist-induced rise in intracellular Ca^{2+} levels are likely mechanism that counteracts activation of neutrophils, resulting in an effective inhibition of CD11b activation and NET formation. CD11b mediates inflammation by regulating leukocyte adhesion and migration and has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity, chemotaxis and cellular activation [43]. Importantly, $sPLA_2$-HDL efficiently inhibited neutrophil adhesion to ICAM-1 coated surfaces under shear stress and potently inhibited neutrophil migratory response towards IL-8. Although NETs are able to trap and kill bacteria and their formation is an important first-line defense against various pathogens, excessive NET production may strongly promote inflammation, leading to endothelial damage, infiltration of further neutrophils into atheromatous lesions, thrombus formation and autoimmunity [35;36].

Of particular interest, we observed that the major $sPLA_2$-HDL associated lysophospholipid, LPC 16:0, was the most effective, whereas other LPCs and free fatty acids showed weak or no inhibitory effects. Given that groups IIA, V and X show a differential hydrolysis of molecular species of phosphatidylcholine [44], $sPLA_2$ modified HDLs with various activities are expected to be formed under inflammatory conditions.

Notably, treatment with native HDL showed only minimal effects on neutrophil function despite its ability to efflux cholesterol. Our results suggest that bidirectional lipid flux (efflux of cholesterol is accompanied by influx of HDL-cholesterylester) mediated by control HDL results in minimal net change in the cholesterol content of the neutrophil membrane as observed by filipin staining. We assume that lysophospholipid-enrichment of HDL generates a particle that more efficiently depletes neutrophil cholesterol. In line with our finding is the observation that that lysophospholipids rapidly enter lipid rafts in the cell membrane promoting raft fission [45] and promote net cholesterol efflux [46], which might explain the increase in cholesterol efflux properties of $sPLA_2$-HDL. In line with that notion, $sPLA_2$-HDL preparations, but not control HDL, markedly disrupted neutrophil lipid rafts. Interestingly, the increased ability of $sPLA_2$-HDL to mobilize cell cholesterol seems to be independent of ABCA1 and SR-BI, proteins known to mediate cholesterol efflux (Supplementary figure X). Moreover, the improved cholesterol efflux ability of $sPLA_2$-HDL strikingly differs between cell types, given that $sPLA_2$-HDL mediated cholesterol efflux of from J774 macrophages was not different from native HDL (Supplementary figure XI). The reason for that surprising observation is not clear, but different lipid raft content and/or lipid raft composition of freshly isolated neutrophils compared with a macrophage cell line might be an explanation.

Of particular interest is the recent observation that a large fraction of phosphatidylcholine moiety of administered reconstituted HDL is rapidly hydrolyzed by $sPLA_2$ isoforms in blood and causes a remarkable increase of HDL-associated lysophospholipid content reaching levels of 300 μmol/L 4 hours post administration [47;48], which raises the possibility that formed lysophospholipids contributed to the potent anti-inflammatory activity of administered reconstituted HDL.

Previous studies supported an important role for a subset of $sPLA_2$ isoforms ($sPLA_2$-IIA, -III, -V, and -X) from initiation and progression to cardiovascular complications in the pathophysiology of atherosclerosis [49]. Hydrolysis of phosphatidylcholine in low-density lipoprotein by $sPLA_2$ isoforms has been implicated in the promotion of atherosclerosis [50;51]. These observations have stimulated interest in the potential value of $sPLA_2$ inhibition as a cardioprotective strategy. Initial studies demonstrated that varespladib, a nonspecific pan-$sPLA_2$ inhibitor, reduced activity of $sPLA_2$-IIA by more than 90%, in addition to lowering low-density-lipoprotein cholesterol and C-reactive protein in patients with stable coronary disease and acute coronary syndrome. Surprisingly, varespladib-induced $sPLA_2$ inhibition did not reduce cardiovascular ischemic complications and resulted in an excess rate of myocardial infarction and stroke in patients with acute coronary syndromes [8]. Moreover, inhibition of $sPLA_2$ showed no overall survival benefit in septic patients [9]. Interestingly, also a large phase III study testing an inhibitor of lipoprotein-associated phospholipase A_2 has failed to lower the risk of cardiovascular events in coronary heart disease patients [52], providing further evidence that the physiologic and pathophysiologic roles of phospholipases are not well understood.

A potential limitation of the present study is that we only assessed the impact of $sPLA_2$ on HDL functionality, while *in vivo* HDL particles are subjected to an array of compositional changes and factors acting in concert. Hydrolysis of HDL phospholipids by $sPLA_2$ alters the structure and composition of HDL particles with the metabolic consequences of increased catabolism. Experimental studies have shown that the addition of LPS to plasma resulted in a marked decrease in cholesterol ester transfer protein activity, leading to changes in HDL levels considered as an adaptive response to preserve or increase HDL [53]. But also other factors alter HDL composition and function under inflammatory conditions. The concentrations of the acute phase protein SAA markedly increase during the acute-phase reaction. SAA associates with HDL, altering structure and function of HDL [54]. Interestingly, overexpression of SAA in the absence of generalized inflammation does not result in changes of HDL cholesterol or apoA-I levels *in vivo* [55] whereas overexpression of $sPLA_2$ results in alterations of HDL metabolism *even* in the absence of generalized inflammation [56]. Although increased $sPLA_2$ and decreased CETP activities might be beneficial for host defense in the setting of acute infection, replacement of HDL-apolipoproteins by SAA might contribute to increased incidence of cardiovascular disease [54].

In conclusion, our data provide novel evidence that the increased cholesterolmobilizing activity and suppression of rise in intracellular Ca^{2+} levels of $sPLA_2$-HDL might effectively counteract agonist-induced activation of neutrophils. Our results raise the possibility that

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sPLA$_2$-induced modification of HDL composition and function modulates neutrophil trafficking and effector responses during inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

C5a	complement component 5a
FFA	free fatty acid
fMLP	N-formyl-lmethionyl-l-leucyl-l-phenylalanine
HDL	high-density lipoprotein
ICAM-1	intracellular adhesion molecule-1
IL-8	interleukin 8
NET	neutrophil extracellular traps
LPC	lysophosphatidylcholine
PC	phosphatidylcholine
PMNL	polymorphonuclear leukocytes
sPLA$_2$	secretory phospholipase A$_2$
sPLA$_2$-HDL	secretory phospholipase A$_2$ treated HDL

References

[1]. Vadas P, Pruzanski W. Induction of group II phospholipase A2 expression and pathogenesis of the sepsis syndrome. Circ.Shock. 1993; 39:160–167. [PubMed: 8490995]

[2]. Murakami M, Lambeau G. Emerging roles of secreted phospholipase A(2) enzymes: an update. Biochimie. 2013; 95:43–50. [PubMed: 23022039]

[3]. Nevalainen TJ. Serum phospholipases A2 in inflammatory diseases. Clin.Chem. 1993; 39:2453–2459. [PubMed: 8252715]

