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## Scavenger receptor SR-BI in macrophage lipid metabolism

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### Abstract

**OBJECTIVE**—To investigate the mechanisms by which macrophage scavenger receptor BI (SR-BI) regulates macrophage cholesterol homeostasis and protects against atherosclerosis.

**METHODS and RESULTS**—The expression and function of SR-BI, was investigated in cultured mouse bone marrow-derived macrophages (BMM). SR-BI, the other scavenger receptors SRA and CD36 and the ATP-binding cassette transporters ABCA1 and ABCG1 were each distinctly regulated during BMM differentiation. SR-BI levels increased transiently to significant levels during culture. SR-BI expression in BMM was reversibly down-regulated by lipid loading with modified LDL; SR-BI was shown to be present both on the cell surface as well as intracellularly. BMM exhibited selective HDL CE uptake, however, this was not dependent on SR-BI or another potential candidate glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1). SR-BI played a significant role in facilitating bidirectional cholesterol flux in non lipid-loaded cells. SR-BI expression enhanced both cell cholesterol efflux and cholesterol influx from HDL, but did not lead to altered cellular cholesterol mass. SR-BI-dependent efflux occurred to larger HDL particles but not to smaller HDL<sub>3</sub>. Following cholesterol loading, ABCA1 and ABCG1 were up-regulated and served as the major contributors to cholesterol efflux, while SR-BI expression was down-regulated.

**CONCLUSION**—Our results suggest that SR-BI plays a significant role in macrophage cholesterol flux that may partly account for its effects on atherogenesis.

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#### Conflict of interest

There are no conflicts of interest or potential conflicts of interest.

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## Keywords

SR-BI; ABCA1; ABCG1; cholesterol efflux; macrophage; HDL

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## 1. Introduction

Class B Scavenger Receptor Type 1 (SR-BI) is a CD36-related cell surface glycoprotein involved in lipid metabolism<sup>1,2</sup>. SR-BI is recognized mainly as an HDL receptor, which mediates selective uptake of HDL cholesteryl esters (CE) into cells. SR-BI is most abundantly expressed in liver and steroidogenic tissues, where it functions to deliver HDL CE for cholesterol excretion and for steroid hormone synthesis, respectively<sup>3,4</sup>. SR-BI can also facilitate cholesterol efflux from cells including macrophages<sup>5</sup>. In addition to its roles in cholesterol metabolism, other important functions for SR-BI have been proposed, such as platelet aggregation<sup>6</sup>, oxidative stress<sup>7</sup>, endothelial nitric oxide synthase activation<sup>8</sup> and apoptosis<sup>9</sup>. Hepatic SR-BI plays a pivotal role in HDL cholesterol clearance from plasma and consequently plasma HDL cholesterol levels<sup>10</sup>. Hepatic SR-BI expression is also an important positive regulator of the rate of macrophage-to-feces reverse cholesterol transport (RCT)<sup>11</sup>. In line with this concept, SR-BI has been shown to play an anti-atherogenic role in mice<sup>12,13,14</sup>.

Studies have shown that liver specific SR-BI null mice develop less atherosclerosis than that from whole body SR-BI null mice, suggesting an atheroprotective function of SR-BI in peripheral tissues<sup>15</sup>. SR-BI is detected in cultured human monocyte-derived macrophages and atherosclerotic lesions<sup>16</sup>. An athero-protective role of macrophage SR-BI has been suggested from studies performed using bone marrow transplantation. Inactivation of macrophage SR-BI promotes advanced atherosclerotic lesion development in apolipoprotein E-deficient mice<sup>17</sup>, as well as in LDL receptor-deficient mice<sup>18,19</sup>. However, macrophage SR-BI may have dual effects on atherogenesis in that it is reported to promote early lesion development while inhibiting more advanced lesions<sup>19</sup>. The mechanisms by which macrophage SR-BI modulate atherosclerosis are unclear.

Macrophage foam cell formation with CE accumulation plays a key role in the formation of atherosclerotic lesions. Cholesterol efflux from macrophages, mediated by the ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) plays a key role in preventing foam cell formation. On the other hand, the role of SR-BI in macrophage cholesterol efflux is uncertain. In bone marrow transplantation studies, one study reported that SR-BI plays a significant role in efflux from peritoneal macrophages (MPM)<sup>19</sup>, while a second study failed to show an SR-BI effect on efflux<sup>17</sup>. Another study in which cholesterol efflux to human serum was measured, indicated a minimal role for macrophage SR-BI in efflux<sup>20</sup>. Previous studies have demonstrated that SR-BI stimulates the bidirectional flux of free cholesterol between HDL and SR-BI-expressing cells rather than efflux alone<sup>21</sup>. To investigate potential functions of SR-BI in macrophage lipid metabolism, the regulation of SR-BI expression and other cholesterol transporters as well as their roles in cholesterol transport were studied during the differentiation of bone marrow-derived macrophages (BMM). Our results suggest that SR-BI plays a significant role in macrophage cholesterol flux that may contribute to the protective effects in atherogenesis.

## 2. Materials and Methods

### 2.1. Animals

SR-BI-deficient mice (SR-BI-null) were obtained from M. Krieger<sup>4</sup>. SR-BI homozygous (SR-BI<sup>-/-</sup>) and wild type (WT) mice (both 1:1 mixed C57BL/6 × 129 backgrounds) were

bred from a common mating pair of SR-BI heterozygous (*SR-BI*<sup>+/-</sup>) mice. ABCG1-deficient mice (ABCG1-null) from Deltagen were backcrossed to a C57BL/6 background (>99.9% C57BL/6 background). For all animal experiments, 8- to 12-week-old male mice, weighing 20–25 g, were used. All animal experiments were approved by the Veterans Affairs Medical Center, Institutional Animal Care and Use Committee.

