



High Density Lipoprotein Stimulated Migration of Macrophages Depends on the Scavenger Receptor Class B, Type I, PDZK1 and Akt1 and Is Blocked by Sphingosine 1 Phosphate Receptor Antagonists

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Abstract

HDL carries biologically active lipids such as sphingosine-1-phosphate (S1P) and stimulates a variety of cell signaling pathways in diverse cell types, which may contribute to its ability to protect against atherosclerosis. HDL and sphingosine-1-phosphate receptor agonists, FTY720 and SEW2871 triggered macrophage migration. HDL-, but not FTY720-stimulated migration was inhibited by an antibody against the HDL receptor, SR-BI, and an inhibitor of SR-BI mediated lipid transfer. HDL and FTY720-stimulated migration was also inhibited in macrophages lacking either SR-BI or PDZK1, an adaptor protein that binds to SR-BI's C-terminal cytoplasmic tail. Migration in response to HDL and S1P receptor agonists was inhibited by treatment of macrophages with sphingosine-1-phosphate receptor type 1 (S1PR1) antagonists and by pertussis toxin. S1PR1 activates signaling pathways including PI3K-Akt, PKC, p38 MAPK, ERK1/2 and Rho kinases. Using selective inhibitors or macrophages from gene targeted mice, we demonstrated the involvement of each of these pathways in HDL-dependent macrophage migration. These data suggest that HDL stimulates the migration of macrophages in a manner that requires the activities of the HDL receptor SR-BI as well as S1PR1 activity.

Citation: Al-Jarallah A, Chen X, González L, Trigatti BL (2014) High Density Lipoprotein Stimulated Migration of Macrophages Depends on the Scavenger Receptor Class B, Type I, PDZK1 and Akt1 and Is Blocked by Sphingosine 1 Phosphate Receptor Antagonists. PLoS ONE 9(9): e106487. doi:10.1371/journal.pone.0106487

Editor: Olivier Kocher, Harvard Medical School, United States of America

Received: March 3, 2014; **Accepted:** August 4, 2014; **Published:** September 4, 2014

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Funding: This research was supported by a grant in aid (T 7239) and a Program Grant (PRG 6502) from the Heart and Stroke Foundation of Canada. AA is a recipient of a graduate scholarship from Kuwait University, Faculty of Medicine. LG is a recipient of a graduate scholarship from the Equal Opportunities Canada-Chile Program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Macrophages are phagocytic cells that play a key role in innate host defense against invading pathogens, environmental agents and in clearance of modified/damaged host cells/molecules [1]. Macrophages also play a key role in the development of atherosclerotic vascular disease. Atherosclerosis is characterized by the accumulation of cholesterol-engorged macrophages within the walls of arteries. These so called foam cells appear to be the most abundant cells within atherosclerotic plaques. Atherosclerosis is triggered by the retention of low density lipoprotein (LDL) in the walls of arteries, subsequent modification of LDL, for example by oxidation, and engulfment of modified LDL by macrophages [2–4]. Macrophage endocytosis of modified LDL is mediated by scavenger receptors such as the class A types I and II and CD36 proteins, in a manner that is not regulated by the accumulation of cellular cholesterol [1,3]. This leads to the accumulation of large amounts of intracellular cholesterol stored in cytoplasmic cholesterol ester droplets, giving the cells a characteristic foamy appearance.

A characteristic feature of macrophages in atherosclerotic plaques is their relative inability to migrate. Recently this has been linked to increased cellular cholesterol in macrophage-derived foam cells in atherosclerotic plaques as a consequence of

endocytosis of modified lipoproteins [5–7]. In addition, hypoxia has also been implicated in reduced macrophage migration [8]. Migration of macrophages and dendritic cells, related phagocytic antigen presenting cells, has recently been shown to be critical for their egress out of plaques, a key step in the regression of atherosclerotic plaques [9–14]. Atherosclerotic plaque regression, or the reversal of pre-established atherosclerotic plaques, is an important goal in the design of anti-atherosclerosis therapies which would be administered to patients with pre-established disease [15]. Thus mechanisms for inducing macrophage migration in response to appropriate chemotactic factors which could lead to their egress out of atherosclerotic plaques are important for designing novel therapeutics aimed at stimulating atherosclerotic plaque regression. Animal models and human studies have both demonstrated that atherosclerotic plaque regression can be achieved by decreasing the concentration of circulating LDL and increasing the concentration of circulating high density lipoproteins (HDL) [11,13,16–19].

An inverse relationship between circulating levels of HDL and coronary heart disease has been reported in numerous clinical and epidemiological studies [20–22]. HDL particles as well as HDL associated proteins and lipids were shown to exert a broad scope of potentially anti-atherogenic effects [23–25]. These include the ability to mediate reverse cholesterol transport from atheroscle-

rotic plaque resident foam cells to the liver [26–28]. HDL also exhibits various anti-inflammatory and anti-oxidative properties [23–25]. Short term weekly infusions of reconstituted HDL particles resulted in rapid and significant regression of coronary atherosclerosis in patients with acute coronary syndrome [16]. Similar, though more striking, results have been obtained in apolipoprotein (apo) E knockout (KO) mice injected with reconstituted HDL [29,30]. A study of atherosclerotic plaque regression in mice has reported significant alterations in the expression of a variety of genes in inflammatory cells in the regressing plaques, including significantly increased expression of the scavenger receptor class B, type I (SR-BI) [13].

SR-BI is a high affinity HDL receptor that mediates selective HDL lipid uptake [31]. Data from genetically altered mice demonstrates that overexpression of SR-BI in livers protects against atherosclerosis while knockout of SR-BI either in all tissues or in bone marrow derived cells promotes atherosclerosis [32–34]. The interaction of HDL with SR-BI leads to both bi-directional lipid exchange between the bound particle and cells [31,35] as well as the activation of various signaling pathways [23,25,36]. HDL induced activation of protein kinase C (PKC) was reported in Chinese hamster ovary-derived cells overexpressing SR-BI, and PKC activity, in turn, appears to increase the selective lipid uptake activity of SR-BI [37–39]. HDL dependent signaling mediated by SR-BI is also well demonstrated in endothelial cells, where the interaction of HDL with SR-BI activated various signaling pathways such as the PI3K-Akt, p38 MAPK and ERK1/2 resulting in induction of endothelial nitric oxide synthase (eNOS) and cell migration but suppression of adhesion molecules expression and apoptosis [25,40]. Studies in endothelial cells demonstrated that cholesterol efflux, cholesterol binding to the C-terminal transmembrane domain of SR-BI and the adaptor protein, PDZK1, were all required for HDL-induced signaling [25,41] (reviewed in [42,43]). In contrast little is known about HDL-SR-BI induced signaling in macrophages.

PDZK1 (Postsynaptic Density Protein (PSD-95)/Drosophila Discs-Large (Dlg)/Tight-Junction Protein (ZO1)) is a 519 amino acid, 63 kDa adapter protein that contains four PDZ protein-protein interaction domains [44–46]. The first and third PDZ domain of PDZK1 interact with the last three amino acids, AKL, of SR-BI's C-terminal cytoplasmic tail [45,47,48]. Knockdown of PDZK1 or deletion of the last three amino acids of SR-BI's C-terminal tail has been shown to impair HDL-dependent signaling in endothelial cells [40,41].

Some of the atheroprotective actions of HDL may involve the delivery of bioactive lipids to cells [49–51]. For example, sphingosine-1-phosphate (S1P) and related lysosphingolipids carried by HDL exert atheroprotective effects on the endothelium [51–55]. Reconstituted HDL containing apo A1, phosphatidyl choline and S1P can efficiently deliver S1P to S1P receptors on cells [56,57]. S1P signaling is mediated by a family of five G-protein coupled receptors (GPCR's), the S1P receptors (S1PR's) 1–5, encoded by distinct genes [58,59]. In macrophages, S1P suppresses proinflammatory cytokine production in response to lipopolysaccharide, apparently through S1PR1 [60]. In endothelial cells, S1P increases eNOS activity and abundance and induces cell migration and survival via S1PR1 and S1PR3 [61] while HDL associated S1P inhibits the expression of adhesion molecules via S1PR1 and partially via S1PR3 [40] and improves endothelial barrier function via S1PR1 [62]. S1P induced endothelial cell migration involves Rho kinase [61,63], p38 MAPK [63,64] and PI3-Akt-Rac [65–67] pathways. S1P can be supplied exogenously to cells or generated intracellularly [58,59]. S1P in the circulation is carried primarily by HDL [51–53,55]. This, coupled with SR-

BI's ability to mediate selective lipid uptake from HDL into cells, suggests that SR-BI might mediate HDL signaling in part by mediating the transfer of S1P from HDL into cells.

