ROLE OF HDL AND S1P IN MACROPHAGE SIGNALLING

McMaster University

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AN EXAMINATION OF THE ROLE OF SPHINGOSINE-1-PHOSPHATE IN HIGH DENSITY LIPOPROTEIN MEDIATED PROTECTION OF MACROPHAGES AGAINST APOPTOSIS

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LAY ABSTRACT

Atherosclerosis, is a disease where in the artery walls thicken due to cholesterol build-up, is the major underlying cause for cardiovascular diseases, which is currently a leading cause of death in many populations. We believe that HDL, the "good" cholesterol and S1P, a small molecule carried by HDL, can help prevent the progress of atherosclerosis by preventing macrophages, cells that absorb the cholesterol, from dying. We attempt to prove this by providing S1P or HDL to macrophages that are made to undergo cell death. Results show that both HDL and S1P can protect cells against cell death induced by many factors. However, HDL can protect against certain cell death inducing stimuli without the need for S1P and more research is required to fully understand HDL's protective role in atherosclerosis. Understanding how HDL elicits atheroprotective signalling in macrophages will help in finding new drugs and therapies to reduce atherosclerosis-based deaths across the world.

ABSTRACT

Prevention of macrophage apoptosis in advanced atherosclerotic lesions can help stop atherosclerosis progression to vulnerable plaques. High density lipoprotein (HDL) can protect macrophages from apoptosis that has been induced by a variety of agents. We hypothesize that this is the consequence of the sphingolipid, sphingosine-1-phosphate (S1P), specifically carried by HDL, and transferred to S1P receptor 1 (S1P1) on the cells via the HDL receptor, scavenger receptor class B type 1 (SR-B1).

Apoptosis was induced in murine peritoneal macrophages from wild type and different knockout mice with, tunicamycin, thapsigargin, staurosporine, or UV irradiation. Apoptosis was measured by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) or with cleaved caspase-3 (CC3) staining. Treatment of cells with HDL or S1P protected them against apoptosis induced by a variety of stimuli. In contrast, pre-treatment of HDL with S1P lyase, which irreversibly cleaves S1P, eliminated the ability of HDL to protect macrophages. Inhibition of SR-B1's lipid transport activity reduced HDL dependant protection against apoptosis. Furthermore, HDL dependent protection against apoptosis induced by tunicamycin was prevented when the S1P receptor S1P1 was knocked out. However, this protection was not prevented when apoptosis was induced by staurosporine.

These results suggest that the HDL mediated protection of macrophages against apoptosis is multi-faceted and one approach may involve SR-B1 mediated delivery of S1P from HDL to the S1P1. Understanding the mechanisms by which HDL elicits atheroprotective signalling in macrophages will provide insight into new targets for therapeutic intervention in atherosclerotic disease.

ACKNOWLEDGEMENT

I would not have been able to achieve any of this work without the help and guidance of my supervisor Dr. Bernardo Trigatti, my colleagues and friends here at the Trigatti lab, and my family. Words cannot express the gratitude I have for my supervisor who has enabled me to work at this lab and provide help and advice no matter the time or day. Any issues I had dreaded over during my time at the lab was always put to ease at every one on one meeting I had with him each week.

Special thanks to the previous members of the Trigatti lab, especially Dr. Leticia Gonzalez, Dr. Kristina Durham, Dr. Pei Yu, and Melissa MacDonald, who taught me all the essential lab skills and advised me when I first started work at this lab. I would also like to give my eternal gratitude to the current lab members, especially Alexander Qian, Yak Deng, and Ting Xiong for their assistance and support with mice work. I would like to also acknowledge Aric Huang, Vi Dang, and Monica De Paoli from Dr. Geoff Werstuck's lab for being my inspirational role models, and for providing new insights with regards to my lab work.

Lastly, I thank my family and friends for keeping me going throughout all my years in academia. Getting this far was only possible thanks to each and everyone of them.

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LIST OF ABBREVIATIONS

apo	Apolipoprotein
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CVD	Cardiovascular disease
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FPLC	Fast protein liquid chromatography
HDL	High density lipoprotein
HRP	Horseradish peroxidase
KO	Knockout
NCLPDS	Newborn calf lipoprotein deficient serum
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PDZK1	Post-synaptic density protein/Drosophila disc-large protein/Zonula occludens protein containing 1
PI3K	Phosphatidylinositol-3-kinase

PVDF	Polyvinylidene fluoride
S1P	Sphingosine 1 phosphate
S1P1	Sphingosine 1 phosphate receptor 1
S1PL	Sphingosine 1 phosphate lyase
SDS	Sodium disulfide
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMC	Smooth muscle cell
SR-B1	Scavenger receptor class B member 1
SS	Staurosporine
TBS	Tris buffered saline
TdT	Terminal deoxynucleotidyl transferase
Th	Thapsigargin
Tm	Tunicamycin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPR	Unfolded protein response
UT	Untreated
UV	Ultraviolet
WT	Wildtype

DECLARATION OF ACADEMIC ACHIEVEMENT

The experiments in this thesis were conducted by Kevin Chathely. Melissa McDonald and Yak Deng handled the breeding and housing of the mice utilized in this thesis. Two figures included in this thesis have already been published. **Figure 4.8 A** has been published in the journal *Atherosclerosis*, titled "PDZK1 in leukocytes protects against cellular apoptosis and necrotic core development in atherosclerotic plaques in high fat diet fed ldl receptor deficient mice" by Pei Yu, et al[1]. **Figure 4.8 B** has been published in a Data Article titled "Data on leukocyte PDZK1 deficiency affecting macrophage apoptosis but not monocyte recruitment, cell proliferation, macrophage abundance or ER stress in atherosclerotic plaques of LDLR deficient mice" referring to the previous paper[2]. My supervisor, Dr. Bernardo Trigatti, and the members of my supervisory committee, Dr. Geoff Werstuck, and Dr. Jon Schertzer have provided guidance throughout this project.

CHAPTER 1:INTRODUCTION

1.1. Cardiovascular disease & atherosclerosis

Disease of the heart and blood vessels or "Cardiovascular Disease" (CVD), was the most common underlying cause of death in 2013, and is still a leading cause of death in developed nations today[3]. In Canada specifically, CVD accounted for 20% of all deaths during the year 2013[4]. Based on data from the National Health and Nutrition Survey from 2013 to 2014, it is estimated that more than 1 in 3 adults in America (92.1 million) had one or more types of CVD[3]. Moreover, by 2030 approximately 40% of the US adult population is projected to have some form of CVD[3,5]. Along with this rise in cases of CVD, the direct medical costs for CVD will also rise accordingly. Within the span of 20 years from 2010 to 2030, direct medical costs for CVD are estimated to triple in amount, from $273 billion to $818 billion[5]. Taking these statistics into account, the need for a better understanding of CVD and its underlying mechanism has become crucial for tackling its rising prevalence and costs.

Although there are many forms of CVD, one major underlying pathology of CVD is atherosclerosis, where artery walls thicken due to accumulation of plaque in its inner walls[6]. This can occur due to multiple factors such as, elevation of modified low-density lipoproteins (LDL), hypertension, infectious microorganisms (eg. Chlamydia pneumoniae), sheer stress, free radicals, and toxins produced as a result of smoking[7]. These risk factors cause a complex endothelial dysfunction that then triggers inflammatory responses. When the response fails to resolve the dysfunction, it may persist, hence atherosclerosis is regarded as an inflammatory disease. At the later stages of atherosclerosis, the plaque becomes unstable and prone to rupture, at this stage the patient has a very high chance of receiving a major cardiac complication such as myocardial ischemia, infarction, or cardiac arrest.

1.2. Stages of atherosclerosis

Atherogenesis, or the formation of atherosclerotic plaques, starts with the endothelial dysfunction which then triggers inflammatory responses leading to the expression of adhesion molecules at the endothelium that recruit white blood cells (leukocytes) such as monocytes and lymphocytes[7,8]. At the same time the endothelium becomes more permeable promoting the entry of LDL into the arterial subendothelial space where they are then modified and retained. To counter the influx of these lipoproteins, the recruited monocytes differentiate into macrophages which then take up the modified lipoproteins, becoming engorged with cholesteryl esters through a process called phagocytosis. Macrophage accumulation and conversion to this lipid rich "foam" cells, named for their foam like appearance, is the first stage in making up the earliest form of lesion known as fatty streaks. These foam cells further drive the inflammatory response by producing high levels of cytokines and growth factors such as interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNFα)[7,8].

In advanced lesions, both macrophage derived foam cells and the outlining endothelial cells around the plaque may undergo apoptosis, which is a form of programmed cell death. This may be due to a variety of stresses including oxidative and endoplasmic reticulum (ER) stress[9,10]. When neighbouring phagocytic cells fail to clear apoptotic foam cells, they accumulate giving rise to secondary necrosis and the formation of a lipid rich, cell free necrotic core[9,11]. In parallel to these events, smooth muscle cells (SMCs), proliferate and elevate collagen and elastin levels[7,8]. With the help of these extracellular matrix molecules, SMCs create a fibrous cap that covers the plaque. At this point, the plaque is fully developed and may be prone to rupture at any point in time. Although the mechanism behind plaque stability and rupture is not fully

understood, it is known that a plaque with a fibrous cap that is too thin due to a lack of SMCs and collagen, while having an excess of macrophages and a large necrotic core size, is highly prone to rupture[12–14]. Foam cells also play a part in destabilizing the fibrous cap by releasing matrix degrading proteinases such as matrix metalloproteinase[15]. In patients at this stage, plaques may impede blood flow due to arterial narrowing or break apart and obstruct distal narrower arteries, also known as embolism. Moreover, when a plaque ruptures acute coronary occlusion may occur as thrombosis and the coagulation cascade system attempt to resolve the newly perceived hole in the arterial wall[13,16]. This then leads to major cardiovascular complications as the thrombus formation threatens the start of a myocardial ischemia, or infarction.

1.3. Role of macrophage apoptosis in atherosclerosis

Macrophage apoptosis plays a central role in atherosclerosis, both in mitigating and promoting the inflammation depending on the stage of atherosclerosis progression. In the earlier stages of atherosclerosis, macrophages take up the excess lipoproteins accumulating in the arterial walls through endocytosis. These lipoproteins such as LDL get modified in various ways such as oxidation and glycation during the progression of atherosclerosis[17,18]. This influx of saturated fatty acids and oxidized lipids can lead to stiffening of the ER membrane within the macrophages. This leads to disruptions in the ER's function of protein modification and folding, which results in an accumulation of misfolded proteins in the ER[19,20]. This triggers the unfolded protein response (UPR), where in three signalling pathways known as, protein kinase R-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) signal the cell to either help overcome the ER stress or aim towards apoptosis[9,21]. Hence, prolonged ER stress is one method in which foam cells undergo apoptosis, and in early lesions, the dead cells are quickly cleared away and safely dealt with by other

macrophages or phagocytes through a process known as efferocytosis[22,23]. However, in advanced lesions, efferocytosis fails to keep up with the level of apoptotic macrophages[23]. This defective clearance results in an increased level of inflammation and the formation of the necrotic core in the lesion[9,22]. Hence, macrophage apoptosis in the early stages of atherosclerosis can be seen as beneficial and atheroprotective, which is in contrast to the later stages of atheroma progression where it becomes detrimental. Therefore, finding methods to prevent macrophages from undergoing apoptosis in the later stages of atherosclerosis can be a useful tool in impeding atherosclerosis progression.

1.4. High Density Lipoprotein

Of the major groups of lipoproteins, the high density lipoprotein (HDL) has been shown to have many atheroprotective and anti-inflammatory effects on numerous cell types involved in atherogenesis[24,25]. Moreover, HDL has always been a popular target of study when dealing with atherosclerosis due to clinical and epidemiologic studies showing a high inverse correlation between HDL cholesterol (HDL-C) level and coronary heart disease[26,27]. However, clinical studies that attempted to increase HDL-C have been unsuccessful, and recent studies have concluded that increasing HDL-C has no affect on reducing the risk of coronary heart disease[28,29]. This suggests that HDL *cholesterol* may not itself play a direct role in protection against coronary heart disease. One possibility is that one or more other properties of HDL particles (other than its cholesterol cargo) may be responsible for protection against coronary heart disease.

HDL as a class of lipoproteins is made up of a spectrum of particles of different size, protein, and lipid compositions (see **Figure 1.1**). All HDL also share common characteristics such as the presence of apoA1. However, both the protein and lipid compositions of HDL

particles can vary with particle size, as well as during physiological states such as, post atherosclerotic plaque rupture[30,31]. Aside from categorizing HDL based on composition it can also be divided into subclasses based on its size, and studies suggest that patients with CVD have an increased level of small HDL particles while having lower levels of the larger HDL subclass[32,33]. Understanding the complexity of HDL and further analysis of its interactions with other systems, especially the immune system, could provide an effective method in using HDL as a biomarker for evaluating CVD risk amongst patients in advance.

One of the most well-known methods to which HDL directly affects atherosclerosis is through its ability to mediate reverse cholesterol transport (RCT). RCT begins with the production of nascent HDL by the liver and intestine, which then travels through the plasma collecting cholesterol and phospholipids from peripheral tissues, thereby forming a mature HDL[34,35]. This mature HDL can then further collect more cholesterol from cells such as macrophages through transporters such as ATP binding cassette subfamily A member 1 (ABCA1) and the HDL receptor, scavenger receptor class B type 1 (SR-B1), as well as through passive diffusion[34]. This load of cholesterol is then transported back into the liver via hepatic SR-B1 where it is catabolized, and safely disposed through biliary secretion[34]. Although RCT involves the collection of cholesterol throughout the body, the ability to safely efflux cholesterol from macrophages involved in atherogenesis, makes HDL play a crucial role in impeding atherosclerosis. Aside from its role in RCT, HDL is also known to have many atheroprotective properties such as being anti-thrombotic, anti-oxidative, anti-inflammatory, and anti-apoptotic towards many cell types associated with atherosclerosis, however the underlying mechanisms behind these roles are not yet fully understood[35,36].