## 2.2. Bone marrow macrophage (BMM) preparation

Murine BMM were obtained and cultured by standard procedures<sup>22</sup>. Isolated cells were suspended and cultured in RPMI 1640 containing 50 IU of penicillin G per ml, 50 µg of streptomycin per ml, 2 mM glutamine, 10% fetal bovine serum, and 15% (vol/vol) L-cell conditioned medium (LCM).

## 2.3. Flow Cytometry

Flow cytometric analysis of BMM cell-surface marker expression was carried out as described by Yona et al.<sup>22</sup>. Attached cells were dissociated by incubation at room temperature with an enzyme-free cell dissociation buffer (Gibco) for 25 min. Trypan blue positivity was less than 5% for all samples. Cells were stained with rat anti-mouse CD11b (Mac1, Invitrogen), or F4/80 (AbD Serotec) or control rat IgG, followed by FITC-conjugated chicken anti-rat IgG antibody (Molecular Probes). Flow cytometry was performed using a FACScalibur cytometer (Becton Dickinson, Cowley, U.K.).

## 2.4. Western blotting

Total cell proteins (10 µg) were separated on a 4%–20% polyacrylamide gradient gel, transferred to PVDF membranes and immunoblotted with rabbit anti-mouse SR-BI (Novus, NB400-104), rat anti-mouse CD36<sup>23</sup>, goat anti-mouse SRA (R&D Systems, AF1797), mouse anti-human ABCA1 (gift from M. Hayden), rabbit anti-mouse ABCG1 (Novus, NB400-132), or mouse anti-β-actin (Sigma, A5441). Immunoblots were visualized by the Amersham<sup>TM</sup> ECL<sup>TM</sup> Western Blotting Detection Reagents (GE Healthcare).

## 2.5. Cellular lipid and protein determinations

Lipids were extracted as described previously<sup>24</sup> and aliquots were assayed for total and free cholesterol content using a colorimetric kit (Wako). Cell protein was determined using BCA<sup>TM</sup> Protein Assay Kit (Thermo Scientific).

## 2.6. Biotinylation of cell surface SR-BI

Biotinylation of cell surface proteins was carried out using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) as described previously<sup>25</sup> followed by isolation using Streptavidin Iron Oxide Particles (Sigma). The supernatant (intracellular proteins) and the pellet (surface proteins) were analyzed by SDS-PAGE and immunoblotting using the rabbit anti-mouse SR-BI (Novus, NB400-104) antibody and quantified by densitometry.

## 2.7. Isolation and labeling of lipoproteins

HDL ( $d = 1.063\text{--}1.21$  g/mL) was isolated from C57BL/6 mouse plasma or human plasma by density gradient ultracentrifugation<sup>26</sup>. Human HDL was subfractionated to obtain HDL<sub>2b</sub> ( $d = 1.09\text{--}1.11$  g/mL) and HDL<sub>3</sub> ( $d = 1.13\text{--}1.18$  g/mL). Protein concentrations were determined by the method of Lowry et al.<sup>27</sup>. HDL<sub>2b</sub> was radiolabeled by the iodine monochloride method and HDL-associated CE was traced with non-hydrolyzable [1,2(n)-<sup>3</sup>H]cholesteryl oleoyl ether (Amersham Biosciences)<sup>25</sup>. Lipoproteins were analyzed by SDS-PAGE and non-denaturing gradient gel electrophoresis. LDL ( $d = 1.019\text{--}1.063$  g/mL) was isolated from human plasma<sup>26</sup>. Acetylated LDL (acLDL) was prepared as described previously<sup>28</sup>.

## 2.8. HDL cell binding, association and selective uptake

HDL cell association, selective CE uptake and 4°C binding assays were performed as described previously<sup>25</sup>. BMM were incubated with <sup>125</sup>I/[<sup>3</sup>H]CEt labeled HDL<sub>2b</sub> (10 µg/mL) in serum-free medium containing 0.5% fatty acid-free BSA for 4 h at 37°C. Cell association of <sup>125</sup>I-HDL protein uptake was determined. The non-iodide, trichloroacetic acid-soluble <sup>125</sup>I in cell medium corresponding to degraded <sup>125</sup>I-apolipoprotein was assayed. Selective lipid uptake is defined as <sup>3</sup>H - (<sup>125</sup>I cell-associated + <sup>125</sup>I degraded) and represents the uptake of cholesteryl ester that cannot be accounted for by the internalization of intact particles. SR-BI-specific values were calculated as the difference between the values for WT and SR-BI-null cells.

## 2.9. RT-PCR

PCR amplification of mouse glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1) was carried out using the following gene specific primers: forward primer: tactaagtggacagccaggaggatg, reverse primer: gagcagctctgtgtctgattgcag, (GenBank accession no. [NM\\_026730](#), nt. 56-266, 211bp). GAPDH was used as a control house keeping gene.

