

Regulation of macrophage SR-BI by lipoproteins and inflammatory stimuli

Regulation of macrophage SR-BI by lipoproteins and inflammatory stimuli

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Abstract

In atherosclerotic plaques, macrophages ingest modified LDL and turn to foam cells. Others have shown that SR-BI expression levels inversely correlated with cellular cholesterol levels, and is independent of well characterized cholesterol sensing pathways; SREBP and LXR. Thus the mechanism of regulation of SR-BI is unclear. In this study, we showed that treating macrophage with agents known to increase cellular cholesterol levels, namely acLDL, LDL, M β CD:Cholesterol, resulted in reduction in HMGCoAR mRNA and SR-BI expression levels. In contrast, acLDL did not reduce SR-BI mRNA levels in macrophages from acLDL SR-A KO mice, demonstrating that acLDL mediate suppression of SR-BI was dependent on SR-A. Fucoidan, a known competitive inhibitor of acLDL binding to SR-A, and subsequent degradation, also suppressed SR-BI expression levels. Unlike acLDL, however, fucoidan induced mRNA levels corresponding to the pro-inflammatory genes iNOS and IL-6 mRNA levels, and its effects were not altered by the lack of SR-A. Instead, fucoidan mediated stimulation of iNOS and IL-6 and suppression of SR-BI mRNA levels was prevented by an anti-CD14 blocking antibody, demonstrating that the fucoidan mediated effects were dependent on CD14. Interleukin-15, a pro-inflammatory cytokine that binds to a distinct receptor, also induced iNOS and IL-6 mRNA levels and reduced SR-BI expression, suggesting that inflammatory signaling in general can reduce SR-BI expression levels. Treatment of macrophages with the lipoproteins acLDL, LDL or HDL suppressed the induction of iNOS and IL-6 mRNA by fucoidan or IL-15. Macrophages foam cells in an atherosclerotic

plaque may have reduced SR-BI due to exposure with modified LDL or inflammatory cytokines or both in an atherosclerotic plaque. SR-BI expression in macrophages protects against atherosclerosis development. Our data suggests that modified lipoproteins as well as inflammatory stimuli suppress SR-BI expression in macrophages and this may contribute to their pro-apoptotic properties.

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List of Abbreviations

Acyl-CoA cholesteryl acyl transferase	ACAT
Acetyl LDL	acLDL
Bovine serum albumin	BSA
Cholesterol ester	CE
PBS supplemented with 1 mM CaCl ₂	cPBS
Damage-associated molecular pattern	DAMP
Dulbecco's Modified Eagle Medium	DMEM
Enhanced chemiluminescence	ECL
Endoplasmic reticulum	ER
familial hypercholesterolemia	FH
high-density lipoprotein	HDL
high-density lipoprotein cholesterol	HDL-C
Hydroxymethylglutaryl CoA reductase	HMGCoAR
Horseradish peroxidase	HRP
Knock out	KO
low-density lipoprotein	LDL
low-density lipoprotein cholesterol	LDL-C
low-density lipoprotein receptor	LDLR
Lipopolysaccharide	LPS
Lipoteichoic acid	LTA
Muramyl dipeptide	MDP
Mouse peritoneal macrophages	MPM
Niemann-Pick C protein 1	NCP1
Niemann-Pick C protein 2	NCP2
Newborn calf serum	NCS
Pathogen-associated molecular pattern	PAMP
Phosphate buffered saline	PBS
Phorbol 12-myristate 13-acetate	PMA
Roswell Park Memorial Institute Medium	RPMI
SREBP cleavage-activating protein	SCAP
Scavenger receptor class A	SR-A
Scavenger receptor class A isoform I	SR-AI

Scavenger receptor class A isoform II	SR-AII
Scavenger receptor class A isoform III	SR-AIII
Scavenger receptor class B type I	SR-BI
Scavenger receptor cystein rich	SRCR
Sterol regulatory element-binding protein	SREBP
Unesterified cholesterol	UC

1. Introduction

1.1 Scavenger receptors and atherosclerosis

Both epidemiological and intervention studies revealed that, in humans, plasma cholesterol levels are major risk factors for developing atherosclerosis, where low-density lipoprotein cholesterol (LDLC) level correlates directly and high-density lipoprotein cholesterol (HDLC) level correlates inversely with atherosclerosis¹. In humans, LDL is the major cholesterol transporting lipoprotein². Cells of the body endocytose LDL using LDL receptors (LDLRs), and utilized this cholesterol source for plasma membrane construction and steroid hormone production^{2,3}. At the same time, this keeps the concentration of circulating LDLs low. A human genetic disease called familial hypercholesterolemia (FH), is caused by mutations producing defective LDLRs that do not clear circulating LDLs. Thus in FH patients, the LDL's lifetime in the plasma is increased 2-3 fold and LDL accumulates to high levels. These LDLs are eventually cleared⁴. This clearance is partially carried out by macrophages, leading to the accumulation of LDL derived cholesterol in unintended parts of the body^{3,4}. Atherosclerosis is the unintended accumulation of cholesterol in the vessel wall.

FH patients have extensive atherosclerosis and experience heart attacks early in life. Studies from FH patients showed plasma LDL levels played a critical role in developing atherosclerosis. However, in FH, there appears to be a paradox. How does LDL cholesterol get inside these macrophages when they do not have functional LDLRs? It turned out that these LDL were chemically modified to a form that is no longer

recognized by parenchymal cell LDLR, instead these LDL are endocytosed by so called “scavenger receptors” (SR) on macrophages. To isolate SRs, different modified forms of LDL were created. Acetylated LDL is a chemically modified LDL form by reacting LDL with acetyl anhydride at low temperature⁵. This effectively masks the positive charge on ϵ amino groups of lysines in LDL’s protein component, making acetylated LDL more anionic compared to LDL. AcLDL was chosen as a model of modified LDL because it is not recognized by LDLR⁶. Later researchers found that macrophages have binding sites that facilitated the endocytosis and lysosomal degradation of acLDL, leading to massive lipid accumulation and formation of foam cells, the major constituents of an atherosclerotic plaque. Binding assays with acLDL “fished out” more than one SRs. Currently, numerous SRs have been described and distributed into 8 classes based on structural similarities⁷.

Scavenger receptor class A (SR-A) was the first receptor identified to bind and facilitate endocytosis of acLDL⁶. SR-A is the major receptor responsible for endocytosis of acLDL⁸. SR-A also binds a broad range of polyanionic ligands including oxLDL, Apo-AI or ApoE in lipid free or lipid associated form⁹, advanced glycation end product-modified proteins in diabetics¹⁰, β -amyloid fibrils (a component of Alzheimer plaque), and may be involved in other diseases. In vivo, the process of acetylation of LDL to form acLDL is unlikely, and oxidized LDL is the physiologically relevant form of modified LDL leading to atherosclerosis^{2,3}.

The study of FH provided a framework for atherosclerosis studies, but atherosclerosis is not limited to FH patients. In addition to accumulation of LDL derived cholesterol, inflammatory molecules are produced within atherosclerotic plaques at all stages of their development¹¹. Currently, atherosclerosis is considered a chronic inflammatory disease of arteries characterized by continuous recruitment of blood monocytes into the vessel wall followed by their transformation into macrophage foam cells¹¹. During monocyte differentiation into macrophages and thereafter, scavenger receptor A (SR-A) expression is induced⁵. In vitro experiments have shown that SR-A mediated 80% of acLDL degraded by macrophages⁸. Furthermore, SR-A and another distinct scavenger receptor CD36, together mediated 90% of the uptake of modified LDL by macrophages¹².

1.2 Scavenger Receptor family of integral proteins

The functional diversity of SRs is remarkable. SRs can be regarded as a family of pattern recognition receptors (PRR), recognizing a broad range of ligands, a characteristic that is essential for processing myriads of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern DAMPs. PAMPs are structures conserved among microbial species, and DAMPs are endogenous molecules released from damaged cells¹³ and includes modified lipoproteins. SR-A for example, recognizes bacterial cell wall components, muramyl dipeptide (MDP), lipoteichoic acid (LTA) of gram-positive bacteria, lipopolysaccharide (LPS) of gram-negative bacteria, apoptotic cells and modified lipoproteins.

Studies with SR-A knock out (KO) mice has emphasized its role in immune recognition. SR-A KO mice are more susceptible to bacterial infection and endotoxin shock, due to lack of bacteria and endotoxin clearance by macrophages lacking SR-A¹⁴⁻¹⁷. It has been suggested that SRs facilitate the clearance of pathogens while other families of PPRs with overlapping ligand binding trigger inflammation, and together they cooperate to mount an appropriate immune response¹⁸.

SRs can also be regarded as a set of lipoprotein receptors. The scavenger receptor class B type I (SR-BI) mediates cholesterol transport between cells and HDL. SR-A and CD36 on macrophages facilitate the clearance of modified LDL. As such, SRs also play a major role in lipoprotein metabolism, cholesterol homeostasis and atherosclerosis. It is important to distinguish between signaling events due to direct SR engagement and signaling events due to endocytosed cholesterol or bioactive lipids.

1.3 Molecular structure and important motifs of SR-A

SR-As are trimeric integral membrane glycoproteins, predominantly expressed by macrophages. There are three isoforms of SR-A that arise from alternative splicing of a single gene product¹⁹. SR-AI and SR-AII were first cloned from a bovine lung library^{20, 21}, and SR-AIII was later discovered from PMA treated human THP-1 cells¹⁹. Before cloning of SR-A, the presence of this receptor on cells was verified by binding and degradation of acLDL⁶. Macrophages do express other receptors that mediate the binding of acLDL, these receptors are CD36, SR-BI²² and others^{23 24}. Thus it was not until after 1990 when SR-A was cloned that its specific functions could be analyzed²⁰.

Bovine SR-AI, II, III coding sequences contain 1359, 1074, 1164 base-pairs encoding proteins with 453, 358, 388 amino acids respectively. SR-AIII is trapped in the endoplasmic reticulum (ER) and does not contribute to lipid uptake, although when expressed together with SR-AI or II, it appears to exert a dominant negative effect on acLDL degradation¹⁹. SR-AI and SR-AII each form homotrimers and have molecular masses of 220 kDa and 115 kDa respectively. The calculated molecular mass of an SR-AI monomer is 50 kDa, with N-glycosylation contributing an additional 20 kDa^{19; 20}. The SR-AI monomer can be divided into 6 domains. At the C-terminal extracellular end is the scavenger receptor cystein rich (SRCR) domain (residues 344-453). The SRCR domain is highly conserved across species²⁵. Putative functions include binding of cell associated ligands and cell to cell interactions.

Following the SRCR domain is the collagenous domain (residues 272-343). This domain facilitates recognition of ligands relevant to atherosclerosis including modified LDL, type I collagen and other polyanions. The SR-AII monomer consists of 349 amino acids. The 110 amino acid C-terminus of SR-AI is replaced by a 6 amino acid C-terminus in SR-AII. Despite this difference, SRAI and SR-AII has very similar binding activities, demonstrating that the SRCR domain is not required for ligand binding. The collagenous domain of each monomer consists of 24 uninterrupted Gly-X-Y repeats which form a triple helix with the other two monomers, similar to the triple helix of collagen, which has extended Gly-X-Y repeats. Fourteen of the 24 Y residues in the Gly-X-Y repeats, are either prolines or lysines, making this a positively charged region. SRA binding is due to

this stretch of positively charged lysines. Cultured cells over-expressing truncated bovine SR-A lacking the collagenous domain cannot bind and degrade acLDL²⁶. Similar truncation experiments with SR-AII demonstrate that the region containing Lys at positions 327, 334, 337 and 340 is the binding site with Lys 337 being essential²⁷.

Next, is the alpha helical coiled-coil domain (residues 151-271). Within its amino acid sequence are 2 sets of highly conserved heptad repeats with hydrophobic leucines or isoleucines at positions 1 and 4. When the monomer forms an α -helix with 3.5 residues per turn, these hydrophobic residues line up on one side of the helix and form a hydrophobic face. The hydrophobic face provides inter-helical interaction that is essential for trimer formation. The trimeric conformation is essential for ligand recognition and binding in the collagenous domain. In fact, SR-A KO mice were generated by disrupting exon four of the SR-A gene, which codes for the alpha helical coiled-coil domain¹⁴. Histidine 260 in this domain is crucial for ligand dissociation²⁸ as is described later. The alpha helical coiled-coil domain facilitates serum dependent, cation independent adhesion to tissue culture plastic^{29, 30}. The popular SR-A antibody 2F8 binds an epitope in this domain³¹ and interferes with cell adhesion.

The alpha helical coiled-coil domain is linked to the transmembrane domain (residues 51-76) by a spacer domain (residue 77-150). The final domain is the amino-terminal cytoplasmic domain (residues 1-50) which contains Ser21 and Ser49 that are phosphorylated upon ligand binding³². Different SR-A ligands have been shown to

produce different phosphorylation patterns that affect the rate receptor internalization^{32 33}.

1.4 Mechanism of SR-A mediated endocytosis

Macrophage SR-A directly associates with both caveolin1 and clathrin and utilizes both the caveolae/lipid raft as well as clathrin dependent endocytosis pathways^{34, 35 28, 36, 37}. A single SR-A ligand has been shown to trigger different downstream signaling events depending on the method of endocytosis²⁰.

When acLDL is added to SR-A expressing cells, within 2 min, SR-A /acLDL complex starts to cluster and concentrates in the caveolae and the clathrin coated pits. In 10 min the SRA acLDL complexes are encircled by an endosome and transported inside the cell. Vacuolar ATPases on the endosome translocates protons into the lumen, and the pH inside the endosome starts to drop. Histidine has a pKa of 6.0 and as the pH drops below this, histidines at position 260 in the α -helical coiled coil domain of each SRA monomer become protonated. The protonated, positively charged histidines of each monomer repels each other and SR-A's trimeric structure is disrupted, causing SRA to dissociate from its ligand²⁸. The receptor is transported to the trans-Golgi network from where they are recycled back to the plasma membrane. The acLDL containing endosomes, on the other hand, merge with lysosomes and their contents are degraded^{28, 36}. It has been shown that lysosomes are long lived structure that accepts endosomes continuously³⁸. The cholesterol esters (CE) carried by acLDL are hydrolyzed by lysosomal acid lipase, and by an unknown mechanism, the unesterified cholesterol (UC) is

transported to Niemann-Pick C protein (NPC)-1 containing vesicle. It has been speculated that NPC2 in the vesicle lumen carries UC to NPC1 on vesicle membrane, where a vesicle containing UC emerges. At least half of UC is transported to the trans-Golgi network that then gets transported to the ER or the plasma membrane. The ER contains both endocytosed and endogenously synthesized cholesterol.

UC is potentially toxic and its level is carefully monitored by sterol regulatory element-binding protein (SREBP) / SREBP cleavage-activating protein (SCAP) system³⁹. In brief, newly synthesized SREBPs are embedded in the ER associated with the SREBP cleavage activating protein (SCAP). SCAP contains a sterol sensing motif. When the cholesterol level is low, conformation changes in SCAP allow it to escort SREBP to the Golgi. Subsequent cleavage of SREBP by Site-1 protease and Site-2 protease in the Golgi frees the amino terminal nuclear SREBP (nSREBP) domain. nSREBP translocates to the nucleus and activates transcription by binding to sterol response elements (SREs) in the promoter or enhancer regions of more than 30 genes for lipid synthesis and uptake. SREBP-2 is one of three isoforms of SREBPs, and preferentially transcribes genes involved in cholesterol synthesis, one of which is HMGCoA reductase, the enzyme that catalyzes the rate limiting reaction of de novo cholesterol synthesis. When cellular cholesterol levels are sufficient, SCAP prevents SREBP translocation and HMG-CoA reductase is kept low. Excess unesterified cholesterol is re-esterified by Acyl-CoA cholesteryl acyl transferase (ACAT) in the ER and stored as cytoplasmic lipid droplets³⁸.

1.5 Dynamics of acLDL uptake by mouse peritoneal macrophages

Macrophage foam-cells are the major constituent of an atherosclerotic plaque, and incubation of macrophages with modified LDL has been used to study the mechanism of cholesterol loading. Here we summarize some of these results. MPMs bind and degrade acLDL with half-maximal values of 5 $\mu\text{g}/\text{mL}$ (at 4°C) and 25 $\mu\text{g}/\text{mL}$ (at 37°C) respectively⁶. The cellular uptake process and degradation of acLDL are both saturable. The start of acLDL binding to its degradation reaches completion in 1 hr. Inhibiting the degradation of acLDL inside the cell does not reduce the rate acLDL uptake, nor did preloading the cells with massive amount of cholesterol. Inversely, cholesterol starving of MPM by incubation in lipoprotein deficient serum for 48 hrs, did not increase the rate of acLDL uptake. Thus acLDL uptake is not regulated by cellular cholesterol levels.