[4]. Lin MK, Farewell V, Vadas P, Bookman AA, Keystone EC, Pruzanski W. Secretory phospholipase A2 as an index of disease activity in rheumatoid arthritis. Prospective double blind study of 212 patients. J.Rheumatol. 1996; 23:1162–1166. [PubMed: 8823686]

[5]. Pruzanski W, Vadas P. Secretory synovial fluid phospholipase A2 and its role in the pathogenesis of inflammation in arthritis. J.Rheumatol. 1988; 15:1601–1603. [PubMed: 3070027]

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[6]. Koenig W, Vossen CY, Mallat Z, Brenner H, Benessiano J, Rothenbacher D. Association between type II secretory phospholipase A2 plasma concentrations and activity and cardiovascular events in patients with coronary heart disease. Eur.Heart J. 2009; 30:2742–2748. [PubMed: 19666896]

[7]. Rosenson RS, Hurt-Camejo E. Phospholipase A2 enzymes and the risk of atherosclerosis. Eur.Heart J. 2012; 33:2899–2909. [PubMed: 22802388]

[8]. Nicholls SJ, Kastelein JJ, Schwartz GG, Bash D, Rosenson RS, Cavender MA, Brennan DM, Koenig W, Jukema JW, Nambi V, Wright RS, Menon V, Lincoff AM, Nissen SE. VISTA-16 Investigators, Varespladib and cardiovascular events in patients with an acute coronary syndrome: the VISTA-16 randomized clinical trial. JAMA. 2014; 311:252–262. [PubMed: 24247616]

[9]. Abraham E, Naum C, Bandi V, Gervich D, Lowry SF, Wunderink R, Schein RM, Macias W, Skerjanec S, Dmitrienko A, Farid N, Forgue ST, Jiang F. Efficacy and safety of LY315920Na/S-5920, a selective inhibitor of 14-kDa group IIA secretory phospholipase A2, in patients with suspected sepsis and organ failure. Crit.Care Med. 2003; 31:718–728. [PubMed: 12626975]

[10]. Gijon MA, Perez C, Mendez E, Crespo M. Sanchez. Phospholipase A2 from plasma of patients with septic shock is associated with high-density lipoproteins and C3 anaphylatoxin: some implications for its functional role. Biochem.J. 1995; 306(Pt 1):167–175. [PubMed: 7864806]

[11]. Dashti M, Kulik W, Hoek F, Veerman EC, Peppelenbosch MP, Rezaee F. A phospholipidomic analysis of all defined human plasma lipoproteins. Sci.Rep. 2011; 1:139. [PubMed: 22355656]

[12]. Catapano AL, Pirillo A, Bonacina F, Norata GD. HDL in innate and adaptive immunity. Cardiovasc.Res. 2014; 103:372–383. [PubMed: 24935428]

[13]. Marsche G, Saemann MD, Heinemann A, Holzer M. Inflammation alters HDL composition and function: implications for HDL-raising therapies. Pharmacol.Ther. 2013; 137:341–351. [PubMed: 23246719]

[14]. Birner-Gruenberger R, Schittmayer M, Holzer M, Marsche G. Understanding high-density lipoprotein function in disease: Recent advances in proteomics unravel the complexity of its composition and biology. Prog.Lipid Res. 2014; 56C:36–46. [PubMed: 25107698]

[15]. Tietge UJ, Maugeais C, Lund-Katz S, Grass D, deBeer FC, Rader DJ. Human secretory phospholipase A2 mediates decreased plasma levels of HDL cholesterol and apoA-I in response to inflammation in human apoA-I transgenic mice. Arterioscler.Thromb.Vasc.Biol. 2002; 22:1213–1218. [PubMed: 12117740]

[16]. Leber B, Spindelboeck W, Stadlbauer V. Infectious complications of acute and chronic liver disease. Semin.Respir.Crit.Care.Med. 2012; 33:80–95. [PubMed: 22447263]

[17]. Donnelly LE, Barnes PJ. Defective phagocytosis in airways disease. Chest. 2012; 141:1055–1062. [PubMed: 22474147]

[18]. Guasti L, Dentali F, Castiglioni L, Maroni L, Marino F, Squizzato A, Ageno W, Gianni M, Gaudio G, Grandi AM, Cosentino M, Venco A. Neutrophils and clinical outcomes in patients with acute coronary syndromes and/or cardiac revascularisation. A systematic review on more than 34,000 subjects. Thromb.Haemost. 2011; 106:591–599. [PubMed: 21866299]

[19]. Medzhitov R, Janeway C Jr. Innate immunity. N.Engl.J.Med. 2000; 343:338–344. [PubMed: 10922424]

[20]. Soehnlein O. Multiple roles for neutrophils in atherosclerosis. Circ.Res. 2012; 110:875–888. [PubMed: 22427325]

[21]. Holzer M, Trieb M, Konya V, Wadsack C, Heinemann A, Marsche G. Aging affects high-density lipoprotein composition and function. Biochim.Biophys.Acta. 2013; 1831:1442–1448. [PubMed: 23792422]

[22]. Holzer M, Zangger K, El-Gamal D, Binder V, Curcic S, Konya V, Schuligoi R, Heinemann A, Marsche G. Myeloperoxidase-derived chlorinating species induce protein carbamylation through decomposition of thiocyanate and urea: novel pathways generating dysfunctional high-density lipoprotein. Antioxid.Redox Signal. 2012; 17:1043–1052. [PubMed: 22462773]

[23]. Holzer M, Wolf P, Inzinger M, Trieb M, Curcic S, Pasterk L, Weger W, Heinemann A, Marsche G. Anti-psoriatic therapy recovers high-density lipoprotein composition and function. J.Invest.Dermatol. 2014; 134:635–642. [PubMed: 23985995]

[24]. BLIGH EG, DYER WJ. A rapid method of total lipid extraction and purification. Can.J.Biochem.Physiol. 1959; 37:911–917. [PubMed: 13671378]

[25]. Schratl P, Royer JF, Kostenis E, Ulven T, Sturm EM, Waldhoer M, Hoefler G, Schuligoi R, Lippe IT, Peskar BA, Heinemann A. The role of the prostaglandin D2 receptor, DP, in eosinophil trafficking. J.Immunol. 2007; 179:4792–4799. [PubMed: 17878378]

[26]. Balenga NA, Aflaki E, Kargl J, Platzer W, Schroder R, Blattermann S, Kostenis E, Brown AJ, Heinemann A, Waldhoer M. GPR55 regulates cannabinoid 2 receptor-mediated responses in human neutrophils. Cell Res. 2011; 21:1452–1469. [PubMed: 21467997]

[27]. Luschnig-Schratl P, Sturm EM, Konya V, Philipose S, Marsche G, Frohlich E, Samberger C, Lang-Loidolt D, Gattenlohner S, Lippe IT, Peskar BA, Schuligoi R, Heinemann A. EP4 receptor stimulation down-regulates human eosinophil function. Cell Mol.Life Sci. 2011; 68:3573–3587. [PubMed: 21365278]