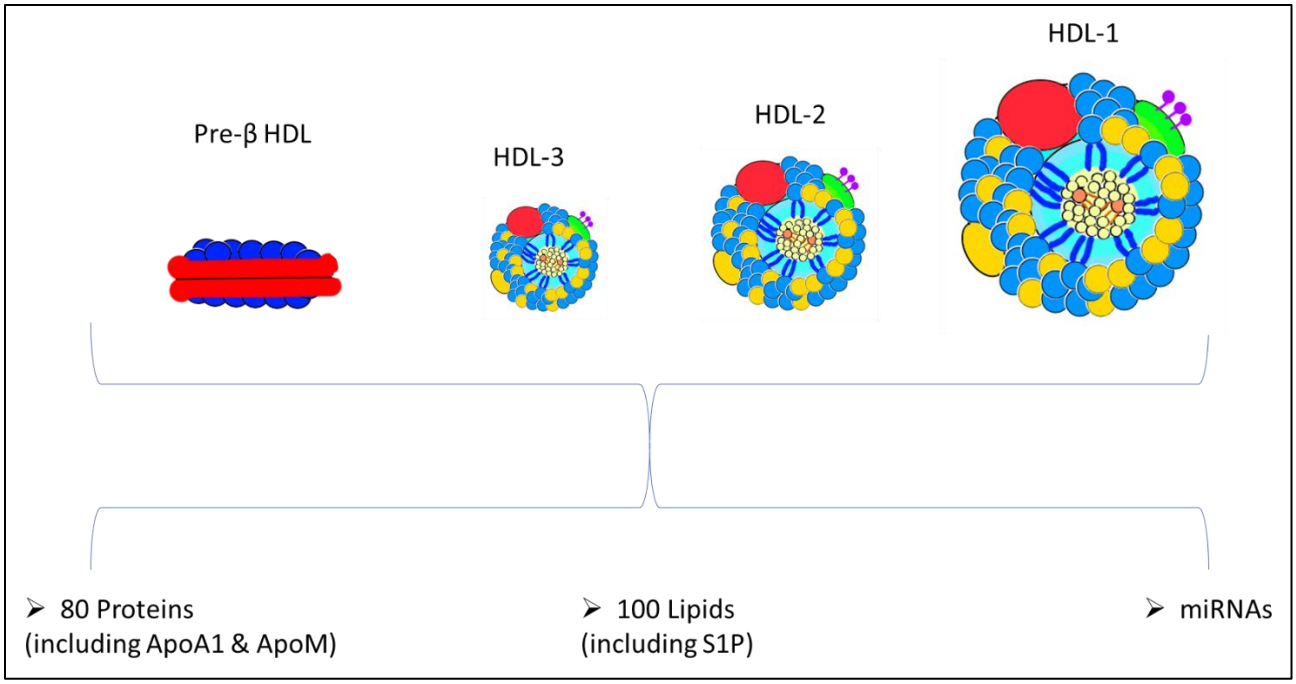


Figure 1. 1: HDL subclasses and composition.

HDL is a complex class of particles, with varying compositions including more than 80 proteins, and hundreds of lipid species[37]. Proteins include, a wide variety of apolipoproteins such as ApoA1 and ApoM, which carries the sphingolipid sphingosine-1-phosphate (S1P). HDL can also be divided into subclasses based on its size & shape. The smaller, more discoidal HDL is referred to as pre-β or "nascent" HDL, and after accumulation of cholesteryl esters it then acquires a spherical shape and is known as α-HDL. α-HDL is classified as either HDL- 1, 2 or 3 based on its size (the largest being HDL-3 and the smallest being HDL-1).

1.5. Sphingosine-1-Phosphate

HDL houses many lipids, of which, sphingosine-1-phosphate (S1P), a bioactive sphingolipid, has been known to play diverse roles that may protect against CVD similar to HDL. Some of these roles include regulation of, cell growth, differentiation, survival, lymphocyte trafficking, and of vascular integrity[25,38,39]. In granulosa cells, S1P has been found to prevent apoptosis induced by oxidative stress[40]. In endothelial cells sphingosine kinase-1 (which creates S1P) enhances endothelial cell survival through the activation of PI-3K/Akt and regulation of Bcl-2 family of apoptosis regulatory proteins[41]. S1P is created through the phosphorylation of sphingosine by sphingosine kinases[25,42]. Sphingosine kinases are ubiquitously expressed and come in two isoforms (Sphk1 & Sphk2). Sphk1 catalyzes S1P formation at the plasma membrane, while Sphk2 forms S1P in the mitochondria, ER, and nucleus[42]. S1P can be converted back to sphingosine by sphingosine phosphatases, or be broken down by S1P lyase (S1PL) into phosphoethanolamine and hexadecenal (see **Figure 1.2**) [25,38,43]. S1P binds to five G protein-coupled receptors (S1P1, S1P2, S1P3, S1P4, S1P5) which vary in distribution amongst many cell types with S1P1-3 being expressed widely, S1P4 being expressed mainly in lymphatic/hematopoietic tissue, and S1P5 being limited to the central nervous system and spleen[44]. The highest concentration of S1P is found in the blood with HDL being the major carrier of S1P in the plasma[38,45,46]. This HDL associated S1P is thought to be carried by apoM, a component said to be present amongst ~5% all HDL particles[47]. Although this apolipoprotein is limited to a small subpopulation of HDL they have been known to protect LDL against oxidation and stimulate cholesterol efflux more efficiently than HDL lacking apoM[47]. However, more research is required to fully understand the direct link between S1P and HDL as other studies

also suggest that apoM may not be the only carrier or molecular partner for S1P since there does not seem to be a correlation between apoM and S1P in human plasma or in HDL[48–50].

Some roles of S1P can seem contrary to each other and this could be due to functional distinctions between "free" S1P and HDL- associated S1P[24]. For example, free S1P has a tendency to participate in pro-inflammatory, vasoconstrictive, and other processes that could also have adverse effects[24]. Meanwhile, reported effects of HDL-associated S1P are non-deleterious, potentially beneficial for cardiovascular homeostasis[24]. Moreover, the five S1P receptors elicit distinct signaling patterns in response to S1P, leading, in some cases, to opposing outcomes. This can be seen in S1P's regulation of mast cell function as activation of S1P1 promotes migration of mast cells while in contrast S1P2 halts the migration and promotes mast cell degranulation instead[51].

Two drugs targeting the S1P pathway have been developed with one approved for use. LX2931 is an oral inhibitor of S1PL which has been developed as a potential treatment for rheumatoid arthritis and is currently in phase 2 of clinical trials[51,52]. As S1P plays a role in lymphocyte trafficking, inhibiting S1PL disturbs the S1P gradient and prevents this movement of lymphocytes from the lymphoid tissue to the blood, hence alleviating autoimmune and inflammatory disorders, which in this case affects the joints[51]. Fingolimod (FTY720), an analogue of S1P, has been approved for treatment of multiple sclerosis, an inflammatory disorder where in immune system targets the central nervous system[53]. Fingolimod, in the long term, acts as an indirect functional antagonist specifically to S1P1 through prolonged receptor internalization and partial degradation[54]. Since S1P1 is involved in promoting lymphocyte trafficking, the downregulation of this receptor leads to a strong immunosuppressive effect which is beneficial in cases like multiple sclerosis.

Moreover, since S1P plays a role in promoting cell survival, Fingolimod also has potential for anti-cancer therapy, as it also inhibits Sphk1 which catalyzes S1P formation as mentioned earlier[55]. One study has shown inhibition of Sphk1 by Fingolimod triggers prostate cancer cell apoptosis in vitro[55]. Both the S1PL and S1P1 inhibitors alter the S1P gradient whether by increasing S1P level in the lymphoid tissues through the inhibition of the lyase or through modulating the S1P1 receptor. There is still much to be explored in the field of S1P, as its downstream signalling pathways, especially the pleiotropic affects it has on the immune system, is still largely unknown.

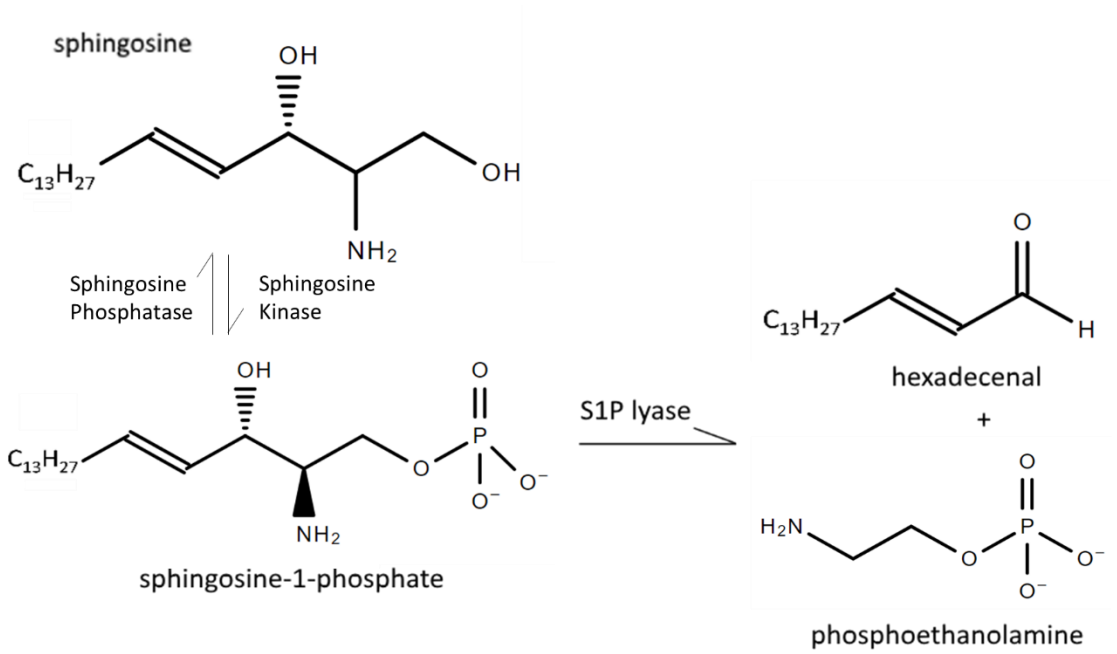


Figure 1. 2: Overview of S1P synthesis and breakdown by S1P lyase.

Sphingosine is phosphyralted by sphingosine kinases (Sphk1 & Sphk2) and S1P is converted back to sphingosine by sphingosine phosphatase. S1P lyase irreversibly cleaves S1P at C2-C3 carbon-carbon bond to form a hexadecenal and phosphoethanolamine.

1.6. HDL and macrophage interactions

Recent work done in our lab has shown that HDL can stimulate directional migration of macrophages in-vitro, as well as protect macrophages from apoptosis induced by oxidative and ER stress that has been generated by a variety of agents[1,56,57]. This HDL dependent signalling in macrophages involves the HDL receptor SR-B1, its adaptor protein PDZK1 (post-synaptic density protein/Drosophila disc-large protein/Zonula occludens protein containing 1), and the G-protein coupled receptor S1P1[1,2,56,57]. Treatment of macrophages from wild type mice with HDL or with the S1P1 agonist SEW2871, stimulated macrophage migration. Meanwhile, macrophages from mice lacking SR-B1, or from mice lacking PDZK1 both failed to migrate when treated with HDL[56]. Furthermore, treatment with the S1P1 antagonist W146, inhibited macrophage migration during the treatment of both HDL and SEW2871[56]. Similarly, treatment of macrophages with either HDL or SEW2871 suppressed apoptosis in response to different treatments, and this ability of HDL was lost in macrophages that lacked PDZK1 or S1P1[1,57]. Likewise, HDL treatment of macrophages resulted in increased Akt phosphorylation but this increase was not seen when either PDZK1, S1P1 or Akt1 were not expressed[1,56,57]. The observation that HDL dependent signaling requires S1P1 for Akt phosphorylation, macrophage migration, and protection against apoptosis, suggests the possibility that HDL's effects may have been mediated by S1P, the known ligand of S1P1 (**see Figure 1.3**).

In conclusion, HDL plays a pivotal role in protection against atherosclerosis progression, and further research on HDL and macrophage interaction can lead to findings that can better elucidate the mechanisms by which HDL elicits atheroprotective signalling in macrophages will provide insight into new targets for therapeutic intervention.

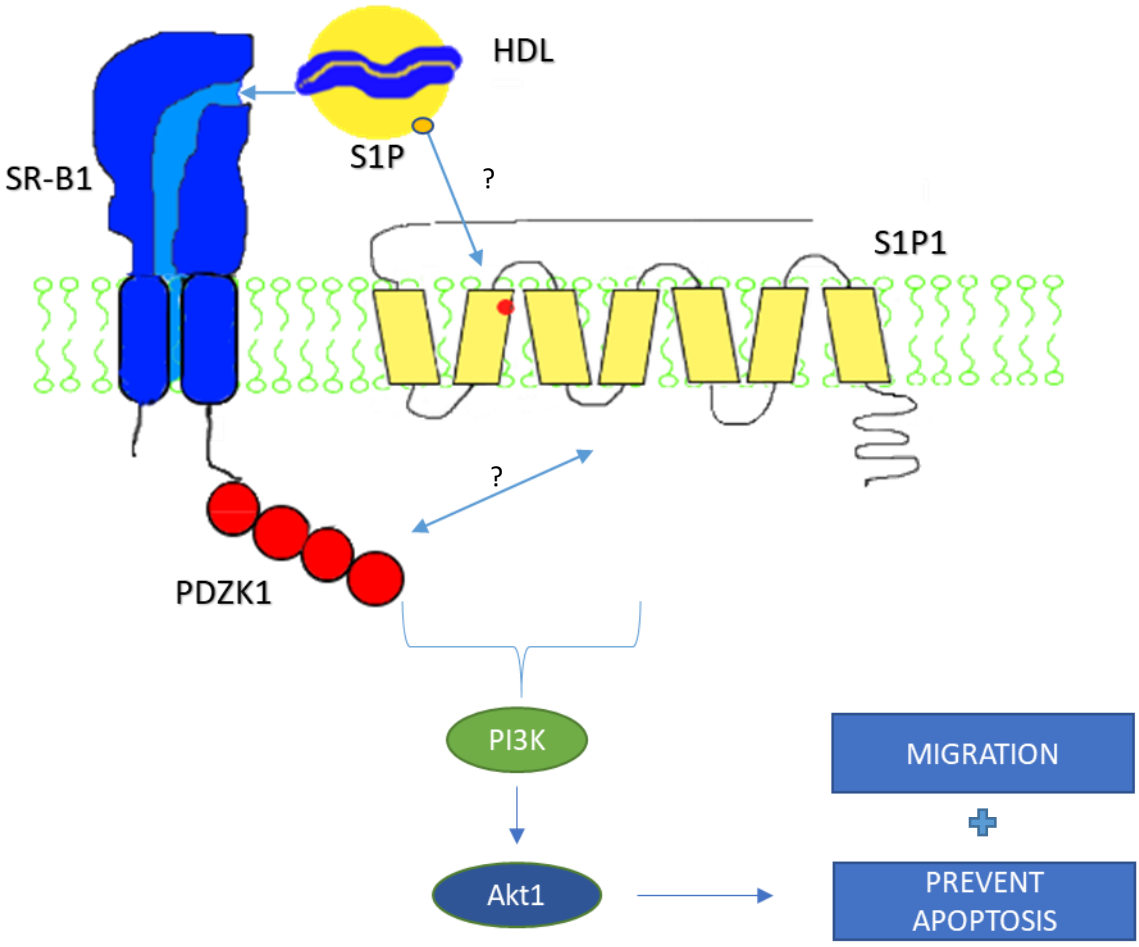


Figure 1. 3: Working model for HDL mediated signaling in macrophages.

HDL along with its associated S1P binds to SR-B1 which leads to the activation of PDZK1 and S1P1 signaling pathways. This leads to the phosphorylation of Akt1 via PI3K and other diverse signaling pathways to promote macrophage migration as well as protection against apoptosis induced by ER stress inducing agents.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

We hypothesize that HDL signalling, which confers protection against apoptosis in macrophages, is mediated by the transfer of S1P carried by HDL onto S1P1 via SR-B1.

2.2. Objectives

1. Determine whether HDL can protect macrophages against apoptosis induced by different stressors
2. Test whether S1P can protect macrophages from apoptosis in a similar fashion to HDL
3. To examine the effects of S1P lyase on HDL's ability to supress apoptosis in macrophages.
4. Assess the involvement of SR-B1 associated lipid transport in the HDL mediated protection of macrophages against apoptosis.
5. Assess the involvement of PDZK1 in the HDL mediated protection of macrophages against apoptosis.