Excess cholesterol is stored in the cytoplasm as CE droplets, giving macrophages a foamy appearance. Effective competitive inhibitors of acLDL binding and degradation such as fucoidan, maleyl-LDL, maleyl-albumin, dextran sulfate are all negatively charged, and reflects the role of negative charges in binding to the positively charged region in the collagenous domain of SR-A^{6, 20}.

After SR-A was cloned in 1990, transfection of the SR-A gene into Chinese hamster ovary (CHO) cells, made it possible to study SR-A specific functions without interference from other receptors^{20, 21, 40}. CHO cells transfected with SR-AI showed high affinity binding ($K_d \sim 5 \mu\text{g protein}/\text{mL}$) and degradation ($K_m \sim 1 \mu\text{g}/\text{mL}$) of acLDL. Malelyl BSA, polyI and polyvinyl sulfate were good inhibitors of acLDL degradation, while LDL,

BSA, PolyC were not^{20, 21, 40}. As expected, prolonged incubation of CHO cells expressing SR-AI with acLDL led to cholesterol accumulation.

In 1997, SRA KO mice were generated by insertion of a neomycin resistance gene cassette into exon four of SR-A gene. This exon encodes the alpha-helical coiled coil domain that is essential for the formation of a functional SR-A homotrimer. Thioglycolate elicited MPMs isolated from SR-A KO mice degraded 70% to 80% less acLDL compared to WT MPM^{8, 14}.

Through competition experiments with acLDL, several polyanionic molecules were identified as SR-A ligands. However, as new SR receptors are been discovered, with ligand binding overlap with SR-A, the specificities of these ligands for SR-A have been questioned.

1.6 Scavenger Receptor Class B Type I (SR-BI)

SR-BI is a multifunctional receptor and its expression prevents atherosclerosis development. Although SR-BI recognizes both native and modified lipoproteins^{22, 41, 42} and various non-lipoprotein polyanionic ligands shared among SRs, SR-BI is a physiological relevant receptor for HDL and is expressed in tissue active in HDL CE selective uptake, such as steroidogenic tissues and liver^{41, 43, 44}. SR-BI is also expressed by macrophages and in atherosclerotic plaques^{45, 46}. SR-BI's function in liver and steroidogenic tissues are well documented⁴⁷ and research into its roles for macrophages are emerging. In brief, hepatic SR-BI drives reverse cholesterol transport, an important

process for peripheral tissue to shuttle excess cholesterol via HDL to the liver for excretion in feces. SR-BI of steroidogenic tissues utilizes HDL-CE for hormone production. The regulation and function of macrophage SR-BI are less known.

1.7 SR-BI gene

Currently there are 3 members in the class B scavenger receptor family, 1) SR-BI, 2) CD36 and 3) lysosomal integral membrane protein II (LimpII is a lysosomal protein) encoded by distinct genes. These proteins are grouped together based on structural similarities. These include the presence of 2 transmembrane domains and relatively short cytoplasmic N- and C-terminal tails, with the majority of the protein forming a large extracellular loop that is heavily glycosylated⁴⁸. The SR-BI gene is located on chromosome 12 in humans and 5 in mice. Two splice variants SR-BI have been described in the literature namely SR-BI and SR-BII^{49,50}. In mice, SR-BI mRNA is the spliced product of 13 exons, in which the stop codon is located in the 12th exon, and the entire transcript translates into a protein with 509 amino acid residues⁴⁸. Alternatively in SR-BII, exon 12 is excluded and an alternative stop codon in exon 13 is used instead^{49,50}. The SR-BII protein contains 506 amino acid residues. The protein sequence differences between SR-BI and SR-BII protein is found in their C-terminal cytoplasmic tails, which are coded by exon 12 for SR-BI and exon 13 for SR-BII. Functionally, SR-BII facilitated selective lipid uptake from HDL and cholesterol efflux to HDL are four fold less efficient than SR-BI⁵⁴. In macrophages, SR-BI is the major isoform, and SR-BI mRNA represents

70% of total SR-BI/SR-BII transcripts⁵¹. The major interaction between HDL and SR-BI appears to be provided by protein-protein interactions. SR-BI binds ApoAI with high affinity (Kd of 5-8 $\mu\text{g}/\text{mL}$), the major protein component on HDL⁵². Crosslinking experiments showed that SR-BI has high affinity for the Apo-AI C-terminal domain (residue 148-243) and the N-terminal domain (residue 1-86). SR-BI also recognized an unrelated class A α -helix motif, suggesting that binding of other apolipoproteins such as LDL, may be facilitated by possessing this common motif^{22, 52}.

1.8 SR-BI functions learned from mouse studies

Genetically modified mice, that are deficient in, or express less than normal amounts of SR-BI have the following characteristics; reduced ability to clear plasma HDL, increased plasma cholesterol due to abnormally large cholesterol rich HDL, increase atherosclerosis, and depleted lipid storage in steroidogenic tissues^{41, 43, 44, 53, 54}.

Conversely, hepatic over expression of SR-BI has the opposite effects^{55, 56}. In addition to facilitating RCT, hepatic SR-BI expression levels also regulate the rate of RCT⁵⁶. The functional importance of SR-BI with respect to atherosclerosis can be seen on ApoE SR-BI DKO mice. These mice have accelerated atherosclerosis and do not survive beyond 8 weeks of age. The cause of death is occlusive atherosclerosis in their coronary arteries that is not seen in ApoE KO control mice, and these occlusions manifest as myocardial infarctions and heart failure.

SR-BI facilitates selective uptake of CE and other lipids from HDL by cells, as well as the redistribution of FC between cells and HDL^{46, 57}. Therefore SR-BI mediates bi-directional cholesterol transport between HDL and cells, a process driven by the cholesterol concentration gradient. Thus SR-BI expressed by macrophages in atherosclerotic plaques may protect against atherosclerosis by promoting the efflux of accumulated cholesterol from foam-cells to HDL in both humans⁴⁵ and mice⁴⁶. In addition to SR-BI, macrophages also express ABCA1 and ABCG1. These are ATP powered cholesterol pumps that provide alternative RCT pathways. ABCA1 facilitates cholesterol efflux to lipid free and lipid poor ApoAI. People with dysfunctional ABCA1 (Tangier disease) or ABCA1 KO mice have virtually no HDL⁵⁸, thus ABCA1 is essential for the formation of mature HDL. In contrast, SR-BI and ABCG1 facilitate cholesterol efflux to larger HDL particles but not to lipid free ApoAI. The contribution of macrophage SR-BI to cholesterol efflux has been assessed. In vitro experiments with cholesterol loaded J774 macrophages or cholesterol loaded mouse peritoneal macrophages and the SR-BI inhibitor BLT-1, suggest that SR-BI contributed 6% and 10% toward cholesterol efflux to serum⁵⁹. In vitro experiment with MPM isolated from SR-BI KO mice showed that cholesterol efflux to HDL was reduced by 24% compared to MPM of WT mice⁶⁰. Another study isolated BMM from WT, SR-BI KO mice and injected them intra-peritoneally into WT mice, and found no difference in cholesterol efflux to serum and feces between mice injected with SR-BI and WT macrophages, suggesting that macrophage SR-BI may not participate in RCT in vivo⁶¹. It is possible that cholesterol efflux was not observed

because these macrophages were not cholesterol loaded, and SR-BI facilitates bidirectional free cholesterol efflux between HDL and cells down a concentration gradient⁴⁶.

Bone marrow transplantation has allowed researchers to gain insights into the role of macrophage specific proteins on the development of atherosclerosis. For example, to study the role of SR-BI on atherosclerosis, mice that are prone to atherosclerosis, such as the ApoE^{-/-} mice, can be exposed to lethal doses of radiation, upon which their bone marrow cells are killed. The bone marrow populations can then be reconstituted with bone marrow from SR-BI^{-/-} mice. The macrophage populations that eventually arise from the donor bone marrow will not express SR-BI, allowing examining the role of macrophage specific SR-BI. A set of ApoE^{-/-} recipient mice that carry bone marrow from ApoE^{-/-} SR-BI^{+/+} are used as comparison.

Lethally irradiated ApoE^{-/-} mice reconstituted with ApoE^{-/-} SR-BI^{-/-} bone marrow (ApoE^{-/-} SR-BI^{-/-} → ApoE^{-/-}) showed 86% increase in aortic sinus atherosclerosis compared to control (ApoE^{-/-} SR-BI^{+/+} → ApoE^{-/-})⁶². This increase in atherosclerosis was not due to reduced cholesterol efflux, since In vitro experiments with thioglycolate elicited MPM isolated from these mice, showed that SR-BI deficiency did not reduce their ability to efflux cholesterol, which is supported by lack of differences between their lipid profiles. Another study showed that SR-BI^{-/-} → LDLR^{-/-} mice on Western type diet for 4 month have 70% increase in aortic sinus atherosclerosis compared to WT → LDLR^{-/-}

/- control without affecting plasma cholesterol levels⁶³. A similar study showed that SR-BI^{-/-} → LDLR^{-/-} mice on Western type diet for 10 weeks have 50% increase in aortic sinus atherosclerosis and 10% increase necrotic core content compared to WT → LDLR^{-/-} control⁶⁰. Results from these studies demonstrate that macrophage SR-BI has a profound effect on atherosclerosis development. In addition to potential roles in cholesterol efflux, SR-BI may also suppress inflammation and apoptosis as well as mediate HDL stimulated macrophage migration. One study showed that SR-BI KO mice are more prone to LPS induced endotoxemia and death⁶⁴. LPS Treatment resulted in aberrant inflammatory cytokines production. This is attributed to the combination of reduced clearance of LPS by hepatic SR-BI, and reduced adrenal selective lipid uptake from HDL resulting in the deficiency in anti-inflammatory corticosterone secretion. Corticosterone supplement reduced sensitivity of SR-BI KO mice to LPS. In a related study, SR-BI KO mice were found to be more prone to septic death. When BMM were isolated from these mice and treated with LPS, they produced more NO and inflammatory cytokines TNF α , IL-6⁶⁴ compared to WT BMM. Corticosterone supplement did not rescue SR-BI KO mice from septic death, which suggests that SR-BI has direct anti-inflammatory roles, and in the context of atherosclerosis may protect macrophages by reducing inflammatory signaling and cytokine secretion.

In our lab, we have evidence suggesting that HDL via SR-BI suppresses macrophage apoptosis and induces migration. This involves the activation of PI3K/Akt and mitogen activated protein kinase signaling pathways, leading to migration and

decreased levels of the pro-apoptotic protein Bim (Trigatti unpublished data). It is well accepted that apoptosis of macrophage foam cells in plaque release pro-inflammatory molecules, and inflammation is a major driving force for atherosclerosis progression. Because of the importance of macrophage SR-BI for protection against atherosclerosis, we have investigated how it is regulated by other scavenger receptor ligands which may participate in pro-apoptotic pathways.

We have investigated the regulation of SR-BI by acLDL and by fucoidan, both known to be scavenger receptor ligands. Our findings suggest that both suppress SR-BI expression although by different pathways. AcLDL appears to suppress SR-BI by mediating cellular cholesterol loading in an SR-A dependent manner. In contrast, although fucoidan has been reported to bind to SR-A, it appears to suppress SR-BI expression via CD14. Because fucoidan also induces expression of the inflammatory genes iNOS and IL-6 via CD14, we have tested a distinct inflammatory mediator, IL-15 and demonstrate that it also suppresses SR-BI expression. These data suggest that strategies to prevent SR-BI suppression by cholesterol loading or inflammatory signaling may reduce development of atherosclerosis.

2. Materials and Methods

2.1 Materials

2.1.1 Mice

SR-A KO mice on a C57BL6/J background were kindly provided by Dr. Dawn Bowdish (McMaster University, Hamilton, ON). Wild type C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbour, ME) and bred in house.

2.1.2 Reagent or material, Product number (Supplier)

Fucoidan from *Fucus vesiculosus*, F5631 (Sigma)

Methyl- β -cyclodextrin, C4555 (Sigma)

Human HDL, BT-914 (Biomedical Technologies, Inc)

Human LDL, BT-903 (Biomedical Technologies, Inc)

Human Acetylated LDL, BT-906 (Biomedical Technologies, Inc)

Rat anti mouse F4/80 antibody, ab6640 (Abcam)

Alexa488 conjugated Rabbit anti rat antibody, A21210 (BD Pharmingen)

Rabbit anti mouse SR-BI antibody, NB-400-101 (Novus Biologicals)

HRP conjugated donkey anti rabbit IgG, 711-035-152 (Jackson Immunoresearch)

Rabbit anti mouse p38 MAPK, 9212 (Cell Signaling Technology)

Rat anti mouse CD14, 557896 (BD Pharmingen)

Rat anti mouse CD16/CD32 (Fc γ III Receptor), (eBioscience)

Recombinant mouse IL-15, 447-ML (R&D Systems)

8 Well Coverglass, 155411 (Nunc)

Tissue culture dishes (Greiner Bio-One)

Fetal bovine serum (PAA)

QuantiTect Reverse Transcription Kit, 205313 (Qiagen)

RNeasy Mini Kit, 74104 (Qiagen)

Pierce BCA Protein Assay Kit, 23225 (Thermo Scientific)

UltraPure DNase/RNase-Free Distilled Water, 10977 (Gibco)

DMSO (Sigma)

Platinum SYBR Green qPCR SuperMix-UDG, 11733-046 (Invitrogen)

Formaldehyde (Sigma)

PVDF (Perkin Elmer)

Triton-X 100 (Sigma)

DMEM (McMaster University Center for gene therapeutics media prep core facility)

RPMI (McMaster University Center for gene therapeutics media prep core facility)

NP-40 (USB)

Western Lightning Chemiluminescent Reagent (Perkin Elmer)

Tween 20 (Sigma)

Tris (Bioshop)

Phosphate buffered saline (McMaster University Center for gene therapeutics media prep core facility)

KBR (Caledon)

Newborn Calf Serum (Gibco)

DTT (Roche)

Agarose (Bioshop)

Complete Mini Protease Inhibitor Cocktail Tablet, 11836153001 (Roche)

0.2 μ M filter (Sarstedt)

L-glutamine (Gibco)

Penicillin/streptomycin (Gibco)

Vacuum filter unit 0.22 μ m, 83.1824.001 (Sarstedt)

BSA (Sigma)

CaCl₂ (BHD)

2.2 Methods

2.2.1 Preparation of newborn calf lipoprotein-deficient serum (NCLPDS)

To 320 mL of newborn calf serum, added 107.7g KBr and stirred at 4°C until dissolved. Spun the NCS-KBr at 4°C at 24,000Xg for 48 hrs in a Beckmen Coulter Ti-70 centrifuge rotor. Afterward, the NCLPDS-KBr separated into two fractions. The top white fractions contained lipoproteins and was discarded. The bottom brown yellow fractions were collected into dialysis tubes with 12-14K MW cut off, and dialyzed at 4°C with 6 liters of 0.9% NaCl 8 times over 48 hours period to eliminate KBr. The dialyzed product or NCLPDS was sterile filtered with 0.2 μ M filter, and its protein concentration was determined. The NCLPDS protein concentration was adjusted to 70 μ g/mL, and stored at -20°C.

2.2.2 Cell lines and culture conditions

2.2.2.1 Murine macrophage cell line Raw264.7

Murine macrophage cell line Raw264.7 cells were purchased from ATCC. Raw264.7 cells were maintained in DMEM supplemented with 10% fetal calf serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (medium D10) in 5%CO₂ at 37°C. For experiments, Raw264.7 cells were washed 3X with warm PBS, collected and re-seeded at a density of 4X10⁵ cells/cm² in DMEM supplemented with 3% newborn calf lipoprotein-deficient serum (NCLPDS), 2 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (medium D3) for 24 hr before addition of treatments. Treatments were prepared in medium D3.

2.2.2.2 Human monocytic cell line THP-1 and its differentiation

Human monocytic cell line THP-1 cells were purchased from ATCC. THP-1 cells were maintained in (medium R10) in 5%CO₂ at 37°C. For differentiation, THP-1 cells were seeded at a density of 1.57X10⁵ cells/cm² in medium R10 supplemented with 100 nM PMA for 72 hr, after which cells were maintained in medium R10 without PMA

2.2.2.3 Murine fibroblast cell line L929

Murine fibroblast cell line L929 cells were purchased from ATCC. L929 cells were maintained in medium D10 in 5%CO₂ at 37°C.