## 2.10. Cholesterol flux determination

Cellular cholesterol efflux was determined as described<sup>29</sup>. Briefly, cells were labeled with 0.2 µCi/mL [<sup>3</sup>H] cholesterol (35–50 Ci/mmol, Amersham Biosciences) for 48 h, washed, and equilibrated in serum-free medium containing 0.2% fatty acid-free BSA for 16 h. Cells were then incubated for 5 h at 37°C in 0.2% fatty acid-free BSA medium without or with mouse HDL, human HDL<sub>2b</sub> or HDL<sub>3</sub> (40 µg/mL), human apoA-I (10 µg/mL) for 5 h. Efflux was calculated as the percentage of counts in the medium relative to total counts. For some experiments, counts were converted into ng cholesterol efflux per mg cell protein using the determined [<sup>3</sup>H] specific activity of cellular cholesterol.

Cholesterol influx was determined as described<sup>30</sup>. Briefly, HDL<sub>2b</sub> was labeled with [<sup>3</sup>H]cholesterol (20 µCi/mg HDL protein) during an overnight incubation at 4°C. The radiolabeled HDL in serum-free medium containing 0.2% fatty acid-free BSA was incubated with cells at day 5 of culture. After incubation and washing, cells were lysed in 0.1N NaOH and [<sup>3</sup>H] cholesterol present in the cells determined. Counts were converted into ng cholesterol influx per mg cell protein using the [<sup>3</sup>H] specific activity of the cholesterol in the radiolabeled HDL.

## 2.11. Statistical analysis

Statistics and best-fit curves were calculated with Graphpad's Prism software. Data were expressed as means ± SEM. Results were analyzed by Student's *t*-test.

## 3. Results

### 3.1 Expression of cholesterol transporters during BMM differentiation in culture

Bone marrow derived macrophages have been widely used as a convenient and useful model cell system to study macrophage function<sup>31,32</sup>. We confirmed the differentiation of isolated bone marrow cells into macrophages by staining with two markers of macrophage differentiation, Mac1 and F4/80. Freshly isolated bone marrow cells from WT, SR-BI-null and ABCG1-null mice (day 0) were largely positive for Mac1 (about 50–70% of cells) and to a lesser extent for F4/80 (about 10–20% of cells) (Supplemental Fig.1). During culture in LCM, cells attached and the expression of both markers increased so that by day 5 virtually

all attached cells expressed both Mac1 (>99%) and F4/80 (98–99%) and were classified as BMM. Similar results were seen for each of the three genotypes.

The regulation of SR-BI and other key cholesterol transporters was examined during BMM differentiation. The scavenger receptors SR-BI, CD36, SRA and ATP-binding cassette transporters ABCA1, ABCG1 each showed different protein expression profiles during macrophage differentiation (Fig. 1). SR-BI levels showed an up-regulation during the early stages of culture (2–5 days) with a maximal level of expression at day 5. After day 5, SR-BI levels decreased. CD36 expression increased moderately during culture, declining only at day 13. SRA was highly expressed until day 5 before declining. Interestingly, the closely related ABCA1 and ABCG1 were regulated differently from one another during differentiation. ABCA1 expression reached a maximum at day 13, whereas ABCG1, the other major transporter involved in cholesterol efflux, reached a maximum by day 7 before declining.

To assess whether SR-BI regulation during BMM differentiation was reversible, cell culture medium was changed on day 16 to 2% lipoprotein-deficient serum (LPDS)-containing medium and cultured for a further 24 h before SR-BI analysis on day 17. As shown in Figure 2A, SR-BI was markedly up-regulated by LPDS, indicating that the down-regulation of SR-BI during culture was reversible and not the result of irreversible differentiation-induced gene suppression. Cholesterol loading of day 5 cells for 24 h using the SRA ligand acLDL markedly decreased SR-BI expression in a dose dependent manner (Fig. 2B). The down-regulation of SR-BI by acLDL was associated with an increase in cellular total cholesterol, free cholesterol, and particularly esterified cholesterol (Fig. 2D). In contrast to SR-BI, ABCA1 and ABCG1 were markedly up-regulated by acLDL (Fig. 2B,C), consistent with their known LXR-mediated regulation by oxysterols<sup>33</sup>.

### 3.2 SR-BI-independent selective cholesteryl ester uptake in BMM

The ability of SR-BI to mediate selective lipid uptake into BMM was investigated using <sup>125</sup>I/[<sup>3</sup>H]CEt-labeled HDL. BMM cells were used on day 5 of culture, at which time SR-BI expression had reached a maximum. Human HDL<sub>2b</sub> protein cell association was slightly but significantly reduced in SR-BI-null cells compared to WT cells (Fig. 3A). Relatively small amounts of protein degradation were observed in both cell types. Compared to protein uptake, high rates of cellular CE uptake were shown. Interestingly, no difference in CE uptake or the calculated selective CE uptake was found between SR-BI-null and WT cells. Similar results were obtained using mouse HDL (Supplemental Fig. 2) and human HDL<sub>3</sub> (data not shown) that showed also no evidence of SR-BI-dependent HDL CE uptake in BMM between day 5 and 9.

To address the question why SR-BI does not play a role in CE uptake in BMM, HDL binding to cells was analyzed at 4°C. As shown in Figure 3B, no difference in HDL binding was observed between SR-BI-null and WT cells. The cell surface expression of SR-BI also was analyzed by cell surface protein biotinylation, SR-BI was distributed approximately equally between the cell surface and intracellular compartments (Fig. 3C). These results indicate that the inability of SR-BI to enhance HDL binding and selective uptake of CE is not due to a lack of SR-BI at the cell surface of BMM.