CHAPTER 3:METHODS AND MATERIALS

3.1. Materials

Sphingosine-1-phosphate (S1P) was purchased from Avanti Polar Lipids, Inc (Alabaster, Alabama). Tunicamycin was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Staurosporine was acquired from Toronto Research Chemicals (North York, ON, Canada). HDL were purchased from Alfa Aesar (Ward Hill, MA, USA) or extracted manually from human plasma (see below). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless indicated otherwise.

3.2. Mice

All animal experiments were pre-approved by the McMaster University Animal Research Ethics Board in accordance with Canadian Council on Animal Care guidelines. Sources of mice: C57BL6/J (used for all experiments unless stated otherwise); B6129SF2/J (control for *Pdzk1*$^{-/-}$); *Pdzk1*$^{-/-}$: The Jackson Laboratory (Bar Harbor, ME, USA). LysM$^{Cre/Cre}$ S1P1$^{lox/lox}$ mice were described previously[57]. Mice were bred and housed in the TaARI Animal Facility with automatic watering and free access to food (standard chow diet).

3.3. HDL preparation

0.75 ml of 0.2 M NaEDTA at a pH 7.4 was added per 50 ml of human plasma. Plasma was then adjusted from its initial density of 1.006 g/ml, to a final density of 1.063 g/ml with potassium bromide (KBr), using the following formula:

$$\text{Mass of KBr (in grams)} = \frac{\text{initial volume (in ml)} \times (\text{final density} - \text{initial density})}{1 - (0.312 \times \text{final density})}$$

Plasma containing KBr was then loaded onto 39 ml Quickseal tubes (Beckman Coulter, Mississauga, ON, Canada; Cat # 342414), balanced, sealed, and centrifuged in a Ti 70.1 rotor at 55,000rpm for 15.2 hours at 4^{o}C with maximum acceleration. After the centrifugation lipoproteins less dense than HDL (LDL, IDL, VLDL, Chylomicrons/remnants) floated up to the top of the tube were removed and the HDL rich plasma was recovered after cutting the tubes with a tube cutter under the lipoprotein bands. The HDL rich plasma was recovered and adjusted to a density of 1.215g/ml, to which freshly prepared 20 mM Butylated Hydroxytoluene (44 mg of BHT in 10 ml of 95 % ethanol) is added at 1µl/ml of sample, and centrifuged as before to float up the HDL. Afterwards, the HDL was dialyzed against LDL buffer (0.9% NaCl, 0.0001% EDTA, pH: 7.4) at 4^{o}C for 8 changes (3 hours per change). Prior to dialysis, as well as between changes, nitrogen gas was bubbled through the dialysis buffer for 20 min. After dialysis, each sample was filter sterilized using a 0.2-micron low protein binding filter and protein concentrations were determined by Lowry assay.

3.4. HDL analysis

HDL was fractioned by gel filtration fast-protein liquid chromatography (FPLC) using an AKTA system with a Tricorn Superose 6 HR10/300 column (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada). Cholesterol levels on each fraction were analyzed by the Infinity Cholesterol enzymatic assay kit (Thermo Fisher Scientific, Ottawa, ON, Canada; Cat. #TR13421) to measure total cholesterol. Afterwards, the fractions that resided inside the cholesterol peaks where pooled by sets of five fractions starting from fraction 27 to fraction 51. This was done by taking 2.1 µl from each fraction. These 5 pools of samples were subjected to SDS-PAGE immunoblotting with either goat anti-human ApoA1 (Midland Bioproducts, 71107) or mouse monoclonal ApoM (8F12) antibody (Cell Signaling Technology, Danvers, MA, USA;

Cat. #5709). Pooling was done by taking 10µl from each fraction, and 1.54 x 10^{-06} mmoles of HDL (~2.1µl from each pool) was then loaded. HRP conjugated, rabbit-anti-goat (Novus, NB7352) was used as the secondary antibody and was detected using ECL Western Blotting reagent kit (Pierce; 32106).

3.5. Cells and cell culture

Peritoneal macrophages were elicited to enter the peritoneal cavity utilizing 1 ml of 10% thioglycolate broth and harvested after 4 days past the initial injection time. Mice were anesthetized using isoflurane gas and euthanized humanely using CO_2 and cervical dislocation. Peritoneal cells were then collected by peritoneal lavage using 10 ml of phosphate buffered saline (PBS) with 5mM Ethylenediaminetetraacetic acid (EDTA), spun down in a centrifuge at 500× g for 5 minutes at 4ºC. The cells are then resuspended in 2ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/mL penicillin and 50 U/mL streptomycin. Cells were then washed again in this manner once more, and then macrophages were counted using a hemocytometer. Macrophages were then plated in either 8-well chambered slides at 1.5 x 10^5 cells per 0.8cm^2 well for fluorescent staining-based assays, or in 6-well culture plate at 2 x 10^6 cells per 9.5 cm^2 well for western blot assays. Macrophages were cultured in incubators at 37ºC with a humidified atmosphere of 5% CO_2, with DMEM supplemented as before at a volume of 500µl for 8-well slides, or 1ml for 6-well plates for 2 hours to allow macrophages to adhere. The cells were then washed with DMEM with no additions in sets of three, and then the medium was changed into DMEM with 10% FBS for an overnight period (16-18 hours). Cells were monitored after each media change to check for abnormalities in macrophage health (e.g. deterioration is denoted by granularity around the nucleus), or for general contamination. On the next day, the cells were washed again as before

and the media was replaced with in DMEM supplemented with 3% newborn calf lipoprotein deficient serum (NCLPDS), 2 mM L-glutamine, 50 µg/mL penicillin and 50 U/mL streptomycin, 16 hours prior to treatment. All treatments were conducted over a period of 24 hours unless indicated otherwise. Immediately after 24 hours, cells in 8-well slides were fixed onto the slides with 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature, washed with PBS twice, and then underwent apoptosis staining (as described in section *3.6*). Cells in 6-well plates were washed in cold sterile PBS 3 times before applying 80µl of RIPA buffer (50 mMTris-HCl at pH 7.4 containing 150 mM NaCl, 1% Triton x-100, 1% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) supplemented with protease inhibitors (1 mM PMSF, 1 µP/mL pepstatin A, 1 mg/mL leupeptin, and 2 µ/mL aprotinin) and phosphatase inhibitors (phosSTOP, Roche Diagnostics, Mannheim, Germany). Cells are then immediately lysed, through physical disruption, within 10 minutes while the plate was kept on ice. Cell lysates are then collected and cell debris was spun down at 4000rpm in a microcentrifuge for 10 minutes and removed. Protein concentration was then determined using the BCA protein assay kit (Pierce Biotechnology, IL, USA) following the manufacturer's instructions. Samples were denatured with sample buffer (15 mM Tris-HCL, at pH 6.8 containing 3 % SDS, 5 mM DTT, 0.429 % bromophenol blue, and 10 % glycerol), for 5 minutes at 95^{o}C, and stored at -20^{o}C, until used for Western Blotting (see section *3.7*).

3.6. Apoptosis staining and analysis

Apoptosis was detected by either terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) or cleaved caspase 3 (CC3) immunofluorescence staining. CC3 staining was as follows: Cultured cells treated and fixed in-vitro (see *3.5*), were permeabilized with ice-cold 0.2% Triton X-100 in PBS (100µl/well) for 10 minutes. The

chambers were then removed and the slides were washed with PBS twice at 5 minutes each in a coplin jar. Afterwards, cells were incubated under dark conditions, with anti-CC3 (Asp175) antibody (Cell Signaling Technology, Danvers, MA, USA; Cat. #9661) diluted 1:200 in PBS supplemented with 3% goat serum, for 18 hours at 4 °C. Slides are then washed 3 times at 5 minutes each with PBS and then incubated with Alexa 488-labeled F (ab')$_2$ fragment of goat anti-rabbit IgG (H + L) (1:500 in PBS) for 1 h at room temperature. TUNEL assays were conducted utilizing ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Millipore, Etobicoke, ON, Canada; Cat. #S7110) following the manufacturer's instructions. After staining with either method, the slides were washed in PBS and counterstained with DAPI (300nM; Invitrogen) for 5 minutes. The slides were then washed and mounted with microscope slide cover glass by using 3 drops of PermaFluor^{TM} aqueous mounting medium (Thermo Scientific, Ottawa, ON, Canada; Cat. # TA-030-FM). After mounting the slides, they were imaged using a Zeiss Axiovert 200M inverted fluorescence microscope (Carl Zeiss Canada Ltd. Toronto, ON, Canada) using a 40x objective. 3-4 images are taken from the corners of each well. Each image is composed of 2 shots, 1 for each filter utilized. TUNEL positive or CC3 associated nuclei were then quantified using Image J software. An average % of TUNEL or CC3 positive/associated cells was then taken for each well. When n was based per mouse, an average was taken amongst all wells representing the same treatment.

3.7. Western Blotting

Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), utilizing 12% acrylamide gel (unless stated otherwise), and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked at room temperature for 1 hour with 5% non-fat milk in tris-buffered saline with 1% tween (TBS-T, 20ml). All primary antibodies were incubated at a

1:1000 dilution in 5% bovine serum albumin (BSA) in TBS-T overnight at 4°C. The next day, membranes were incubated with secondary antibodies; either HRP-donkey anti-mouse IgG for Mouse primary mab or HRP-donkey anti-rabbit IgG for rabbit primary antibody at a 1: 5000 dilution in 5% non-fat milk in TBS-T for 1 hour at room temperature. Membranes were incubated with HRP substrate (ECL Western Blotting reagent kit, Pierce; 32106) before band detection in the ChemiDoc imaging system.

3.8. Statistical analysis

Data was analyzed utilizing GraphPad Prism 6 software (San Diego, CA, USA). With the assumption of a Gaussian distribution, parametric tests were primarily used for all stats. For comparison between two groups, student's t-test was used and data with multiple groups, was analyzed with one-way ANOVA with the post-hoc Tukey's multiple comparisons test. For results with multiple treatments and mice models, a two-way ANOVA with the post-hoc Tukey's multiple comparisons test was utilized to better understand the relationship between the 2 independent variables on 1 dependant variable. Data are presented as mean ± SEM and were considered statistically significant if $p < 0.05$.

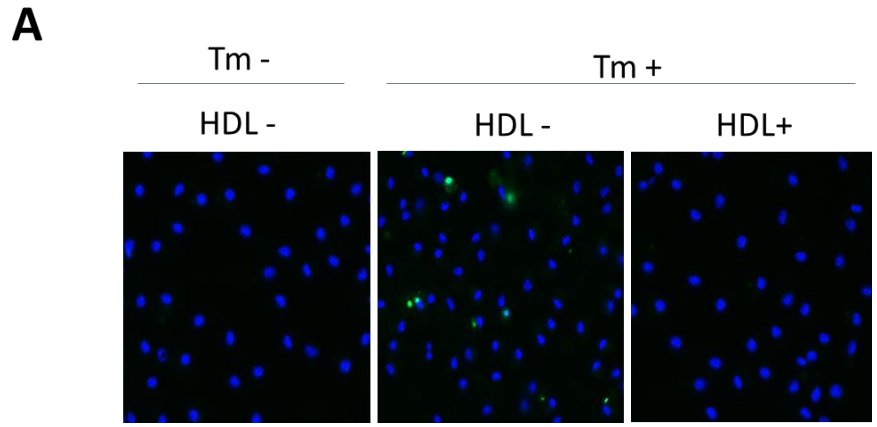
CHAPTER 4: RESULTS

HDL protects macrophages against apoptosis induced by various stimuli

To test the dose dependence of HDL mediated protection against apoptosis, macrophages were treated with tunicamycin (Tm) in the presence of varying concentrations of HDL (5-75μg/mL). Cells treated with Tm at 10μg/ml in the absence of HDL was included as a control for maximal apoptosis[1]. Treatment of macrophages with tunicamycin caused a significant increase of TUNEL positive cells **(Figure 4.1 A&B)**. HDL triggered a significant dose-dependent reduction in tunicamycin-induced apoptosis, starting at a concentration of 10μg protein/ml, and a maximal protection at 50 and 75μg protein/ml.

To test for HDL's ability to supress apoptosis, Tm, thapsigargin (Th), staurosporine (SS), and UV irradiation were used to induce apoptosis amongst WT peritoneal macrophages **(Figure 4.2A-D)**. Tm and Th induce apoptosis through ER stress with the former inhibiting protein N-glycosylation and the latter inhibiting ER Ca^{2+}-dependent ATPase. SS induces apoptosis by non-selectively inhibiting protein kinases which in turn disrupts major cell cycle proteins. Meanwhile, UV irradiation damages the cell's DNA and the cell undergoes apoptosis when it cannot repair this damage. Treatments involved 2 main components, the presence or absence of HDL (50μg/ml), and the presence or absence of the apoptotic inducer. For Tm (10μg/ml), Th (5μM), and SS (0.3μM), cells were incubated along with or without HDL for 24 hours prior to detection of apoptotic cells. For UV radiation, cells were pre-treated with HDL for 2 hours, after which they were exposed to varying levels (10-50 mJ/cm^2) of UV irradiation. Immediately afterwards, the media was replaced and cells were incubated with or without HDL for 24 hours. The experiment was conducted with technical replicates of 3-4 for each type of treatment and the experiments were repeated multiple times with cells prepared from different mice to ensure

reproducibility. Apoptosis levels amongst the macrophages were determined by TUNEL staining (refer to Ch *3.6: Apoptosis staining and analysis*). Baseline levels of apoptosis in cells that were incubated in the absence of lipoproteins and apoptosis inducers were low, with approximately 1-2% of cells being TUNEL positive **(Figure 4.2A-D)**. Treatment with HDL alone did not affect the levels of apoptosis of macrophages cultured in the absence of apoptosis inducers. When cells were incubated with ER stress inducers such as tunicamycin **(Figure 4.2A)**, or thapsigargin alone **(Figure 4.2B)**, there was a significant increase in TUNEL positive cells relative to baseline. Similarly, treatment with staurosporine **(Figure 4.2C)**, a non-specific protein kinase inhibitor, resulted in a significant increase in level of apoptosis relative to control. Finally, exposure to UV irradiation also triggered increased apoptosis in a UV-dose dependent manner **(Figure 4.2D)**. In all cases, incubation of cells with HDL resulted in a significant reduction in apoptosis, in response to each treatment (tunicamycin, thapsigargin, staurosporine or UV irradiation) **(Figure 4.2A-D)**. To compare the degree of protection by HDL against different apoptosis inducers, the maximum level of apoptosis was set as 100% and the degree of protection by HDL was calculated as the % maximal apoptosis **(Figure 4.2E)**. HDL treatment induced a significant reduction in apoptosis induced by, Tm (89%), SS (96%), Th (94%), and UV at 50mJ/cm^2 (87%) respectively. This suggests that HDL mediates protection of macrophages against apoptosis induced by various stimuli.