2.2.2.4 Isolation of mouse peritoneal macrophages and culture conditions

Mouse was euthanized by CO₂ asphyxiation. The abdomen was disinfected by soaking in 70% ethanol for about 1 min. The abdominal skin was raised to cause tenting and 10 mL of ice cold PBS was injected using 25-gauge needle into the peritoneal cavity. After 1 min of vigorous shaking of the mouse body, a small incision was made in the abdominal skin along the midline from the lower abdomen to the neck. The abdominal skin is retracted and separated from the peritoneal membrane. The PBS in the peritoneal cavity was recovered with an 18 gauge needle. Typically, 9 mL of PBS is recovered. If during the process any internal organ was punctured, the sample was discarded. The recovered PBS was dispensed into a 50 mL Falcon tube containing 10 mL of RPMI supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (medium R10) and kept on ice for less than 30 min. Cells were pelleted by centrifugation at 500 x g for 5 min. The supernatant was discarded and cells were resuspended in medium R10, and distributed into 8 wells in 24 well plates, and into each well of an 8 well coverglass for immunofluorescence staining (see Immunofluorescent staining for details). Macrophages were cultured for 2 hrs to allowed attachment to the well. Non-attached cells were removed by washing 3 times with warm PBS. The medium is replaced with RPMI supplemented with 3% NCLPDS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin (medium R3) for 24 hrs in 5%CO₂ at 37°C before addition of treatments. Typically, 6X10⁶ cells (excluding red blood cells) were collected from each mouse, with approximately 50% of cells becoming adherent after the washing steps. The final cell density was approximately 375,000 cells per cm².

2.2.3 Preparation of Methyl- β -Cyclodextrin:cholesterol (M β CD:Ch)complexes

M β CD:Cholesterol complexes at a ratio 6:1 has been prepared similarly to a previously described method⁶⁵. Briefly, cholesterol was dissolved in chloroform:methanol 1: 1 (v:v) in a glass tube, and adequate amount of was transferred to a glass media bottle. The chloroform and methanol was evaporated by flushing the bottle with 0.2 μ M filtered N₂ gas. In a separate glass media bottle M β CD was dissolved in media to a final concentration of 2.5 mM. Media with 2.5 mM M β CD:Cholesterol was added to the cholesterol containing bottle. Bottles containing M β CD and M β CD:Cholesterol were sonicated in a bath sonicator at room temperature for 30 minutes, with periodical banging of the bottle to dislodge dried cholesterol that was stuck to the wall. The M β CD:Cholesterol will turn into a cloudy mixture. Both M β CD and M β CD:Cholesterol medium were incubated on a platform rocker at 37°C overnight. Before use, the medium were filtered through a 0.45 μ M filter for sterilization and removal of excess cholesterol crystals.

2.2.4 SDS-PAGE and immunoblotting

Cells were washed 3 times with ice cold PBS and moved to a 4°C cold room. Cells were lysed in ice-cold lysis buffer consisting of 20 mM Tris-HCl pH 7.4 containing 100 mM NaCl, 0.5% w/v NP-40, 0.2% Triton X-100, and 1 tablet of phosphatase inhibitor (Complete Mini Protease Inhibitor Cocktail Tablet, Roche) per 50 mL. Nuclear fractions were discarded by centrifugation at 14,000 rpm for 10 min at 4°C. The protein concentrations of the remaining cell lysates were measured using the BCA protein assay

(BCA Protein Assay Kit, Pierce, USA). Cell lysates containing 50ug of proteins were run on 12.5% SDS-PAGE then transferred to polyvinylidene fluoride membranes (Amersham, BS, UK). SR-BI protein bands were stained with SR-BI isoform specific primary antibody NB-400-101 (1:1000, Novus Biologicals). Total P38 MAPK protein bands were used as loading control and stained with P38 MAPK primary antibody (1:1000, Cell Signaling Technology). Both SR-BI and total P38 MAPK bands were detected by secondary HRP conjugated donkey anti rabbit IgG 711-035-152 (1:35000, Jackson ImmunoResearch). The membranes were visualized using enhanced chemiluminescence kit (ECL), and densitometry on band intensities were performed using Biorad Gel Doc system. Results are reported relative to control untreated cells.

2.2.5 RT-PCR

Total RNA was isolated using the Rneasy Mini kit (Qiagen), following the manufacturer's instructions. Total RNA was converted to cDNA using Quantitect Reverse Transcription kit (Qiagen) following the manufacturer's instructions. After completion, the cDNA reactions were diluted 10 fold. Each RT-PCR reaction contained 3 µl of diluted cDNA reaction product, 5 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 2 µl of 1 µM forward and reverse primer mix, and was loaded into a well of a 384 well plate. The primer sequences were selected using Pubmed's primer-BLAST. Primers were synthesized by Invitrogen. RT-PCR reactions were run on an Applied Biosystems 7900HT Fast Real-Time PCR System. The $\Delta\Delta C_t$ method was employed to quantify changes in transcript levels⁶⁵.

2.2.6 Immunofluorescent staining

At room temperature, cells that were plated onto 8 well coverglass were washed 3X with PBS, fixed in 2.5% paraformaldehyde in PBS for 30 min, washed 3X in PBS supplemented with 1 mM CaCl₂ (cPBS), and blocked in cPBS supplemented with 0.1% BSA for 1 hr, followed by incubation in F4/80 primary antibody diluted 200X in cPBS supplemented with 0.1% BSA at 37°C for 1hr. Cells were washed at room temperature 3X in cPBS supplemented with 0.1% BSA for 5 min per wash, once with cPBS, followed by incubation in Alexa488 conjugated secondary antibody diluted 1000X in cPBS supplemented with 0.1% BSA at 37°C for 1hr. Cells were washed at room temperature 3X in PBS supplemented with cPBS 0.1% BSA for 5 min per wash, once with cPBS, followed by counter staining with 300 nM DAPI. , Cell were washed 3X with cPBS. cPBS containing 0.5 μM ascorbate was added and cells were imaged using a Zeiss Axiovert 200 inverted fluorescent microscope.

2.2.7 Statistical Analysis

Data are presented as mean ± SEM. Groups were compared with Student's *t*-test.

3 Results

3.1 Treatment of Raw264.7 murine macrophages with acLDL or fucoidan reduced SR-BI protein.

AcLDL and fucoidan are commonly used ligands for a variety of SRs⁶. To test the effects of SR ligands on the levels of SR-BI protein in macrophages, we treated Raw264.7 murine macrophages for 24 hrs with increasing concentrations of either fucoidan or acLDL, and measured SR-BI protein levels by immunoblotting (Fig 1). Studies by others have shown that SR-A is the major receptor for AcLDL and accounts for greater than 80% of acLDL degradation by macrophages⁸. Fucoidan is a competitive inhibitor of acLDL degradation and a ligand for SR-A. Both fucoidan and acLDL treatments resulted in reduced levels of SR-BI protein after 24 hrs of treatment (Fig 1A and B). AcLDL at a concentration of 25 µg/mL reduced SR -BI protein levels by ~30% while 50 or 100 µg/mL acLDL reduced SR-BI protein levels by ~70% compared to control untreated cells (Fig 1A, lane 1, 5, 6, 7). Fucoidan also led to reduced SR-BI protein levels although to a lesser extent. Fucoidan at 25 µg/mL led to ~40% reduction in SR-BI protein and increasing the concentration of fucoidan to 50 or 100 µg/mL did not lead to further reductions (Fig 1A, lane 1, 2, 3, 4). Thus the lowest concentration of fucoidan tested in Figure 1A and B appeared to already be saturating. Therefore lower fucoidan concentrations were tested in a separate experiment (Fig 1C, D). In this experiment, cells were incubated without treatment (control) or with fucoidan between 3 and 50 µg/mL. Treatment with 3 µg/mL fucoidan did not affect SR-BI protein levels, whereas treatment of cells with

fucoidan at 6 $\mu\text{g}/\text{mL}$ or greater reduced SR-BI protein levels by approximately 30% compared to control untreated cells. Therefore, the threshold dose of fucoidan to trigger a reduction in SR-BI protein levels appeared to fall between 3 and 6 $\mu\text{g}/\text{mL}$ (Fig 1D).

3.2 Validation of RT-PCR primers.

In order to test if the reductions in SR-BI protein were the result of altered levels of transcripts from the SR-BI gene, we set out to test SR-BI transcript levels by quantitative RT-PCR, employing the $\Delta\Delta\text{Ct}$ method to quantify changes in transcript levels⁶⁶. The primer sequences were selected using Pubmed's primer-BLAST.

To verify that the PCR cycle numbers at which the threshold PCR product was obtained (Ct value) varied directly with the amount of starting template, RNA was extracted from the liver of a wild type mouse, and used for cDNA synthesis. A tenfold serial dilution series from the cDNA synthesis reaction was made, and 3 μl from each dilution were run in triplicate RT-PCR reactions. Standard curves were produced by plotting Ct values against fold dilution (Fig S1). From these standard curves, PCR amplification efficiencies of CypA and GAPDH primer pairs were calculated to be 93% and 69% respectively. Using regression analysis, the R^2 value for both primer pairs are almost 1.00.

To analyze RT-PCR data quantitatively, a reference gene is required as a loading control, which all other data are normalized to. Housekeeping genes are commonly

used as loading controls because their expression levels are relatively constant. The expression of three commonly used housekeeping genes, glyceraldehyde phosphate dehydrogenase (GAPDH), cyclophilin A (CypA) and β -actin were tested in Raw264.7 cells treated for 3 hrs with the following; fucoidan (25 $\mu\text{g}/\text{mL}$), M β CD:Cholesterol (2.5 mM methyl- β -cyclodextrin complexed to 0.42 mM cholesterol), fucoidan (25 $\mu\text{g}/\text{mL}$) plus M β CD:Cholesterol (2.5 mM:0.42 mM), 2.5 mM M β CD alone, fucoidan (25 $\mu\text{g}/\text{mL}$) plus 2.5 mM M β CD, and control untreated cells incubated in parallel. Three independent experiments were carried out in parallel. Data from one representative experiment is shown (Fig S2). We chose 3 hrs as a treatment time to avoid potential complications of cytotoxicity seen occasionally with 24 hr treatments of high concentrations of fucoidan (data not shown). RNA was isolated and equal amounts were used to prepare cDNA. Equal volumes of the reverse transcriptase reactions were used as templates for triplicate RT-PCR reactions using Sybr green to detect amplification products. The amplification plots (Fig S2, panels A-C) and dissociation curves (Fig S2, panels D-F) for GAPDH, CypA, and β -actin are shown. The dissociation curves in all cases consisted of a single major peak indicating a single major amplification product. The amplification plots for GAPDH across all treatments were most similar, whereas those for CypA and β -actin displayed some variability. We therefore chose GAPDH as the most appropriate loading control for Raw264.7 cells treated under these conditions.

A similar analysis was performed for GAPDH and CypA expression in thioglycolate elicited peritoneal macrophages isolated from wild type mice (Fig S3). Macrophages

were treated for 3 hrs as described above with an additional treatment of acLDL (100 µg/mL). Equal amounts of total RNA were used for cDNA synthesis. Equal volumes of the reverse transcriptase reactions were used as templates for real time PCR as above. The amplification plots (Fig S3, panels A-B) and dissociation curves (Fig S3, panels C-D) are shown. As above, the dissociation curves consisted of a single major peak in each case indicative of a single major amplification product. In the peritoneal macrophages, however, the amplification curves for CypA across all treatments were most similar, whereas those for GAPDH displayed some variability. Therefore we chose CypA as the loading control for analyses of transcript levels in mouse peritoneal macrophages. Other primer pairs (Table S1) have been validated in a similar way (data not shown).

3.3 Treatment of Raw264.7 murine macrophages with AcLDL or fucoidan reduced SR-BI mRNA.

To test if the reduction in SR-BI protein levels by fucoidan or acLDL in Figure 1 are accompanied by decreased SR-BI mRNA levels, we treated Raw264.7 macrophages for 3 hrs with acLDL at 100 µg/mL and fucoidan at 25 µg/mL, and monitored SR-BI mRNA levels using RT-PCR. We also treated macrophages with LDL (100 µg/mL) as a control lipoprotein which does not bind to SR-A, but is known to deliver cholesterol to macrophages via the LDL receptor. Hydroxymethylglutaryl CoA reductase (HMGCoAR) mRNA levels were used to evaluate cholesterol loading since HMGCoAR expression is tightly and inversely regulated by cellular sterol levels³⁹. Both acLDL and LDL treatments reduced HMGCoAR mRNA levels to 65% and 46% compared to control

untreated cells respectively, whereas fucoidan had no effect (Fig 3A). acLDL and LDL appeared to slightly reduce SR-BI mRNA levels, although the differences from control untreated cells did not reach statistical significance (Fig 3C). Fucoidan has been reported to induce iNOS activity in a manner dependent of SR-A⁶⁷. Thus iNOS mRNA levels were measured as well. Fucoidan treatment induced iNOS mRNA level 7 fold compared to control untreated cells, whereas acLDL and LDL had no effect (Fig 3B). Fucoidan treatment also reduced SR-BI mRNA levels to 66% of control untreated cells (Fig 3C). We suspected the lack of effect of acLDL on SR-BI expression observed in panel C might be the result of the short incubation time used. Therefore we tested both acLDL and fucoidan treatments for longer time periods of 6 and 24 hrs (Fig 3D). Indeed, longer treatment periods with acLDL and fucoidan reduced SR-BI mRNA levels further. With 6 hr and 24 hr treatments, acLDL reduced SR-BI mRNA level to 80% and 40% compared to control untreated cells respectively. At 6 hr and 24 hr, fucoidan reduced SR-BI mRNA levels to 50% and 36% of controls, respectively. This data demonstrates that both acLDL and fucoidan are able to reduce SR-BI transcript levels in RAW264.7 macrophages. Although both are reportedly ligands of SR-A, they clearly have different effects on the cells. acLDL treatment decreased HMGCoAR mRNA levels consistent with cholesterol loading by these particles, whereas fucoidan did not. In contrast, fucoidan treatment induced iNOS gene expression suggesting that it was triggering inflammatory signaling pathways, whereas acLDL did not. This raises the possibility that the molecular pathway(s) by which acLDL or fucoidan lead to reductions in SR-BI may also be different.

3.4 SR-BI is induced upon differentiation of monocytic cells into macrophages

The THP1 cell line was originally established from a human acute monocytic leukemia⁶⁸. These cells are grown in suspension, and upon exposure to Phorbol 12-myristate 13-acetate (PMA), become adherent and differentiate to macrophage-like cells⁶⁹. To determine if SR-BI expression is altered upon monocyte to macrophage differentiation, THP1 cells were exposed to 100 nM PMA for 3 days after which the media was replaced with fresh media lacking PMA and cells were cultured for up to an additional 7 days. Total RNA was isolated from each sample and equal amounts of RNA were used to prepare cDNA. Equal volumes of the cDNA preparations were then used as templates for RT-PCR using Sybr green to detect amplification products. Primer pairs for human β -actin, CD68, SR-A and SR-BI were used (see Table S1). β -actin mRNA levels increased by 7 fold after 3 days of exposure to PMA and continued to increase up to 27 fold by day 4, after which they appeared relatively stable up to day 10 (Fig 3A). These changes are consistent with alterations in cell shape and adherence as the cells differentiate from round non-adherent myeloid cells to adherent macrophages⁶⁹. CD68 is a lysosomal membrane protein, highly expressed by macrophages and commonly used as a macrophage marker⁷⁰. CD68 mRNA increased by 134 fold by day 3 and continued to increase up to 600-800 fold by days 8-10 of differentiation (Fig 3B). SR-AI mRNA levels increased over 4000-fold by days 3-4 but then dropped to between 1000 to 2000 fold higher than untreated THP-1 cells by days 8-10 of differentiation (Fig 3C). SR-BI mRNA levels increased 38 fold after 3 days in PMA and continued to increase up to 136 fold at

day 4 after which they appeared relatively stable up to day 10 (Fig 3D). These data demonstrate that SR-BI mRNA levels become induced upon macrophage differentiation of THP-1 cells. However because we detected fluctuations in SR-BI mRNA levels on different days after differentiation, and because of the possibility that treatment of cells could affect the differentiation program itself leading to indirect effects rather than direct effects on SR-BI gene expression, we chose not to continue with the THP-1 macrophages for our studies.

3.5 Verification of cells collected from peritoneal cavity by immunofluorescent staining for macrophage marker F4/80.

In order to verify our findings from studies of RAW 264.7 cells in a more physiologically relevant cell system we prepared primary macrophages from mice. Because we observed that fucoidan treatment induced iNOS gene expression and may therefore activate macrophages, we avoided using agents such as thioglycollate to elicit or recruit macrophages to the peritoneal cavity since such agents themselves may also activate macrophages. Instead, we collected resident mouse peritoneal macrophages (MPM), by lavage of the peritoneal cavity, followed by 2hr incubation to allow macrophages to attach to tissue culture dishes. The non-attached cells were removed by washing. To verify that the remaining cells were macrophages, these cells were immuno-stained with a primary antibody against the macrophage-specific cell surface marker F4/80, followed by a green fluorescent secondary antibody, and finally

counterstained with blue fluorescent DNA stain, DAPI to reveal the nuclei. Typical results are shown in Fig 4. Virtually all adhering cells stained for F4/80 (Fig 4B). Cells incubated with the secondary antibody only, had no green fluorescence, indicating that the secondary antibody bound to the F4/80 primary antibody specifically (Fig 4A). Fibroblasts may be collected from the peritoneal cavity along with macrophages⁷¹ and retained even after several washing steps. Therefore we tested if the F4/80 antibody could discriminate between macrophages and fibroblasts. Mouse CCL-1 (L929) fibroblasts were cultured and stained as above for F4/80 following the same staining procedure (Fig 4C, 4D). Our results showed that mouse CCL-1 fibroblasts did not stain for F4/80 (Fig 4C), consistent with another study⁷². We concluded that the cells we collected are mainly macrophages.