[28]. Sabroe I, Hartnell A, Jopling LA, Bel S, Ponath PD, Pease JE, Collins PD, Williams TJ. Differential regulation of eosinophil chemokine signaling via CCR3 and non-CCR3 pathways. J.Immunol. 1999; 162:2946–2955. [PubMed: 10072545]

[29]. Konya V, Blattermann S, Jandl K, Platzer W, Ottersbach PA, Marsche G, Gutschow M, Kostenis E, Heinemann A. A Biased Non-Galphai OXE-R Antagonist Demonstrates That Galphai Protein Subunit Is Not Directly Involved in Neutrophil, Eosinophil, and Monocyte Activation by 5-Oxo-ETE. J.Immunol. 2014; 192:4774–4782. [PubMed: 24733850]

[30]. Konya V, Philipose S, Balint Z, Olschewski A, Marsche G, Sturm EM, Schicho R, Peskar BA, Schuligoi R, Heinemann A. Interaction of eosinophils with endothelial cells is modulated by prostaglandin EP4 receptors. Eur.J.Immunol. 2011; 41:2379–2389. [PubMed: 21681739]

[31]. Sturm GJ, Schuligoi R, Sturm EM, Royer JF, Lang-Loidolt D, Stammberger H, Amann R, Peskar BA, Heinemann A. 5-Oxo-6,8,11,14-eicosatetraenoic acid is a potent chemoattractant for human basophils. J.Allergy Clin.Immunol. 2005; 116:1014–1019. [PubMed: 16275369]

[32]. Heinemann A, Ofner M, Amann R, Peskar BA. A novel assay to measure the calcium flux in human basophils: effects of chemokines and nerve growth factor. Pharmacology. 2003; 67:49–54. [PubMed: 12444303]

[33]. Kirchner T, Moller S, Klinger M, Solbach W, Laskay T, Behnen M. The impact of various reactive oxygen species on the formation of neutrophil extracellular traps. Mediators Inflamm. 2012; 2012:849136. [PubMed: 22481865]

[34]. Schratl P, Heinemann A. Differential involvement of Ca2+ and actin filament in leukocyte shape change. Pharmacology. 2009; 83:131–140. [PubMed: 19092285]

[35]. Doring Y, Drechsler M, Wantha S, Kemmerich K, Lievens D, Vijayan S, Gallo RL, Weber C, Soehnlein O. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. Circ.Res. 2012; 110:1052–1056. [PubMed: 22394519]

[36]. Doring Y, Manthey HD, Drechsler M, Lievens D, Megens RT, Soehnlein O, Busch M, Manca M, Koenen RR, Pelisek J, Daemen MJ, Lutgens E, Zenke M, Binder CJ, Weber C, Zernecke A. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. Circulation. 2012; 125:1673–1683. [PubMed: 22388324]

[37]. Peng Y, Akmentin W, Connelly MA, Lund-Katz S, Phillips MC, Williams DL. Scavenger receptor BI (SR-BI) clustered on microvillar extensions suggests that this plasma membrane domain is a way station for cholesterol trafficking between cells and high-density lipoprotein. Mol.Biol.Cell. 2004; 15:384–396. [PubMed: 14528013]

[38]. Drobnik W, Borsukova H, Bottcher A, Pfeiffer A, Liebisch G, Schutz GJ, Schindler H, Schmitz G. Apo AI/ABCA1-dependent and HDL3-mediated lipid efflux from compositionally distinct cholesterol-based microdomains. Traffic. 2002; 3:268–278. [PubMed: 11929608]

[39]. Favari E, Zanotti I, Zimetti F, Ronda N, Bernini F, Rothblat GH. Probucol inhibits ABCA1-mediated cellular lipid efflux. Arterioscler.Thromb.Vasc.Biol. 2004; 24:2345–2350. [PubMed: 15514211]

[40]. Nieland TJ, Penman M, Dori L, Krieger M, Kirchhausen T. Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI. Proc.Natl.Acad.Sci.U.S.A. 2002; 99:15422–15427. [PubMed: 12438696]

[41]. Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997; 387:569–572. [PubMed: 9177342]

[42]. Feig JE, Shamir R, Fisher EA. Atheroprotective effects of HDL: beyond reverse cholesterol transport. Curr.Drug Targets. 2008; 9:196–203. [PubMed: 18336237]

[43]. Solovjov DA, Pluskota E, Plow EF. Distinct roles for the alpha and beta subunits in the functions of integrin alphaMbeta2. J.Biol.Chem. 2005; 280:1336–1345. [PubMed: 15485828]

[44]. Pruzanski W, Lambeau L, Lazdunsky M, Cho W, Kopilov J, Kuksis A. Differential hydrolysis of molecular species of lipoprotein phosphatidylcholine by groups IIA, V and X secretory phospholipases A2. Biochim.Biophys.Acta. 2005; 1736:38–50. [PubMed: 16122976]

[45]. Tanaka T, Sano R, Yamashita Y, Yamazaki M. Shape changes and vesicle fission of giant unilamellar vesicles of liquid-ordered phase membrane induced by lysophosphatidylcholine. Langmuir. 2004; 20:9526–9534. [PubMed: 15491182]

[46]. Hara S, Shike T, Takasu N, Mizui T. Lysophosphatidylcholine promotes cholesterol efflux from mouse macrophage foam cells. Arterioscler.Thromb.Vasc.Biol. 1997; 17:1258–1266. [PubMed: 9261255]

[47]. Levels JH, Pajkrt D, Schultz M, Hoek FJ, van Tol A, Meijers JC, van Deventer SJ. Alterations in lipoprotein homeostasis during human experimental endotoxemia and clinical sepsis. Biochim.Biophys.Acta. 2007; 1771:1429–1438. [PubMed: 17980169]

[48]. Drew BG, Carey AL, Natoli AK, Formosa MF, Vizi D, Reddy-Luthmoodoo M, Weir JM, Barlow CK, van Hall G, Meikle PJ, Duffy SJ, Kingwell BA. Reconstituted high-density lipoprotein infusion modulates fatty acid metabolism in patients with type 2 diabetes mellitus. J.Lipid Res. 2011; 52:572–581. [PubMed: 21224289]

[49]. Mallat Z, Lambeau G, Tedgui A. Lipoprotein-associated and secreted phospholipases A(2) in cardiovascular disease: roles as biological effectors and biomarkers. Circulation. 2010; 122:2183–2200. [PubMed: 21098459]

[50]. Sato H, Kato R, Isogai Y, Saka G, Ohtsuki M, Taketomi Y, Yamamoto K, Tsutsumi K, Yamada J, Masuda S, Ishikawa Y, Ishii T, Kobayashi T, Ikeda K, Taguchi R, Hatakeyama S, Hara S, Kudo I, Itabe H, Murakami M. Analyses of group III secreted phospholipase A2 transgenic mice reveal potential participation of this enzyme in plasma lipoprotein modification, macrophage foam cell formation, and atherosclerosis. J.Biol.Chem. 2008; 283:33483–33497. [PubMed: 18801741]

[51]. Boyanovsky BB, van der Westhuyzen DR, Webb NR. Group V secretory phospholipase A2-modified low density lipoprotein promotes foam cell formation by a SR-A- and CD36-independent process that involves cellular proteoglycans. J.Biol.Chem. 2005; 280:32746–32752. [PubMed: 16040605]