A
Tm -
Tm +
HDL -
HDL -
HDL+

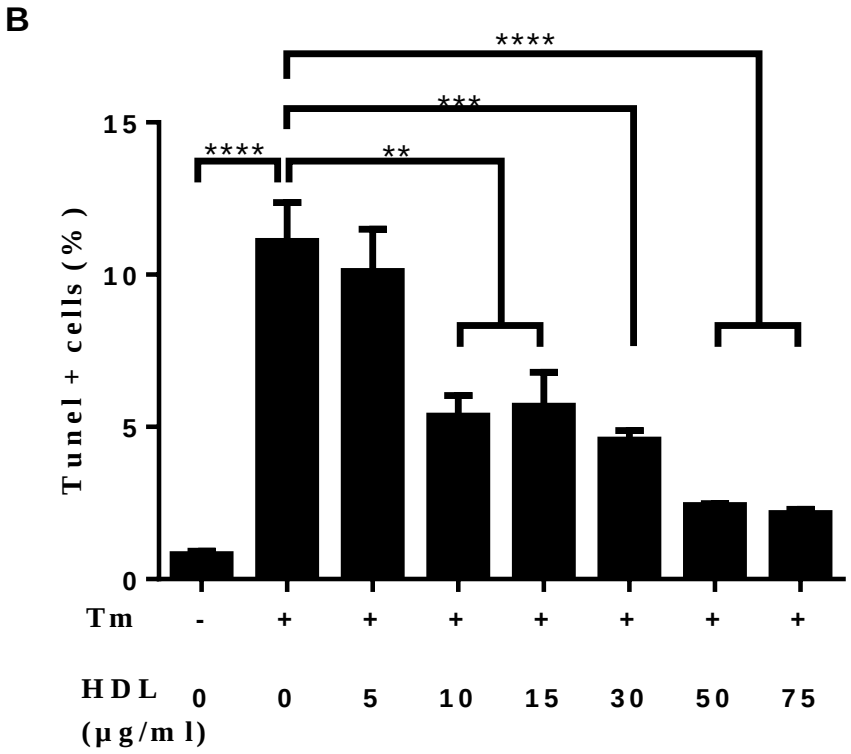

B
****
***
**
****
15
10
5
0
Tunel + cells (%)
Tm - + + + + + + +
HDL (µg/ml) 0 0 5 10 15 30 50 75

Figure 4. 1: HDL protects macrophages from apoptosis in a dose dependant manner.

Thioglycolate-elicited murine peritoneal macrophages were incubated with tunicamycin (Tm, 10μg/mL) in the absence or presence of varying concentrations of HDL (5 – 75μg/ml). After 24 hrs of incubation, cells were fixed and stained for DNA cleavage (TUNEL) and nuclei (DAPI). A: Representative images of macrophages from WT mice in the presence or absence of Tm and HDL (50μg/ml). DNA cleavage was seen in green; and nuclei were seen in blue. B. Quantification of TUNEL-positive nuclei as a percentage of total nuclei. Results (n=3 where cells were isolated from 1 mouse) are shown as mean ± SEM. **p<0.01, ***p<0.001, ****p<0.0001; 1-way ANOVA w/ Tukey's multiple comparisons test.

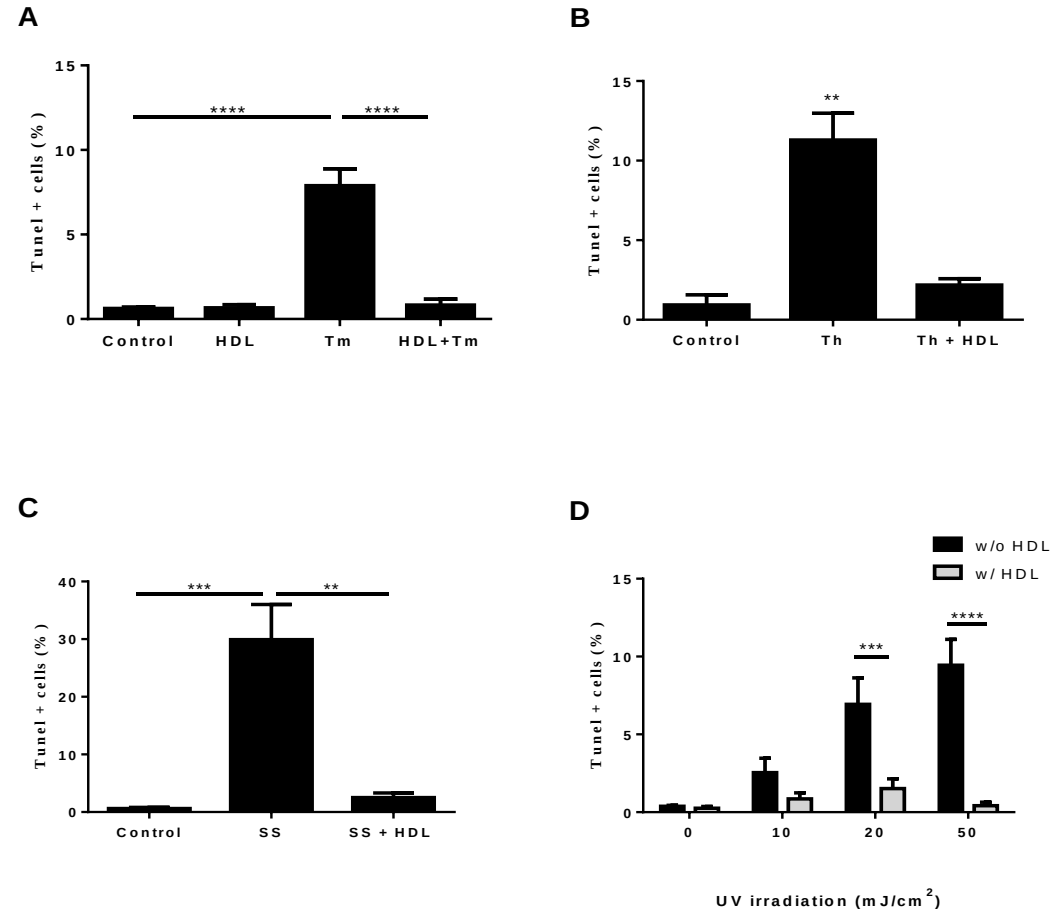

A
Tunel + cells (%)
15
10
5
0
****
****
Control
HDL
Tm
HDL+Tm
B
Tunel + cells (%)
15
10
5
0
**
Control
Th
Th + HDL
C
Tunel + cells (%)
40
30
20
10
0
***
**
Control
SS
SS + HDL
D
w/o HDL
w/ HDL
Tunel + cells (%)
15
10
5
0
***
****
0
10
20
50
UV irradiation (mJ/cm2)
E
% of maximal apoptosis
150
100
50
0
****
Tm
Tm + HDL
% of maximal apoptosis
150
100
50
0
****
SS
SS + HDL
% of maximal apoptosis
150
100
50
0
**
Th
Th + HDL
% of maximal apoptosis
150
100
50
0
****
UV (50mJ/cm2)
UV + HDL

Figure 4. 2: **HDL supresses apoptosis induced by various stimuli in WT murine peritoneal macrophages.**

Thioglycolate-elicited murine peritoneal macrophages were incubated with or without **A.** tunicamycin (Tm, 10μg/mL), **B.** thapsigargin (Th, 5μM), and **C.** staurosporine (SS, 0.3μM) in the presence or absence of, HDL (50μg/mL) as indicated. **D.** Alternatively, cells were pre-incubated with or without HDL for 2 hrs and then exposed to UV radiation at 10, 20, and 50mJ/cm^2 in the absence of HDL, and after the media was changed, cells were incubated with or without HDL for a further 24 hrs. **A-D** TUNEL-positive nuclei were quantified as a percentage of total nuclei. Results (n=3-4 where cells were isolated from 1 mouse) are shown as mean ± SEM. **E.** Average % reduction in apoptosis achieved by HDL treatment across all stimuli. Each apoptotic inducer elicits varying levels of apoptosis, so to allow for side by side comparison, results for each inducer are expressed relative to the maximal apoptosis seen (set as 100%). Results are shown as mean ± SEM (Tm: n=11 mice, SS: n= 7, where cells were isolated from 2 mice and plated in 3-4 replicates, Th: n=3 mice, UV: n=7 mice). **p<0.01, ***p<0.001, ****p<0.0001; (**A-C.** 1-way ANOVA w/ Tukey's multiple comparisons test. **D.** 2-way ANOVA w/ Tukey's multiple comparisons test. **E.** Unpaired Student's t-test).

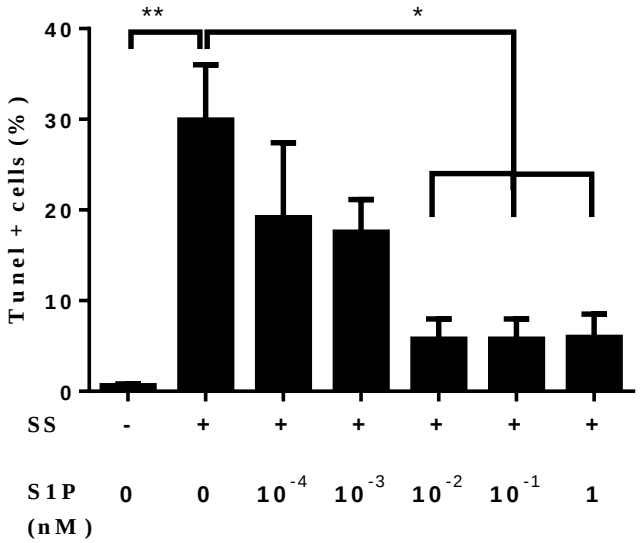
S1P protects macrophages against apoptosis induced by staurosporine and this is partially mediated by the S1P receptor S1P1

To test the effects of sphingospine-1-phosphate (S1P), a bioactive lipid known to be carried by HDL, on macrophage apoptosis, macrophages were treated with staurosporine (0.3μM) in the absence or presence of S1P ranging from 0.1pM to 1nM. Treatment of macrophages with SS caused a significant increase of TUNEL positive cells **(Figure 4.3A)**. S1P showed a significant dose-dependent protection of macrophages against SS induced apoptosis starting at a concentration of 10pM (81% decrease).

To test the involvement of the S1P receptor 1 (S1P1) in S1P mediated protection against apoptosis, macrophages were collected from either the wild type (C57BL6) mice, or $S1P1^{MKO}$ mice which lack the S1P1 receptor in all myeloid cells. These macrophages were then treated with SS in the absence or presence of S1P. SS treatment induced similar levels of apoptosis in WT and $S1P1^{MKO}$ macrophages **(Figure 4.3B)**. Treatment with 1nM S1P, corresponding to the highest concentration used in the dose response curve of Figure 4.3A, induced a similar level of protection (84%) of WT macrophages as that seen in Figure 4.3A. In contrast, 1nM S1P treatment reduced the average level of SS-induced apoptosis by only 54% in macrophages lacking S1P1, suggesting that the anti-apoptotic effects of S1P may be at least partially mediated by S1P1. Surprisingly, when higher concentrations of S1P were used, the level of protection of WT macrophages against SS-induced apoptosis was reduced. This was even more pronounced in S1P1-deficient macrophages. In combination with the results of Figure 4.3A, this suggests that S1P exhibits a U-shaped dose response pattern with regards to suppression of SS-induced apoptosis. The mechanism driving this phenomenon is currently unclear. In $S1P1^{MKO}$ cells, 1nM S1P was able to partially (n.s.) protect against SS-induced apoptosis although the level of

protection was less than that seen in WT cells, with a decrease in apoptosis by 55% vs 84%. Similar to WT cells, higher S1P concentrations were even less effective at protecting against SS-induced apoptosis in S1P1^{MKO} cells, with 100nM S1P even elevating apoptosis level (by 43%) past that of SS alone. Together these results suggest that for SS-induced apoptosis, S1P-dependant protection may be only partially mediated by S1P1 and is lost at higher S1P concentrations.

A


**
*
40
30
20
10
0
Tunel + cells (%)
SS - + + + + + +
S1P 0 0 10-4 10-3 10-2 10-1 1
(nM)

B

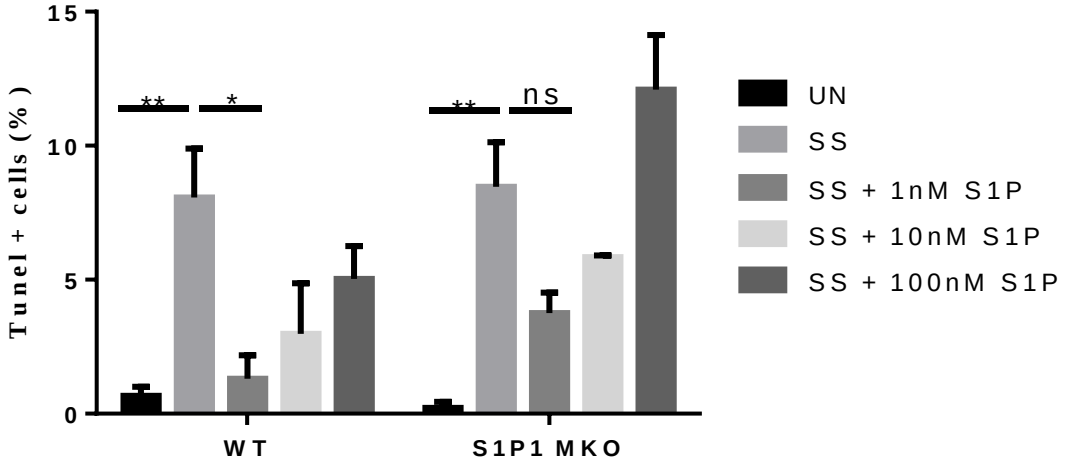

15
10
5
0
Tunel + cells (%)
** *
** ns
WT
S1P1 MKO
UN
SS
SS + 1nM S1P
SS + 10nM S1P
SS + 100nM S1P

Figure 4. 3: S1P protects macrophages from SS-induced apoptosis in a U-shaped dose response pattern and is partially mediated by S1P1.