3.6 acLDL, fucoidan and cholesterol loading with cyclodextrin cholesterol reduced SR-BI mRNA in mouse resident peritoneal macrophages.

Earlier in figure 1 and 2, we showed that the treatment of Raw264.7 murine macrophages with acLDL or fucoidan resulted in reduced SR-BI protein and mRNA levels. Similar experiments were carried out using resident MPM from wild type mice. We treated resident MPM from wild type mice with fucoidan (25 µg/mL), acLDL (100 µg/mL) and cholesterol (0.42 mM complexed to 2.5 mM MβCD) for 3 hrs and measured the HMGCoAR, iNOS and SR-BI mRNA levels using quantitative RT-PCR as described above. MβCD is a donut shaped structure with a hydrophobic core. MβCD:cholesterol

complexes are formed when cholesterol is solubilized within the hydrophobic core of M β CD^{65, 73}. This complex acts as a cholesterol donor allowing for cholesterol loading of cells without binding to SR-A. acLDL treatment reduced HMGC α AR mRNA levels to 40% of those in untreated control cells (Fig 5A). Similarly cholesterol complexed to M β CD reduced HMGC α AR mRNA levels to approximately 60 % of control untreated cells. In this particular experiment, fucoidan appeared to increase HMGC α AR levels however the difference did not reach statistical significance. As observed for RAW264.7 cells, fucoidan greatly induced iNOS mRNA levels (approximately 100-fold) whereas neither acLDL nor cholesterol complexed to M β CD had significant effects (Fig 5B). The basal levels of iNOS were readily detectable in untreated Raw264.7 cells (Fig 2B) but not in untreated MPM (Fig 5B). All three treatments resulted in reduced SR-BI mRNA levels. Fucoidan, acLDL and cholesterol complexed to M β CD reduced SR-BI mRNA levels to 47%, 51%, 23% compared to control untreated cells respectively (Fig 5C). acLDL and fucoidan treatments had similar effect on MPM and Raw264.7 macrophages with subtle differences. acLDL treatment was able to substantially reduce SR-BI in resident MPM's after a 3 hr treatment, while Raw264.7 required longer treatment times (Fig 5C compared to 2C, 2D). acLDL and cholesterol complexed to M β CD were alike in that they both reduced HMGC α AR mRNA levels, did not induce iNOS, and reduced SR-BI mRNA levels. In contrast, fucoidan treatment induced iNOS but did not reduce HMGC α AR mRNA levels. Together, this data is consistent with our findings in RAW264.7 cells suggesting the possibility that acLDL and fucoidan may be reducing SR-BI expression via

different pathways; acLDL by mediating cholesterol loading and fucoidan by activation of inflammatory signaling.

3.7 AcLDL and fucoidan both reduced SR-BI but through different pathways.

It is well established that SR-A is the major receptor for acLDL, and that fucoidan is an effective competitor for the degradation of acLDL⁶. However, neither acLDL nor fucoidan bind exclusively to SR-A⁷⁴. To begin to determine if acLDL and fucoidan acted through the same or different pathways to reduce SR-BI mRNA, we tested the effects of treating macrophages with both agents (Fig 6, 7). MPM were treated for 3 hrs with no additions or with increasing concentrations (6, 12.5 and 25 µg/mL) of fucoidan in the absence or in the presence of acLDL (at 100 µg/mL). Quantitative RT-PCR was used to measure the expression of iNOS and interleukin 6 (IL-6) as markers of inflammatory gene expression, as well as HMGCoAR and SR-BI. When MPM were treated with increasing concentrations of fucoidan, both iNOS (Fig 6A) and IL-6 were induced. AcLDL treatment alone had no effect on either iNOS or IL-6 mRNA levels, but appeared to reduce fucoidan's ability to induce iNOS at all fucoidan concentrations tested (Fig 6A) and IL-6 at 6 and 12.5 µg/mL but not 25 µg/mL fucoidan (Fig 6 B). It is not clear how acLDL suppressed the induction of iNOS or IL-6 mRNA by fucoidan. One possibility is that acLDL may act as to compete with fucoidan for binding to the same receptor however later experiments suggest this is not likely the case (addressed below). AcLDL treatment reduced HMGCoAR mRNA levels to 54% of control, indicative of increased cellular

cholesterol. As observed in Fig 6, fucoidan appeared to increase HMGCoAR levels but the differences did not reach statistical significance (Fig 6C). When MPM were treated with acLDL combined with increasing concentrations of fucoidan, a trend towards increasing HMGCoAR mRNA was observed, consistent with fucoidan being a competitive inhibitor of acLDL degradation⁶. Fucoidan at 6 µg/mL led to an approximately 40 % reduction in SR-BI mRNA and increasing the concentration of fucoidan to 12.5 or 25 µg/mL did not lead to further reduction. AcLDL alone at 100 µg/mL slightly reduced SR-BI to 82% of control (Fig 6D). Combining acLDL and fucoidan treatments did not reduced SR-BI mRNA level beyond the levels observed with fucoidan treatment alone.

In a complementary experiment, we treated wild type MPM with increasing concentrations (0, 25, 50 or 100 µg/ml) of acLDL either in the absence or in the presence of a single concentration (6 µg/mL) of fucoidan (Fig 7). As in Fig 6, we measured IL-6, HMGCoAR and SR-BI gene expression. AcLDL alone at any of the concentrations tested have no effect on IL-6 gene expression (Fig 6A). On the other hand, fucoidan alone greatly induced IL-6 mRNA levels (Fig 7A). Consistent with findings observed in Fig 6 A and B, acLDL appeared to reduce fucoidan's ability to induce IL6 mRNA levels, in an acLDL concentration dependent manner. Fucoidan alone at 6 µg/mL appeared to slightly increase HMGCoAR mRNA levels, however as in Fig's 5A and 6C, the differences did not reach statistical significance (Fig 7B). AcLDL reduced HMGCoAR levels to between 26 and 40 % of untreated controls; with fucoidan appearing to increase HMGCoA mRNA levels slightly (but not statistically significantly) in acLDL treated cells

(Fig 7B), consistent with the results observed in Fig 6C. AcLDL at 25 $\mu\text{g}/\text{mL}$ led to an approximately 25 % reduction in SR-BI mRNA and increasing the concentration of acLDL up to 100 $\mu\text{g}/\text{mL}$ did not lead to further reductions (Fig 7C). Fucoidan alone at 6 $\mu\text{g}/\text{mL}$ reduced SR-BI levels to $\sim 70\%$ of control (Fig 7C). The addition of acLDL at any of the concentrations tested did not significantly reduce SR-BI mRNA levels further (Fig 7C).

3.8 The inflammatory cytokine IL-15 reduces macrophage SR-BI

The observation that acLDL was able to reduce the ability of fucoidan to stimulate the induction of iNOS (Fig 6 A) and IL-6 (Fig 6B and 7A) initially suggested the possibility that acLDL could be competing with fucoidan for binding to the same receptor, such as SR-A. This prompted us to examine the specificity of the acLDL effect by using an unrelated inflammatory mediator to stimulate IL-6 expression in resident peritoneal macrophages. We chose IL-15 because it signals in macrophages through a distinct receptor consisting of one or more subunits of the IL-15R α , IL-2/15R β and the common cytokine receptor γ_c ^{75, 76}, and has not been reported to interact with SR's. This allowed us to test if acLDL might exert a general effect on inflammatory signaling. We treated MPM with increasing concentrations of acLDL (0, 25, 50, 100 $\mu\text{g}/\text{mL}$) in the presence or absence of recombinant murine IL-15 (0.5 $\mu\text{g}/\text{mL}$). As observed in Figs 6B and 7A, acLDL treatments alone had no effect on IL-6 expression. On the other hand, IL-15 treatment increased IL-6 mRNA levels by at least 100-fold (Fig 8A). Surprisingly, acLDL reduced the ability of IL-15 to induce IL-6 by approximately 50%. IL-15 itself did not affect HMGC α R expression nor did it affect the ability of acLDL to reduce HMGC α R mRNA levels (Fig 8B).

acLDL reduced SR-BI mRNA levels by between 25 and 30 %. Surprisingly IL-15 alone reduced SR-BI mRNA levels by approximately 35% and the addition of acLDL led to a trend toward further reduction in SR-BI mRNA; however this trend did not reach statistical significance (Fig 8C). Importantly, the data in Figures 5-8 suggests that acLDL, rather than inducing inflammatory gene expression, suppresses activation of pro-inflammatory gene expression s by different mediators (fucoidan and IL-15). Although the mechanisms are not clear, it appears unlikely that the effects of increasing acLDL on the ability of fucoidan to induce IL-6 are the consequence of competition for binding to a common receptor. These results also demonstrate that IL-15, a distinct inflammatory mediator, is able to bring about a reduction in SR-BI mRNA levels in mouse macrophages, suggesting inflammatory signaling in general plays a role in modulating SR-BI expression.

3.9 The ability of acLDL, but not fucoidan to reduce SR-BI mRNA is dependent on SR-A.

To test the hypothesis that acLDL mediated effects on HMGCoAR and SR-BI expression, and on suppression of fucoidan's ability to stimulate iNOS and IL-6, may all be mediated by SR-A, we compared these parameters in resident MPM's from wild type and SR-A knockout mice. We also included other types of lipoproteins (HDL and LDL) to determine which if any of these effects were specific for acLDL. In WT MPMs (Fig 9A, red bars) acLDL and LDL treatments reduced HMGCoAR mRNA to 26% and 40% respectively, while HDL had no effect (Fig 9A). In MPM lacking SR-A expression (Fig 9A, blue bars), the effect of acLDL on HMGCoAR was greatly diminished, whereas LDL treatment reduced HMGCoAR mRNA to similar levels in both WT and SR-A KO MPM. This is consistent the

requirement for SR-A for endocytosis acLDL but not LDL which is endocytosed by the LDL receptor. Thus acLDL is unable to load cells with cholesterol in the absence of SR-A, whereas cholesterol loading by native LDL is unaffected. As observed in other experiments, fucoidan led to slight increases in HMGCoAR levels. As seen in Fig 9B and C, none of the lipoproteins, added alone, affected expression levels of IL-6 or iNOS in MPM from either WT or SR-A KO mice. Fucoidan induced IL-6 (Fig 9B) and iNOS (Fig 9C) mRNA even in absence of SR-A. Furthermore, the fucoidan induction of iNOS and IL-6 mRNA levels were reduced by all three lipoproteins (acLDL, LDL and HDL) both in WT and in SR-A KO macrophages. Thus it appears that the suppression of fucoidan induced iNOS and IL-6 is not specific to acLDL and is not dependent on SR-A. More importantly, fucoidan's induction of iNOS and IL-6 expression is mediated by a receptor other than SR-A contrary to what has been published by Nakamura et al⁶⁷. AcLDL reduced SR-BI in WT MPM to ~70% of untreated control. This effect was completely lost in SR-A KO MPM. In contrast, fucoidan reduced SR-BI mRNA in both WT and SR-A KO MPM. Thus the ability of acLDL to reduce SR-BI mRNA levels is dependent on SR-A. This, together with the observation that cholesterol loading of macrophages also leads to reduced SR-BI suggests that acLDL mediated, SR-A dependent suppression of SR-BI is likely the consequence of cholesterol loading of macrophages. In contrast, the ability of fucoidan to reduce SR-BI mRNA levels is independent of SR-A.

3.10 Fucoidan down-regulation of SR-BI mRNA requires CD14

Although Nakamura et al⁶⁷ reported that fucoidan mediated the induction of iNOS activity via a pathway that involved SR-A, our data suggests that SR-A is not required. In contrast, Freeman and co-workers⁷⁴ reported that fucoidan can stimulate the tyrosine phosphorylation of several proteins including PI3K and induce the production of TNF α by macrophages in a manner that is dependent on CD14 and not SR-A⁷⁴. To test the role of CD14 in fucoidan mediated induction of iNOS and IL-6 and suppression of SR-BI, we treated WT MPM and with an anti-CD14 blocking antibody, 4C1. Because we used the whole antibody and macrophages possess Fc receptors that bind to the Fc portions of antibodies, we pre-treated macrophages with an Fc receptor blocker to prevent binding of the anti-CD14 antibody to the macrophage Fc receptor. Control cells were also treated with the Fc receptor blocker to ensure that it did not interfere with the ability of fucoidan to alter expression of the genes of interest. The Fc receptor blocker did not affect the ability of fucoidan to strongly induce IL-6 or iNOS (Fig 10A, B), to slightly increase HMGCoAR (Fig 10C) or to suppress SR-BI (Fig 10D). In contrast, the anti-CD14 blocking antibody substantially reduced the ability of fucoidan to induce IL-6 and iNOS by 94% and 53% respectively. Similarly, the anti-CD14 blocking antibody completely suppressed the ability of fucoidan to reduce SR-BI mRNA levels (Fig 10D). This result demonstrated that fucoidan mediated the induction of iNOS and IL-6 and the suppression of SR-BI via CD14.

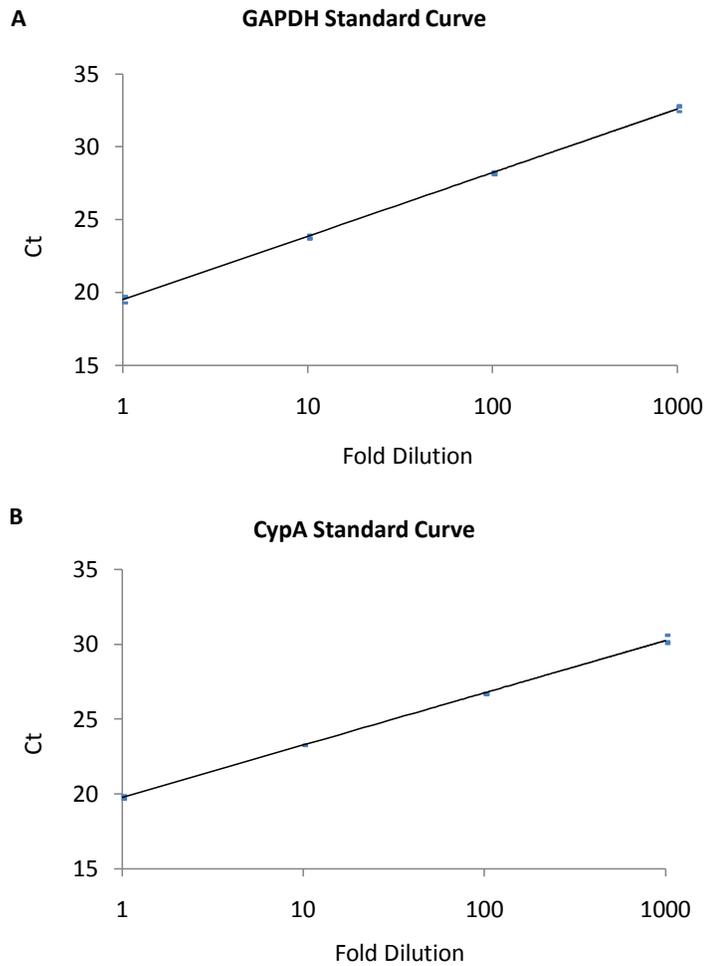


Fig S1. Primer set validation of housekeeping genes in Raw264.7 macrophages.

Raw264.7 macrophages were seeded at a density of 4×10^5 cells/cm² in RPMI supplemented with 3% NCLPDS and cultured for 24 hours. The media was removed and fresh media was added for 3 hrs. Cells were harvested, total RNA was prepared and cDNA was synthesized as described in the Methods section. Serial 10-fold dilutions of the cDNA synthesis reaction were generated and 3 μ l was subjected to RT-PCR for (A) GAPDH or (B) CypA. The cycle numbers at which the amount of product reached the threshold (Ct) are plotted versus the fold dilution of template cDNA.