A candidate molecule for mediating selective lipid uptake is GPIHBP1, which is able to mediate selective lipid uptake from HDL in transfected CHO cells<sup>34</sup>. RT-PCR analysis showed that whereas GPIHBP1 mRNA was expressed in heart and liver, no GPIHBP1 mRNA was detected in either BMM or MPM (Supplemental Fig. 3); strongly indicating that GPIHBP1 was also not responsible for selective lipid uptake in these cells.



### 3.3 Role of SR-BI and other transporters in cholesterol flux

Cellular cholesterol efflux to mouse HDL, human HDL<sub>2b</sub> or HDL<sub>3</sub> or apoA-I was determined in BMM from WT, SR-BI-null, and ABCG1-null mice. The size of mouse HDL and HDL<sub>2b</sub> was shown to be larger than that of HDL<sub>3</sub> (Supplemental Fig. 4). In the absence of lipid loading, cholesterol efflux to the larger mouse HDL and human HDL<sub>2b</sub> was more efficient than to the smaller human HDL<sub>3</sub>. Mouse HDL and human HDL<sub>2b</sub> induced significantly higher cholesterol efflux from SR-BI WT BMM than from SR-BI-null BMM (Fig. 4A), showing that SR-BI played a significant role in cholesterol efflux to these particles. Interestingly, no SR-BI-dependent cholesterol efflux to human HDL<sub>3</sub> was observed. There was also a substantial level of cholesterol efflux that was independent of SR-BI, likely by a process designated as aqueous diffusion<sup>20</sup>. Very low levels of cholesterol efflux to apoA-I were observed in the absence of cholesterol loading (Fig. 4A, C), consistent with the observed low expression of ABCA1. In non lipid-loaded cells, cholesterol efflux to various HDL particles in ABCG1-null BMM was similar to that in WT BMM (Fig. 4C), indicating that ABCG1 did not play a significant role in cholesterol efflux in the absence of lipid loading. Cholesterol efflux to HDL on day 5 was also compared to efflux on day 3 and day 7 (Supplemental Fig. 5A). In line with the expression level of SR-BI in BMM during differentiation, maximal SR-BI-dependent efflux was seen in day 5 BMM, although the correlation between SR-BI expression and efflux was relatively weak. One possible explanation for this might be differences in the intracellular distribution of SR-BI at the different days in culture. No ABCG1-dependent cholesterol efflux to HDL was observed in non lipid-loaded cells on any of the indicated days in culture (Supplemental Fig. 5B).

In lipid-loaded cells, SR-BI did not play a significant role in cholesterol efflux as indicated by similar efflux values to various HDLs from BMM with or without SR-BI (Fig. 4B). In contrast, cholesterol efflux to apoA-I in lipid-loaded cells was dramatically increased compared to non lipid-loaded cells (Fig. 4B, D), suggesting a marked increase in efflux by ABCA1, recognized as the major contributor to apoA-I-mediated cholesterol efflux in macrophages and known to be up-regulated by lipid loading. After lipid loading, cholesterol efflux to mouse HDL, HDL<sub>2b</sub> or HDL<sub>3</sub> was significantly higher in WT BMM than that in BMM lacking ABCG1, indicating a significant contribution of this transporter to cellular cholesterol efflux (Fig. 4D). Unlike efflux facilitated by SR-BI, ABCG1 promoted cholesterol efflux to both large and small HDL particles. This data is consistent with previous study that ABCG1 facilitated cholesterol efflux to large and small rHDL particles<sup>35</sup>.

To determine whether SR-BI-mediated cholesterol efflux results in a net efflux of cellular cholesterol mass, total cholesterol and free cholesterol levels in BMM were measured after incubation with HDL. HDL treatment for 8 h did not alter cellular cholesterol content, and there was no significant difference in cholesterol content between SR-BI WT and SR-BI-null BMM (Supplemental Fig. 6). These results suggest that SR-BI-induced cholesterol efflux to HDL does not produce a net change in cholesterol mass in macrophages under these conditions.

Since SR-BI mediated efflux is part of a bidirectional process, we investigated HDL cholesterol influx into BMM. The role of SR-BI in cholesterol influx was examined by incubating BMM with [<sup>3</sup>H]cholesterol-labeled HDL for 3 h (Fig. 5). HDL induced significantly higher cholesterol influx to SR-BI WT BMM than to SR-BI-null BMM, indicating that SR-BI expression enhances cholesterol influx. SR-BI is known to mediate bidirectional flux in transfected COS-7 cells through both a low- and high-affinity component<sup>21</sup>; we therefore measured [<sup>3</sup>H]cholesterol flux between cells and HDL at concentrations up to 500 µg/mL HDL. We observed significantly more influx (Supplemental Fig. 7A) and efflux (Supplemental Fig. 7B) in SR-BI WT than SR-BI-null BMM, especially

at higher concentrations of HDL. Interestingly, the quantity and the concentration-dependence of influx and efflux were comparable, consistent with a bidirectional process.

#### 4. Discussion

The major finding of the present study is that SR-BI in cultured bone marrow macrophages plays a significant role in facilitating bidirectional cholesterol flux under non lipid-loaded conditions. We show for the first time that SR-BI is transiently induced during BMM differentiation in culture and although not active in promoting HDL CE selective uptake, does mediate bidirectional cholesterol flux between macrophages and HDL. Under these conditions, there were very low levels of ABCA1- or ABCG1-mediated efflux to either apoA-I or HDL. Our results suggest that SR-BI plays a significant role in macrophage cholesterol exchange, a function that may contribute to its effects on atherogenesis.