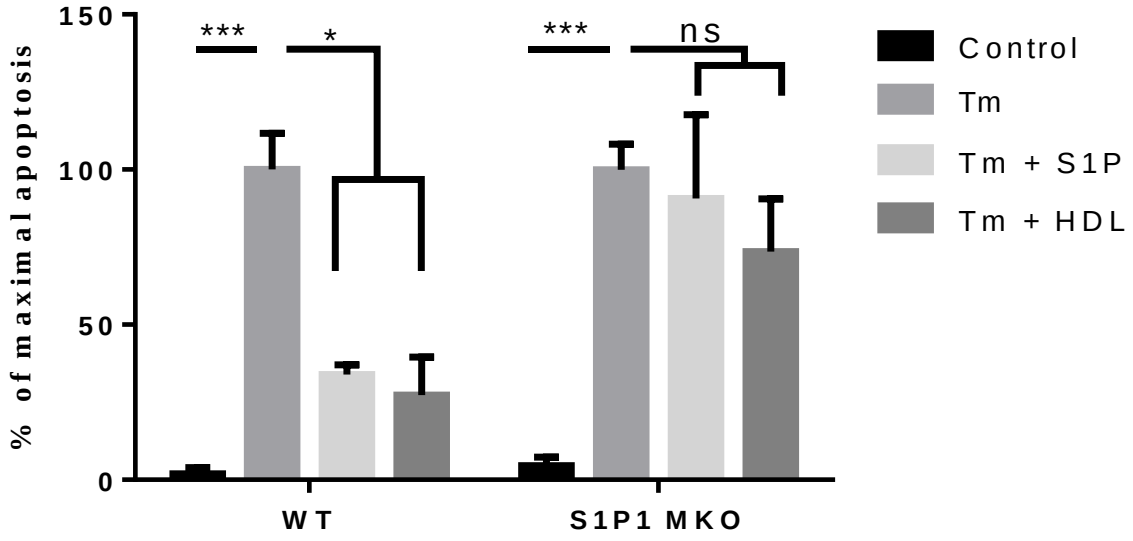
A. Thioglycolate-elicited murine peritoneal macrophages were incubated with staurosporine (SS, 0.3μM) in the absence or presence of varying concentrations of S1P as indicated. **B.** Macrophages from either wild type or macrophage selective S1P1 deficient ($S1P1^{MKO}$ or $LysM^{Cre/Cre}$; $S1P1^{lox/lox}$) mice were incubated with SS in the absence or presence of S1P at the indicated concentrations. **A** & **B.** After 24 hrs of incubation, cells were fixed and stained for DNA cleavage (TUNEL) and nuclei (DAPI). TUNEL-positive nuclei were quantified as a percentage of total nuclei. Results (n=3-4 where cells were isolated from 1 mouse) are shown as mean ± SEM. *$p<0.05$, **$p<0.01$; (**A.** 1-way ANOVA w/ Tukey's multiple comparisons test. **B.** 2-way ANOVA w/ Tukey's multiple comparisons test).

S1P1 is required for HDL-dependent protection against Tm-induced apoptosis but not against SS induced apoptosis

Since S1P can also confer protection against apoptosis in a manner similar to HDL, I investigated whether the S1P1 receptor was required for HDL dependent protection against apoptosis. To test this, macrophages from WT and $S1P1^{MKO}$ mice were treated with tunicamycin (10μg/ml) either in the presence or absence of HDL (50μg/ml) or S1P (1nM) for 24hrs. Apoptosis levels amongst the macrophages were determined by TUNEL staining. Tm increased apoptosis in both WT and $S1P1^{MKO}$ macrophages **(Figure 4.4A)**. Within the WT macrophages, co-treating with either the HDL or S1P, significantly decreased the level of apoptosis. In contrast, neither HDL nor S1P were able to reduce Tm-induced apoptosis in $S1P1^{MKO}$ cells. This suggests that S1P1 is required for HDL and S1P to confer protection against apoptosis induced by the ER stressor tunicamycin.

This experiment was also repeated with SS (0.3μM) to verify results using a different apoptotic inducer. SS increased apoptosis in both WT and $S1P1^{MKO}$ macrophages **(Figure 4.4B)**. Within the WT macrophages, co-treating with either the HDL or S1P, significantly decreased the level of apoptosis as seen before. However, within the $S1P1^{MKO}$ cells, in contrast to the Tm-induced apoptosis experiment, HDL successfully reduced SS-induced apoptosis. S1P statistically failed to reduce SS-induced apoptosis, although the average apoptosis level was lowered as seen before within the $S1P1^{MKO}$ cells (**Figure 4.3)**. This suggests that HDL can confer protection against SS induced apoptosis in macrophages independent of S1P or its receptor S1P1.

A


150
100
50
0
% of maximal apoptosis
***
*
***
ns
Control
Tm
Tm + S1P
Tm + HDL
WT
S1P1 MKO

B

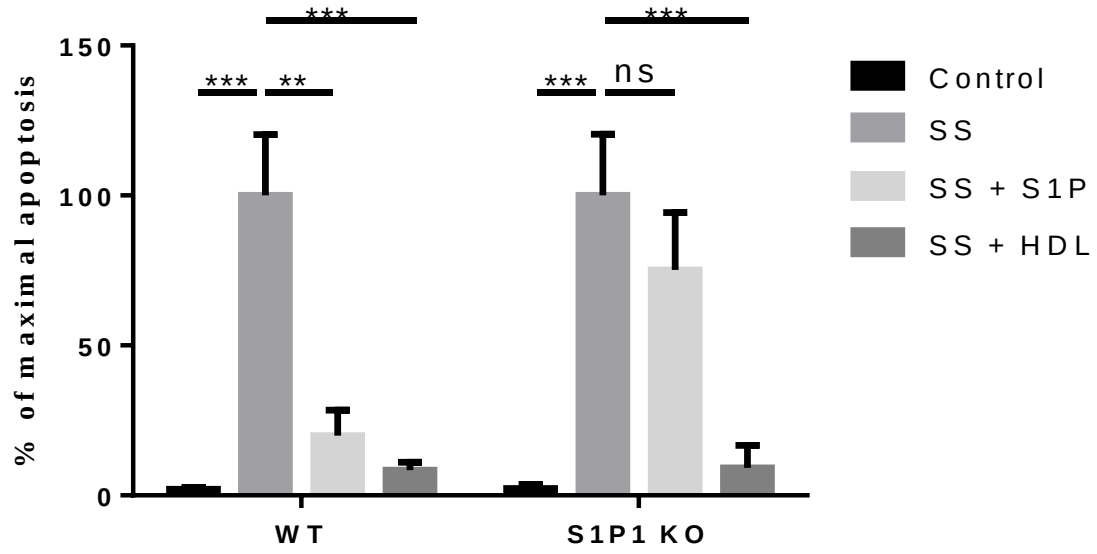

150
100
50
0
% of maximal apoptosis
***
***
***
**
***
ns
Control
SS
SS + S1P
SS + HDL
WT
S1P1 KO

Figure 4. 4: HDL requires S1P1 to mediate protection of macrophages from Tm induced apoptosis but not against SS induced apoptosis.

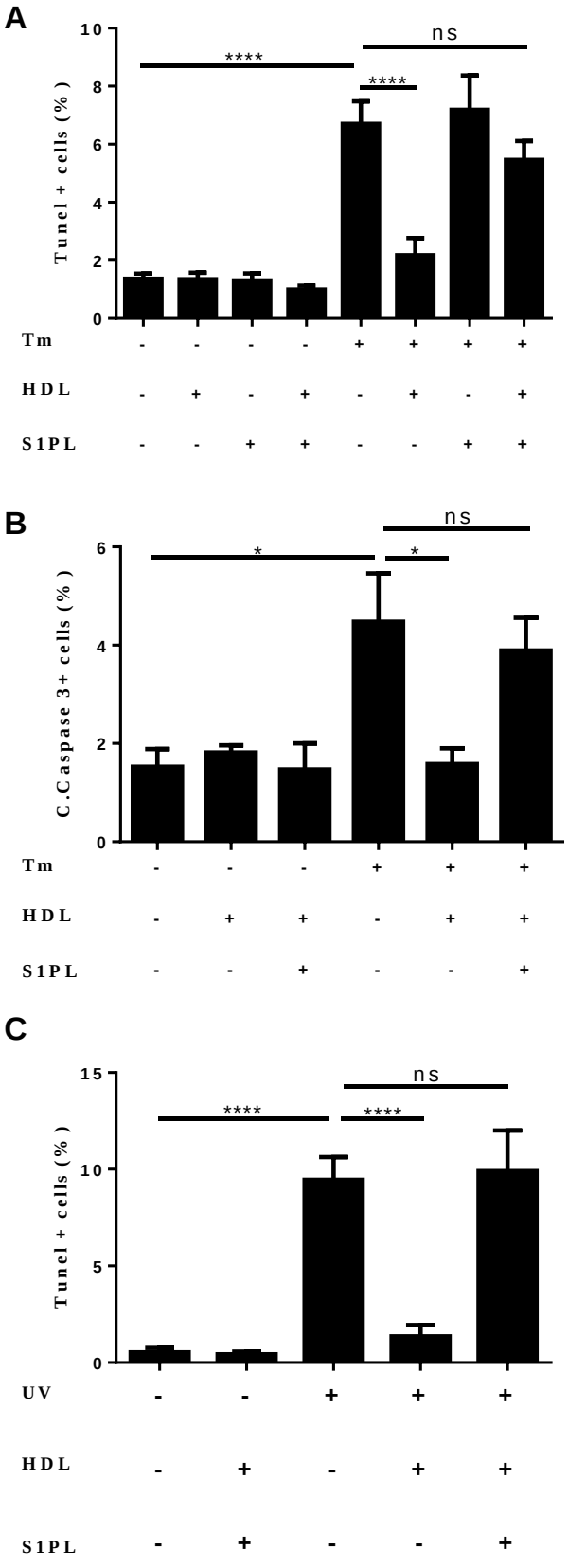
Thioglycolate-elicited peritoneal macrophages from either WT or $S1P1^{MKO}$ mice were incubated with **A.** tunicamycin (Tm, 10μg/ml) or **B.** staurosporine (SS 0.3μM) in the presence or absence of HDL (50μg/mL) or S1P (1nM) as indicated. **A & B.** After 24 hrs of incubation, cells were fixed and stained for DNA cleavage (TUNEL) and nuclei (DAPI). TUNEL-positive nuclei were quantified as a percentage of total nuclei and to allow for better side by side comparison, results for each apoptotic inducer are expressed relative to the maximal apoptosis seen (set as 100%). Results (n=4 where cells were isolated from 1 mouse) are shown as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001 (2-way ANOVA w/ Tukey's multiple comparisons test).

Effects of S1P Lyase treatment on HDL's ability to supress apoptosis in macrophages

S1P lyase (S1PL), which irreversibly cleaves S1P to phosphoethanolamine and hexadecenal, was utilized to determine if HDL's ability to protect macrophages against apoptosis was dependent on S1P. HDL was pre-treated for 1hr with S1PL (0.02µg/1mg of HDL protein), and then added to cells along with tunicamycin or after UV irradiation, and apoptosis levels were evaluated 24 hrs later by TUNEL or CC-3 staining. For controls, HDL was incubated in parallel without S1PL, or in some cases S1PL was incubated in parallel without HDL prior to addition to cells. Neither HDL, S1PL alone or S1PL + HDL triggered apoptosis in macrophages **(Figure 4.5A)**. Tunicamycin treatment significantly increased apoptosis as measured by TUNEL staining. S1PL alone did not affect the level of tunicamycin-induced apoptosis. When tunicamycin was incubated together with control HDL, there was a significant protection against apoptosis as seen previously. However, when tunicamycin was incubated with HDL and S1PL, there was no protection seen. Similar results were observed when apoptosis was evaluated by CC3 staining **(Figure 4.5B)**, or when apoptosis was induced by UV irradiation instead of Tm treatment **(Figure 4.5C)**. Therefore, HDL loses the ability to protect against apoptosis when S1PL is added, suggesting that HDL's ability to protect against apoptosis is dependant on S1P.

As an alternative to the binary approach in studying apoptosis achieved with the TUNEL assay, an apoptotic marker was measured through western blot analysis (see Methods). Poly (ADP-ribose) polymerase (PARP) are a family of proteins involved in ADP-ribosylation which is important in many cellular functions such as DNA repair, gene regulation and apoptosis. When cells undergo apoptosis, caspases cleave PARP (~113kDa) into 2 parts (24 kDa & 89 kDa)[58]. Cell lysates of macrophages, treated with the SS in the absence or presence of HDL or S1PL + HDL, were analyzed for PARP as a potential marker for apoptosis. No bands were observed

when cleaved PARP specific antibody was utilized (data not shown). Faint cleaved PARP bands were seen with full length PARP antibody, but the levels of cleaved PARP were inadequate to quantify properly **(Figure 4.5D)**. This suggests that cleavage of PARP may not be crucial for apoptosis amongst murine macrophages. TUNEL and CC3 assays for SS induced apoptosis have yet to be conducted to determine HDL's dependence on S1P.


A
Tunel + cells (%)
10
8
6
4
2
0
****
****
ns
Tm - - - - + + + +
HDL - + - + - + - +
S1PL - - + + - - + +
B
C.Caspase 3+ cells (%)
6
4
2
0
*
*
ns
Tm - - - + + +
HDL - + + - + +
S1PL - - + - - +
C
Tunel + cells (%)
15
10
5
0
****
****
ns
UV - - + + +
HDL - + - + +
S1PL - + - - +

D

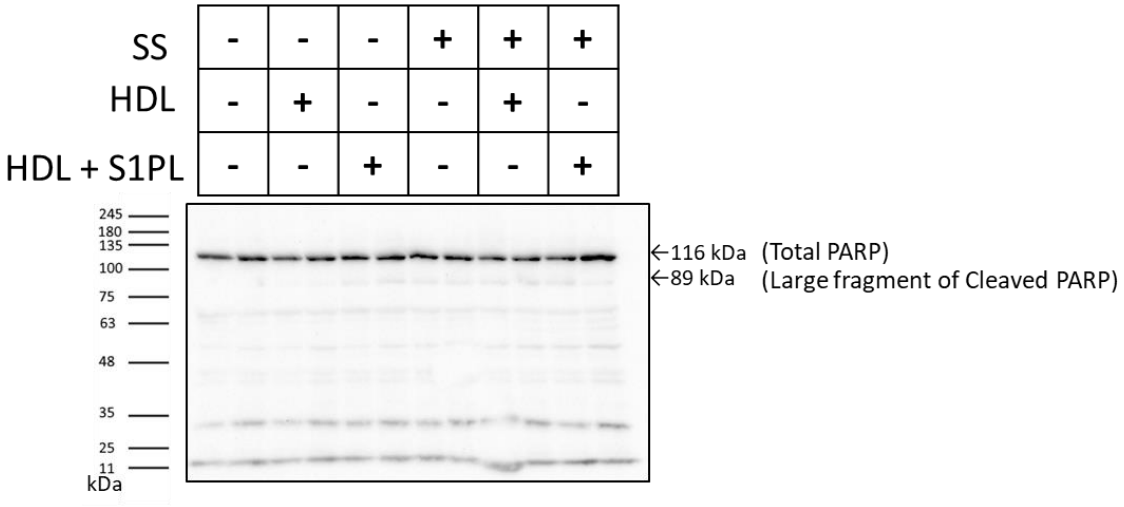

SS - - - + + +
HDL - + - - + -
HDL + S1PL - - + - - +
245
180
135
100
75
63
48
35
25
11
kDa
←116 kDa (Total PARP)
←89 kDa (Large fragment of Cleaved PARP)

Figure 4. 5: S1P lyase treated HDL fails to protect macrophages from apoptosis induced by tunicamycin or UV irradiation.