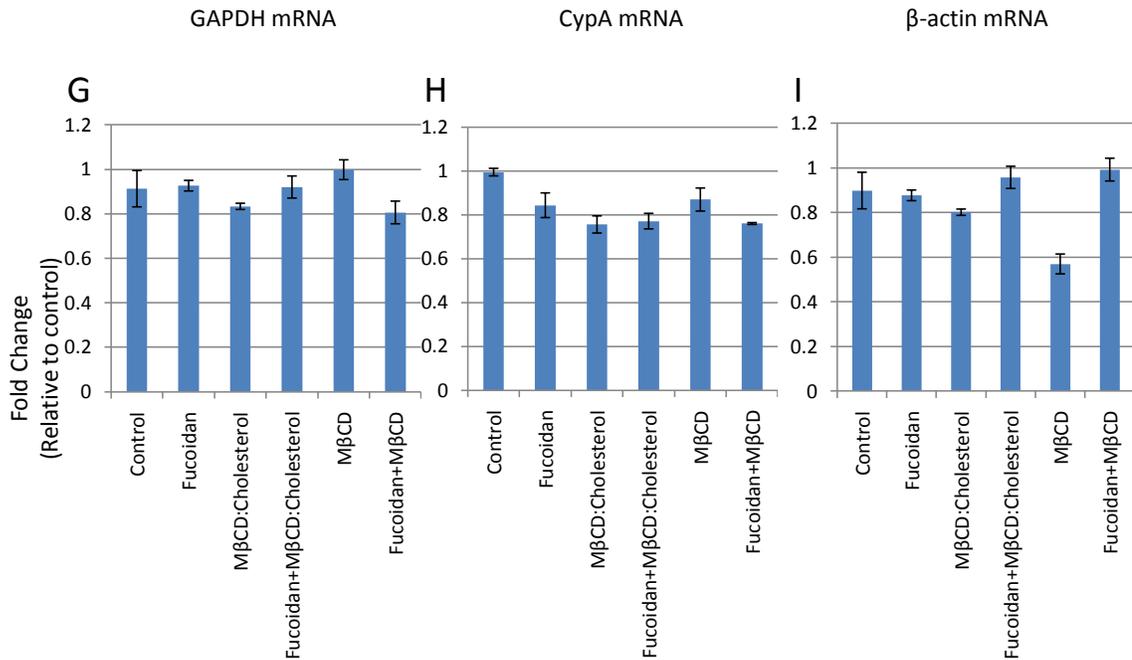


Fig S2. Primer set validation of housekeeping genes in Raw264.7 macrophages.

Raw264.7 macrophages were cultured as in described in the legend to Fig S1 prior to treatment for 3 hrs as follows: μ g Control (no treatment), Fucoidan (25 μ g/mL), M β CD:Cholesterol (methyl- β -cyclodextrin complexed to cholesterol at molar ratio of 2.5 mM : 0.42 mM), Fucoidan (25 μ g/mL) plus M β CD:Cholesterol (methyl- β -cyclodextrin complexed to cholesterol at molar ratio of 2.5 mM : 0.42 mM), M β CD (methyl- β -cyclodextrin 2.5 mM), Fucoidan (25 μ g/mL) plus M β CD (methyl- β -cyclodextrin 2.5 mM). Total RNA was prepared from each sample and 1 μ g was converted to cDNA. The completed cDNA reactions were diluted 10 fold and 3 μ l was used for RT-PCR. Every sample was run in triplicate RT-PCR reactions. Amplification plots (A-C) and dissociation curves (D-F) are shown for GAPDH (A,D), CypA (B,E) and β -actin (C,F). Each trace represents one PCR reaction. Relative fold changes of (G) GAPDH (H) CypA and (I) β -actin mRNA's. The Ct values obtained for each RT-PCR reaction were converted to 2^{CT} . The 2^{CT} values were then normalized to a single 2^{CT} value of one of the control RT-PCR reactions, and $2^{\Delta CT}$ or fold change relative to control were derived. Three independent experiments were carried out in parallel. Data from one representative experiment is shown.

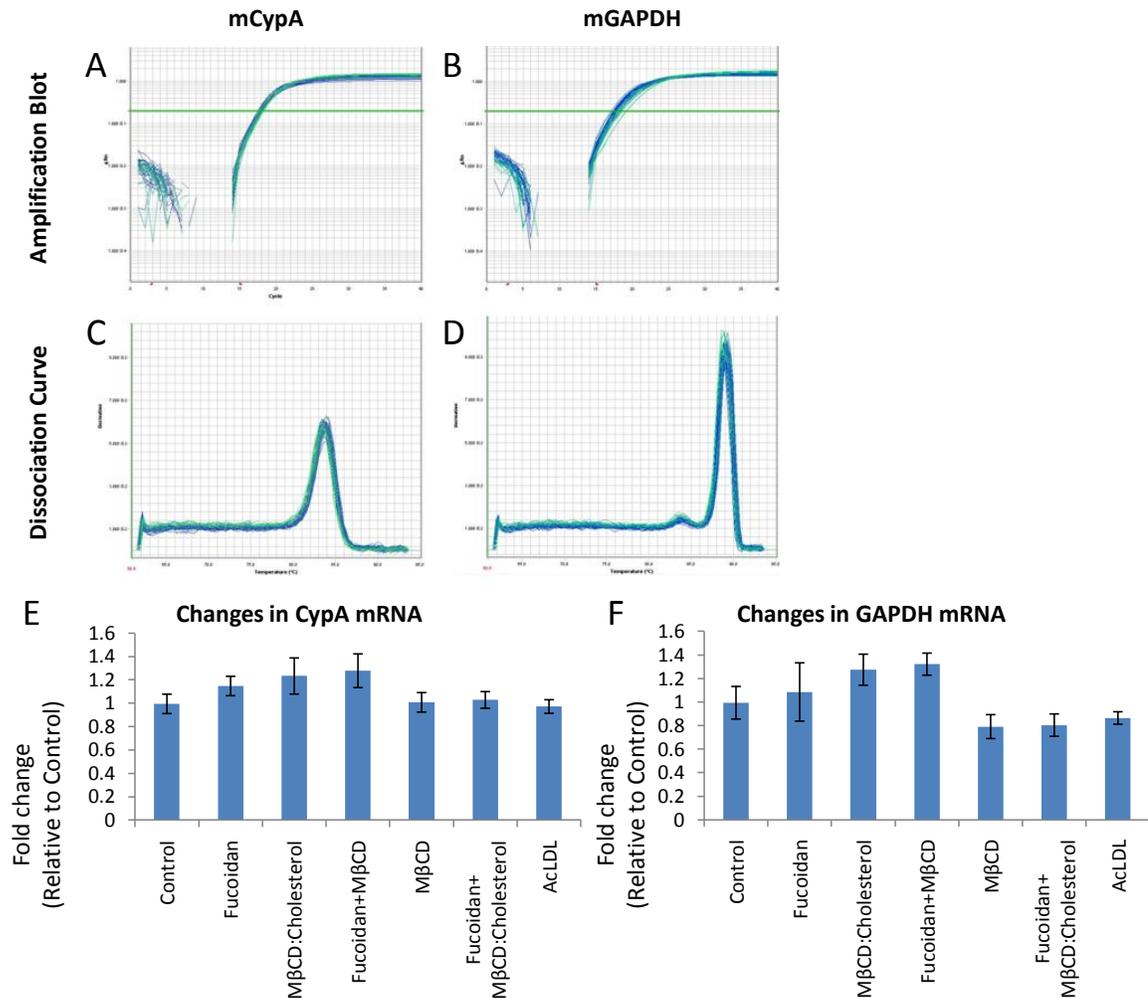


Fig S3. Primer set validation of housekeeping genes using elicited mouse peritoneal macrophages.

Thioglycolate elicited mouse peritoneal macrophage were collected from two female C57/BL6 mice. Cells from each mouse were grown separately in RPMI supplemented with 10% FBS for 3 days, then RPMI supplemented with 3% NCLPDS for 4 days before cells were treated for 3 hours as follows: μg Control (no treatment), fucoïdan (25 $\mu\text{g}/\text{mL}$), M β CD:cholesterol (methyl- β -cyclodextrin complexed to cholesterol at ratio of 2.5 mM : 0.42 mM), fucoïdan (25 $\mu\text{g}/\text{mL}$) plus M β CD (methyl- β -cyclodextrin 2.5 mM), M β CD (methyl- β -cyclodextrin 2.5 mM), fucoïdan (25 $\mu\text{g}/\text{mL}$) plus M β CD (methyl- β -cyclodextrin complexed to cholesterol at molar ratio of 2.5 mM : 0.42 mM), AcLDL (100 $\mu\text{g}/\text{mL}$). Total RNA was prepared from each sample and 1 μg was converted to cDNA. The completed cDNA reactions were diluted 10 fold and 3 μl was used for RT-PCR. Every sample was run in triplicate RT-PCR reactions. Amplification plots (A,B) and dissociation curves (C,D) CypA and GAPDH are shown. Each trace represents one PCR reaction. Relative fold changes of (E) CypA and (F) GAPDH were determined as described in the legend to Figure S2. The average $2^{\Delta\text{CT}}$ from two mice are shown. Error bars represent the ranges.

Target gene	Primer Pair Sequence	Amplicon Size (bp)	Amplification Efficiency (%)	R ² value
Mouse SRBI Forward	CAGGCTGTGGGAAGCTCTAGC	248	95	0.99
Mouse SRBI Reverse	GAAAAAGCGCCAGATACAGC			
Mouse HMG-CoA reductase F	GGGAGCATAGGCGGCT	60	96	0.98
Mouse HMG-CoA reductase R	TGCGATGTAGATAGCAGTGACA			
Mouse Cyclophilin A F	GGCCGATGACGAGCCC	64	93	1
Mouse Cyclophilin A R	TGTCTTTGGAAGTTTGTCTGCAA			
Mouse GAPDH F	ACCACAGTCCATGCCATCAC	452	69	1
Mouse GAPDH R	TCCACCACCCTGTTGCTGTA			
Mouse Inos F	TCCTGGAGGAAGTGGGCCGAA	195	69	1
Mouse Inos R	CTCCACGGGCCCGGTAAGTCA			
Mouse Bactin F	ACCAACTGGGACGATATGGAGAAG	214	n/a	n/a
Mouse Bactin R	TACGACCAGAGGCATACAGGGACAA			
Mouse IL-6 F	TCTCTGCAAGAGACTTCCATCCAGT	71	n/a	n/a
Mouse IL-6 R	AGTAGGGAAGGCCGTGGTTGTCA			
Human B-actin F	CACTCTCCAGCCTTCCTTC	90	n/a	n/a
Human B-actin R	GGATGTCCACGTCACACTTC			
Human SRBI F	CTGGCAGAAGCGGTGACT	97	n/a	n/a
Human SRBI R	CAGAGCAGTTCATGGGGATT			
Human CD68 F	CCACCAGCCAGGGACCCTCA	463	n/a	n/a
Human CD68 R	GCTGCGTGGGGGAAGGACAC			
Human SR-AI F	CACGAGGATTTCCAGGTCCAA	186	n/a	n/a
Human SR-AI R	CGACCAGTCGAAGTTTCGTAAATG			

Table 1. Mouse primer pairs for RT-PCR

Primers sequences were selected using Pubmed's primer-BLAST. Standard curves for selected primers were produced from RT-PCR using 10 fold serial dilutions of starting cDNA. PCR amplification efficiencies and R² values were calculated from the standard curves. Standard curves for GAPDH and CypA are those shown in Fig S1 A and B.

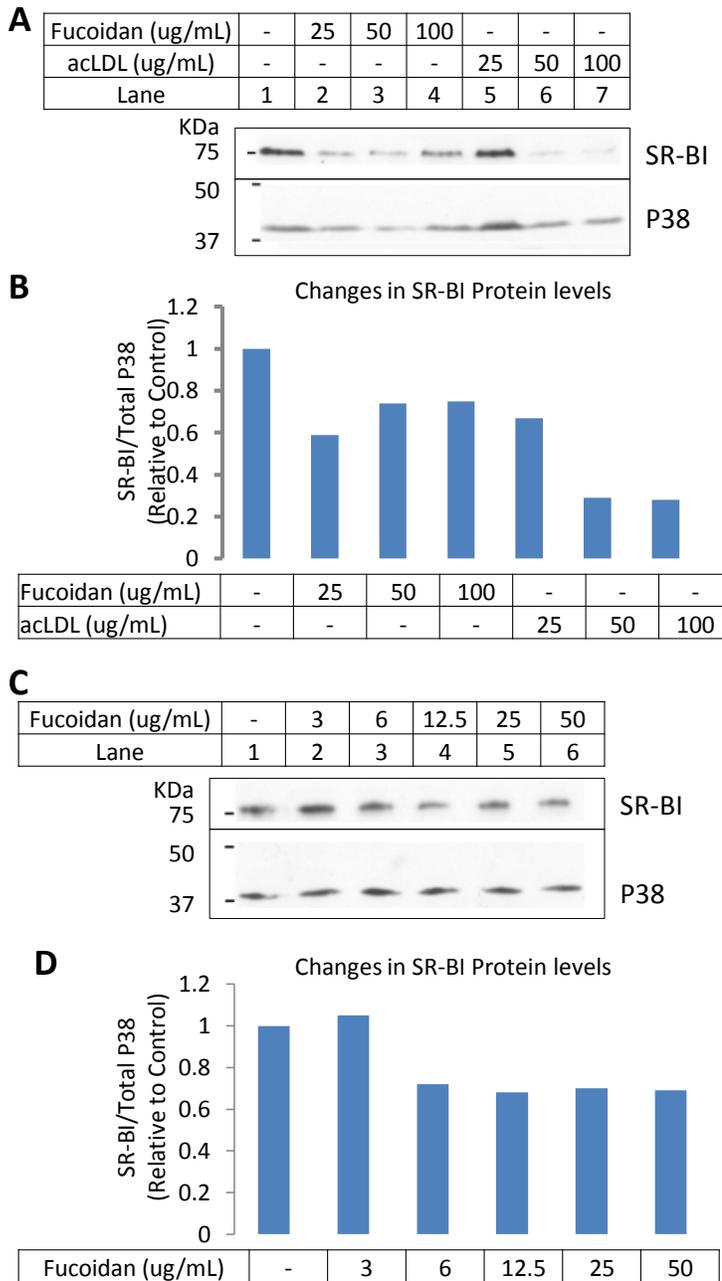


Fig 1. acLDL and Fucoidan reduced SR-BI protein levels in Raw264.7 macrophages

Raw264.7 cells were plated in 10 cm dishes at a density of 4×10^5 cells/cm² in DMEM supplemented with 3% NCLPDS. After 24 hours the medium was replaced with fresh medium containing either no additions (lane 1) or the indicated concentrations of fucoidan (A, lanes 2-4, and C lanes 2-6) or acLDL (A, lanes 5-7). After 24 hrs, the cells were lysed and 50µg of proteins were analyzed by 12.5% SDS-PAGE and immunoblotting for either SR-BI or p38 mitogen activated protein kinase. Representative immunoblots are shown in A and C. SR-BI band intensities were determined and normalized to p38 band intensities using a Gel Doc system (BioRad). A representative experiment is shown. This experiment was repeated once, and similar results were obtained.

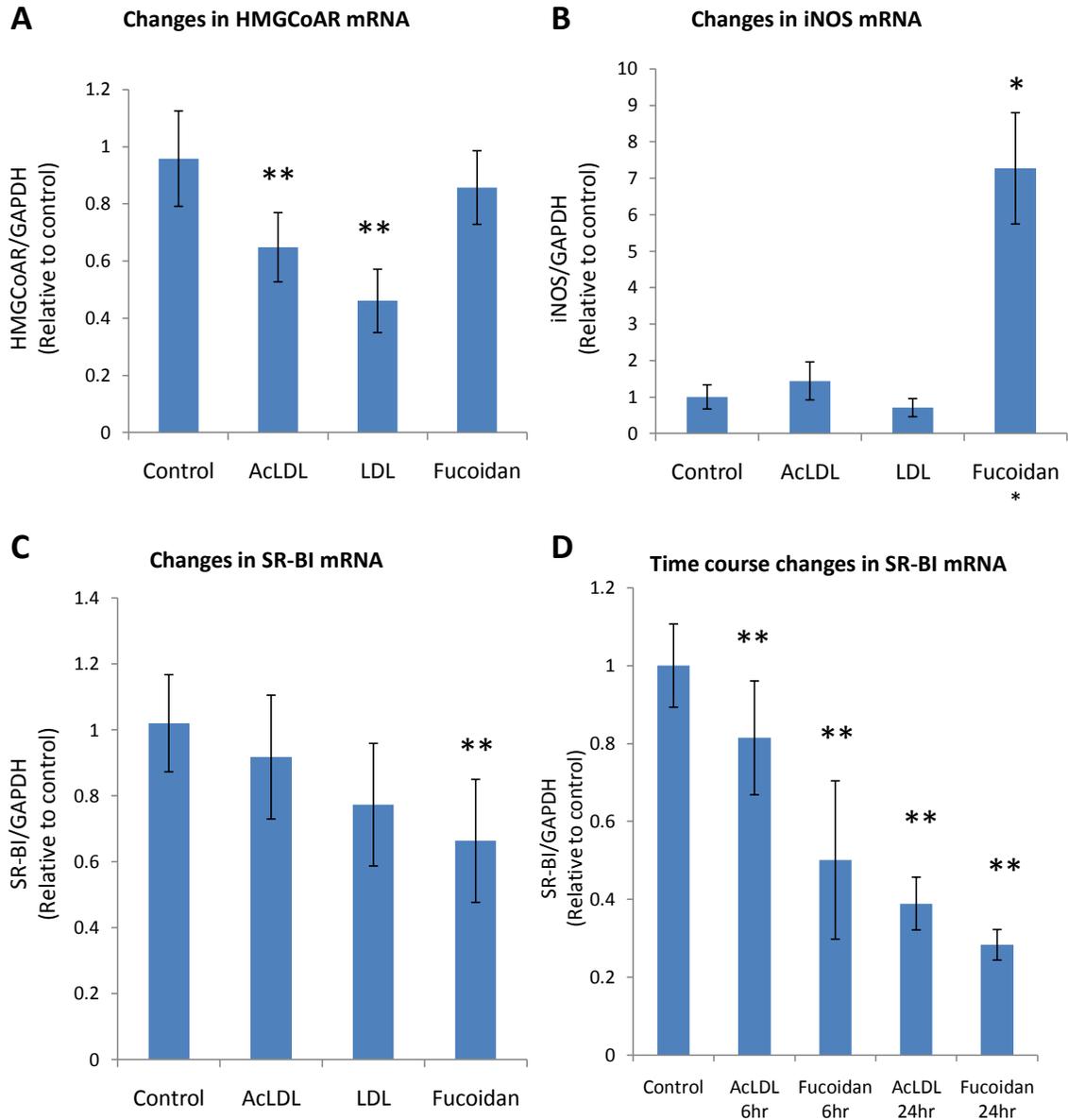


Fig 2. AcLDL and fuoidan reduced SR-BI mRNA in Raw264.7 cells.