[52]. O'Donoghue ML, Braunwald E, White HD, Steen DP, Lukas MA, Tarka E, Steg PG, Hochman JS, Bode C, Maggioni AP, Im K, Shannon JB, Davies RY, Murphy SA, Crugnale SE, Wiviott SD, Bonaca MP, Watson DF, Weaver WD, Serruys PW, Cannon CP, SOLID-TIMI 52 Investigators. Effect of darapladib on major coronary events after an acute coronary syndrome: the SOLID-TIMI 52 randomized clinical trial. JAMA. 2014; 312:1006–1015. [PubMed: 25173516]

[53]. Masucci-Magoulas L, Moulin P, Jiang XC, Richardson H, Walsh A, Breslow JL, Tall A. Decreased cholesteryl ester transfer protein (CETP) mRNA and protein and increased high density lipoprotein following lipopolysaccharide administration in human CETP transgenic mice. J.Clin.Invest. 1995; 95:1587–1594. [PubMed: 7706465]

[54]. Artl A, Marsche G, Lestavel S, Sattler W, Malle E. Role of serum amyloid A during metabolism of acute-phase HDL by macrophages. Arterioscler.Thromb.Vasc.Biol. 2000; 20:763–772. [PubMed: 10712402]

[55]. Hosoai H, Webb NR, Glick JM, Tietge UJ, Purdom MS, de Beer FC, Rader DJ. Expression of serum amyloid A protein in the absence of the acute phase response does not reduce HDL cholesterol or apoA-I levels in human apoA-I transgenic mice. J.Lipid Res. 1999; 40:648–653. [PubMed: 10191288]

[56]. Tietge UJ, Maugeais C, Cain W, Grass D, Glick JM, de Beer FC, Rader DJ. Overexpression of secretory phospholipase A(2) causes rapid catabolism and altered tissue uptake of high density lipoprotein cholesteryl ester and apolipoprotein A-I. J.Biol.Chem. 2000; 275:10077–10084. [PubMed: 10744687]

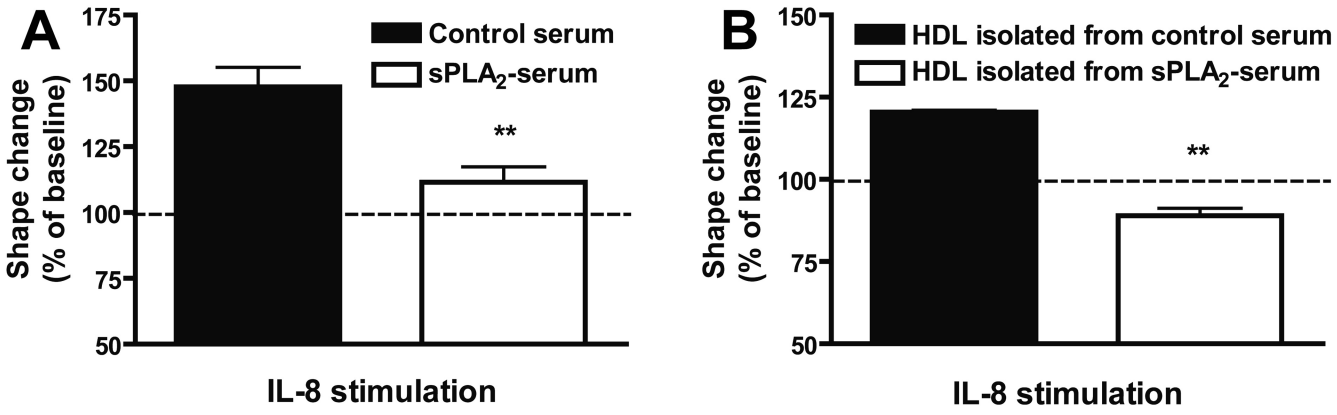

Figure 1. $sPLA_2$-treated serum inhibits neutrophil activation

Freshly isolated human serum was incubated in the absence or presence of $sPLA_2$ type III for 24 hours at 37°C. (A) Treated and untreated serum (20 % v/v) was subsequently added followed by stimulation with IL-8 (3 nmol/L) for 4 min. (B) HDL was isolated from $sPLA_2$ treated serum and control serum and subsequently added to human neutrophils (125 μg HDL-protein/ml, 15 min at 37°C) followed by stimulation with IL-8 (3 nmol/L) for 4 min. Neutrophil shape change was assessed by flow cytometry. Values are expressed as % of baseline. The results are shown as mean ± SD of three separate experiments using PMNL from different healthy donors. Statistical analysis was performed using Student's t-test. *$p < 0.05$, **$p < 0.01$ *versus* control.

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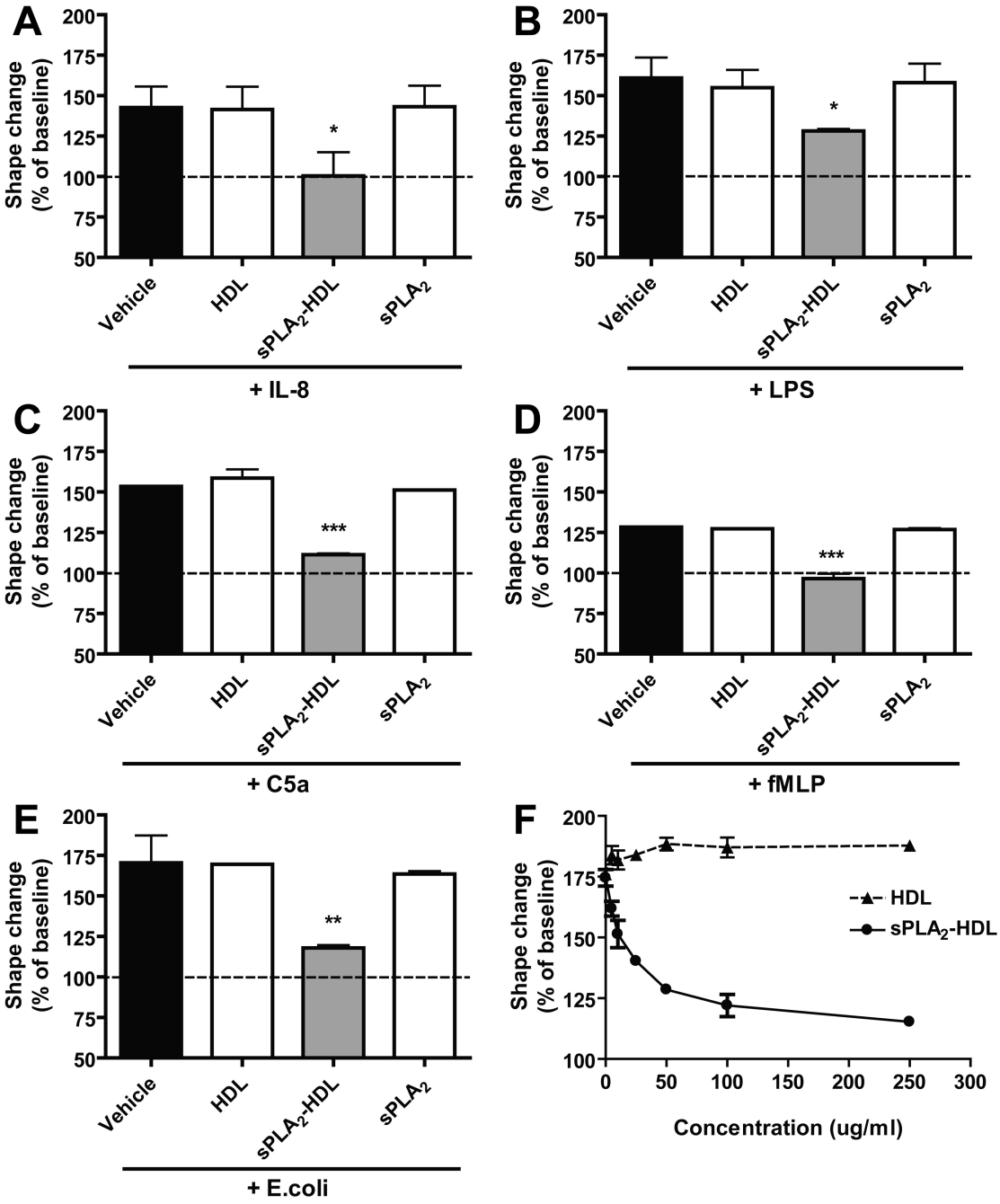