A. Thioglycolate-elicited murine peritoneal macrophages were incubated with or without tunicamycin (Tm, 10μg/mL) in the absence of additional treatments, or in the presence of untreated HDL, S1PL alone, or S1PL-treated HDL, untreated HDL (50μg/mL), S1PL (0.001μg/ml), or S1PL treated HDL. After 24 hrs of incubation, cells were fixed and stained for DNA cleavage (TUNEL) and nuclei (DAPI). TUNEL-positive nuclei were quantified as a percentage of total nuclei. Results are shown as mean ± SEM (n = 12 where cells were isolated from 4 mice and plated in triplicates except for S1PL alone and S1PL + Tm treatments, where n= 6 and cells were isolated from 2 of the 4 mice and plated in triplicates). **B.** Cells were treated and fixed as described in A, but stained for CC3 and DAPI. CC3-positive cells were quantified as a percentage of total nuclei. Results (n=3-4 where cells were isolated from 1 mouse) are shown as mean ± SEM. **C.** Macrophages were exposed to UV irradiation (50mJ/cm^2), in the presence or absence of, untreated HDL (50μg/mL), or HDL treated with S1PL. After 24 hrs of incubation, cells were fixed and stained for DNA cleavage (TUNEL) and nuclei (DAPI). TUNEL-positive nuclei were quantified as a percentage of total nuclei. Results (n=8 where cells were isolated from 2 mice and plated in quadruplicates) are shown as mean ± SEM. **D.** Levels of complete and cleaved PARP amongst macrophages treated with SS in the presence and absence of HDL and S1PL as indicated. Treatments were done in duplicates, over 24hr time period. Proteins were separated on a 10% SDS-PAGE acrylamide gel and blotted onto PVDF membrane. Blocked in 5% skim milk and imaged for full and cleaved PARP utilizing PARP primary antibody (Cell Signalling; Cat#:9542). *$p<0.05$; ****$p<0.0001$; 1-way ANOVA w/ Tukey's multiple comparisons test.

Characterization of HDL

Given that S1PL treatment eliminated the ability of HDL to protect against apoptosis, I wanted to determine if S1PL treatment affected the gross structural properties of HDL. HDL that was treated with S1PL or control HDL incubated in parallel without S1PL, were first subjected to size exclusion chromatography. Afterwards, each fraction was analyzed for protein concentration (by UV absorption), and total cholesterol content. Both sets of HDL showed identical protein elution based on UV absorption **(Figure 4.6A)**. Similar to the protein content, the cholesterol contents were identical to each other. Most of the total cholesterol was detected from fractions 27-51, with the highest point of the peaks at fractions 38 – 39 **(Figure 4.6B)**. This experiment was repeated two more times with different batches of HDL, with identical results (results not shown).

We also analyzed the distribution of apolipoprotein (apo) A1 and apoM through western blot analysis (see Methods) of the specific range of fractions where HDL cholesterol was found. Results showed that apoA1 was mainly distributed in the pools from fraction 32 to 46 for both samples **(Figure 4.6C)**. HDL treated with S1PL was also seen to have a slight trace of apoA1 protein within the fraction pool 47-51 which was not present in the control HDL. Moreover, apoM was found only within two sets of pools stretching from fractions 32 – 41 in both samples **(Figure 4.6D)**. These results suggest a fairly equivalent distribution of proteins, and cholesterol. Therefore, I conclude that S1PL treatment of HDL seems to have no major effects on the structure of HDL as measured by size exclusion chromatography.

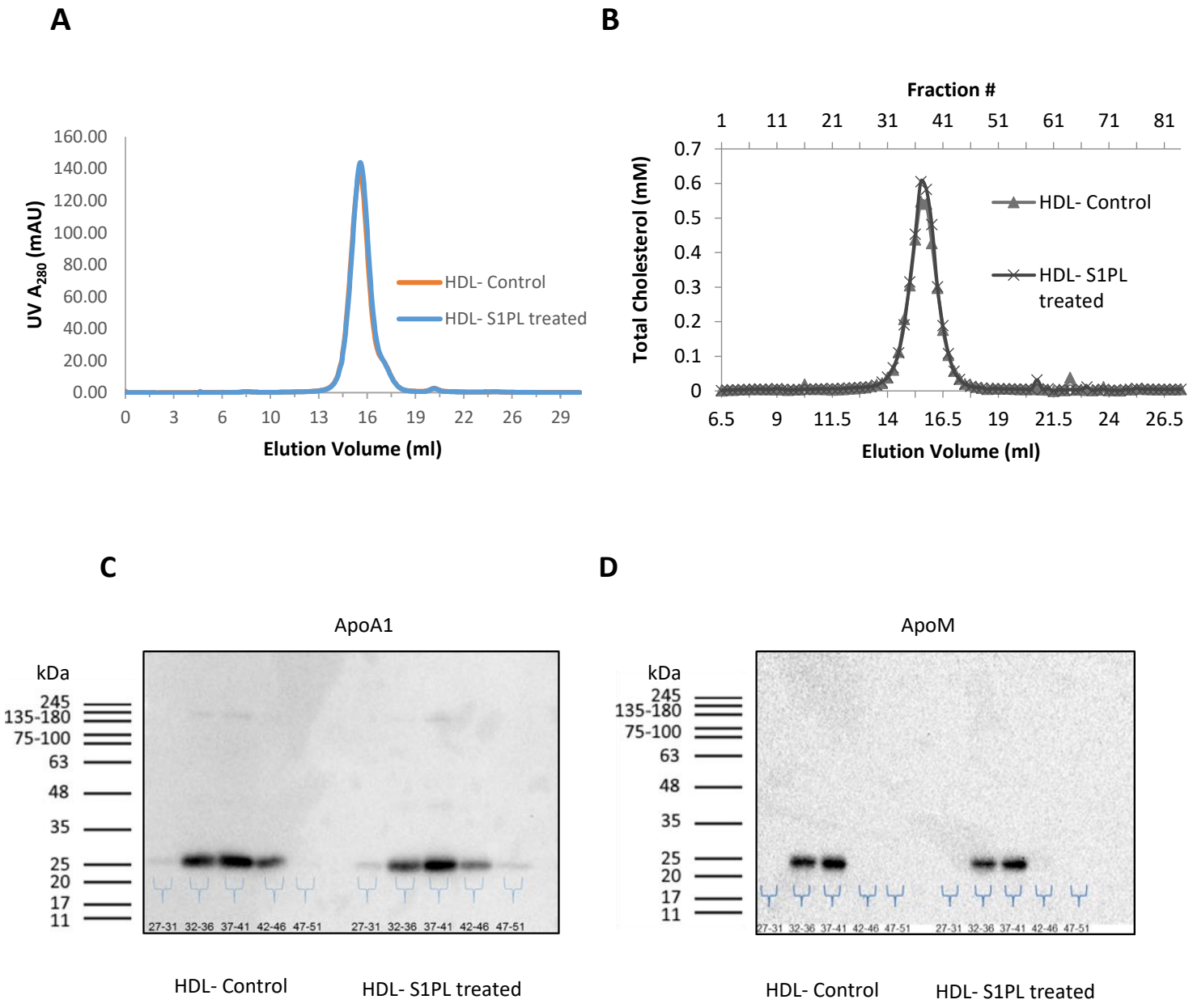

A
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
UV A280 (mAU)
0
3
6
10
13
16
19
22
26
29
Elution Volume (ml)
HDL- Control
HDL- S1PL treated
B
Fraction #
1
11
21
31
41
51
61
71
81
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Total Cholesterol (mM)
6.5
9
11.5
14
16.5
19
21.5
24
26.5
Elution Volume (ml)
HDL- Control
HDL- S1PL treated
C
ApoA1
kDa
245
135-180
75-100
63
48
35
25
20
17
11
27-31
32-36
37-41
42-46
47-51
HDL- Control
HDL- S1PL treated
D
ApoM
kDa
245
135-180
75-100
63
48
35
25
20
17
11
27-31
32-36
37-41
42-46
47-51
HDL- Control
HDL- S1PL treated

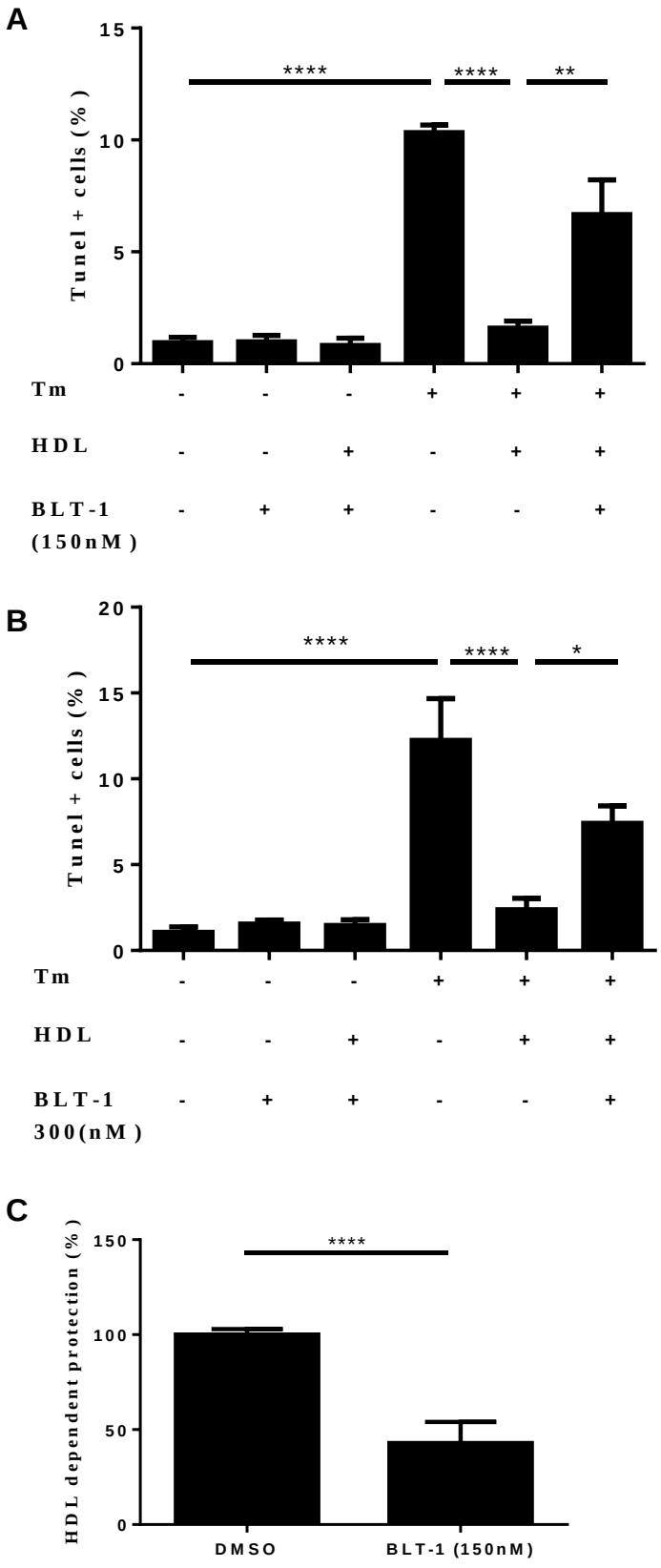
Figure 4. 6: Characterization of HDL- Control vs. HDL- S1PL treated.

HDL was incubated for (1 hour at 37ºC) either with 0.02µg of S1PL per mg of HDL protein, or without S1PL. Afterwards, 250µg of S1PL-treated or control HDL was subjected to size exclusion chromatography on a Tricorn Superose 6 HR 10/300 column using an Akta FPLC system. **A.** UV absorbance profile of eluted protein. Blue: S1PL-treated HDL; Orange: Control-HDL. **B.** Total cholesterol content of each fraction. (×) S1PL-treated HDL; (▲) Control-HDL. Immunoblotting for **C.** ApoA1 and **D.** ApoM in pooled fractions. Fractions from size exclusion FPLC were pooled in sets of 5 from 27 to 51 as indicated. Proteins were separated on a 12% SDS-PAGE acrylamide gel and blotted onto PVDF membrane. Results from A and B are representative of 3 independent analyses.

Inhibiting SR-B1's lipid transport activity attenuates HDL mediated protection of macrophages against apoptosis

To test if the lipid transport activity of the HDL receptor, SR-B1, was required for HDL's ability to protect against apoptosis, cells were incubated for 30 min at 37^{o}C with the small molecule, BLT-1, an inhibitor of SR-B1-mediated lipid transport but not HDL binding. Control cells were treated with DMSO vehicle. Cells were then treated with Tm in the continued absence or presence of BLT-1 and HDL as indicated. Apoptosis was quantified by TUNEL staining as before. BLT-1 treatment, alone or with HDL, in the absence of Tm did not affect the level of apoptosis compared to the control **(Figure 4.7A)**. Tm significantly increased the apoptosis levels compared with the control condition, and this increase was prevented by HDL in control DMSO treated cells. However, treatment with 150nM BLT-1 attenuated HDL's ability to confer protection against tunicamycin. This experiment was repeated with 300nM BLT-1 with similar results **(Figure 4.7B)**.

Figure 4.7C shows that in three independent experiments, treatment with 150 nM BLT-1 reduced the ability of HDL to protect against apoptosis by 57%. For this analysis, HDL dependent protection was calculated as the difference in apoptosis between Tm treated and Tm + HDL treated cells, and this value was set at 100 %. The effect of BLT-1 on HDL-dependent protection was calculated as the relative difference between HDL dependent protection in the presence of BLT-1 to that in the absence of BLT-1. Similarly, in two independent experiments treatment with 300 nM BLT-1 reduced the level of HDL dependent protection by 53% percent (data not shown). This suggests that HDL requires a functioning SR-B1, capable of lipid transport, for full protection of macrophages against apoptosis.


A
Tunel + cells (%)
15
10
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**
Tm - - - + + +
HDL - - + - + +
BLT-1 (150nM) - + + - - +
B
Tunel + cells (%)
20
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Tm - - - + + +
HDL - - + - + +
BLT-1 300(nM) - + + - - +
C
HDL dependent protection (%)
150
100
50
0
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DMSO
BLT-1 (150nM)

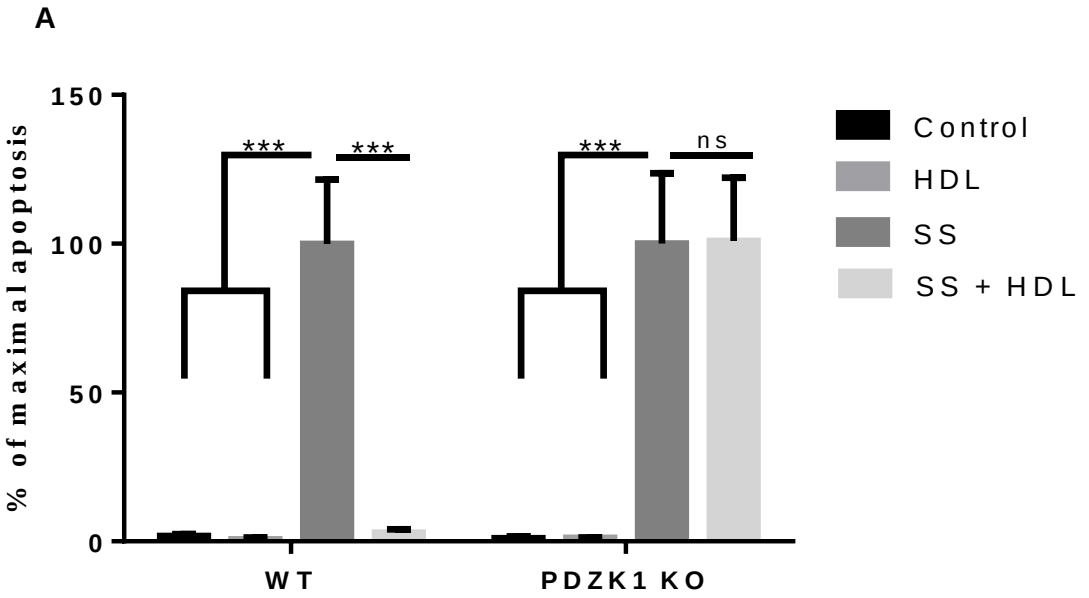
Figure 4. 7: Inhibiting SR-B1's lipid transport activity attenuates HDL mediated protection of macrophages against apoptosis.