Raw264.7 macrophages were plated at a density of 4×10^5 cells/cm² in RPMI supplemented with 3% NCLPDS. After 24 hours the medium was replaced with fresh medium containing either no additions, 100 µg/mL AcLDL, 100 µg/mL LDL or 25 µg/mL fuoidan as indicated. Total RNA was isolated after 3 hrs (A-C) or 6 or 24 hrs (D) and equal amounts were used for cDNA synthesis. The completed cDNA reaction solutions were diluted 10 fold and used for RT-PCR. Each sample was run in triplicate RT-PCR reactions using primers for A) HMGCoAR, B) iNOS, or (C and D) SR-BI and GAPDH as a loading control. The averages \pm standard errors of three independent samples for each condition are shown. Statistically significant difference: * = $p < 0.05$, ** = $p < 0.01$ relative to control.

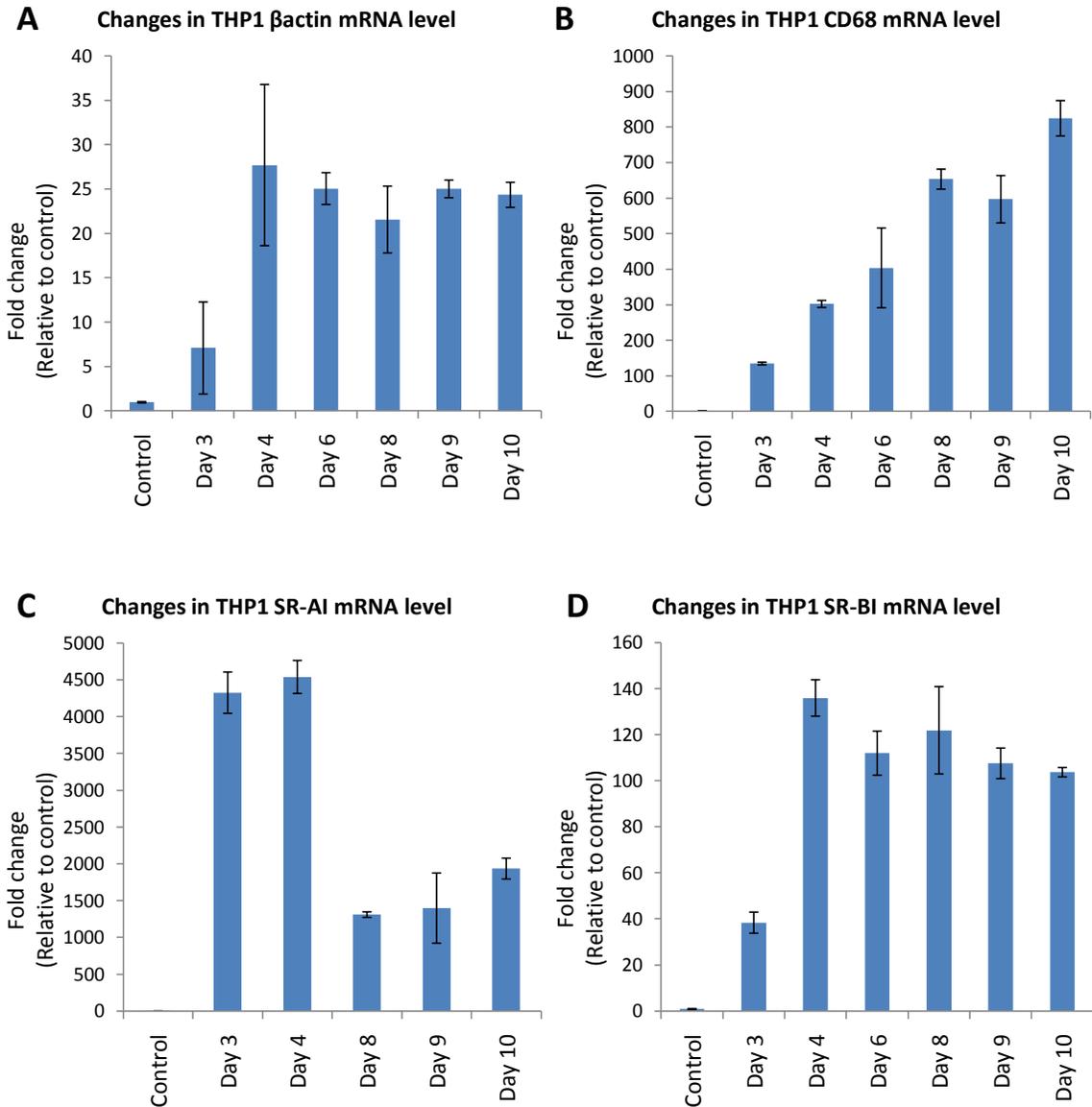


Fig 3. Gene expression changes during PMA induced THP1 monocyte differentiation to macrophage.

THP1 cells were seeded in a 24 well plate at a density of 1.57×10^5 cells/cm² in DMEM supplemented with 10% FBS and 100 nM PMA. After 3 days, the media was replaced with fresh media lacking PMA. At the indicated time points, cells were harvested and total RNA was prepared. 1 μ g of total RNA was used for cDNA synthesis. The complete cDNA synthesis reactions were diluted 10 fold and used for RT-PCR. Every sample was run in triplicate RT-PCR reactions. A) β -Actin, B) CD68, C) SR-AI and D) SR-BI mRNA levels were analyzed. The Ct values obtained for each RT-PCR reaction were converted to 2^{Ct} . The 2^{Ct} values were then normalized to a single 2^{Ct} value of one control RT-PCR reaction, and $2^{\Delta Ct}$ or fold change relative to control were derived. The averages \pm standard errors of three RT-PCR reactions of each sample are shown.

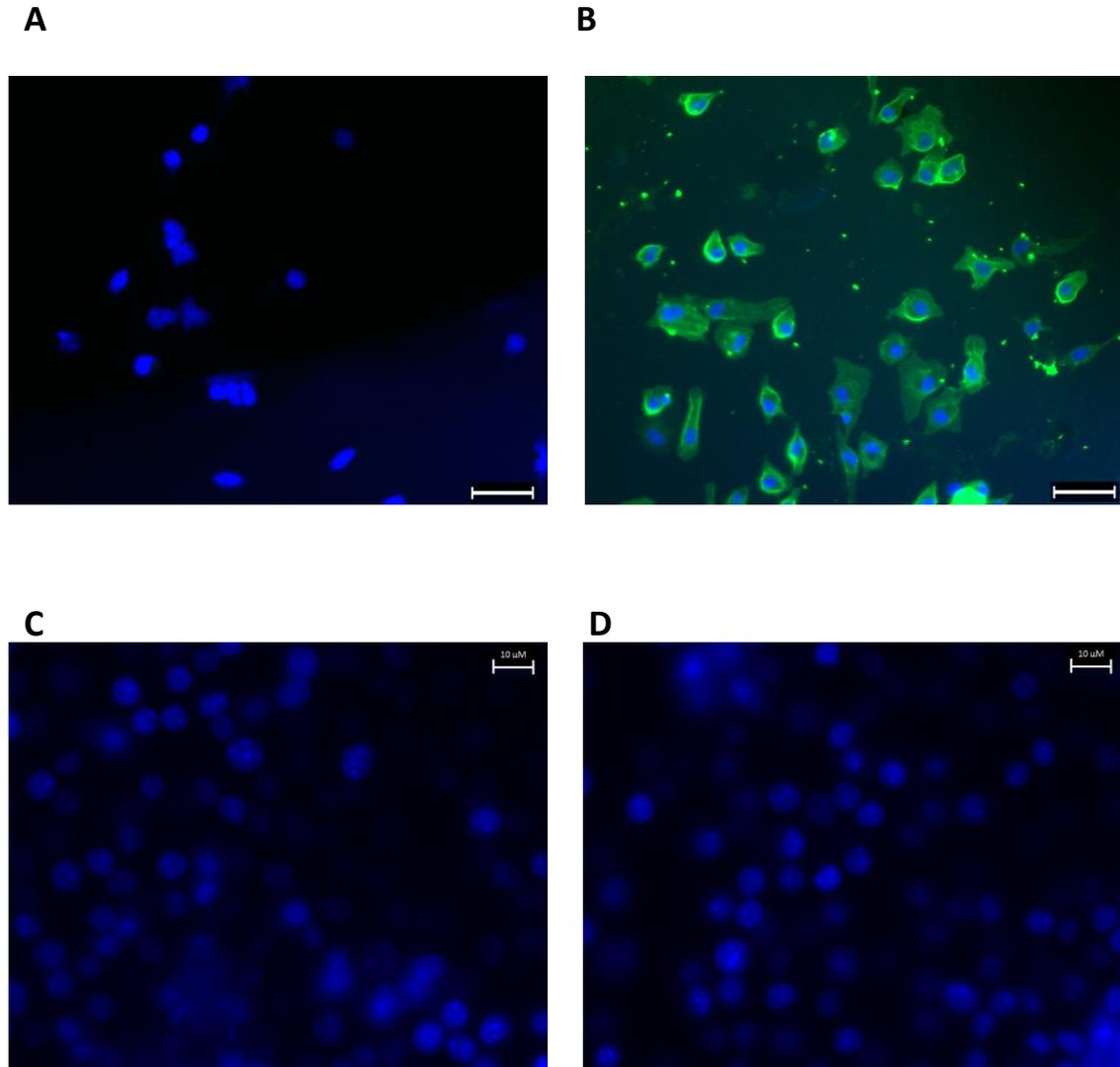


Fig 4. F4/80 staining of resident mouse peritoneal macrophages and CCL-1 fibroblasts.

Resident mouse peritoneal macrophages or mouse CCL-1 fibroblasts were plated on to glass chamber slides in RPMI supplemented with 3% NCLPDS, cultured for 24 hr, then fixed and stained with either Alexa 488 conjugated goat anti rat secondary antibody alone (A, C) or with rat-anti-mouse F4/80 primary antibody, followed by Alexa488 conjugated goat-anti-rat secondary antibody (green) (B,D). Nuclei were stained with DAPI (blue). A, B) Mouse peritoneal macrophages stained. Scale bar = 50 μm . C, D) Mouse CCL-1 fibroblasts. Scale bar = 10 μm .

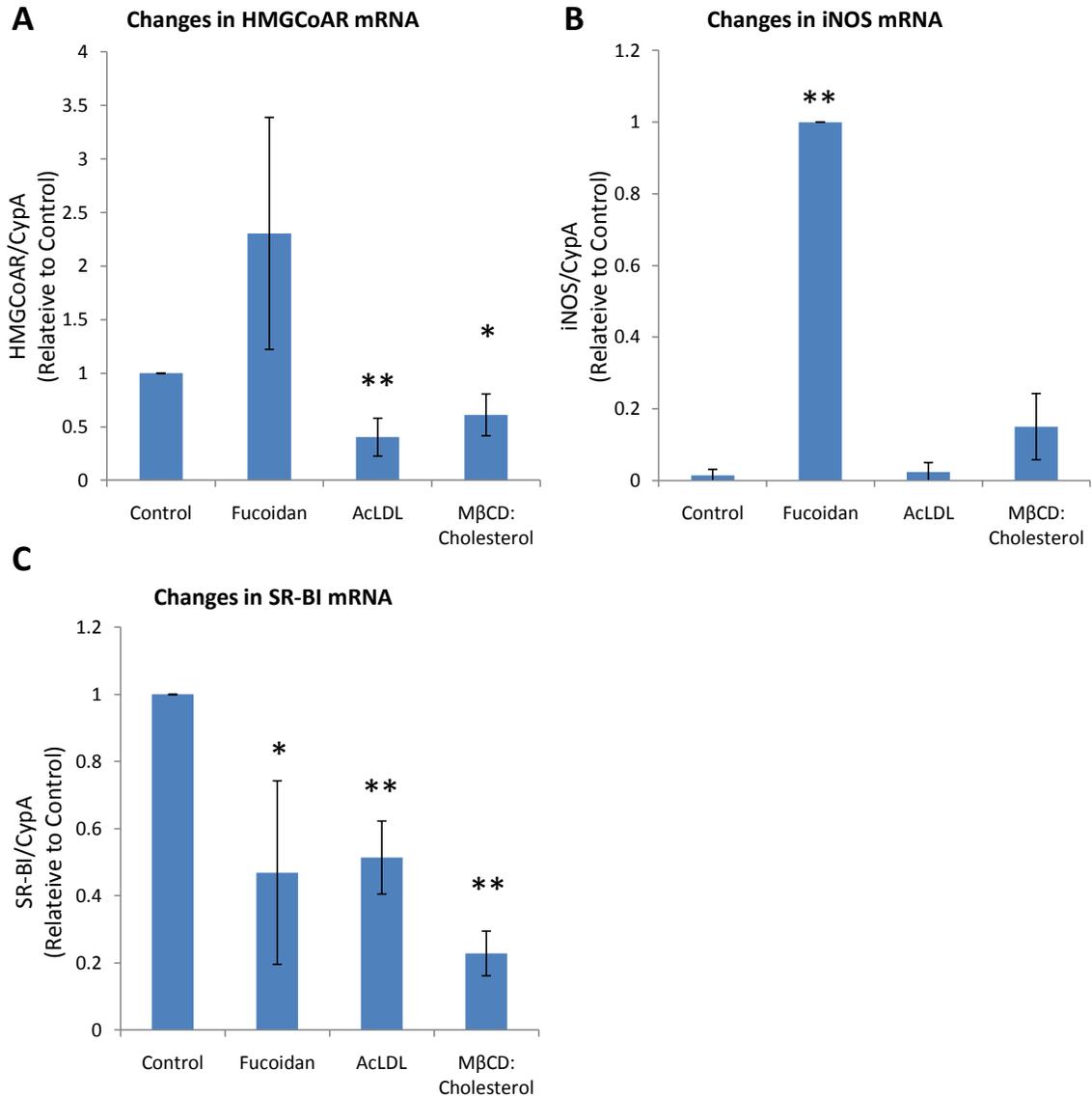


Fig 5. AcLDL, fucoidan and cholesterol loading with MβCD:cholesterol complex reduced SR-BI mRNA in mouse resident peritoneal macrophage.

Resident mouse peritoneal macrophages (MPM) from 3 male C57/BL6 mice were cultured in RPMI supplemented with 10% FBS for 2 hrs to allow attachment of MPM, after which non-attached cells were washed off three times with warm PBS. MPM were then grown in RPMI supplemented with 3% NCLPDS for 24 hours before the media was removed and replaced with fresh media containing either no additions (control), fucoidan (25 ug/mL), AcLDL (100 μg protein/mL), cholesterol complexed to MβCD (ratio of 0.42 to 2.5 mM). RT-PCR was performed as described in the legends to Figs 2 and 3 and the Methods section for A) HMGCoAR, B) iNOS, C) and SR-BI, using CypA as a loading control. The averages ± standard errors of three independent samples for each condition are shown. Statistically significant difference: * = $p < 0.05$, ** = $p < 0.01$ relative to control.