Given the ability of HDL to stimulate the migration of endothelial cells and the role of SR-BI and S1P receptors in that process, and given the importance of macrophage migration for atherosclerotic plaque regression, we hypothesized that HDL might also directly stimulate the migration of macrophages and that this might be mediated by SR-BI and S1PR's. We demonstrate that HDL can directly induce the migration of macrophages in a manner dependent on both SR-BI and on PDZK1. HDL stimulated migration also depends on S1PR1 activity, and is sensitive to the inactivation of PI3K-Akt1, p38 MAPK, ERK1/2, PKC and Rho kinase signaling pathways, known to be downstream of S1PR1. These data suggest that HDL stimulated macrophage migration may be one pathway by which HDL protects against atherosclerosis development and promotes plaque regression, and that this process is mediated by a signaling pathway involving SR-BI, PDZK1, S1PR1 and Akt1.

Materials and Methods

Ethics statement

All procedures involving mice were approved by the McMaster University Animal Research Ethics Board and were in accordance with the guidelines of the Canadian Council on Animal Care. All mice used were euthanized humanely under general anesthesia.

Materials

All materials for cell culture, alexafluor-488-phalloidin and SYBR green were from Invitrogen/Life Technologies Inc. (Burlington ON, Canada). HDL, acetylated LDL (AcLDL) and apoA1 were from Biomedical Technologies, Inc (Stoughton MA, USA). Corning Transwell inserts, cell culture plastic-ware and Camco Quik Stain II were from VWR International (Mississauga, ON Canada). W146 and VPC23019 were from Avanti Polar Lipids (Alabaster AL, USA). Wortmannin, LY294002, SB203580, PD98059, Go6976 and Ro31-8220 were from EMD Millipore Corp. (Billerica, MA, USA). FTY720, SEW2871 and Y-27632 were from Cedarlane Labs (Burlington, ON Canada). Rat tail collagen I and FITC-labeled anti-rabbit IgG were from BD Biosciences (Mississauga, ON, Canada). Reagents for RNA isolation and cDNA synthesis were from Qiagen Inc. (Toronto, ON, Canada). All other suppliers were as indicated. All other reagents, including primers for qRT-PCR were from Sigma-Aldrich Canada Co. (Oakville, ON, Canada).

Mice

All mice were on a C57BL6 background, except PDZK1 KO mice, which were on a mixed C57BL/6J X 129S6 background. C57BL6 mice were bred from founders purchased from the Jackson Laboratories (Bar Harbor ME, USA). SR-BI KO mice, originally provided by Monty Krieger (Massachusetts Institute of Technology), were backcrossed 10 times onto a C57BL/6J background before interbreeding. Akt1, 2 and 3 KO mice were bred from founders provided by Morris Birnbaum (University of Pennsylvania). PDZK1 KO mice and corresponding WT mice were from Jackson Laboratories. All mice were bred and housed in the Thrombosis and Atherosclerosis Research Institute Division of Comparative Medicine and provided with free access to food and water.

Cells and cell culture

Cells were cultured at 37°C in an atmosphere of 5% CO₂ in air. RAW 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2mM L-glutamine and 50 µg/ml penicillin/streptomycin and were passaged by scraping. Primary mouse peritoneal macrophages (MPM's) were isolated from mice injected intraperitoneally on day 0 with 1ml of 10% thioglycolate. On day 4 the mice were anesthetized with isoflurane gas and euthanized humanely by cervical dislocation and peritoneal cells were collected with 10 ml PBS containing 5mM EDTA. The cells were washed once in DMEM containing 20% FBS and plated in DMEM containing 10% FBS (8×10⁶ cells/10 cm dish). Foam cells were prepared by incubating MPM's with AcLDL (100 µg protein/ml) for 48 hrs. Prior to migration experiments, cells were washed twice with serum free media and cultured for 16 hrs either in the absence of serum or in the presence of 3% newborn calf lipoprotein deficient serum (NCLPDS) [68] as indicated.

Macrophage migration and adhesion assays

Macrophage migration was tested in a chemotaxis assay [5,7] using Transwell inserts with pore size of 5 µm that were pre-coated with rat tail collagen I (110 µg/ml). Cells (4×10⁵) were added to the upper chamber and incubated for 2 hrs at 37°C in media containing 5% NCLPDS. The media was then removed and replaced with media containing 0.5% NCLPDS. These filter inserts were placed in wells containing the same media with either no further additives or with one of the following: HDL or apoA1 (100 µg protein/ml) [69], FTY720 (2 ng/ml) [70] or monocyte chemotactic protein-1 (MCP-1; 100 ng/ml [71], Fitzgerald Industries International, Acton, MA USA). In migration assays testing the effects of immunological or pharmacological inhibitors, the cells were pre-incubated with the inhibitor for 30 min and the migration assays were performed in the continued absence or presence of the inhibitors. The following compounds were used at concentrations previously reported to be effective: anti-SR-BI blocking antibody (KKB-1, 0.5 µg/ml [72], generously provided by Karen Kozarsky, ReGenX Biosciences), BLT-1 (0.3 µM [37,73], ID 5234221, ChemBridge Corp.), VPC23019 (10 µM [74], pertussis toxin (PTX, 100 ng/ml) [75], LY294002 (10 µM [76], SB203580 (1 µM) [77], PD98059 (10 µM) [78], Go6976 and Ro31-8220 (5 µM) [79], W-146 (10 µM) [80], SEW2871 (5nM) [81] and Y-27632 (10 µM) [82]. The following reagents were added as 1000× stock solutions in DMSO: BLT-1, VPC23091, LY294002, SB203850, PD98059, Go6976, Ro31-8220, W-146, and Y-27632. PTX was prepared as a 100 µg/ml stock in sterile water containing 2 mg/ml BSA. SEW2871 was prepared as a 100 mM stock in dimethylformamide. For each experiment, all samples were treated with an equivalent final concentration of solvent vehicle for each reagent used. After 4 hrs, cells on the filters were fixed and stained with CamcoQuick Stain II or with 300 nM 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min and rinsed twice with water. The non-migrated cells on the upper surface of the filters were removed by carefully scraping with a cotton swab and the cells that had migrated to the lower face of each filter were visualized by bright-field or fluorescent microscopy (using a Carl Zeiss Axiovert 200 M inverted microscope with a 10× objective) and counted. Cell adhesion to collagen I was tested by plating the same number of cells (as for migration assays) in each well of a 96 well dish pre-coated with rat tail collagen I under the same conditions as those used to coat the Transwell filters. After 4 hrs non-adhered cells were washed away and adherent cells were fixed, stained as above with DAPI, and cell adhesion was measured by counting DAPI stained nuclei.

Phalloidin staining

Cells were cultured on sterile, untreated glass coverslips, as above, in the presence or absence of HDL (100 µg protein/ml) for different times. The cells were then washed twice with PBS, fixed with 3% paraformaldehyde pH 7.4 for 30 min and permeabilized with 0.1% Triton-X100 for 5 min. at room temperature. F-actin was stained using 25units/ml Alexafluor488 phalloidin in PBS for 30 min at room temperature. The coverslips were then mounted using Vectasheild (Vector Laboratories Canada Inc. Burlington, ON, Canada) and imaged using a Zeiss Axiovert 200 M fluorescence microscope with standard FITC filters using a 40× objective.

SDS-PAGE and immunoblotting

RAW 264.7 cells were serum starved overnight and incubated with HDL (100 µg protein/ml) for different times, washed and lysed in ice cold lysis buffer containing: 0.2xPBS, 0.1% Triton-X100, 1× Phosphatase Inhibitor Cocktail 2 (Sigma Aldrich catalogue number P5726) and protease inhibitors (20 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM APMSF and 10 µg/ml pepstatinA). To prepare total membranes, MPMs were homogenized on ice for 1 min in 20 mM Tris-HCl, pH 7.5 containing 2 mM MgCl₂, 0.25 M sucrose, and protease inhibitors with the concentrations indicated above. Homogenates were centrifuged at 3000xg for 10 min at 4°C and the supernatant was subjected to another centrifugation step at 100,000xg for 1 hr at 4°C. The pellet was then suspended in 10 mM sodium phosphate, pH 7.0 containing the protease inhibitors listed above. After boiling, the samples were subjected to SDS-PAGE followed by immunoblotting with rabbit anti-Akt, rabbit anti-phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA). HRP-conjugated donkey-anti-rabbit and donkey-anti-mouse antibodies (Jackson ImmunoResearch Labs Inc, West Grove PA, USA) were used as secondary antibodies and were detected using Western Lightning ECL reagent kit (PerkinElmer Canada, Woodbridge ON, Canada).

Flow cytometry

SR-BI surface expression was measured in unfixed non-permeabilized MPMs. Macrophages were treated with rat anti-CD16/32 (eBioscience Inc, San Diego, CA, USA) to block FC receptors then incubated with rabbit anti-SR-BI antibody (KKB-1, 0.5µg/ml) followed by a FITC-conjugated anti-rabbit IgG. Cell sorting was performed using BD FACSCalibur instrument and data was analyzed using Cell Quest Pro software [73].