Thioglycolate-elicited murine peritoneal macrophages were pretreated for 30 minutes with or without BLT-1 at **A.** 150 nM or at **B.** 300nM, before the addition of tunicamycin (10 μg/ml) and/or HDL (50 μg/ml) and the continued incubation with or without BLT-1 as indicated. All samples contained the same amount of DMSO vehicle. After 24 hrs of incubation, cells were fixed and stained for DNA cleavage (TUNEL) and nuclei (DAPI). TUNEL-positive nuclei were quantified as a percentage of total nuclei. Results are shown as mean ± SEM (**A.** n=11, where cells were taken from 3 mice, and plated in 3-4 wells; **B.** n= 6, where cells were taken from 2 mice and plated in triplicates). **C.** HDL dependent protection against Tm in DMSO and BLT-1 (150nM) treated cells. Difference between average apoptosis induced by Tm treatment alone and apoptosis induced by Tm + HDL treatment in DMSO treated cells are set as 100% HDL dependent protection. Results (n= 3 mice) are shown as mean ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$; (**A.** & **B.** 1-way ANOVA w/ Tukey's multiple comparisons test. **C.** Student's t-test).

PDZK1 expression is required for HDL mediated protection of macrophages against apoptosis

The adaptor protein, PDZK1, which binds to the C terminal cytoplasmic tail of SR-B1, is required for HDL signaling in macrophages[56]. I tested the involvement of PDZK1 in HDL dependent protection against macrophage apoptosis by examining the effects of HDL in macrophages from *Pdzk1*$^{-/-}$ mice. WT and *Pdzk1*$^{-/-}$ macrophages, were exposed to SS in the presence or absence of HDL. Apoptosis was analyzed using TUNEL 24hrs later. SS induced varying levels of apoptosis between WT and *Pdzk1*$^{-/-}$ cells, so to allow for direct side by side comparison, results for each cell type are expressed relative to the maximal apoptosis seen with SS treatment alone (set as 100%).

No significant difference in percent of TUNEL positive cells were found between control and HDL treatment in either group **(Figure 4.8A)**. SS significantly increased apoptosis levels in both groups compared with the control condition. In WT macrophages this increase was prevented by HDL as expected. However, in *Pdzk1*$^{-/-}$ macrophages there was no significant decrease in apoptosis levels by HDL treatment. Similar results were observed when apoptosis was induced by UV irradiation instead of SS treatment **(Figure 4.8B)**. These results suggest that PDZK1 expression is required for HDL mediated protection of macrophages against apoptosis.


A
% of maximal apoptosis
150
100
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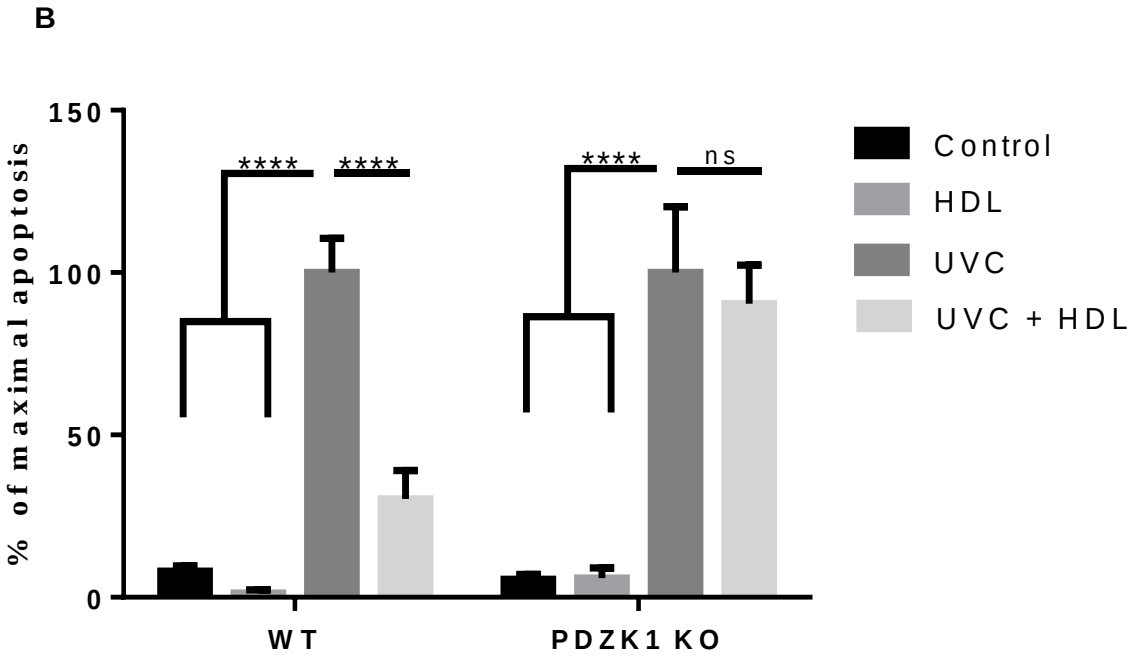
ns
WT
PDZK1 KO
Control
HDL
SS
SS + HDL


B
% of maximal apoptosis
150
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WT
PDZK1 KO
Control
HDL
UVC
UVC + HDL

Figure 4. 8: HDL fails to protect macrophages against apoptosis in *Pdzk1*$^{-/-}$ mice

A. Macrophages from either *Pdzk1*$^{+/+}$ or *Pdzk1*$^{-/-}$ mice were incubated with SS or HDL, as indicated, for 24 hrs. **B.** Macrophages from either *Pdzk1*$^{+/+}$ or *Pdzk1*$^{-/-}$ mice were exposed to UV irradiation at 50mJ/cm^2 to induce apoptosis in the presence or absence of HDL. After UV irradiation, the media was replaced with fresh media with or without HDL as indicated and cells were cultured for 24 hrs. For both **A.** and **B.**, after 24 hrs, cells were fixed and stained for DNA cleavage (TUNEL) and nuclei (DAPI). TUNEL-positive nuclei were quantified as a percentage of total nuclei. Results are shown as mean ± SEM (**A.** n = 3 where cells were isolated from 1 mouse and plated in triplicate. **B.** n = 9 where cells were isolated from 3 mice and plated in triplicate). ***p<0.001; ****p<0.0001; (**A. & B.** 2-way ANOVA w/ Sidak's multiple comparisons test).

CHAPTER 5: DISCUSSION

With regards to atherosclerosis, HDL's involvement is mainly known by its role in RCT where in cholesterol efflux from lipid laden macrophages provides a method to prevent atheroma progression[35]. However, HDL has other atheroprotective effects outside of cholesterol efflux. These are not well established or properly understood. Here we look at HDL's ability to prevent macrophages from undergoing apoptosis and the key components required for successfully mediating this signalling pathway. To start, we tested HDL's ability to protect against apoptosis induced by various stimuli in-vitro. These experiments were conducted on murine peritoneal macrophages obtained from WT mice, and apoptosis was induced utilizing Tm, Th, SS, and UV irradiation. We demonstrate that human HDL at 50µg/ml was able to reduce apoptosis levels by 85-95% within the macrophages, and that this occurs in a dose-dependent manner with regards to apoptosis induced by tunicamycin. This was in line with other studies and with work previously done in our lab that highlight HDL's ability to protect macrophages from apoptosis induced by various stimuli[1,10,57]. However, the exact mechanism starting from the interaction of HDL with the macrophage, to its final act of prevention of stress mediated cell apoptosis is not fully understood. It can be speculated that the bioactive sphingolipid, S1P with its many pleiotropic effects that mirror many of HDL's own inherent effects may play an important role in this system[49]. We demonstrate that S1P at 10pM-1nM, protects against apoptosis induced by staurosporine or tunicamycin, similar to HDL. However, higher does (>10nM) of S1P failed to protect macrophages from apoptosis. This could be due to the fact that physiological S1P levels are tightly regulated in humans at the low nM level with a study reporting the bioactive S1P level in plasma to be around 10nM[59]. This is consistent with our finding that S1P protected up to 10 nM but not at higher doses. Moreover, S1P is also shown to have synergistic effects with

chemotherapeutic drugs and causes cytotoxicity in human brain-metastasized breast cancer (MDA-MB-361) cells[60]. Although the mechanism behind this synergistic effect is not well understood, a similar event may be in occurrence with staurosporine in this experiment as it competes with S1P's otherwise protective role. This may explain the S1P's U-shaped dose dependant protection as well as the trend seen in **Figure 4.3B** where in staurosporine and S1P at 100nM together trigger an even greater level of apoptosis than when staurosporine is treated by itself in the $S1P1^{MKO}$ cells. A treatment of staurosporine with 10mM S1P was also conducted but this yielded near 100% apoptosis which skewed the statistics when comparing between groups (data not shown).

Previous work from our lab implicates S1P1 in mediating HDL signaling in macrophages[56,57]. Hence, we looked into the involvement of S1P1 utilizing $S1P1^{MKO}$ mice. We found that knocking out S1P1 prevented S1P mediated protection against apoptosis induced by tunicamycin, but appeared to only partially impair S1P mediated protection against apoptosis induced by staurosporine. When HDL was used, this discrepancy was more pronounced as knockout of S1P1 completely impaired HDL dependent protection against apoptosis induced by tunicamycin but did not affect HDL dependent protection against apoptosis induced by staurosporine. One possible reason for this discrepancy may be that, staurosporine and tunicamycin trigger apoptosis through different pathways. While staurosporine inhibits a variety of kinases including Erk and Akt and cell cycle proteins, tunicamycin inhibits protein N-glycosylation eliciting the ER stress response[61,62]. Due to staurosporine's non-selective inhibition, its mode of action is unclear compared to tunicamycin and the ER-stress pathway. Regardless, this observation suggests that HDL may engage different pathways to protect against apoptosis. One of these pathways appears to involve S1P1 signaling and is effective at protecting

against apoptosis resulting from ER stress and the UPR (as is the case with tunicamycin induced apoptosis). It is possible that HDL may engage other S1P receptors expressed in macrophages to protect against apoptosis elicited through other pathways (such as staurosporine induced apoptosis). Alternatively, it is plausible for HDL dependent protection against staurosporine induced apoptosis to be S1P/S1P receptor independent (see **Figure 5.1**). Further research will be required to test this.

HDL could also be working independently of S1P to promote cell survival, which may also answer this contrast between staurosporine and tunicamycin. Although S1P can protect against staurosporine induced apoptosis, it may not need to be associated in any way with HDL to do so. Since S1PL irreversibly cleaves S1P[43], without interfering with the HDL size, cholesterol content, and apoA1/M protein levels as demonstrated, it would be a good method to test whether HDL depends on S1P to protect against staurosporine induced apoptosis. Although we demonstrate that HDL is dependant on S1P to mediate protection against tunicamycin-induced apoptosis through the S1PL experiment, we have yet to test it with staurosporine. I had attempted to test this using PARP cleavage as a measure of the activation of apoptosis. However, surprisingly, I found very little PARP cleavage in SS-treated elicited murine peritoneal macrophages. Although there are studies that utilize cleaved PARP as an apoptotic marker to highlight effects of staurosporine, most are done either in other cell types, or in non-murine cell lines such as THP-1[63]. A study by Wang Z, et. Al., has provided support for PARP being dispensable in apoptosis while still being important for genomic stability by utilizing mice lacking genes encoding PARP[64]. Fibroblasts from these PARP deficient mice underwent apoptosis normally when treated with numerous apoptotic inducers. In a similar vein,

macrophages from mice may not require the cleavage of PARP for the execution of apoptosis, which would explain the lack of cleaved PARP in my results.

Similar to tunicamycin, HDL mediated protection of macrophages from UV irradiation was also shown to be dependant on S1P through the use of S1PL. This was not surprising as studies support that UV irradiation triggers ER-stress and a similar apoptotic response as that of tunicamycin[65–67]. For example, it has been shown that Mcl-1, an anti-apoptotic protein that prevents mitochondrial pore formation, is upregulated in response to ER-stress[68]. Along the same line, a recent study has showed that a loss of Mcl-1 was required for UV irradiation to fully trigger apoptosis in a human immortalized cell line[65]. This suggests that HDL depends on S1P to prevent both the tunicamycin and UV irradiation, from triggering ER-stress induced apoptosis within macrophages. With regards to staurosporine induced apoptosis, further assessment is needed for clarifying HDL's independence to S1P in mediating protection, which could potentially shed light to new signalling pathways by which HDL mediates responses in macrophages.

For ER-stress induced apoptosis, my results suggest that SR-B1 plays a role in shuttling HDL associated S1P to S1P1. Although S1P1 is known to be a cell surface receptor, a recent crystal structure analysis of this receptor suggests that S1P must gain access to the binding site located within the transmembrane region as it cannot be accessed from the external milieu[69]. So, we investigated HDL's receptor SR-B1, as it is known to mediate selective uptake of lipids into the plasma membrane of the cells[22,70,71]. Homology modeling of SR-B1 based on LIMP-2, another scavenger receptor protein form the same family, provides support for a hydrophobic tunnel that runs through SR-B1 providing access for lipids to the outer leaflet of the plasma membrane[72]. We speculate that this is the method by which S1P, delivered by HDL enters into

the membrane and reaches the S1P1's inner pocket binding site. To test this, the selective lipid transport inhibitor BLT-1, which covalently modifies a cysteine residue thereby blocking this channel[72–74], was pre-incubated with the cells for 30 minutes prior to the routine HDL treatment with tunicamycin induced apoptosis assay. The results showed that there was a significant decrease, in HDL's ability to protect against tunicamycin induced apoptosis when SR-B1 lipid transport activity is attenuated. The reason for this loss of function could tie into S1P delivery from HDL onto S1P1 via SR-B1. With increased concentration of BLT-1 (300nM vs 150nM) we expected a more complete loss of protection, but it was not seen. This may be due to BLT-1's diminished effect at higher concentration. Studies have reported dose response curves for BLT-1 by looking at lipid uptake in murine cells using fluorescent lipid DiI-HDL[73,74]. Based on their results, BLT-1 was only able to inhibit SR-B1 mediated uptake by a maximum of 80 % at a concentration of 10 μM at which point the dose response curve plateaued, and by 60 % at a concentration of 150 nM[73,74], corresponding to the concentration we used. This level of diminished lipid uptake coincides with the HDL's diminished protection seen in **Figure 4.7B**. Hence increasing the concentration of BLT-1 past 150 nM would provide diminishing effects on the loss of apoptosis protection. The effects of BLT-1, known to block SR-B1's lipid transport activity, suggests that HDL's dependence on SR-B1 to confer protection may involve SR-B1-mediated transport of S1P from HDL into cells. However, it remains to be directly demonstrated whether SR-B1 is able to transport S1P from bound HDL into cells.