Figure 2. $sPLA_2$-treated HDL inhibits IL-8, LPS, C5a, fMLP and E. coli-induced neutrophil activation

Isolated neutrophils were incubated with vehicle, native HDL (100 μg protein/ml), $sPLA_2$-treated HDL (100 μg protein/ml) or $sPLA_2$ alone for 15 min at 37°C, and stimulated with (A) IL-8 (3 nmol/L, 4 min, 37°C) (B) 1 ng/ml LPS in the presence of 2 % serum (90 min, 37°C) (C) C5a (30 nmol/L, 4 min, 37°C) (D) fMLP (5 nmol/L, 4 min, 37°C) or (E) E. coli bacteria (1 h, 37°C, the ratio of neutrophils and bacteria was 1:5). Neutrophil shape change was measured by flow cytometry. Values are expressed as % of baseline. Results shown are mean ± SD. The data represent the results from three to five experiments done using PMNL

from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 *versus* vehicle. (F) Concentration-response curve with $sPLA_2$-treated HDL. Neutrophils were incubated with increasing concentrations of native HDL or $sPLA_2$-treated HDL (0 up to 250 μg/ml) for 15 min at 37°C and stimulated with 1 ng/ml LPS (90 min, 37°C). Neutrophil shape change was measured by flow cytometry and results are shown as mean ± SD (n=3).

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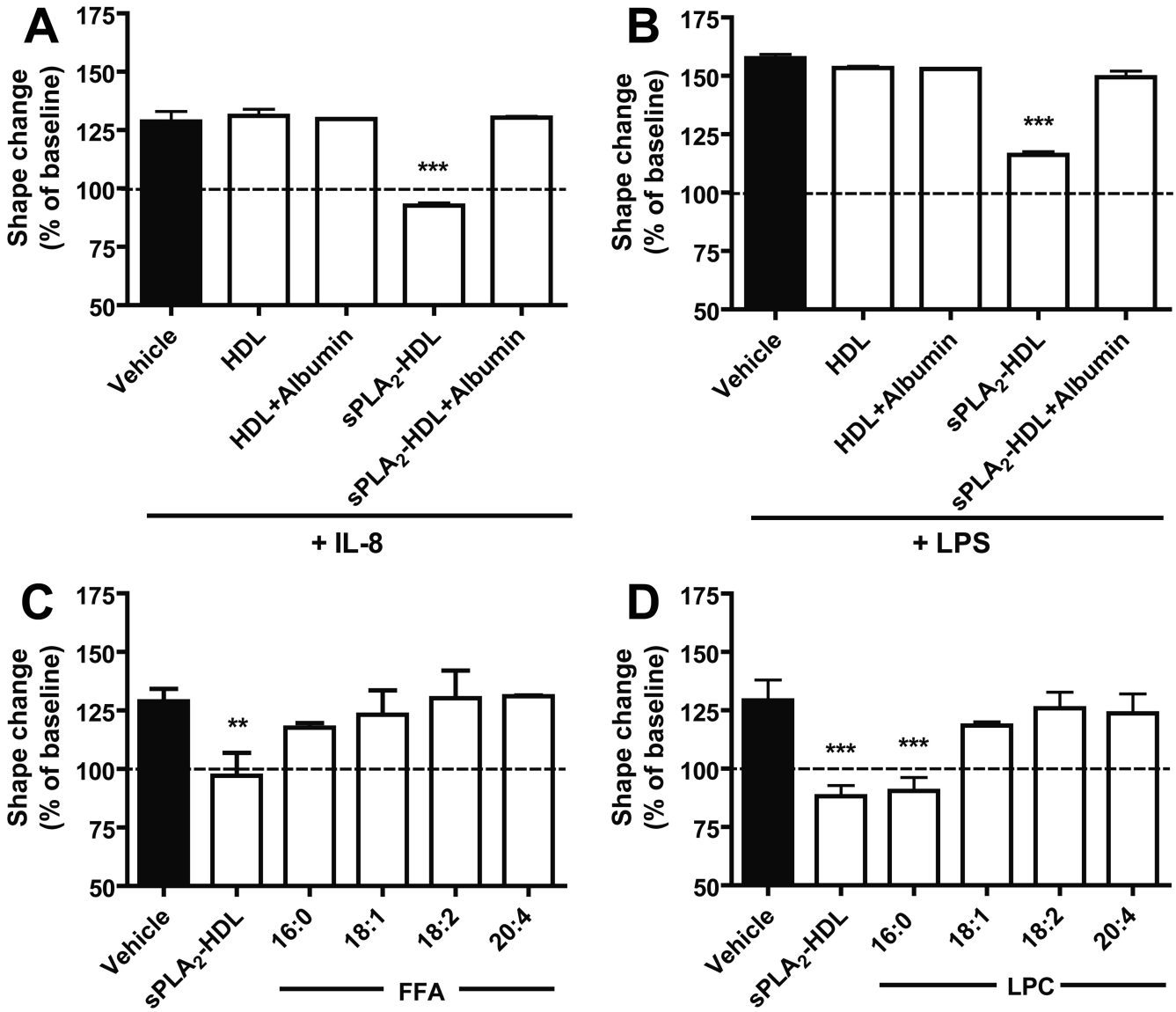

Figure 3. Presence of LPC on sPLA$_2$-treated HDL is necessary for the inhibition of neutrophil shape change

Native or sPLA$_2$-treated HDL were incubated in the presence or absence of 50 mg/ml albumin (1 h, 37°C) and HDL was re-isolated by density gradient ultracentrifugation to remove FFA and lysophospholipids as described in Materials and Methods. 100 μg/ml HDL protein was used in the shape change assay with (A) IL-8 (3 nmol/L, 4 min) or (B) 1 ng/ml LPS (90 min, 37°C). **Enrichment of HDL with FFA and LPC.** Neutrophils were preincubated (15 min, 37°C) with sPLA$_2$-HDL (100 μg/ml protein) and (C) HDL enriched with FFA (16:0, 18:1, 18:2, 20:4) or (D) HDL enriched with LPC (16:0, 18:1, 18:2, 20:4). Neutrophils were then stimulated with IL-8 (3 nmol/L, 4 min, 37°C). Neutrophil shape change was measured by flow cytometry. Values are expressed as % of baseline. Results shown are mean ± SD of three separate experiments using cells from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* vehicle.