Quantitative RT-PCR

Total RNA was extracted from lungs and peritoneal macrophages of wild type mice using RNeasy kit. cDNA was generated from 1 µg of total RNA using Quantitec Reverse Transcription kit. Real-time PCR was performed using the SYBR green detection method as described [83]. Primers were as follows[84]: Mouse S1PR1 forward 5'-ACT TTG CGA GTG AGC TG-3' reverse 5'-AGT GAGCCT TCA GTT ACA GC-3'; S1PR2 forward 5'-TTC TGG AGG GTA ACACAG TGG T-3' reverse 5'-ACA CCC TTT GTA TCA AGT GGC A-3'; S1PR3 forward 5'-TGG TGT GCG GCT GTC TAG TCA A-3' reverse 5'-CAC AGC AAG CAG ACC TCC AGA-3'; GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3' reverse 5'-TCCAC-CACCCTGTTGCTGTA-3'. Results were calculated as described [85].

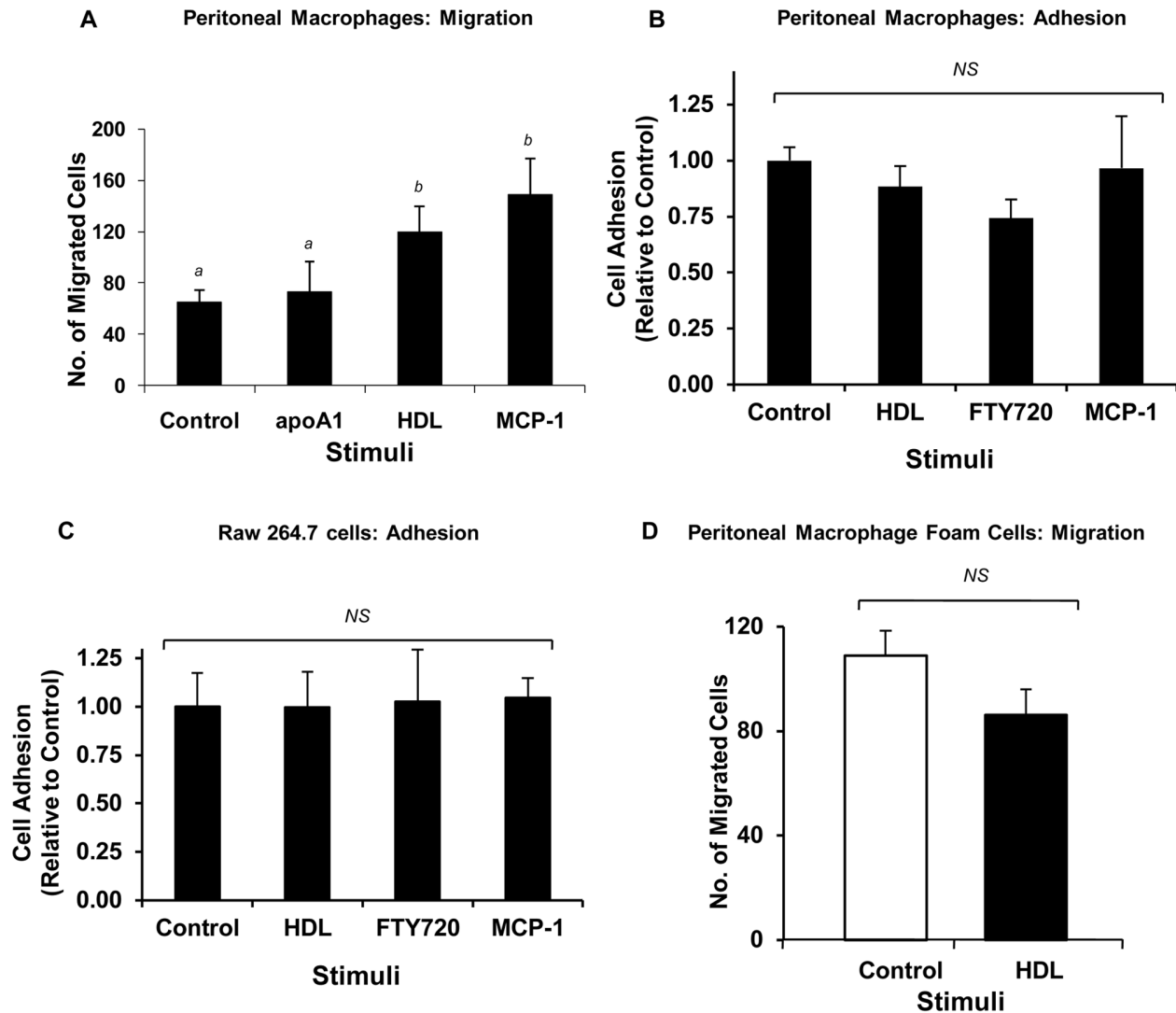


Figure 1. HDL stimulates the migration of macrophages but not of lipid loaded macrophage foam cells. **A.** Wild type mouse peritoneal macrophages were incubated in lipoprotein deficient serum overnight and cell migration in response to no stimulus (control), apoA1(100 μ g/ml), HDL (100 μ g protein/ml) or MCP-1 (100 ng/ml) (each added to the bottom well of the migration assay chamber) was performed as described in the Methods section. The number of migrated cells/well from three independent samples from each group is represented as the mean \pm standard deviation. **B.** Wild type mouse peritoneal macrophages, or **C.** RAW 264.7 cells were incubated in collagen I coated cell culture dishes with either HDL (100 μ g protein/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) under conditions paralleling the migration assay. Cell adhesion was measured by counting DAPI stained nuclei. The degree of cell adhesion was normalized to that in control cells and is represented as the mean \pm standard deviation of triplicates. **D.** Foam cells from wild type mouse peritoneal macrophages were generated by culture for 48 hrs in the presence of AcLDL (100 μ g/ml). Cells were washed and migration in response to HDL was measured as described in the Methods section. The number of migrated cells/well is plotted as the mean \pm standard deviation of triplicates. Statistical analysis was done using one way ANOVA with Holm-Sidak post hoc test (A–C) or Student's T-test (D). Values identified with different letters are statistically significantly different ($P < 0.05$). NS indicates that no statistically significant difference was detected.

doi:10.1371/journal.pone.0106487.g001

Statistical analysis

Data was analyzed with Sigma Plot Software using the Student's T-test for pairwise comparisons between 2 groups, or using one way ANOVA with Holm-Sidak post hoc test or two way ANOVA with Tukey's post hoc test for comparisons of multiple groups where appropriate and was considered statistically significant when $P < 0.05$.

Results

HDL induces macrophage migration in SR-BI and PDZK1 dependent manner

We used a standard three-dimensional Transwell migration assay to examine if HDL might stimulate macrophage migration. In this set up, cells were added to the upper compartment of a cell culture dish, separated from the lower compartment by a collagen I coated membrane with 5 μ m diameter pores. After allowing the cells to adhere to the collagen I coated membrane for 2 hrs, HDL,