Our studies show that scavenger receptors SR-BI, CD36 and SRA and the transporters ABCA1 and ABCG1 are distinctly regulated during macrophage differentiation. SR-BI is up-regulated during BMM culture with maximal level expression at day 5. At this stage, CD36 and SRA are highly expressed, whereas the expression of ABCA1 and ABCG1 is relative low. These results indicate a potential role of SR-BI in maintaining macrophage cholesterol homeostasis in non lipid-loaded cells. Further, our results showed that the reduced SR-BI expression even at later stages of cell culture is highly reversible. This could be relevant in the case of macrophage foam cells that could eliminate their lipid load during atherosclerotic lesion regression<sup>36</sup>. Under such regression conditions in vivo, SR-BI has been shown to be up-regulated in macrophage foam cells within atherosclerotic lesions<sup>36</sup> and therefore would be able to re-exert its function in such cells. On the other hand, lipid-loading of BMM with acLDL markedly decreased SR-BI expression. This is consistent with a previous study of human monocyte-derived macrophages and MPM that demonstrated that sterol loading using acLDL resulted in decreased SR-BI protein and mRNA levels<sup>37</sup>. These results are in contrast with another report that modified lipoproteins such as oxidized LDL and acLDL induced a 5-fold increase in SR-BI mRNA and protein expression in cultured human monocyte-derived macrophages<sup>16</sup>. The explanation for these contrasting results is unclear. As we observed, down-regulation of SR-BI by acLDL is associated with an increase in cellular total cholesterol, free cholesterol, and particularly esterified cholesterol. A previous study by Yu et al. indicated that such sterol-associated changes of SR-BI expression in macrophages are not regulated by LXR or through a SREBP dependent pathway<sup>37</sup>. The mechanisms involved in SR-BI regulation in macrophages remain to be clarified.

SR-BI plays a key role in mediating selective uptake of HDL CE into liver cells and adrenal cells<sup>3,4</sup>. However, selective CE uptake in BMM was shown to be independent of SR-BI, as previously reported in MPM<sup>38</sup>. We and others have reported that SR-BI expression at the cell surface can differ markedly in response to insulin in both hepatocytes<sup>39</sup> and adipocytes<sup>40</sup>, raising the possibility that SR-BI was absent from or expressed at very low levels at the macrophage cell surface. However, our determination that SR-BI is localized to a significant extent on the cell surface of BMM indicates that this is not the case. The reason for the apparent inability of SR-BI to mediate selective lipid uptake in macrophages may be that macrophage SR-BI does not bind to HDL (Fig. 3B). Further work is needed to explain why in macrophages SR-BI is accessible for cell-surface biotinylation but does not enhance HDL binding.

The mechanism of HDL CE uptake in macrophages is also poorly understood. It has been proposed that LXR activation increases HDL<sub>3</sub> CE uptake through the up-regulation of secreted proteins namely, apoE and LPL, that interact with proteoglycans, and this

regulation requires membrane raft domain integrity and may involve caveolae<sup>41</sup>. A potential candidate receptor for mediating HDL CE selective uptake is the GPIHBP1. GPIHBP1 has been reported to bind HDL with high affinity and to mediate selective HDL CE uptake in CHO cells expressing GPIHBP1<sup>34</sup>. However, we have demonstrated that GPIHBP1 expression is not detectable in BMM or MPM, indicating that GPIHBP1 is probably not responsible for mediating the selective uptake of HDL CE in macrophages.

The fact that whole body SR-BI-null mice develop more arterial lesions than liver-specific SR-BI-null mice<sup>15</sup>, suggests that expression of SR-BI in peripheral tissues may fulfill an atheroprotective function. This is supported by bone marrow transplantation experiments which indicate an athero-protective effect of SR-BI in macrophages<sup>17, 18</sup>. To understand this phenomenon, we investigated the contribution of macrophage SR-BI in promoting cholesterol efflux. SR-BI was shown to play a significant role in HDL mediated cholesterol efflux in BMM cells not loaded with cholesterol. Our results also showed that under these conditions large HDL particles, such as mouse HDL and human HDL<sub>2b</sub> promote efflux from BMM more efficiently than that of smaller human HDL<sub>3</sub>. Moreover, SR-BI-dependent cholesterol efflux was evident to mouse HDL and human HDL<sub>2b</sub>, but not to HDL<sub>3</sub>. These findings are consistent with previous studies showing that SR-BI-mediated efflux was positively correlated with the phospholipid content of particles<sup>30</sup>. Since SR-BI - mediated cholesterol efflux to various subfractions of HDL differs, the role of SR-BI in mediating cholesterol efflux could be under-estimated when using whole serum<sup>20, 42</sup> or heterogenous HDL<sup>17</sup> as cholesterol acceptors. This may explain in part the discrepancy among previous studies<sup>17, 19, 20</sup> regarding the role of SR-BI mediated cholesterol efflux in macrophages.