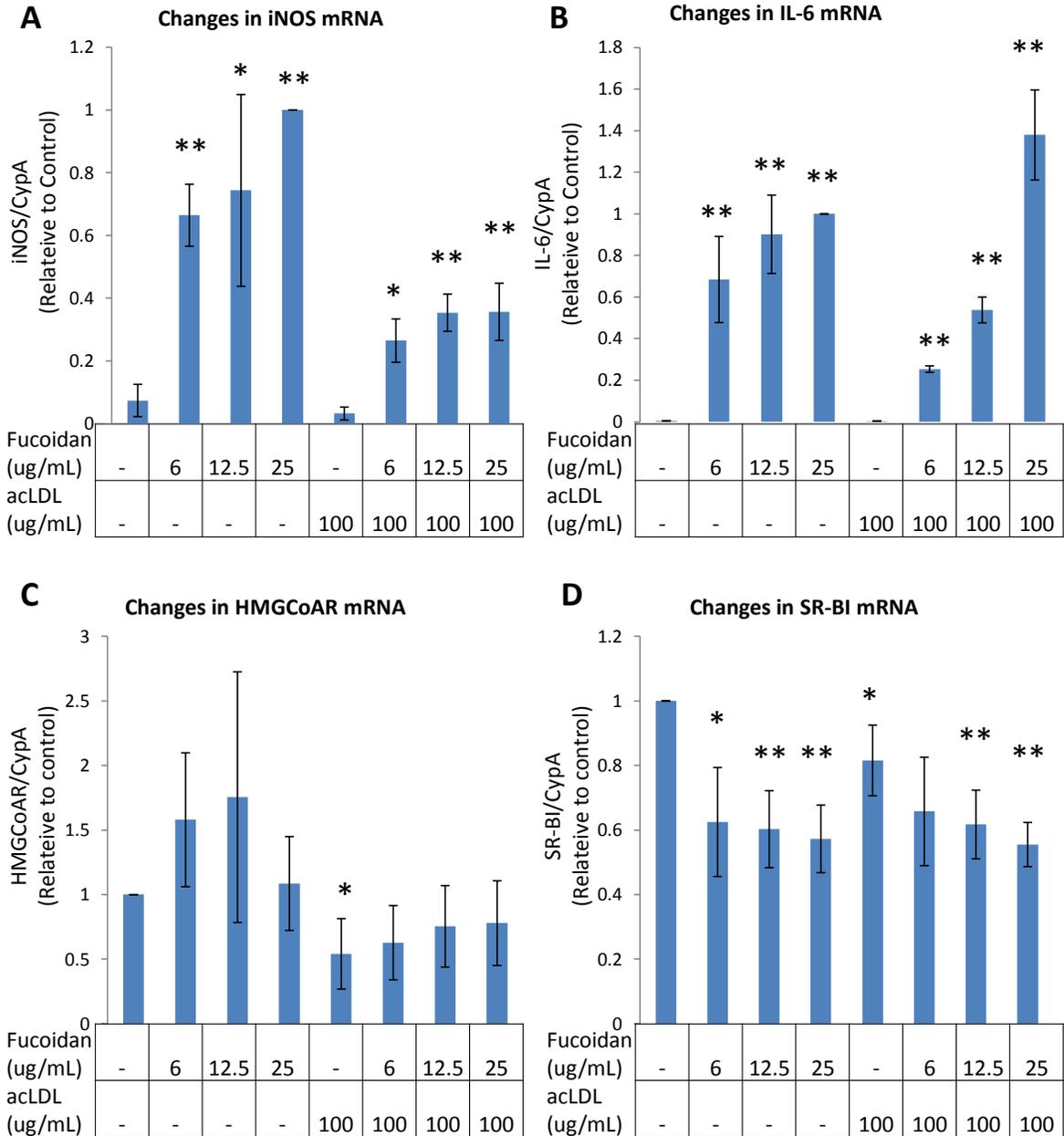


Fig 6. Concentration dependence of fucoidan in the absence or presence of AcLDL on inflammatory genes, HMGCoAR and SR-BI mRNA levels.

Resident mouse peritoneal macrophages from 3 male wild type mice were treated for 3 hrs as described in the legend to Fig 5 and the Methods section without additions or with different concentrations (6, 12.5 and 25 µg/mL) of fucoidan in the absence or presence of AcLDL (100 µg/mL). RT-PCR was performed for (A) iNOS, (B) IL-6, (C) HMGCoAR and (D) SR-BI, using CypA as a loading control. The averages ± standard errors of three independent samples for each condition are shown. Statistically significant difference: * = p < 0.05, ** = p < 0.01 relative to control.

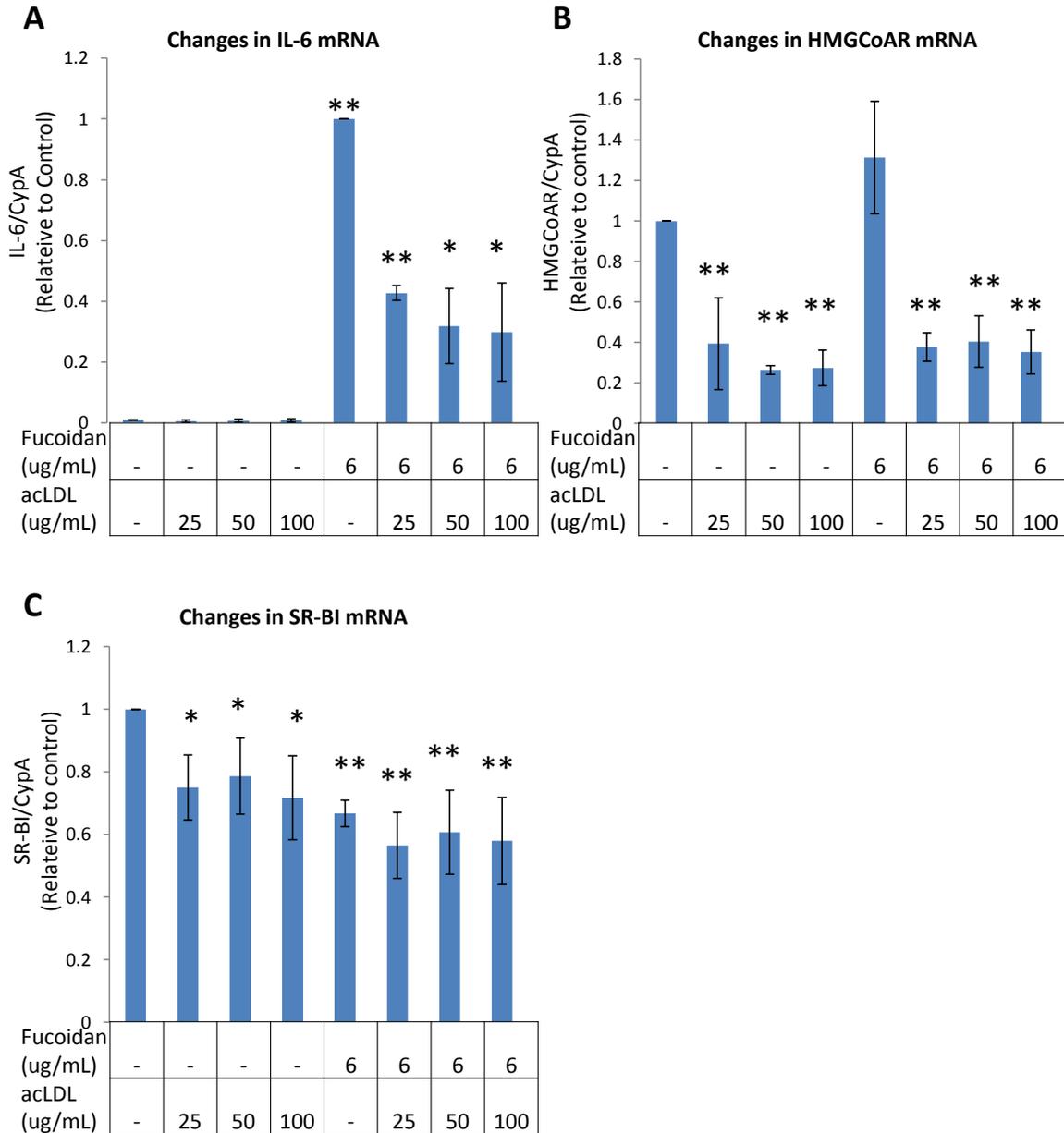


Fig 7. Concentration dependence of acLDL in the absence or presence of fucoidan on IL-6, HMGCAR and SR-BI mRNA levels.

Resident mouse peritoneal macrophages from 3 male wild type mice were treated for 3 hrs as described in the legend to Fig 5 and the Methods section, without additions or with different concentrations (25, 50 and 100 $\mu\text{g/mL}$) of AcLDL in the absence or presence of fucoidan (6 $\mu\text{g/mL}$). RT-PCR was performed for (A) IL-6, (B) HMGCAR and (C) SR-BI, using CypA as a loading control. The averages \pm standard errors of three independent samples for each condition are shown. Statistically significant difference: * = $p < 0.05$, ** = $p < 0.01$ relative to control.

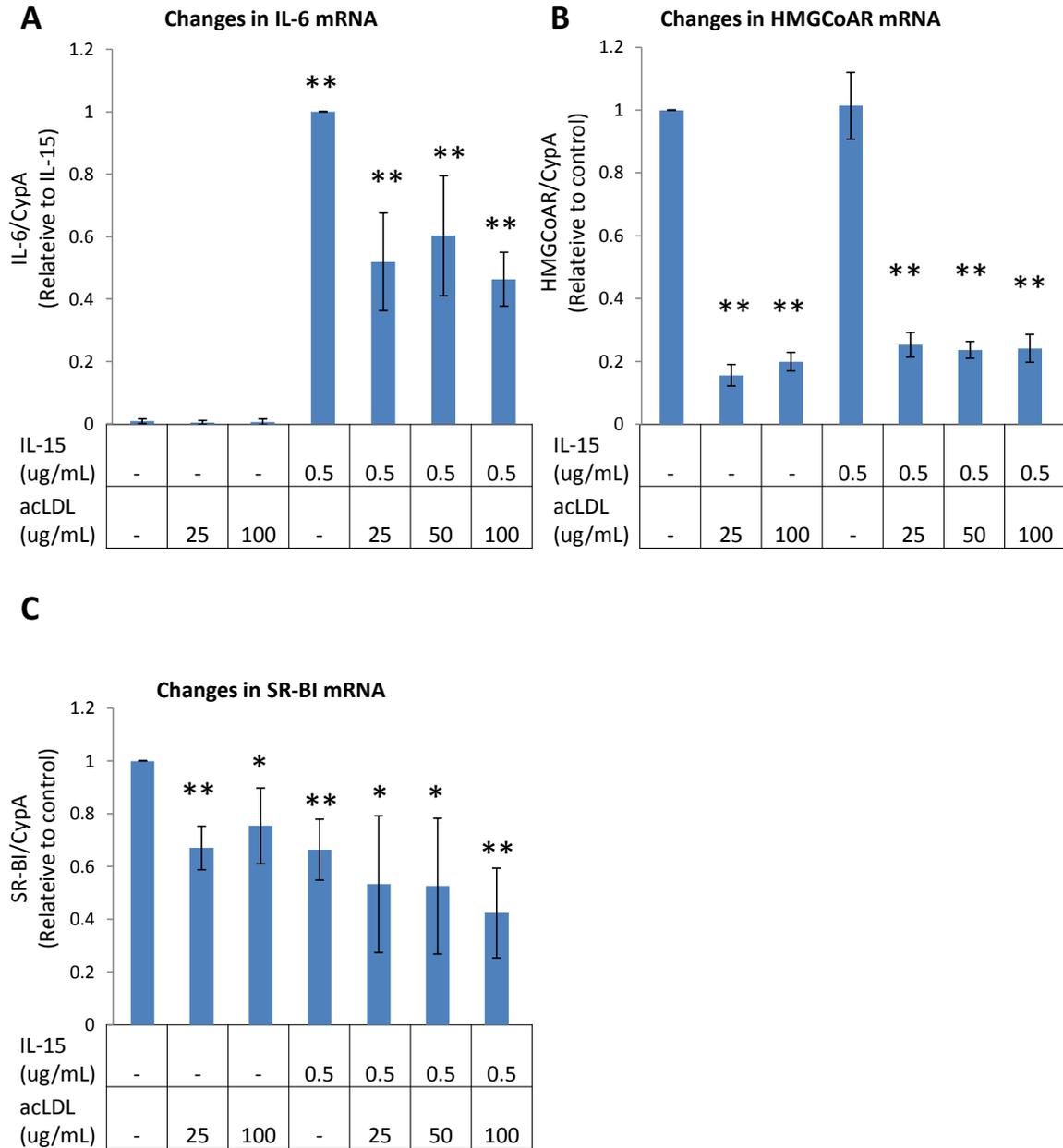
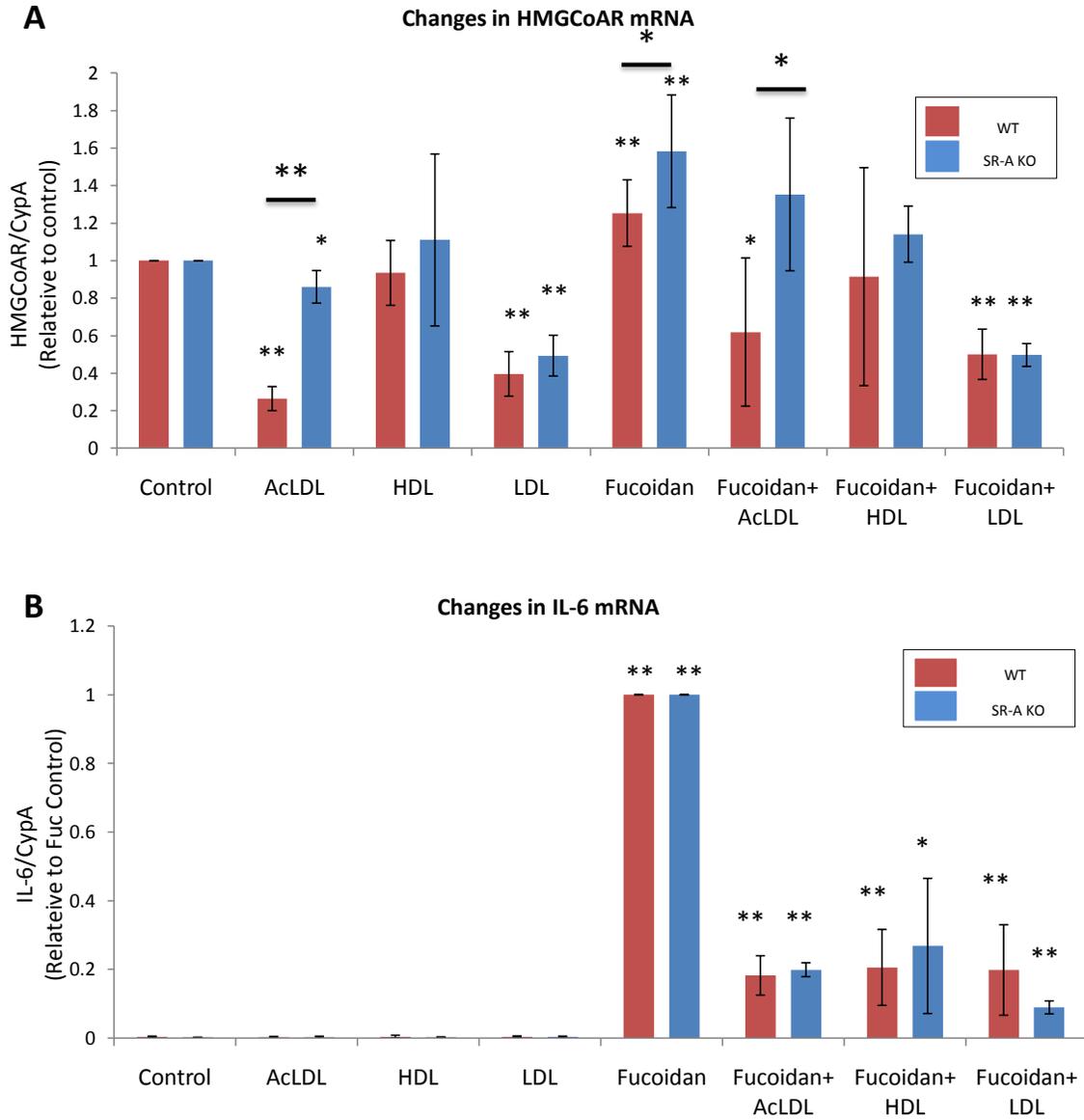


Fig 8 Concentration dependence of acLDL in the absence or presence of IL-15 on IL-6, HMGCoAR and SR-BI mRNA levels.

Resident mouse peritoneal macrophages from 3 male wild type mice were treated for 3 hrs as described in the legend to Fig 5 and the Methods section, without additions or with different concentrations (25 and 100 $\mu\text{g}/\text{mL}$) of acLDL in the absence or presence of IL-15 (0.5 $\mu\text{g}/\text{mL}$). RT-PCR was performed for (A) IL-6, (B) HMGCoAR and (C) SR-BI, using CypA as a loading control. The averages \pm standard errors of three independent samples for each condition are shown. Statistically significant difference: * = $p < 0.05$, ** = $p < 0.01$ relative to control.