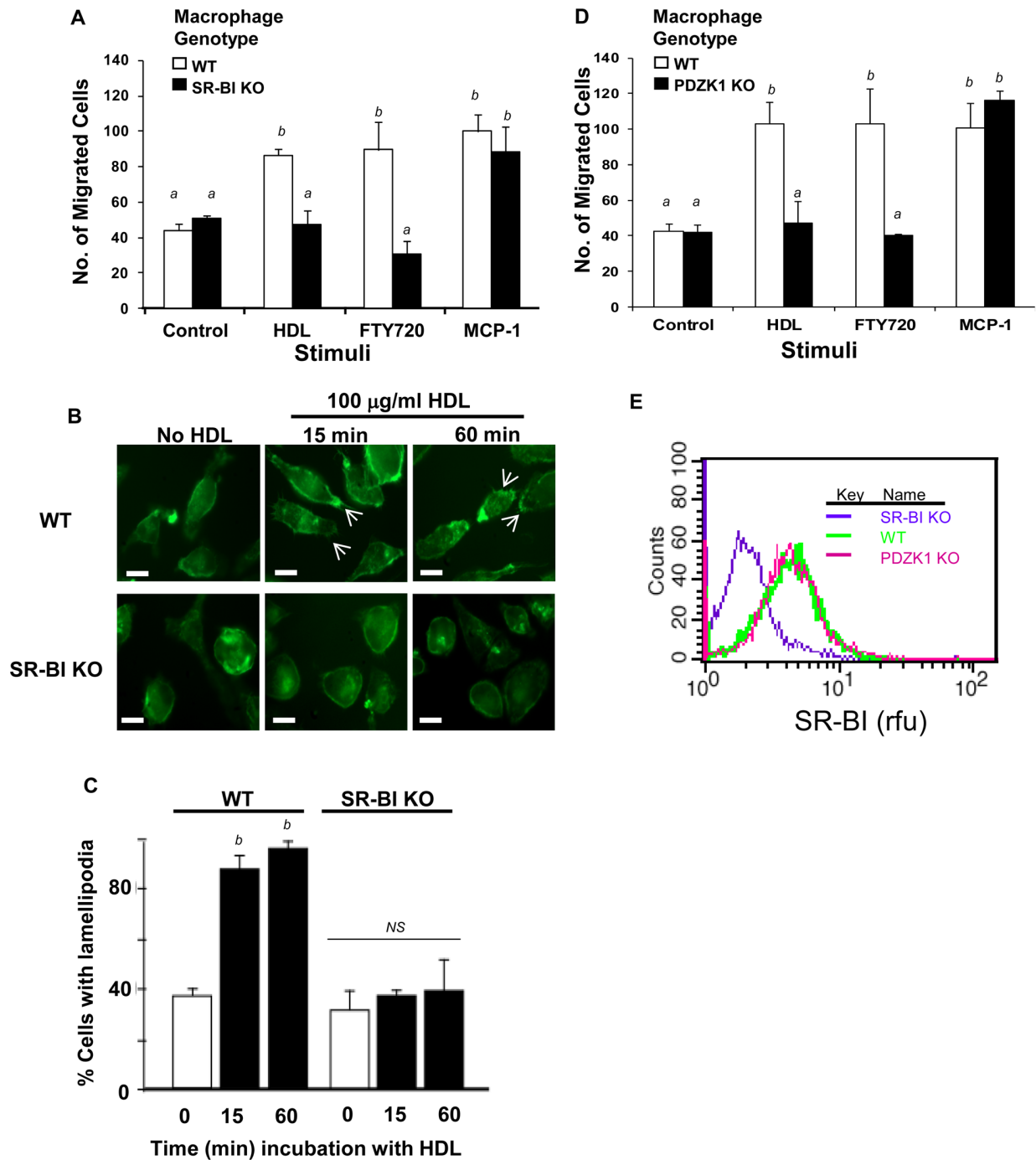


Figure 2. HDL-induced migration is reduced in macrophages deficient in either SR-BI or PDZK1. **A.** Peritoneal macrophages were prepared from either wild type (WT) or SR-BI KO mice, and cultured in the presence of 3% NCLPDS for 16 hrs before analysis. Migration of cells in response to no stimulus (control), HDL (100 μ g protein/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) as described in the legend to Figure 1 and the Methods section. **B.** Peritoneal macrophages from WT or SR-BI KO mice were cultured as described in A, prior to incubation with or without HDL (100 μ g protein/ml) for the times indicated. Actin filaments were visualized by fluorescence microscopy after alexa 488-phalloidin staining. Representative images are shown. Scale bars = 10 μ m. **C.** Numbers of cells with lamellipodia (arrows in B) were counted (~100 cells over 4–5 fields) for cells isolated from three mice from each genotype. **D.** Migration of peritoneal macrophages from WT or PDZK1 KO mice as described for panel A. **E.** Flow cytometry analysis of cell surface SR-BI levels in wild type, SR-BI KO and PDZK1 KO macrophages. Values in A, C and D are means \pm standard deviations of triplicates and are representative of multiple independent assays. Data in E is representative of multiple analyses. Statistical analysis in panels A, C and D was done using two way ANOVA with Tukey post hoc test. Values identified with different letters are statistically significantly different ($P < 0.001$). NS indicates that no statistically significant difference was detected. doi:10.1371/journal.pone.0106487.g002

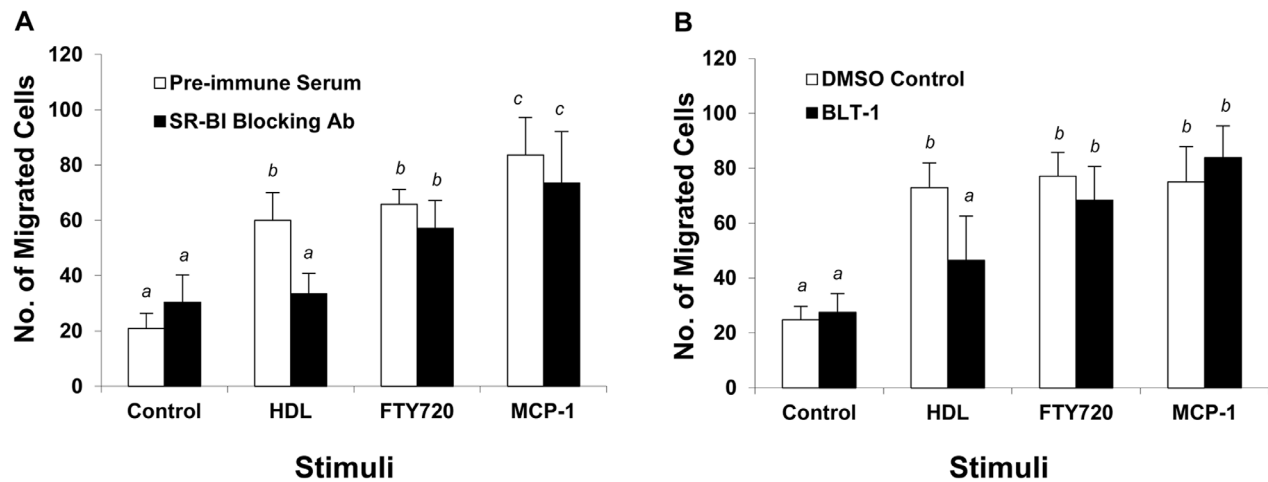


Figure 3. Antibody or small molecule mediated inactivation of SR-BI inhibits HDL dependent but not FTY720 dependent macrophage migration. Mouse peritoneal macrophages were pretreated for 30 min with either **A.** An anti-SR-BI blocking antiserum or pre-immune rabbit serum (0.5 μ g/ml); or **B.** BLT-1 (0.3 μ M), an inhibitor of SR-BI mediated lipid transfer, or DMSO vehicle control. Cell migration in response to HDL (100 μ g protein/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) was measured in the continued presence of the inhibitors as described in the legend to Figure 1 and the Methods section. Data are means \pm standard deviations of 6 replicates. Statistical analysis was done using two way ANOVA with Tukey post hoc test. Values identified with different letters are statistically significantly different ($P < 0.002$). doi:10.1371/journal.pone.0106487.g003

lipid free apoA1, the major protein component of HDL, and MCP1 were added to the lower compartment of individual wells. We also included, as a control, wells in which no stimulus was added. After 4 hrs at 37 $^{\circ}$ C, inserts were removed and the numbers of macrophages that had migrated from the upper to the lower face of the porous membrane were quantified. MCP-1, a well-known chemotactic factor for macrophages, at 100 ng/ml, stimulated a greater than 2-fold increase in migration of wild type mouse peritoneal macrophages from the upper to the lower face of the porous membrane. HDL, but not lipid free apoA1, at 100 μ g protein/ml, induced a similar increase in migration (Figure 1A). To ensure that increased macrophage migration stimulated by either MCP-1 or HDL was not merely the consequence of increased macrophage adhesion to collagen I, which coated the porous membrane of the migration chamber, we tested the effects of each of these on the adhesion of macrophages to collagen I coated wells of a 96 well microtitre dish. Neither HDL, MCP-1, nor the known chemotactic factor FTY720, affected adhesion of either primary mouse peritoneal macrophages (Figure 1B) or RAW 264.7 cells (Figure 1C) to collagen I. In contrast to freshly isolated macrophages, peritoneal macrophages that were lipid-loaded by culture for 48 hrs in the presence of AcLDL failed to migrate in response to HDL (Figure 1D). This is consistent with previously reported findings that cholesterol loading of macrophages reduced their ability to migrate [5,7].

The HDL receptor SR-BI has been reported to mediate HDL signaling leading to migration of endothelial cells (25). To explore if SR-BI was required for macrophage migration in response to HDL we examined the ability of peritoneal macrophages prepared from SR-BI KO mice to migrate in response to HDL (Figure 2A). Unlike macrophages from wild type mice, SR-BI KO macrophages were not able to migrate in response to HDL. Similarly, they were also unable to migrate in response to the S1PR agonist, FTY720, although they showed normal migration in response to MCP-1 (Figure 2A). Exposure of SR-BI expressing wild type mouse peritoneal macrophages to 100 μ g/ml HDL resulted in a relatively rapid (within 15 min) rearrangement of the

actin cytoskeleton and increased the formation of lamellipodia, as revealed by fluorescence microscopic analysis of fixed cells stained with alexa-488 labeled phalloidin (Figure 2B, upper panels, quantified in Figure 2C). Similar results were obtained using murine RAW 264.7 macrophage-like cells (not shown). These alterations in the actin cytoskeleton in response to HDL were not observed in peritoneal macrophages prepared from SR-BI deficient mice (Figure 2B, lower panels, quantified in Figure 2C). Peritoneal macrophages prepared from PDZK1 KO mice were also unable to migrate in response to HDL or to FTY720, although their ability to migrate in response to MCP-1 was unchanged (Figure 2D). Flow cytometry analysis confirmed that as previously reported by others, [86], knockout of PDZK1 did not alter the levels of SR-BI on the cell surface of macrophages (Figure 2E).