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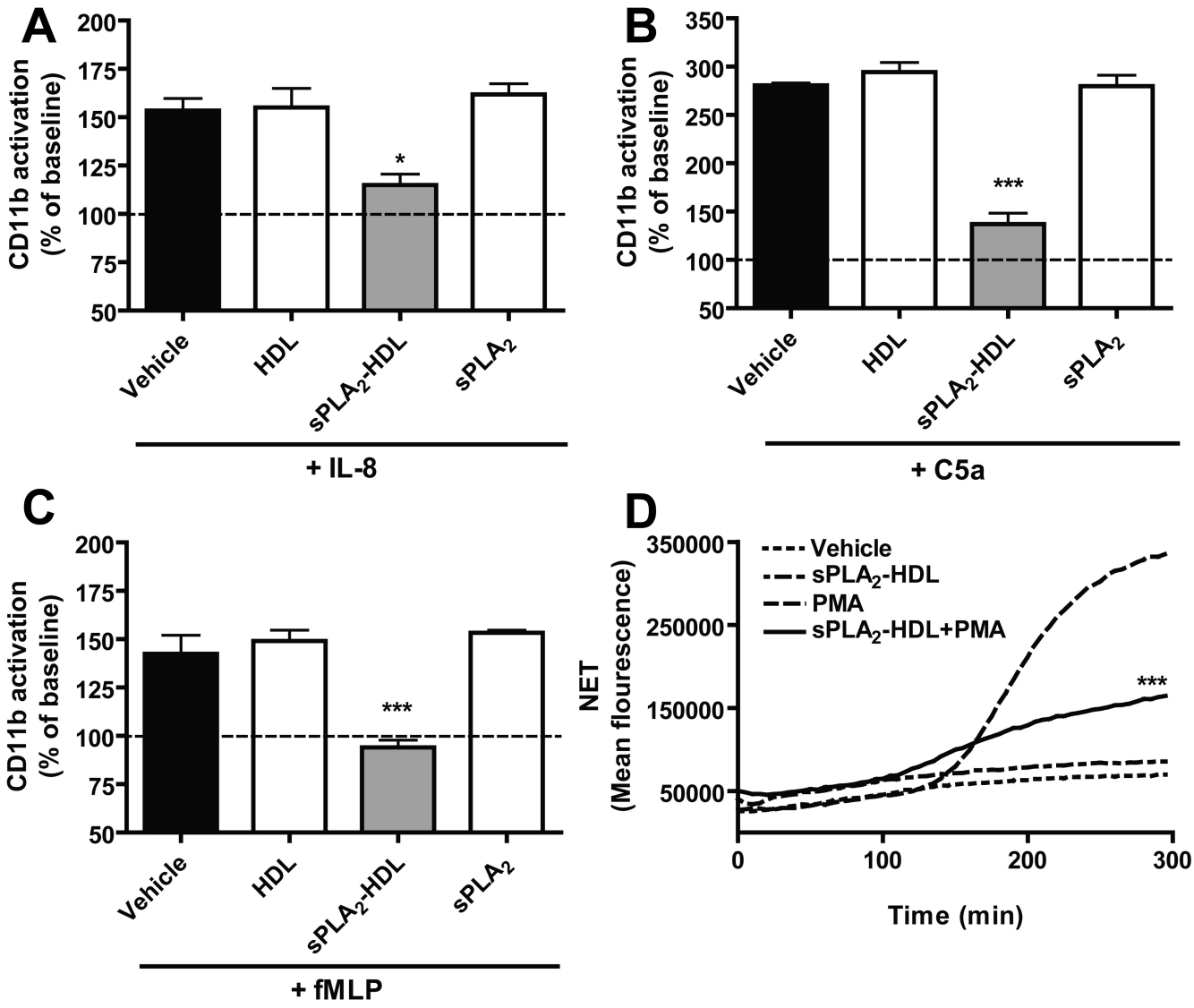

Figure 4. $sPLA_2$-treated HDL inhibits CD11b activation in neutrophils

Isolated neutrophils were preincubated with vehicle, HDL (100 μg protein/ml), $sPLA_2$-HDL (100 μg protein/ml) or $sPLA_2$ alone for 15 min at 37°C in the presence of antibody to the active epitope of CD11b and stimulated with (A) IL-8 (3 nmol/L), (B) fMLP (10 nmol/L) or (C) C5a (30 nmol/L) for 4 min at 37°C. CD11b activation was measured by flow cytometry. Vehicle treated (unstimulated) control was set as baseline and values are expressed as % over baseline. The results are shown as mean ± SD of 4 separate experiments using cells from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* vehicle. **(D)** Isolated neutrophils were treated with vehicle or $sPLA_2$-HDL (100 μg protein/ml) in the presence or absence of 10 nmol/L PMA and NET formation was measured as an increase in fluorescence of the SYTOX Green dye every 10 min for 5 hours at 37°C. Results show a representative experiment of three independent experiments. Values are

expressed as mean fluorescence. *p < 0.05, **p < 0.01, ***p < 0.001 *versus* PMA at 5 hours time point.