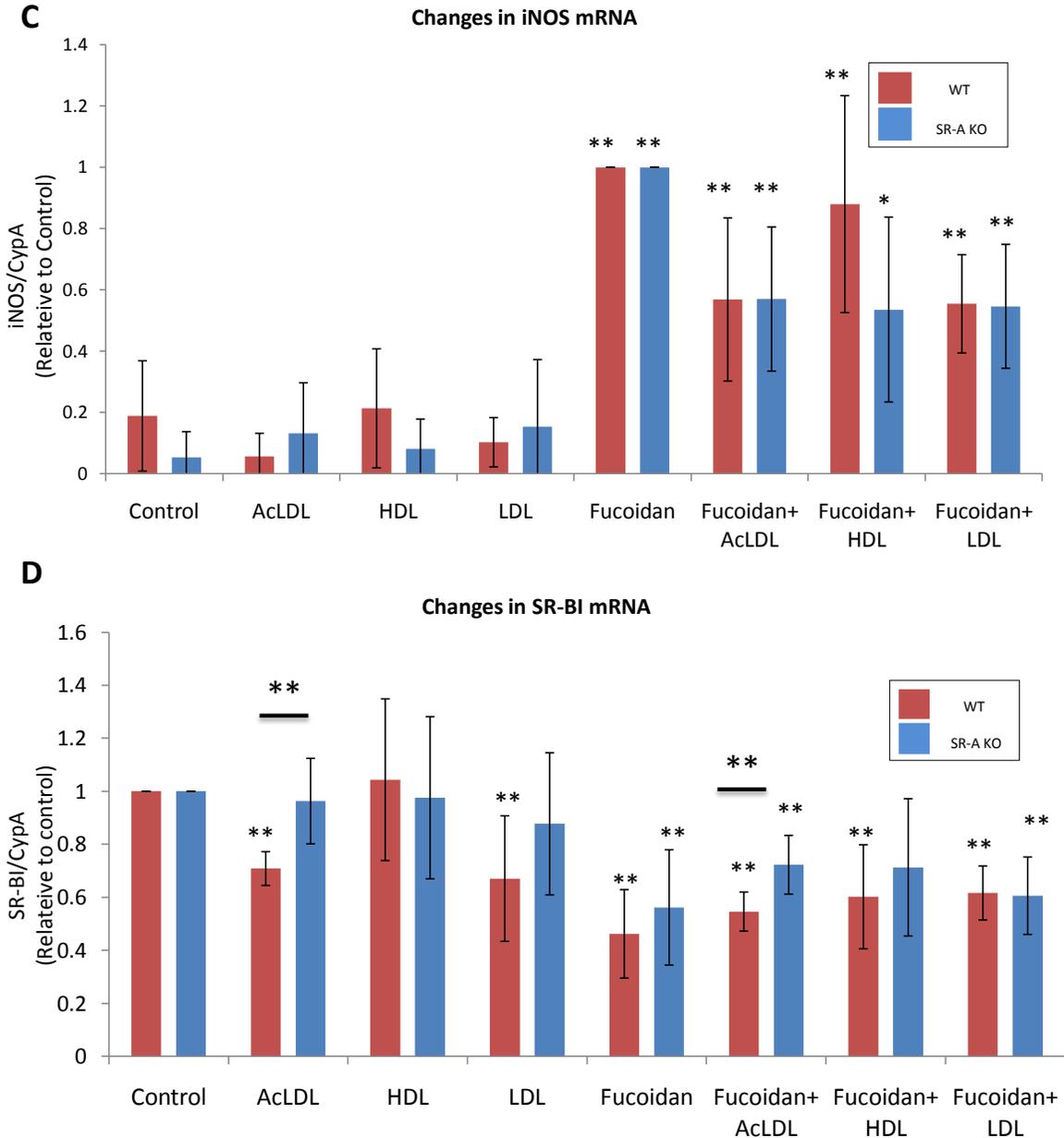


Fig 9. The acLDL but not fucoidan mediated suppression of SR-BI expression is dependent on SR-A.

Resident MPM's from male wild type (n=8) or SR-A KO mice (n=4) were collected, treated in culture for 3 hrs with either no additions, AcLDL, HDL or LDL (each at 100 $\mu\text{g}/\text{mL}$) or fucoidan (25 $\mu\text{g}/\text{mL}$) in the absence or the presence of AcLDL, HDL or LDL (each at 100 $\mu\text{g}/\text{mL}$), and expression levels of (A) HMGCoAR, (B) IL-6, (C) iNOS and (D) SR-BI were analyzed by RT-PCR using CypA as a loading control, as described in the Methods section and the Legend to Fig 5. Results are means \pm standard errors of 8 independent WT samples and 4 independent SR-A KO samples. Statistically significant difference: * = $p < 0.05$, ** = $p < 0.01$ relative to control. * = $p < 0.05$, ** = $p < 0.01$ between genotypes.

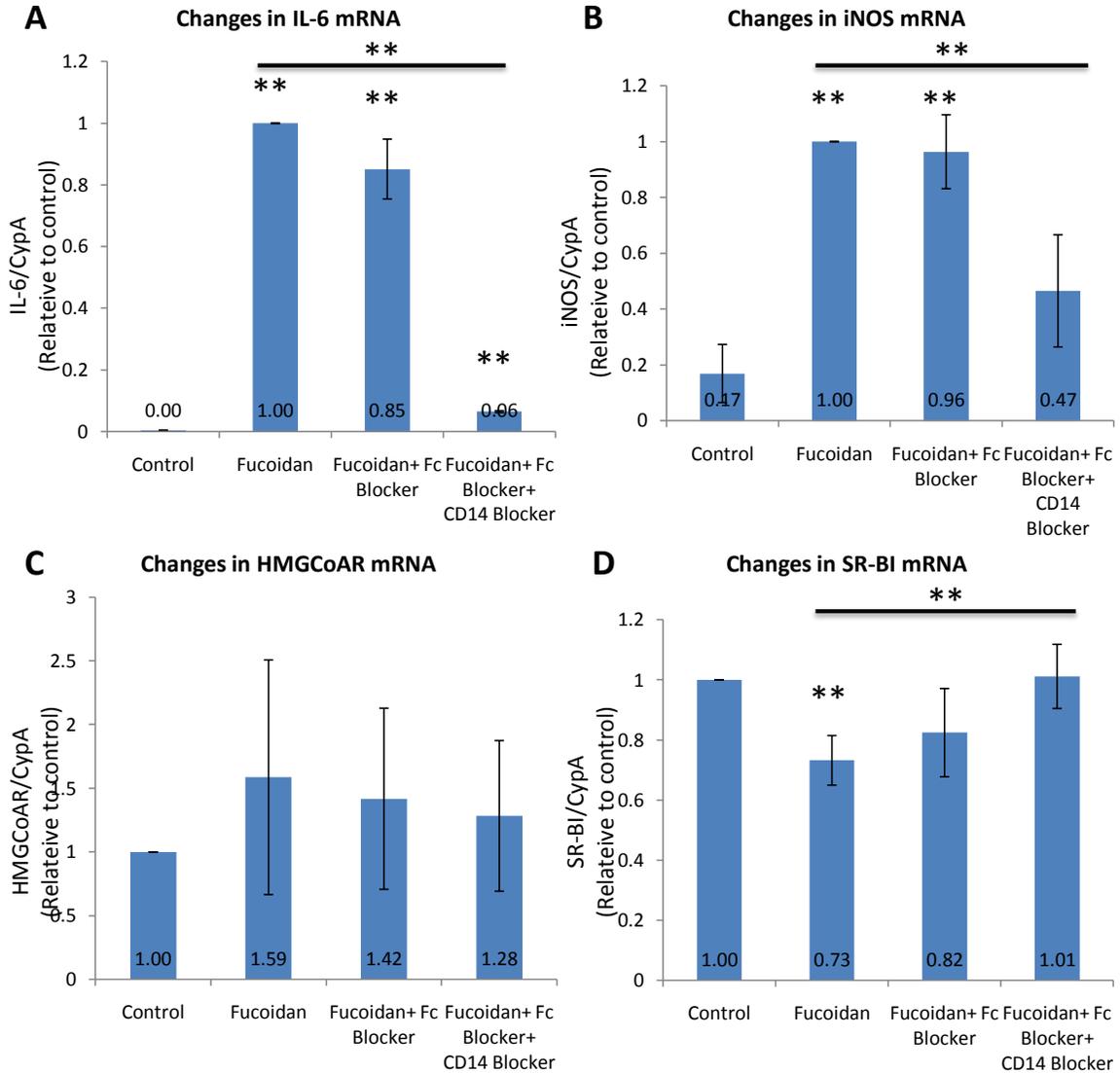


Fig 10. Fucoidan mediated suppression of SR-BI and induction of IL-6 and iNOS mRNA is inhibited by an anti-CD14 blocking antibody.

Resident MPM's from 3 male wild type mice were collected and cultured as described in the Methods section and the legend to Fig 5. Cells were pre-incubated with either no additions or the Fc blocking reagent (5 µg/mL) with or without the anti-CD14 antibody (10 µg/mL) as indicated. Cells were harvested after 3 hrs, and total RNA was prepared and subjected to RT-PCR analysis as described in the Methods section and the legend to Fig 5 for (A) IL-6, (B) iNOS, (C) HMGCoAR, and (D) SR-BI expression using CypA as a loading control. Data are means ± standard errors of three independent samples treated in parallel. Statistically significant difference: * = $p < 0.05$, ** = $p < 0.01$ relative to control. This experiment was repeated with equivalent results.

4. Discussion

In this study, we examined the role of putative SR-A ligands on macrophage SR-BI expression levels and provide evidence that SR-BI expression can be reduced by cholesterol loading or activation of inflammatory signaling. SR-A is the major macrophage receptor for acLDL and mediates acLDL dependent cholesterol accumulation and foam-cell formation^{6,77}. Fucoidan is also widely used as an SR-A ligand, based on its effectiveness as a competitive inhibitor of SR-A mediated acLDL binding and degradation. When macrophages were treated with either acLDL or fucoidan, SR-BI protein levels were reduced (Fig 1) and this reduction is attributable to a concomitant reduction in SR-BI mRNA levels (Fig 2C, 5C, 6D, 7C, 9D). Since fucoidan does not have the means to deliver cholesterol to cells, we interpreted this to imply that binding of SR-A ligands to SR-A may be sufficient to reduce SR-BI expression. However several lines of evidence suggest that this is not the case. Firstly, acLDL treatment decreased HMGCoAR mRNA (Fig 2A, 5A, 6C, 7B, 9A) but had no effect on mRNA expression level of inflammatory molecules such as iNOS and IL6 (Fig 2B, 5B, 6A, 6B, 7A, 9B). Fucoidan on the other hand, did not reduce HMGCoAR mRNA levels but strongly induced iNOS and IL6 mRNA levels. When MPMs were treated with acLDL and fucoidan simultaneously, there were no additive effect on SR-BI mRNA levels (Fig 6D, 7C). Studies by others have also shown that acLDL treatment is incapable of inducing macrophage inflammatory pathways^{67,74}. These differences between gene expression profiles and

the lack of synergistic effects raised the possibility that acLDL and fucoidan may have effected SR-BI expression via different but converging pathways, related to cellular cholesterol loading, and the activation of inflammatory signaling. This is supported by the demonstration that cholesterol complexed to M β CD and LDL (both of which can effect cholesterol loading) both suppressed SR-BI expression whereas HDL, which mediates cholesterol efflux from cells did not (Fig 5C and 9D).

Although Nakamura et al⁶⁷ reported that fucoidan mediated the induction of iNOS activity via a pathway that involved SR-A, we showed that Fucoidan's effect on iNOS, IL-6 and SR-BI mRNA levels was no different between peritoneal macrophages isolated from WT or SR-A KO mice (Fig 9). This demonstrates that SR-A is not required for fucoidan mediated induction of iNOS, IL-6 or suppression of SR-BI. In contrast, Freeman and co-workers⁷⁴ reported that fucoidan can stimulate the tyrosine phosphorylation of several proteins including PI3K and induce the production of TNF α by macrophages in a manner that is dependent on CD14 and not SR-A⁷⁴, thus we tested the involvement of CD14. We showed that fucoidan's effect on IL-6, iNOS and SR-BI mRNA is greatly diminished by an anti-CD14 blocking antibody (Fig 10). Parallels can be drawn between fucoidan and LPS. LPS is the major gram-negative bacteria cell wall component and fucoidan is a cell wall component of an algae. Both fucoidan and LPS can bind SR-A, initiate inflammatory responses by binding to CD14 leading to induction of iNOS and production of inflammatory cytokines such as TNF α and IL-6⁷⁸ (Fig 10). It has been shown that the clearance of LPS by SR-A and SR-BI reduces TLR4 mediated

inflammatory responses^{64, 79}. In an analogous manner, SR-A binding of fucoidan may mediate its clearance rather than signaling. One study has reported that LPS treatment reduced macrophage SR-BI expression levels⁸⁰. Our results demonstrate that fucoidan mediated the induction of iNOS and IL-6 and the suppression of SR-BI via CD14. Like many well established non-lipoprotein ligands of SR-A, fucoidan was identified by its ability to competitively inhibit the binding and degradation of acLDL. Our studies demonstrated that caution must be exercised with respect to the specificity of these putative SR-A ligands.

To further test if inflammatory signaling can result in reduced SR-BI expression, we treated MPM with the pro-inflammatory cytokine IL-15 (Fig 8). We chose IL-15 because we have results showing that mouse with reduced IL-15 have a proportionally decrease in atherosclerosis, and mice over expressing IL-15 have increased atherosclerosis. Also IL-15 signals in macrophages through a distinct IL-15 receptor (IL-15R) consisting of 3 chains, IL-15R α , IL-2/15R β and a common cytokine receptor γ_c ^{75, 76}. Neither IL-15 nor its receptor(s) have been reported to interact with SR's. Similar to fucoidan, IL-15 treatment induced IL-6 mRNA levels⁷⁶ (Fig 8A) and did not influence HMGCoAR mRNA levels (Fig 8B). More importantly IL-15 treatment reduced SR-BI mRNA levels.

Although acLDL did not affect the expression level of iNOS and IL-6, acLDL did suppress the ability of fucoidan and IL-15 to induce these genes. This ability is not unique to acLDL, because fucoidan induction of iNOS and IL-6 mRNA levels were reduced

by other lipoproteins (acLDL, LDL and HDL) both in WT and in SR-A KO macrophages.

Studies by others have shown that 90% of LPS in plasma is lipoprotein bound⁸¹.

Lipoproteins bind to the bioactive lipid A component of LPS and limit its bioavailability to stimulate macrophages⁶⁴. The lipid A component of LPS is structurally similar to phospholipids, facilitating its insertion into lipoproteins. Fucoidan does not contain such hydrophobic domain thus is not likely to be sequestered by lipoproteins. Also, fucoidan's effects on SR-BI mRNA levels were not altered by lipoproteins (acLDL, LDL and HDL). Finally acLDL was also able to suppress IL-15 induction of IL-6. It seems unlikely that this is the result of sequestration of IL-15 by acLDL. Thus it appears that lipoproteins exerted a general suppressive effect on inflammatory signaling. Treating MPM with phospholipid vesicles may tell us whether this effect is apolipoprotein mediated.

The notion that cholesterol loading can result in reduced SR-BI expression is further supported by treating macrophages with agents known to deliver cholesterol. LDLs are the major cholesterol carrying molecule in humans and deliver cholesterol by LDL receptor mediated endocytosis, a distinct pathway from the acLDL / SRA pathway. Cholesterol complexed to M β CD delivers cholesterol to cells independently of surface receptors. Treating macrophages with acLDL, LDL or cholesterol complexed to M β CD did not induce iNOS and IL-6, but decreased HMGCoAR mRNA levels, consistent with an increase in cellular cholesterol levels. More importantly all three cholesterol delivering agents reduced SR-BI mRNA levels. Similar results have been reported by others^{61, 77}. Conversely, Yu et al. showed that cholesterol depletion of cholesterol loaded

macrophage increases SR-BI expression². Yu et al. also showed, however SR-BI expression is not regulated by sterol regulatory element binding proteins (SREBPs) or liver X receptor (LXR)⁷⁷.

MicroRNAs (miRNA) with complementary sequences that bind to the untranslated regions (UTR) on target mRNAs, predispose the miRNA/mRNA complex for degradation. Thus miRNA can regulate mRNA levels. SR-BI's 3'UTR contains a highly conserved miR-223 target site. Remaly and co-workers recently demonstrated that miR-223 delivery to cells significantly reduces SR-BI mRNA levels⁸². This group also recently reported that macrophages expression of miR-223 is induced by cellular cholesterol loading⁸³. Thus it is possible that miR-223 may mediate the suppression of SR-BI by acLDL, LDL and/or cholesterol complexed to M β CD that we have observed. This remains to be tested.

5. References

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