To determine if acute inactivation of SR-BI-mediated HDL binding or lipid transfer activity affected the ability of cells to migrate in response to HDL or FTY720, we tested the effects of pre-treating cells with either an anti-SR-BI blocking antibody, previously reported to prevent HDL binding to SR-BI, or with a small molecule, BLT-1 previously reported to selectively inhibit SR-BI mediated lipid transfer between bound HDL and cells without inhibiting HDL binding to SR-BI [73]. Immunological blockade of SR-BI substantially reduced HDL stimulated migration of peritoneal macrophages from wild type mice (Figure 3A). Treatment with BLT-1 also reduced HDL stimulated migration of peritoneal macrophages from wild type mice (Figure 3B). This suggested that both HDL binding and lipid transfer activities of SR-BI are required for HDL stimulated migration of macrophages. Neither treatment affected the ability of macrophages to migrate in response to FTY720 or to MCP-1 or (Figure 3A, B). Therefore, acute inhibition of SR-BI selectively impairs migration in response to HDL; however unlike macrophages lacking SR-BI expression, those in which SR-BI activity is acutely blocked are still able to migrate in response to the S1PR pro-agonist, FTY720.

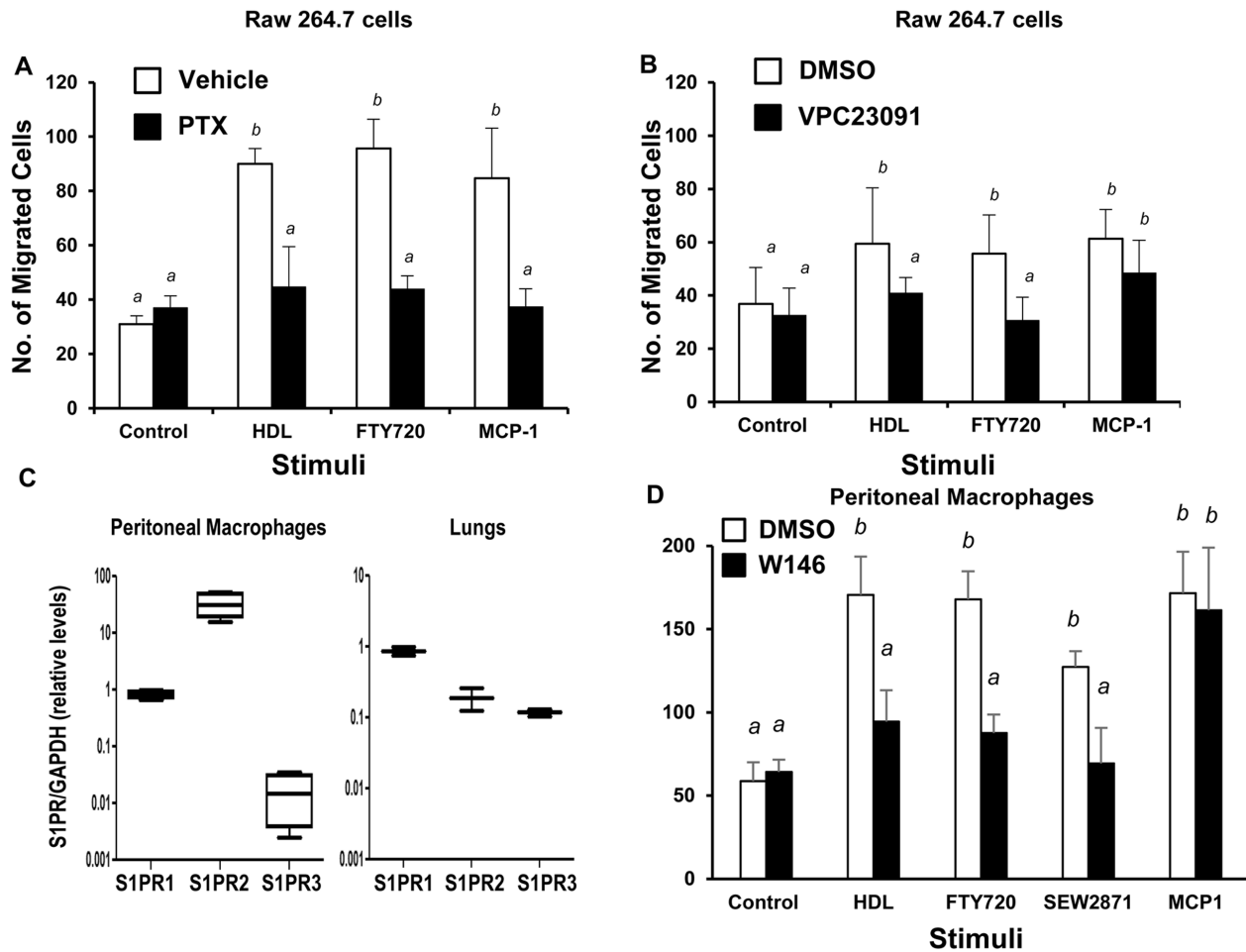


Figure 4. Blockade of S1PR1 prevents HDL stimulated macrophage migration. RAW 264.7 (panels A,B) or mouse peritoneal macrophages (panel D) were pretreated for 30 min with the following: **A.** PTX (100 ng/ml) an inhibitor of $G\alpha_i$ protein coupled receptors; **B.** VPC23019 (10 μ M) an antagonist of S1PR's 1, and 3; or **D.** W-146 (10 μ M) an antagonist specific for S1PR1. Control cells were treated with either 2 μ g/ml BSA or DMSO vehicle as indicated. Cell migration in response to HDL (100 μ g protein/ml), FTY720 (2 ng/ml), MCP-1 (100 ng/ml) and/or SEW2871 (5 nM) was measured in the continued absence or presence of the inhibitors as described in the legend to Figure 1 and the Methods section. **C.** Analysis of expression of S1PR1, 2 and 3 in peritoneal macrophages (left panel) or lung tissue (right panel) from wild type mice. Data are means \pm standard deviations of 6 replicates (A,B) or 3 replicates (C,D). Statistical analysis was done using two way ANOVA with Tukey post hoc test. Values identified with different letters are statistically significantly different ($P < 0.002$). doi:10.1371/journal.pone.0106487.g004

HDL stimulated migration is dependent on a sphingosine-1-phosphate receptor

FTY720 is a pro-agonist of S1PRs and S1P is a component of HDL. We therefore tested the effects of different inhibitors of S1PRs on macrophage migration stimulated by HDL, FTY720, and MCP-1. Like primary wild type mouse peritoneal macrophages (e.g. Figure 2 A), murine RAW 264.7 macrophage like cells were stimulated to migrate by HDL, FTY720 and MCP-1 (Figure 4 A,B). PTX, an inhibitor of the $G\alpha_i$ subunit of heterotrimeric G proteins [87], inhibited the migration of RAW 264.7 cells in response to both FTY720 and, as expected, MCP-1 (Figure 4A), which acts through the chemokine receptor 2, known to be PTX-sensitive [88]. PTX treatment also inhibited migration in response to HDL (Figure 4A), indicating the involvement of a GPCR coupled to $G\alpha_i$ in macrophage responses to HDL. Treatment of RAW 264.7 cells with VPC23019, an antagonist selective for S1PR1 and S1PR3 [89], blocked migration induced by FTY720 and HDL but not by MCP-1 (Figure 4B). We

examined gene expression levels of three S1P receptors, S1PR1, 2 and 3 in mouse peritoneal macrophages and, for comparison, mouse lung tissue. Macrophages expressed S1PR1; S1PR2 levels were greater than 10-fold higher, whereas S1PR3 levels were approximately 100-fold lower than S1PR1 (Figure 4C). This is consistent with other reports of higher expression of S1PR1 than S1PR3 in macrophages [60,90]. On the other hand, in lungs, S1PR1 levels appeared highest and S1PR2 and 3 levels were similar (at almost 10-fold lower than S1PR1). We focused our attention on S1PR1 as a potential mediator of HDL dependent stimulation of migration in macrophages because 1) HDL and FTY720 induced migration was blocked by PTX and VPC23019 which impair S1PR1 and 3 but not S1PR2 signaling [89], 2) S1PR1 expression in macrophages appeared to be much higher than S1PR3, and 3) S1PR2 has previously been reported to negatively regulate macrophage migration [91]. To confirm the involvement of S1PR1 in HDL stimulated macrophage migration, wild type mouse peritoneal macrophages were treated with the