In macrophages not loaded with cholesterol very low levels of cholesterol efflux to apoA-I were observed, indicating that under such conditions ABCA1, which is generally recognized as the major contributor to apoA-I-mediated cholesterol efflux macrophages, does not play a significant role in cholesterol efflux. To assess the contributions of ABCG1 to cholesterol efflux, BMM from WT and ABCG1-null mice were studied. Under non lipid-loaded conditions, efflux to HDL acceptors was similar in WT and ABCG1-null cells, showing a lack of ABCG1 dependent cholesterol efflux. This is in line with the low levels of ABCG1 expression under these conditions. In contrast, under lipid-loaded conditions when ABCG1 is markedly up-regulated, cholesterol efflux to HDL is mediated predominantly by this receptor. This suggests that ABCG1 and SR-BI might contribute to cholesterol regulation at different stages of macrophage foam cell formation. Interestingly, SR-BI was previously shown to inhibit ABCG1-mediated efflux in a transfected cell system<sup>43</sup>. This was proposed to be due to SR-BI mediating selective CE uptake from the acceptor particles. However, an inhibitory effect of SR-BI on ABCG1-mediated efflux was not evident in cholesterol-loaded macrophages. Our findings would also not support such a mechanism in macrophages since macrophage SR-BI does not appear to bind HDL or promote selective CE uptake.

Our studies demonstrate that although SR-BI stimulates cellular cholesterol efflux from macrophages, this is not accompanied by any detectable reduction in cellular cholesterol mass. This can be accounted for by a simultaneous increase in SR-BI-stimulated cholesterol influx from HDL, as shown in other cell types<sup>21</sup>. The inability of SR-BI to promote net efflux of cholesterol from macrophages *in vivo*<sup>42</sup> is consistent with these findings. However, there is increasing evidence for a role of SR-BI in macrophage function. Specific deletion of macrophage SR-BI was shown to increase atherosclerosis in several studies<sup>17-19</sup>. Most recently, the combined deletion of SR-BI and ABCA1 in macrophages was found to markedly increase tissue lipid accumulation and atherosclerosis compared to the single deletion of ABCA1<sup>44</sup>. The mechanism(s) responsible for such effects are unclear but may be related to SR-BI presence in plasma membranes. The consequent stimulation of bidirectional cholesterol exchange might play a protective role in several ways. For



example, increased flux might alter the lipid composition or organization of plasma membranes, especially lipid rafts. SR-BI-mediated flux might also promote the net efflux of minor lipids, such as oxidized sterols, which may contribute to foam cell formation. Alternatively, increased lipid flux or changes in membrane organization may regulate signaling by SR-BI or other membrane receptors. The differential roles of SR-BI, ABCG1 and ABCA1 in cholesterol flux in lipid loaded or non-loaded macrophages are summarized in Table 1.

In conclusion, in the absence of lipid loading, SR-BI contributes significantly to macrophage cholesterol bi-directional flux. Thus, SR-BI may play a significant role in macrophage cholesterol regulation and function, thereby contributing to its effects on atherosclerosis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>ABCA1</b>	ATP-binding cassette transporter A1
<b>ABCG1</b>	ATP-binding cassette transporter G1
<b>acLDL</b>	acetylated low density lipoprotein
<b>apoA-I</b>	apolipoprotein A-I
<b>BMM</b>	bone marrow-derived macrophage
<b>CE</b>	cholesteryl ester
<b>GPIHBP1</b>	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1
<b>HDL</b>	high density lipoprotein
<b>LCM</b>	L-cell conditioned medium
<b>LDL</b>	low density lipoprotein
<b>LPDS</b>	lipoprotein-deficient serum
<b>MPM</b>	mouse peritoneal macrophage
<b>SRA</b>	class A scavenger receptor
<b>SR-BI</b>	class B scavenger receptor type 1

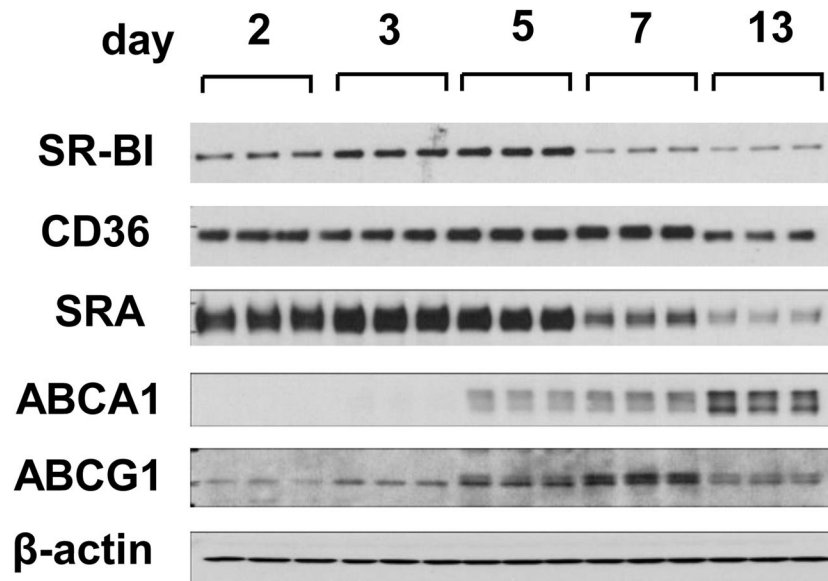
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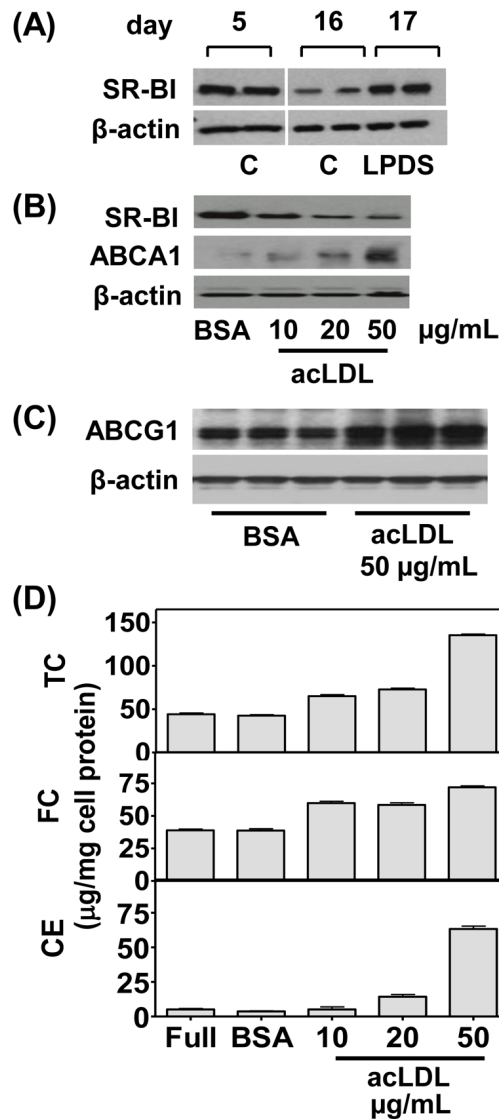
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**Fig. 1.** Gene expression of scavenger receptors and ATP-binding cassette transporters during BMM differentiation and lipid loading. The expression of scavenger receptors SR-BI, CD36, SRA and ATP-binding cassette transporters ABCA1 and ABCG1 was determined by Western blot analysis (10  $\mu$ g cell protein/lane) at the indicated days in culture. The figure is representative of three independent experiments.