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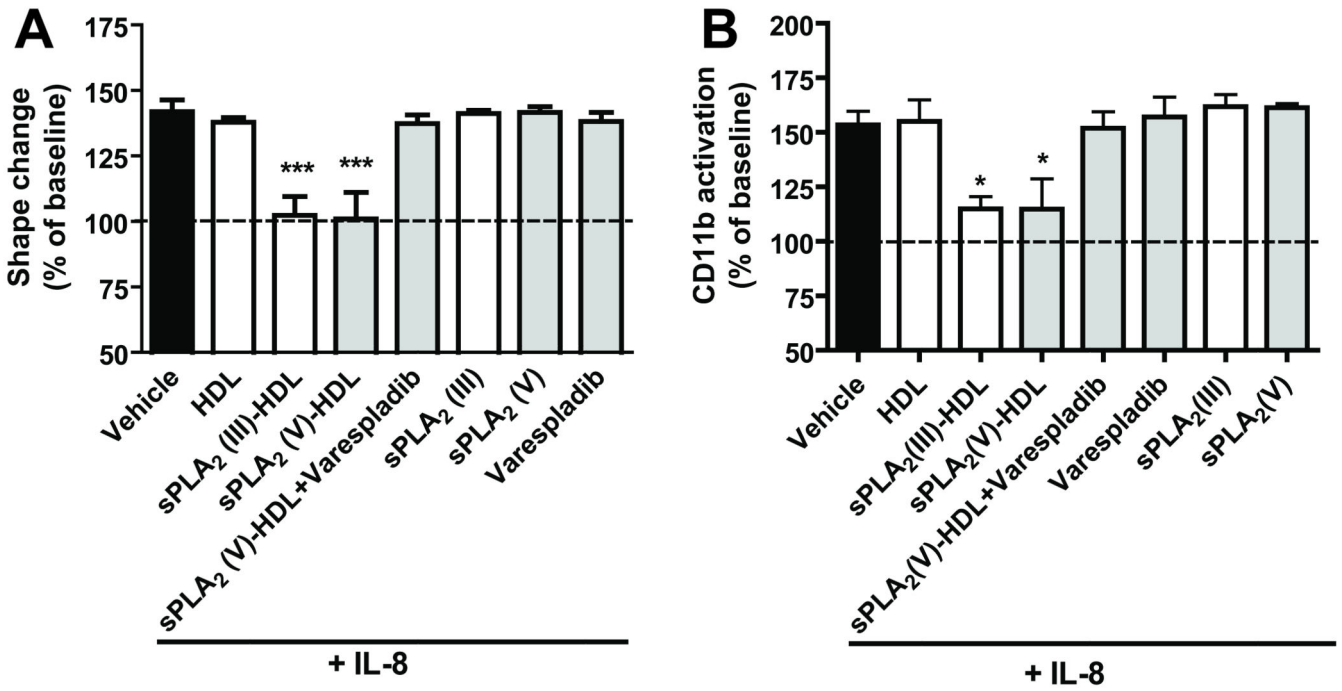

Figure 5. The $sPLA_2$ inhibitor varespladib inhibits the effects of $sPLA_2$-treated HDL on neutrophils

HDL was incubated with 200 ng/ml $sPLA_2$ type III or 400 ng/ml $sPLA_2$ type V in the presence or absence of varespladib (1 μmol/L), overnight, at 37°C. These samples were used for neutrophil treatment. Isolated neutrophils were preincubated (15 min at 37°C) with vehicle (assay buffer), HDL (100 μg protein/ml), $sPLA_2$ (III)-HDL (100 μg protein/ml), $sPLA_2$ (V)-HDL, $sPLA_2$ (V)-HDL + varespladib, varespadib, $sPLA_2$ (III) or $sPLA_2$ (V) alone. Neutrophils were then stimulated with IL-8 (3 nmol/L, 4 min, 37°C) to assess shape change (A) or CD11b activation (B) by flow cytometry. Values are expressed as % of baseline. Results represent means ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* vehicle.

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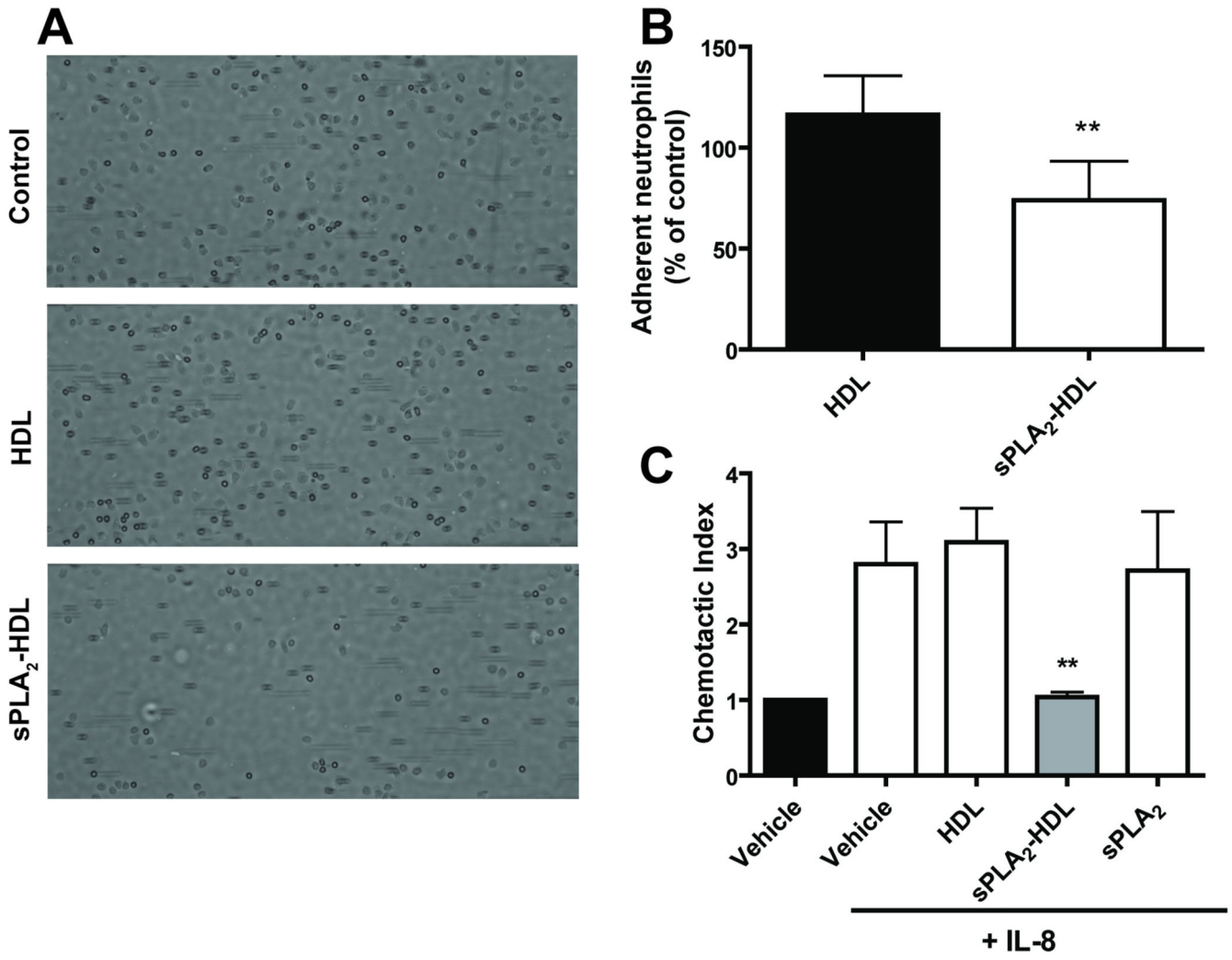

Figure 6. $sPLA_2$-HDL inhibits neutrophil adhesion under flow conditions and neutrophil migration

Isolated human neutrophils were treated with vehicle (assay buffer), HDL (100 μg protein/ml) or $sPLA_2$-HDL (100 μg protein/ml) for 15 minutes at 37°C before perfusion over ICAM-1 coated microchannels. (A) Representative images were taken 5 minutes after the start of the perfusion with neutrophils. (B) Images were quantified by computerized image analysis. Values are expressed as % of vehicle treated control. Data are shown as mean ± SD of 6 experiments with neutrophils from different donors. Statistical analysis was performed using Student's t-test. *$p < 0.05$, **$p < 0.01$ *versus* HDL. (C) Neutrophils were preincubated with vehicle, HDL (100 μg protein/ml), $sPLA_2$-HDL (100 μg protein/ml) or $sPLA_2$ for 15 minutes at 37°C and seeded into the upper wells. Cells were allowed to migrate towards IL-8 (10 nmol/L) for 1 hour at 37°C. Cells that had migrated to the lower chamber were enumerated by flow cytometry. The results are shown as mean ± SD of 3 separate experiments using PMNL from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *$p < 0.05$, **$p < 0.01$ *versus* vehicle.