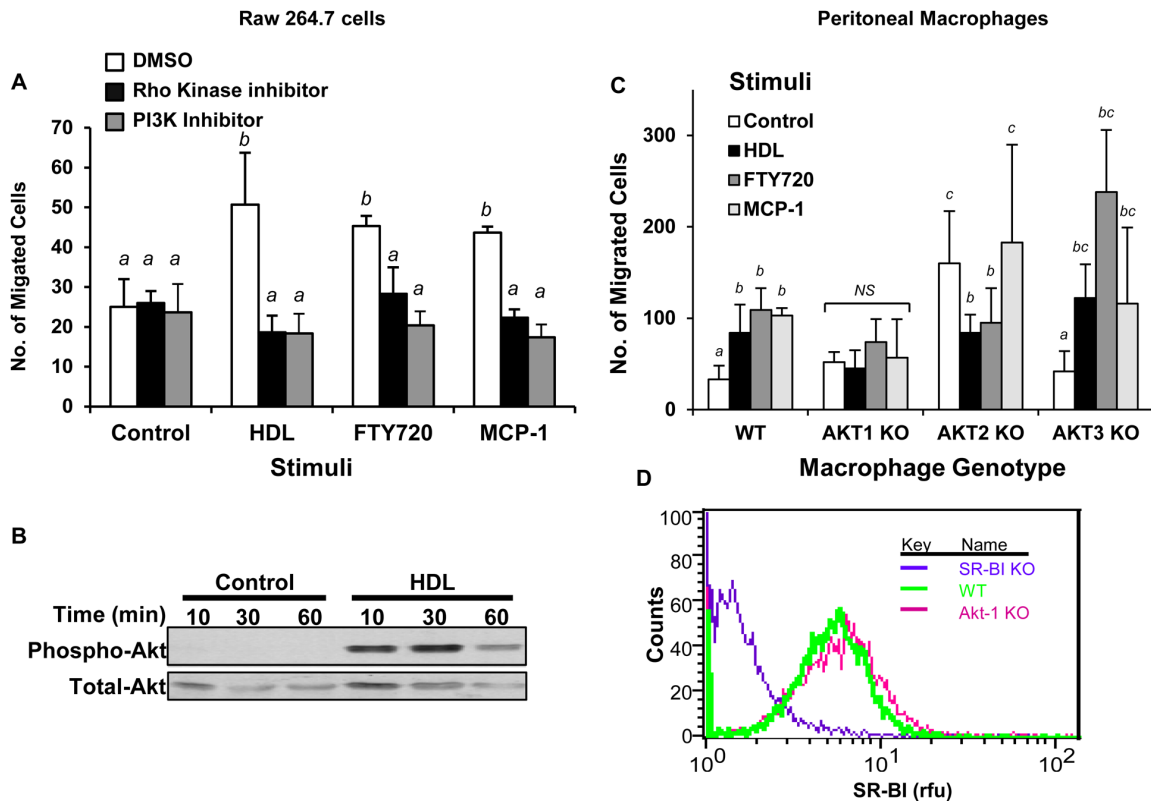


Figure 5. HDL stimulated macrophage migration involves Rho kinase and PI3K-Akt 1 signaling. **A.** RAW 264.7 cells were cultured in media containing 3% NCLPDS for 18 hrs. Cells were pre-incubated with 10 μ M of either the Rho Kinase inhibitor, Y-27632, or the PI3K inhibitor LY294002, or with DMSO vehicle for 30 min and then the migration in response to HDL (100 μ g protein/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) was measured in the continued presence or absence of the indicated inhibitors. **B.** RAW 264.7 cells were serum starved for 18 hrs, washed and treated with or without HDL (100 μ g protein/ml) for 10, 30 or 60 min. Equal amounts of proteins were analyzed by SDS-PAGE and immunoblotting for either phospho-Ser473- or total-Akt. **C.** MPM's were harvested from 6 independent WT, Akt1 KO, Akt2 KO or Akt3 KO mice. Migration in response to no stimulus, HDL (100 μ g protein/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) was measured. Data from A and C are means \pm standard deviations of 6 replicates done over two independent experiments. Values identified with different letters are statistically significantly different ($P < 0.003$, 2 way ANOVA with Tukey post hoc test). **D.** Flow cytometry analysis of cell surface SR-BI levels in wild type, SR-BI KO and Akt1 KO macrophages. Shown are representative histograms of an experiment performed twice. doi:10.1371/journal.pone.0106487.g005

S1PR1-specific agonist, SEW2871, and antagonist W146 (Figure 4D). SEW2871, like HDL and FTY720, stimulated macrophage migration. Furthermore, HDL-, FTY720- and SEW2871-stimulated macrophage migration were blocked by treatment with the S1PR1 specific antagonist, W146 (Figure 4D). In contrast, W146 did not affect migration stimulated by MCP-1 (Figure 4D). Together, these data suggest that S1PR1 is required for HDL stimulated migration of macrophages.

HDL stimulated macrophage migration is mediated by multiple downstream kinases

Cell migration triggered by GPCR signaling has been reported to involve PI3K dependent pathways and PI3K independent pathways involving RhoA and Rho-kinase [92-97]. Consistent with this, pretreatment of RAW 264.7 cells with the Rho kinase inhibitor Y27632 or the PI3K inhibitor LY294002, did not affect basal migration, but reduced migration in response to HDL, FTY720 and MCP-1 (Figure 5A). Wortmannin, another PI3K inhibitor, had similar effects on HDL stimulated migration (data not shown). PI3K and protein kinase B/Akt have been implicated in driving initial steps of cell polarization and migration in response to various stimuli [61,98-102]. HDL (100 μ g protein/ml)

treatment of RAW 264.7 cells induced Akt phosphorylation as early as 10 min, with a peak at 30 min and diminished levels by 60 min (Figure 5B).

To determine which Akt isoform might mediate HDL stimulated migration in macrophages, we examined the ability of HDL to stimulate migration of peritoneal macrophages from KO mice lacking expression of either the Akt 1, 2 or 3 genes. Akt1 KO peritoneal macrophages exhibited slightly elevated basal migration compared to peritoneal macrophages from wild type mice (Figure 5C), but exhibited no increased migration in response to HDL, FTY720 or MCP-1. Akt2 KO peritoneal macrophages exhibited a five-fold increase in migration in the absence of stimulation compared to wild type cells. Migration of Akt2 KO macrophages was substantially reduced by HDL or FTY720, but was not affected by MCP-1, suggesting that Akt2 KO MPMs were still able to respond to both HDL and FTY720, albeit in the opposite manner as did wild type MPMs. On the other hand, HDL, FTY720 and MCP-1 were able to stimulate increased migration of Akt3 KO macrophages (Figure 5C) suggesting that Akt3 was not required for migration in response to these stimuli. The inability of Akt1 KO MPMs to respond to HDL was not due to differences in SR-BI expression levels at the cell surface

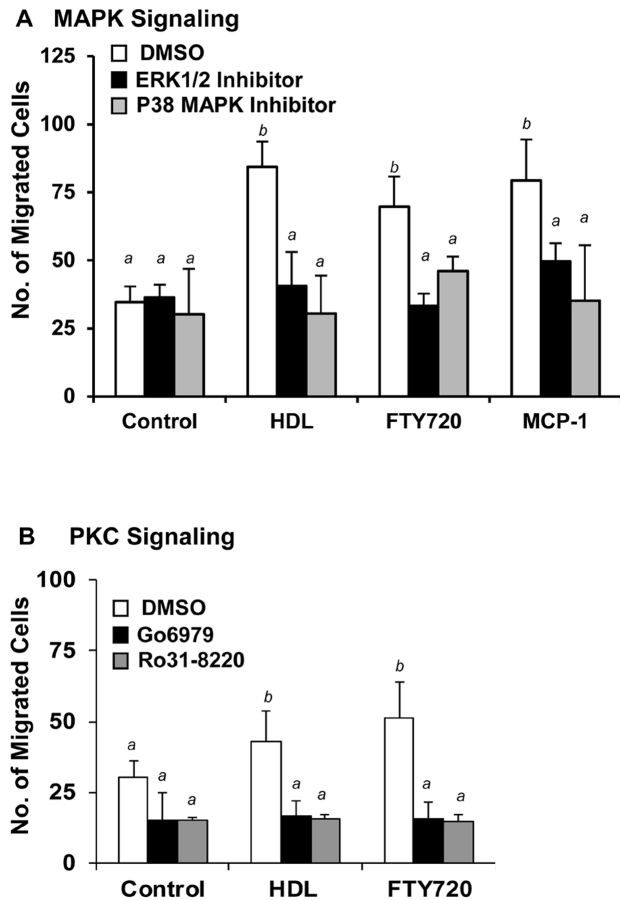


Figure 6. HDL stimulated macrophage migration involves MAP kinase and PKC pathways. RAW 264.7 cells were cultured in media containing 3%NCLPDS for 18 hrs. Cells were pre-incubated with the indicated inhibitors for 30 min and then the migration in response to HDL (100 μ g protein/ml), FTY720 (2 ng/ml) or MCP-1 (100 ng/ml) was measured in the continued presence or absence of the indicated inhibitors. Control cells were treated with DMSO vehicle. **A.** Cells were treated with either 10 μ M of the ERK1/2 pathway inhibitor, PD98059, or 1 μ M of the p38 MAPK inhibitor, SB230580. **B.** Cells were treated with 5 μ M of the PKC inhibitors Ro31-8220 or Go6976. Data are means \pm standard deviations of 3 replicates. Values identified with different letters are statistically significantly different ($P < 0.04$, 2 way ANOVA with Tukey post hoc test). doi:10.1371/journal.pone.0106487.g006

(Figure 5D). Similarly, Akt2 and Akt3 KO MPMs had normal cell surface levels of SR-BI (data not shown).