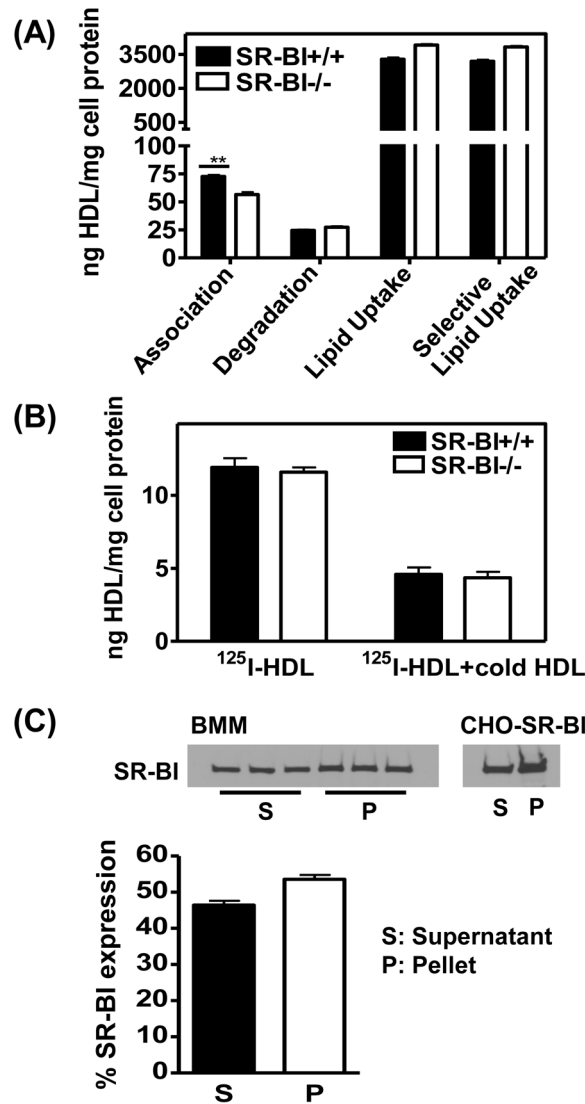




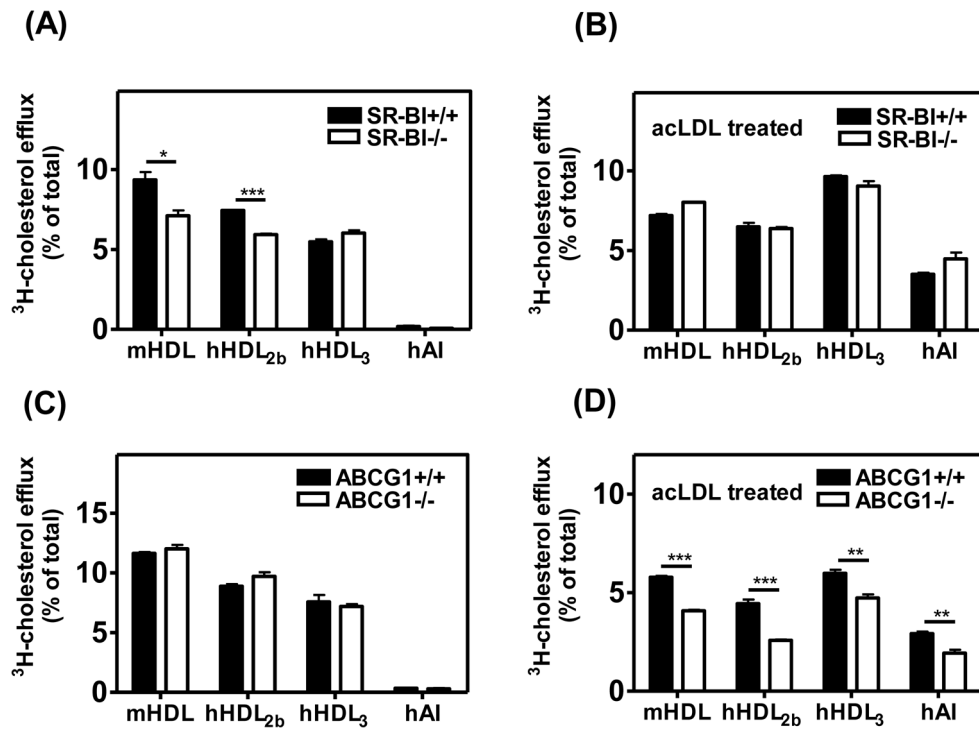
**Fig. 2.**

Expression of cholesterol transporters during lipid loading and depletion.