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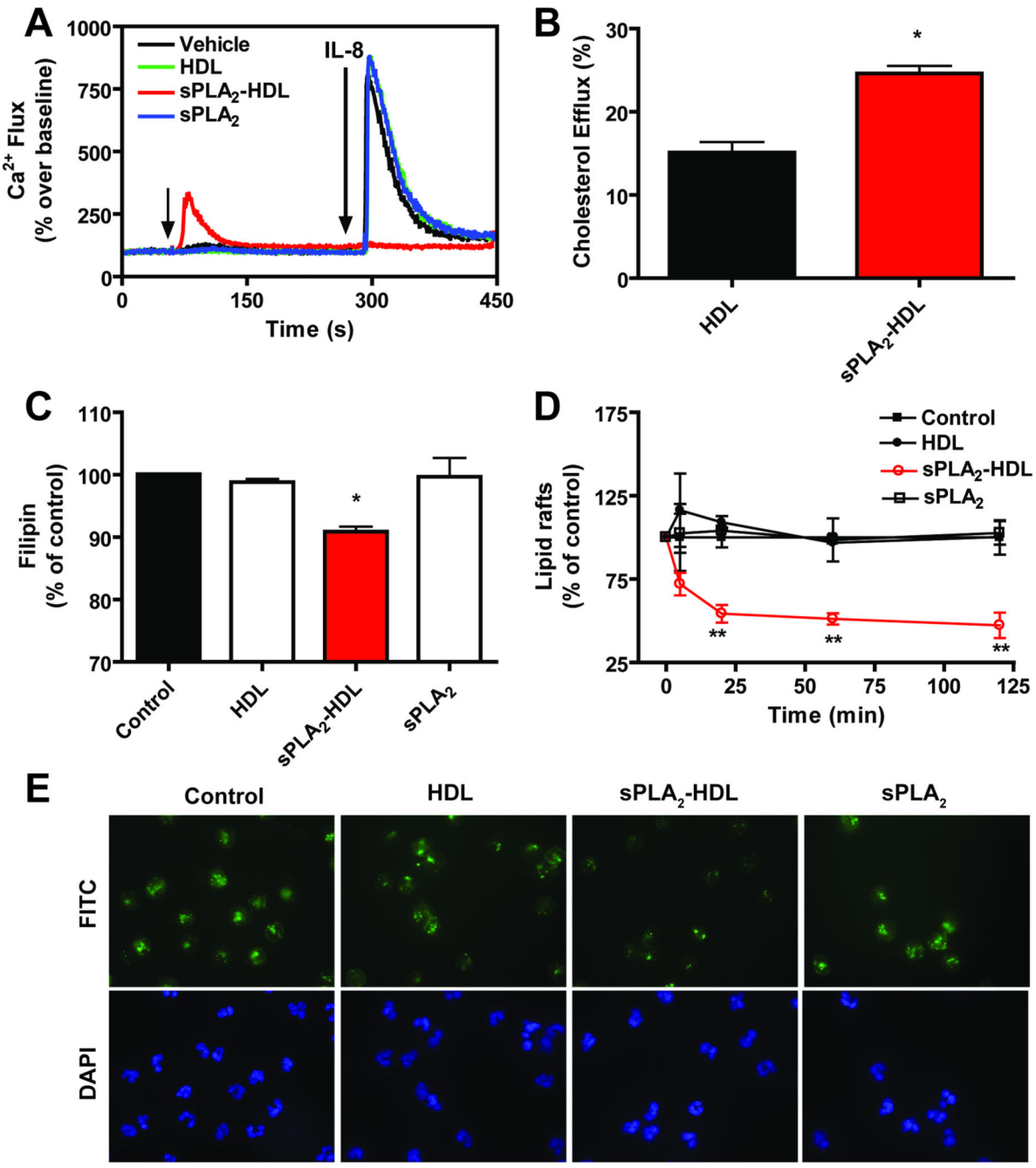

Figure 7. sPLA$_2$-HDL abolishes agonist-induced Ca^{2+} flux and efficiently removes unesterified cholesterol from neutrophils

(A) Baseline Ca^{2+} levels were measured for 1 min and then neutrophils were treated with vehicle, HDL (100 μg protein/ml), sPLA$_2$-HDL (100 μg protein/ml) or sPLA$_2$ for 4 min and Ca^{2+} flux was induced with IL-8 (10 nmol/L). Ca^{2+} flux was detected at room temperature by flow-cytometry as an increase in fluorescence intensity of the Ca^{2+}-sensitive dye Fluo-3-AM in the FL-1 channel. Results show a representative of 4 independent experiments done with cells from different donors. Values are expressed as % over baseline. (B) Isolated neutrophils were preloaded with [^{3}H]-cholesterol for 3 hours and incubated with HDL (100

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μg protein/ml) or $sPLA_2$-HDL (100 μg protein/ml protein) for 60 minutes, and percentage of cholesterol efflux was assessed. Cholesterol efflux is expressed as the radioactivity in the medium relative to total radioactivity in medium and cells. The results are shown as mean ± SD of three separate experiments using PMNL from different healthy donors. Statistical analysis was performed using Student's t-test. *p<0.05, **p<0.01, ***p <0.001 *versus* HDL. (C) Neutrophils were incubated with vehicle, HDL (100 μg protein/ml), $sPLA_2$-HDL (100 μg protein/ml), $sPLA_2$ alone for 30 min at 37°C and stained with Filipin. The amount of unesterified cholesterol was assessed by flow-cytometry. Vehicle treated control was set at 100 % and values are expressed as % of control. Results are shown as mean ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 *versus* control. (D) Neutrophils were incubated with vehicle, HDL (100 μg protein/ml), $sPLA_2$-HDL (100 μg protein/ml) or $sPLA_2$ alone for 5 up to 120 min at 37°C. Lipid raft abundance was determined by assessing FITC-cholera toxin B stained cholesterol-containing microdomains by flow cytometry. Control was set at 100 % and values are expressed as % of vehicle-treated control. Results are shown as mean ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01 *versus* control. (E) Fluorescence microscopy analysis of lipid rafts (green staining). DAPI (blue staining) was used to visualize the nuclei. Original magnification X60 was used. Representative images from 3 independent experiments with neutrophils from different donors are shown.