HDL induced activation of ERK1/2 and p38 MAPK has been reported in vascular smooth muscle cells, endothelial cells and in Chinese hamster ovary cells overexpressing SR-BI [37,103,104]. Therefore we tested the effects of inhibiting these kinase pathways on the ability of macrophages to migrate in response to HDL. Inhibition of the ERK1/2 pathway with PD98059, an inhibitor of ERK1/2 phosphorylation, did not affect basal macrophage migration but prevented macrophage migration in response to HDL, FTY720, and MCP-1 (Figure 6A). Similarly, inhibition of p38 MAPK activity with SB230580 also had no effect on basal macrophage migration but prevented migration in response to HDL, FTY720 and MCP-1 (Figure 6A). This is consistent with reports that both ERK1/2 and p38 MAPK pathways are involved in HDL and S1P induced migration of endothelial cells [61].

HDL also induces the activation of PKC in different cell types [37,38,105], and S1P induced cell migration has been shown to require PKC activity [106]. We therefore tested the involvement of PKC activity using a general PKC inhibitor, Ro31-8220, and an inhibitor of Ca^{2+} -dependent PKC isoforms, Go6976 [107,108]. Both of these inhibitors reduced basal migration and completely blocked the induction of migration of macrophages in response to HDL or FTY720 (Figure 6B), suggesting the involvement of a Ca^{2+} -dependent PKC in HDL or FTY720 stimulated macrophage migration.

Discussion

Macrophages are normally motile cells; however a number of studies have suggested that macrophages in atherosclerotic plaques exhibit impaired migration [5,7]. Under some circumstances, however, migration can be re-established in macrophages in atherosclerotic plaques, and this appears to be important for atherosclerotic plaque regression, e.g. under conditions of elevated HDL levels [10,11,13]. The migration of MPM in response to native and modified lipoproteins has previously been demonstrated, however the underlying mechanisms were not investigated [109]. In the present study we show that HDL, but not lipid free apoA1 induced macrophage migration (Figure 1). This finding is consistent with studies that investigated the role of apoA1 in eNOS activation in endothelial cells, where it was shown that apoA1 is a necessary component of HDL but that lipid free apoA-I is not sufficient for stimulation of eNOS activation [110].

Cell migration is a highly integrated, multistep process that involves dynamic rearrangement of the actin cytoskeleton and an extensive cross talk between signaling molecules at the leading edge of a migrating cell followed by a down regulation of adhesion molecules at the trailing end of the cell, reviewed in [92]. Lamellar extension is one of the early morphological changes in response to chemotactic stimuli [92]. We found that treatment of macrophages with HDL resulted in rapid (within 15 min) formation of lamellipodia in wild type macrophages but not those lacking SR-BI (Figure 2 B,C), consistent with the ability of HDL to stimulate chemotaxis of wild type macrophages but not macrophages in which SR-BI was inactivated or inhibited (Figure 2A, Figure 3), or macrophages that lacked the adaptor protein PDZK1 (Figure 2D) that binds to the C terminus of SR-BI. Our data also demonstrates that HDL induced the migration of macrophages in a manner that is sensitive to antagonists of the G-protein coupled receptor, S1PR1 (Figure 4A, B, D). The ability of an anti-SR-BI blocking antibody and an inhibitor of SR-BI mediated lipid transfer, BLT-1, to inhibit HDL but not FTY720 or MCP-1 induced macrophage migration (Figure 3) demonstrate that HDL binding to SR-BI and SR-BI mediated lipid transfer activities are both required for macrophage migration in response to HDL, but not for migration in response to other chemotactic factors, including the S1P receptor pro-agonist FTY720 or MCP-1. This suggests that SR-BI may be involved in the uptake of HDL associated lipids such as sphingosine and/or S1P and may mediate their delivery to S1PRs, whereas the need for SR-BI activity can be bypassed in the absence of HDL by direct stimulation of S1PR1 with FTY720 (Figure 7). Alternatively, we cannot exclude the possibility that SR-BI mediated cholesterol efflux may also participate in HDL signaling to stimulate macrophage migration as has been suggested for endothelial cells [41] since BLT-1 also inhibits HDL dependent SR-BI mediated cholesterol efflux [111]. Further studies will be required to determine the mechanisms by which HDL binding to SR-BI and SR-BI mediated lipid transfer trigger signaling in macrophages.