Previous work from our lab, as well as my own, has demonstrated that, without the involvement of the scaffold protein PDZK1, HDL is not able to elicit atheroprotective signalling within leukocytes[1,56]. Macrophages from *Pdzk1*$^{-/-}$ mice were utilized for these experiments as the absence of PDZK1 amongst macrophages do not affect the SR-B1 protein levels or its

distribution upon the cell surface[75,76]. This failure to protect macrophages against tunicamycin, staurosporine, and UV induced apoptosis suggests that PDZK1 and its interaction with SR-B1 is also crucial for promoting cell survival.

In conclusion, my results suggest that HDL mediates protection against Tm induced apoptosis of macrophages in an S1P, S1P1, SR-B1, and PDZK1 dependant manner as summarized by the working model shown below **(Figure 5.2)**. HDL also mediates protection against UV irradiation induced apoptosis in a similar manner. My initial hypothesis must still be rejected as HDL was able to protect against staurosporine induced apoptosis without depending on S1P1. Moreover, S1P by itself was able to fully protect macrophages from various apoptotic inducers including staurosporine, but this required S1P1. More work is needed to confirm and to fully understand the underlying mechanisms involved in HDL mediated protection against staurosporine-induced apoptosis. However, these results suggest that HDL may have multiple effects, protecting against apoptosis induced by different stimuli via different pathways. Further elucidation of how HDL and S1P confront various apoptotic situations within macrophages can provide insight into new targets for therapeutic intervention, especially for patients with advanced atherosclerotic lesions.

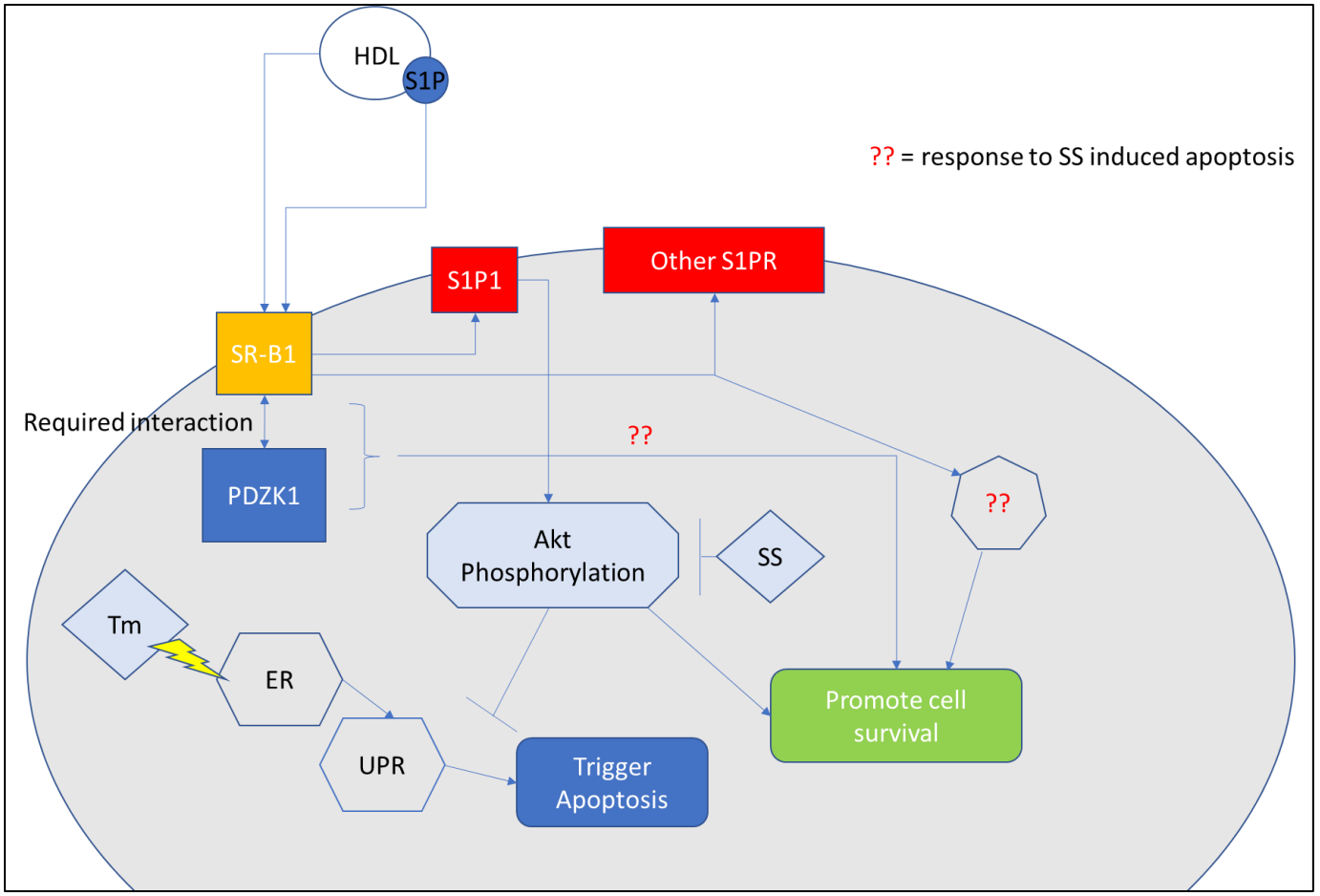
HDL
S1P
?? = response to SS induced apoptosis
S1P1
Other S1PR
SR-B1
Required interaction
??
PDZK1
??
Akt
Phosphorylation
SS
Tm
ER
Promote cell
survival
UPR
Trigger
Apoptosis

Figure 5. 1: Hypothesized internal mechanisms of HDL mediated protection against Tm and SS induced apoptosis.

When a macrophage is exposed to Tm for a prolonged period of time, it inhibits protein N-glycosylation eliciting the ER stress response which eventually leads to cell apoptosis. HDL and S1P can help protect macrophages from this Tm induced apoptosis by triggering the Akt cell survival pathway with the help S1P1 assuming the macrophage has a functioning SR-B1 and PDZK1. However, in a SS induced apoptosis, Akt phosphorylation is inhibited, and S1P fails to rescue the cell if it lacks S1P1. In contrast, HDL can still protect against SS induced apoptosis without needing S1P1, suggesting either a direct pathway downstream of binding to the SR-B1/PDZK1 complex or via another pathway elicited through other S1PRs, to promote overall cell survival.

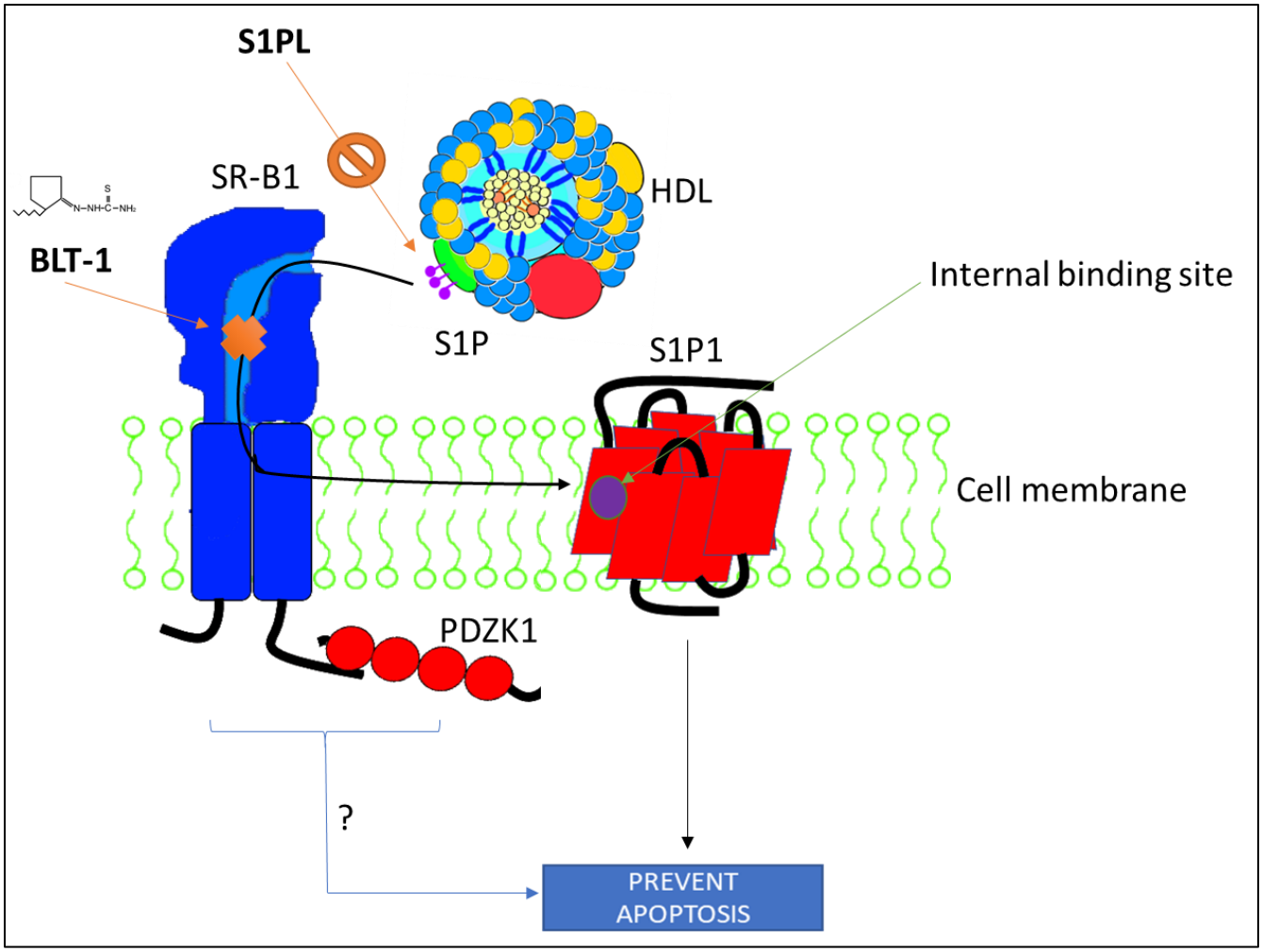
S1PL
SR-B1
HDL
BLT-1
Internal binding site
S1P
S1P1
Cell membrane
PDZK1
?
PREVENT
APOPTOSIS

Figure 5. 2: Re-worked model for HDL mediated signaling in macrophages.

HDL delivers S1P via SR-B1 towards the inner binding pocket found in S1P1 which is ordinarily inaccessible from the external milieu. This triggers downstream signaling pathways to promote macrophage protection against apoptosis induced by ER stress inducing agents. HDL requires S1P as irreversibly removing it with S1PL results in a lack of protection against apoptosis. This protection is also dependant on a functioning SR-B1 and PDZK1 as blocking the lipid transport with BLT-1 or knocking out PDZK1 both deny HDL dependant protection against apoptosis**.** Moreover, based on HDL's ability to protect against SS-induced apoptosis in the absence of S1P1, there must be other factors or pathways involved that requires SR-B1 and PDZK1 which also leads to prevention of apoptosis.

CHAPTER 6: FUTURE WORK

In terms of future work left to be finished, I need to further examine the dichotomy between HDL's behaviour against tunicamycin and staurosporine. With the findings we have demonstrated in mind, I want to evaluate how the S1PL treated HDL would affect the apoptosis induced by staurosporine. This would better clarify HDL's dependence on S1P for all its anti-apoptotic affects on macrophages. Although S1P confers anti-apoptotic affects by itself as well, the underlying mechanism and downstream pathways involved are still unclear. Knocking out other receptors and checking for S1P induced protection may narrow down the initial start of action. This could be done with a similar granulocyte specific knockout or through drugs such as FTY720 that down-regulate or inhibit certain receptors with a decent level of specificity[54]. With regards to elucidating downstream effects, phosphorylation of Akt which triggers the cascade of signalling that promotes cell survival is of great interest. Confirming that staurosporine alters Akt protein levels, and then looking at the Akt levels after the introduction of S1P may provide further evidence for S1P's mode of action. I would go about doing this by treating cells with SS in the absence and presence of S1P (along with appropriate controls), and analyze Akt phosphorylation by immunoblotting for phosphorylated Akt, at both phosphorylation sites (Ser & Thr), and total Akt.

My results with S1PL and with BLT-1 insinuate that S1P and SR-B1 lipid transport activity are required for HDL mediated protection against apoptosis stimulated by tunicamycin and UV irradiation. This suggests that SR-B1 may act by transferring S1P from HDL into the cell membrane where it can gain access to S1P1, which has an S1P binding site buried within the lipid bilayer. To directly test if SR-B1 can mediate the transport of S1P from HDL into cells, fluorescently labeled S1P can be incorporated into HDL and its transport into cells can be monitored by fluorescence microscopy. The only foreseeable problem with this method would be

on how to get these labelled S1P onto the HDL or if possible, label the ones already present on the HDL. HDL is known to have a high affinity for S1P, so incubating the HDL with prelabelled S1P would be enough to get them to associate, similar to S1P fortification of HDL[38,77] . Other than labelling the lipids, one could also use PDZK1 KO or SR-B1 KO murine macrophages and test S1P's anti-apoptotic capabilities. This would at least confirm if SR-B1 and its adaptor protein PDZK1 are crucial in S1P activity. Repeating these experiments along with the original BLT-1 and S1PL experiments but with SS as the inducer of apoptosis is also important in re-affirming key components involved in HDL's alternate pathway in protecting against SS- induced apoptosis.

There are also a few minor adjustments that need to be done to strengthen the experimental design for certain experiments. For example, in all of the S1P experiments there is still the issue of innate source of S1P being produced by the macrophages with their own Sphk. Inhibiting Sphk with its known inhibitors might be important to reduce confounding variables and strengthen the results. Similarly, testing S1PL with S1P instead of HDL is an easy way to confirm the potency of S1PL treatment and whether the incubation times or concentrations utilized are efficient. Repeating certain experiments is also vital for supporting some results that lean towards a trend but was not able to reach significance due to high variability and lack of power in statistics due to low n values (e.g. 4.3B).

Looking further on, we can utilize HDL and S1P to conduct in-vivo experiments on atherosclerotic mouse models, such as LDLR or ApoE KO mice, to study the effects on atherosclerotic progression. These mice models can be placed on a high fat diet to induce atheroma formation, after which they can injected pharmacologically to test whether this affects apoptosis of macrophages, necrotic core, and in turn the plaque size. Depending on the results this could lead to a new atherosclerotic regression model, whereby the focus is on stabilizing the atheroma by

preventing macrophage apoptosis, instead of removing lipids and improving endothelial function. However, this will be more difficult to execute compared to the in-vitro setting as now the treatment of HDL and S1P will impact many other factors not just primarily the macrophages. We can utilize the myeloid specific knockout mice to better elucidate the treatments' effects, but overall limiting confounding variables for this type of study in-vivo will be difficult.

HDL's role in removing excess cholesterol from peripheral tissues, is well documented but these experiments can provide insight to HDL's lesser studied but perhaps equally important role which is in delivering crucial signaling lipids to the tissues so as to mediate other atheroprotective roles. Moreover, HDL as well as S1P's role in preventing apoptosis may also provide newer insights into the field of oncology. In this manner, fully understanding apoptotic pathways and the means to trigger them or prevent them, can further progress mankind's current struggle with both leading causes of death at the same time.

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