**A)** Cell culture medium was changed on day 16 to 2% LPDS-containing medium and cells were then cultured for a further 24 h before SR-BI analysis by Western blotting on day 17. **B,C)** BMM on day 5 were incubated for 24 h in 0.5% BSA medium without or with acLDL at indicated concentrations followed by analysis of SR-BI and ABCA1 (**B**) and ABCG1 in triplicate wells for each condition (**C**). **D)** BMM were incubated for 24 h in L-cell conditioned medium (Full), 0.5% BSA medium or 0.5% BSA medium containing the indicated concentrations of acLDL and cellular lipid content then determined. Values shown are the mean and S.D of triplicate determinations. The figure is representative of three independent experiments.



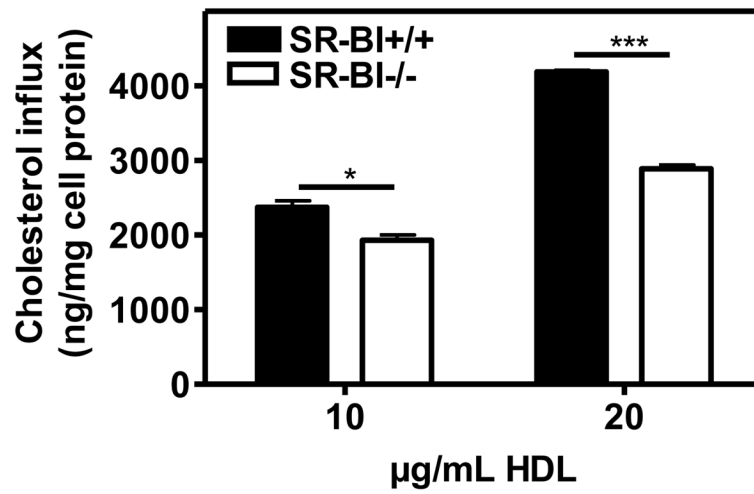
**Fig. 3.** HDL binding and selective lipid uptake in BMM is independent of SR-BI. **A)** Cells at day 5 were incubated with <sup>125</sup>I/[<sup>3</sup>H]CEt labeled HDL<sub>2b</sub> (10 μg/mL) for 4 h at 37°C. HDL cell association, degradation, CE uptake and selective CE uptake were determined. Values shown were the mean and S.D of triplicate determinations. The figure is representative of three independent experiments. **B)** Cells were incubated with <sup>125</sup>I-labeled HDL<sub>2b</sub> (10 μg/mL) in the absence or presence of “cold” unlabeled HDL<sub>2b</sub> (500 μg/mL) for 2 h at 4°C, and HDL cell binding was determined. Values shown were the mean and S.D of triplicate determinations. **C)** Intact BMM cells at day 5 were biotinylated and SR-BI expression in supernatant (S) (intracellular proteins) and Streptavidin pull-down fractions (P) (surface proteins) determined. As a control, CHO cells over-expressing SR-BI were analyzed in parallel. The figure is representative of two independent experiments.



**Fig. 4.**

Role of SR-BI in cholesterol efflux.

BMM from SR-BI WT, SR-BI-null, ABCG1 WT, and ABCG1-null mice were radiolabeled at day 3 for 48 h with [<sup>3</sup>H]cholesterol (0.2μCi/mL) in the absence (A, C) or presence (B, D) of 100 μg/mL acLDL. Cells were then incubated with mouse HDL (40μg/mL), human HDL<sub>2b</sub> (40μg/mL), human HDL<sub>3</sub> (40μg/mL) or apoAI (10μg/mL) for 5 h to elicit removal of cellular cholesterol. Free cholesterol efflux was calculated as [<sup>3</sup>H]cholesterol in medium/[<sup>3</sup>H]cholesterol in medium plus cells. Values shown were the mean and S.D of triplicate determinations. The figure is representative of two independent experiments.



**Fig. 5.** Function of SR-BI in cholesterol influx. BMM cells from SR-BI WT and SR-BI-null mice were incubated on day 5 with 10 or 20 µg/mL [<sup>3</sup>H]cholesterol-labeled HDL<sub>2b</sub> for 3 h at 37°C. Cholesterol uptake was determined. Cholesterol influx was expressed as cell-associated ng cholesterol per mg cell protein. Values shown were the mean and S.D of triplicate determinations.

**Table 1**

Summary of differential roles of SR-BI, ABCG1 and ABCA1 in macrophage cholesterol flux

	Basal BMM		Lipid loaded BMM	
	Influx	Efflux	Influx	Efflux
SR-BI	+	+	-	-
ABCG1	-	-	-	+
ABCA1	-	-	-	+