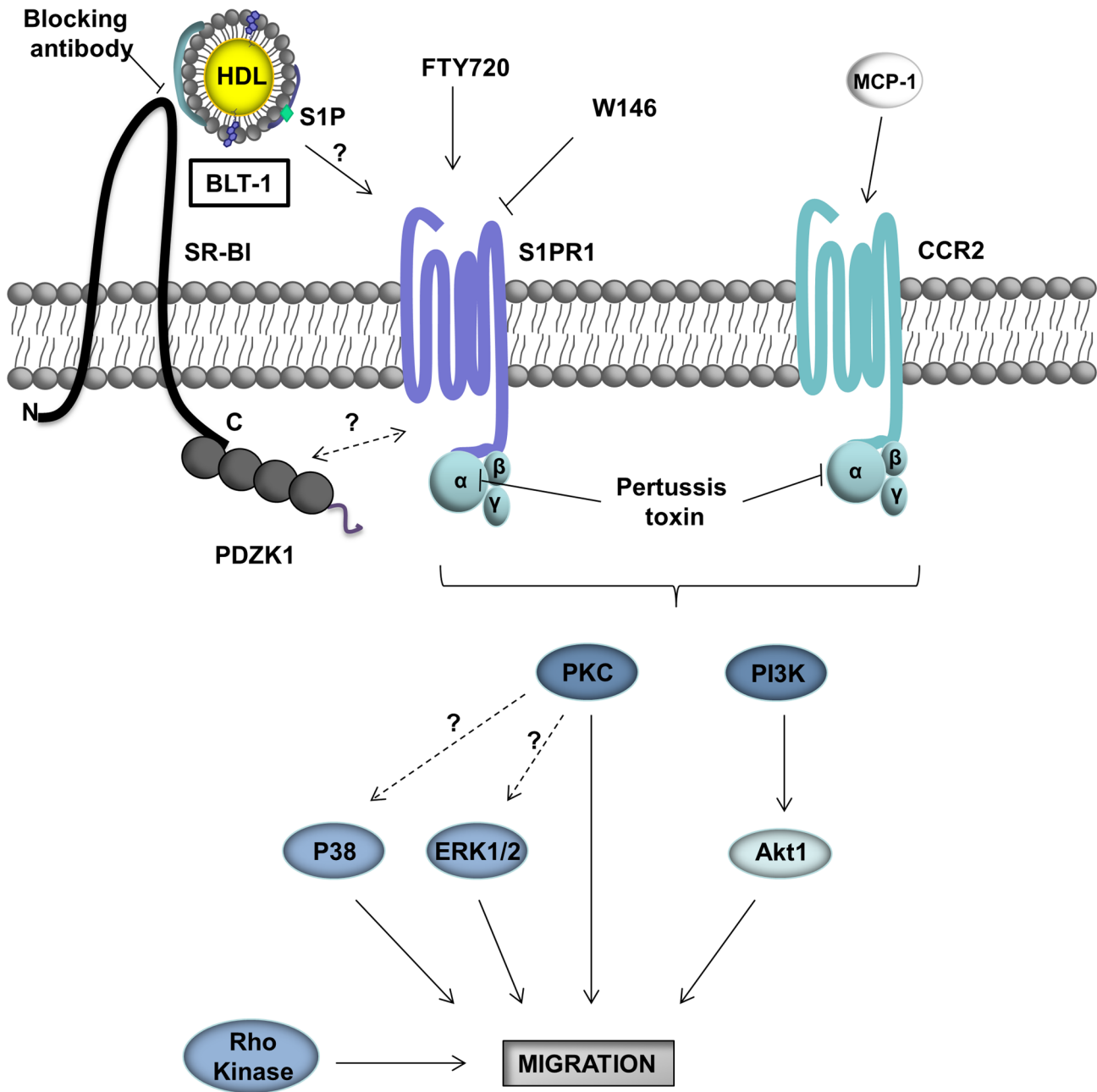


Figure 7. Working model for HDL mediated stimulation of macrophage migration. HDL binding to SR-BI leads to activation of S1PR1 signaling. This may involve transfer of S1P from bound HDL to S1PR1. Inhibition of HDL binding to SR-BI (blocking antibody) or SR-BI-mediated lipid transfer (BLT-1) prevents HDL dependent activation of S1PR1 signaling, but does not affect direct activation of S1PR1 by agonists. Inactivation of expression of SR-BI or PDZK1, on the other hand, inhibits migratory responses to FTY720, through an as yet unknown mechanism. HDL dependent activation of migration is suppressed by inhibition of S1PR1 signaling with FTY720 or W146, which directly antagonize S1PR1, or with PTX, which blocks G α , coupled GPCR's including CCR2 (receptor for MCP-1). Upon appropriate stimulation, S1PR1 (and CCR2) stimulate macrophage migration by activation of diverse signaling pathways including PI3K/Akt1, Rho kinase, PKC, p38 MAPK and Erk1/2 pathways .
doi:10.1371/journal.pone.0106487.g007

Our data suggests that HDL-induced macrophage migration involves an S1PR, likely S1PR1. HDL is the major lipoprotein carrier of plasma S1P [53]. S1P is known to be a modulator of immune cell migration and appears to mediate the migration of endothelial cells treated with HDL [52,58,59]. Therefore we tested the involvement of S1PR activity in HDL dependent macrophage migration. The sphingosine analog FTY720, which is phosphor-

ylated in vivo to FTY720-phosphate [112], a broad spectrum S1PR agonist, induced macrophage migration in a manner that was not diminished by the anti-SR-BI blocking antibody or by BLT-1 (Figure 4). This suggested that neither SR-BI's ability to bind HDL, nor its lipid transfer activity were required for macrophage migration in response to FTY720. Mouse macrophages were reported to express S1PR1 and S1PR2 and lower

levels of S1PR3 [90]. S1PR2 negatively modulates macrophage migration *in vivo* and *in vitro* [91]. A number of lines of evidence implicate S1PR1 as the S1PR that mediates HDL dependent macrophage migration: First, HDL dependent macrophage migration is inhibited by PTX (Figure 3A), an inhibitor of G α_i heterotrimeric G proteins, to which S1PR1 is coupled; second, an S1PR1 specific agonist, SEW2871, stimulates macrophage migration (Figure 3D); third, an S1PR1 specific antagonist, W146, prevents HDL stimulated macrophage migration (Figure 3D); fourth, we have detected S1PR1 but little to no S1PR3 expression in mouse peritoneal macrophages (Figure 4C). S1PR1 has previously been shown to be involved in HDL mediated eNOS activation, cell survival, migration and inhibition of adhesion molecule expression in endothelial cells [40,61]. Our findings suggest that it also plays an important role in SR-BI dependent HDL stimulated migration of macrophages.

Surprisingly, macrophages from either SR-BI KO or PDZK1 KO mice were unable to migrate in response to the S1PR agonist FTY720 (Figure 2A, D). This differed from the results of acutely inactivating SR-BI's HDL binding or lipid transfer activity (Figure 3), which impaired macrophage migration in response to HDL but left migration in response to FTY720 intact. The reasons why SR-BI KO or PDZK1 KO macrophages are unable to migrate in response to FTY720 is not clear and are the subject of on-going studies.

Our data suggests that HDL and the S1PR agonists FTY720 and SEW2871 share common downstream signaling pathways leading to macrophage migration which may suggest the involvement of common upstream signal transducers such as S1PR1. We demonstrate that HDL induced migration is dependent on the activation of PI3K-Akt1 signaling (Figure 5), consistent with the requirement of PI3K-Akt in HDL stimulated migration of endothelial cells [113]. PI3K-Akt signaling is known to be activated by S1PR1 [114]. The Akt family consists of three isoforms: Akt1, 2 and 3, encoded by different genes, all of which are reportedly expressed in macrophages [115]. Different Akt isoforms appear to play distinct roles in HDL stimulated macrophage migration. Interestingly, basal migration was enhanced ~5 fold when Akt2 was knocked out (Figure 5C). This is consistent with previously reported observations that cell migration is enhanced in the absence of Akt2 expression and suggests that Akt2 negatively regulates basal migration [116]. Neither HDL nor FTY720 further increased migration of Akt2 KO macrophages, but rather, both reduced the enhanced basal migration by half (Figure 5C). This suggests that Akt2 KO MPMs could still respond to HDL or FTY720, although their response (reduced migration) was opposite that of wild type macrophages (increased migration) which contained Akt2. Akt3 did not play a significant role in migration in response to HDL, FTY720 or MCP-1 (Figure 5C). On the other hand, HDL induced migration was dependent on Akt1 (Figure 5C). Similar data were observed for FTY720 and MCP-1 stimulated migration. The difference in HDL mediated migration in Akt1, 2, and 3 KO macrophages was not due to differences in expression levels of SR-BI as these cells express similar levels of SR-BI compared to wild type macrophages (Figures 5D and data not shown). Akt isoforms seem to play non-redundant and sometimes opposing roles in cell migration [116] which appear to be cell type specific and depend on the chemotactic agent tested [117–119]. Our data suggests that Akt1 is downstream of HDL and is the key Akt isoform that mediates HDL induced migration of macrophages. Several studies reported the involvement of Akt1 in the development of

atherosclerosis, macrophage inflammation, endothelial cell migration and vascular smooth muscle cell proliferation and protection against apoptosis [117,120–123]. Overall these studies, together with our findings that Akt1 is required for HDL stimulated macrophage migration, are consistent with an atheroprotective function of Akt1. The requirement of Akt1 in HDL induced macrophage migration may suggest that at least some of the atheroprotective actions of HDL are mediated by Akt1.

In addition to the PI3K-Akt1 pathway, we used a chemical approach to demonstrate the involvement of p38 MAPK and ERK1/2 in HDL mediated macrophage migration (Figure 6 A). The involvement of p38 MAPK in HDL induced migration is similar to what has been reported previously for endothelial cells in which p38 MAPK was reported to act downstream of PI3K-Rac [61]. Unlike macrophages in which ERK1/2 appears to play a role in HDL stimulated migration, endothelial cell migration in response to HDL reportedly does not require ERK1/2 [61], suggesting cell type dependent differences in signaling pathways involved in HDL stimulated migration. We also demonstrate, using a chemical approach, the involvement of Ca²⁺-dependent PKCs in macrophage migration in response to HDL, FTY720 and MCP-1 (Figure 6B). HDL has been shown to increase intracellular calcium concentration in diverse cell types in culture, including, Chinese hamster ovary cells [124], human skin fibroblasts [125,126], smooth muscle cells [127] and endothelial cells [54,128]. Whether HDL also increases intracellular calcium in macrophages remains to be determined. Interestingly, Pilon et al. reported that SR-BI expression is increased in human adrenocortical cells in response to PKC activators, resulting in higher lipoprotein binding and specific cholesteryl ester uptake utilized for steroidogenesis [129], and we have previously reported that activation of PKC in transfected Chinese hamster ovary-derived and in HepG2 human hepatoma cells modulates the activity of SR-BI [37,39]. Thus, SR-BI may be both a mediator and/or a target of HDL-dependent PKC activation.

Small GTPases of the Rho family play a key role in actin cytoskeleton organization and cell migration in response to chemokines and cytokines [92–96,130,131]. Blockage of Rho-kinase reduced HDL stimulated migration suggesting the requirement of this pathway in response to HDL (Figure 5A). Similar results were obtained for FTY720 and MCP-1 mediated migration consistent with the importance of the Rho-kinase pathway in macrophage migration in response to these stimuli. Although our data suggests the involvement of different kinases in HDL stimulated macrophage migration (Figure 7), the hierarchy and the interaction between multiple downstream signaling pathways remain to be established.

In conclusion we have demonstrated that HDL induces the migration of macrophages in an SR-BI, PDZK1 and S1PR1 dependent manner as summarized in Figure 7. HDL induced macrophage migration involves the PI3K-Akt1, p38MAPK, ERK1/2, PKC and Rho kinase pathways. HDL mediated macrophage migration may be one mechanism by which HDL contributes to protection against atherosclerosis development and atherosclerotic plaque regression.

Author Contributions

Conceived and designed the experiments: BLT AA LG XC. Performed the experiments: AA LG XC. Analyzed the data: BLT AA LG XC. Wrote the paper: BLT AA XC LG.

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