

# Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION



## **Relative Contributions of ABCA1 and SR-BI to Cholesterol Efflux to Serum From Fibroblasts and Macrophages**

MyNgan Duong, Heidi L. Collins, Weijun Jin, Ilaria Zanotti, Elda Favari and George H. Rothblat

*Arterioscler. Thromb. Vasc. Biol.* 2006;26;541-547; originally published online Jan 12, 2006;

DOI: 10.1161/01.ATV.0000203515.25574.19

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association,  
7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2006 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online  
ISSN: 1524-4636

The online version of this article, along with updated information and services, is  
located on the World Wide Web at:

<http://atvb.ahajournals.org/cgi/content/full/26/3/541>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular  
Biology is online at  
<http://atvb.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, 351 West Camden  
Street, Baltimore, MD 21202-2436. Phone 410-5280-4050. Fax: 410-528-8550. Email:  
[journalpermissions@lww.com](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at  
<http://www.lww.com/static/html/reprints.html>

# Relative Contributions of ABCA1 and SR-BI to Cholesterol Efflux to Serum From Fibroblasts and Macrophages

MyNgan Duong, Heidi L. Collins, Weijun Jin, Ilaria Zanotti, Elda Favari, George H. Rothblat

**Objectives**—Cholesterol efflux is achieved by several mechanisms. This study examines contributions of these pathways to efflux to human serum.

**Methods and Results**—Human fibroblasts were stably transfected with SR-BI while ABCA1 was upregulated. Quantitation of cholesterol efflux to human serum demonstrated that there was efflux from cells without either protein. Expression of ABCA1 produced a small increase in efflux, whereas SR-BI expression had a dramatic impact. To quantitate ABCA1 and SR-BI contribution, fibroblasts were pretreated with Probucol and BLT-1 to, respectively, inhibit these efflux proteins. Exposing SR-BI-expressing fibroblasts to BLT-1 inhibited efflux by 67%. Probucol pretreatment of ABCA1-expressing fibroblasts reduced efflux to serum by 26%. A large fraction of total efflux was uninhibited. For both J774 and mouse peritoneal macrophages, contributions of either ABCA1 or SR-BI to efflux to serum were low, with background/uninhibited efflux contributing from 70% to 90% of total efflux.

**Conclusions**—We have shown that ABCA1-mediated efflux to serum responds to the pool of lipid-free/poor apolipoproteins, whereas phospholipid-containing particles mediate SR-BI efflux. Although SR-BI and ABCA1 contribute to efflux from fibroblasts and cholesterol-enriched macrophages, a large proportion of the total efflux to human serum is mediated by a mechanism that is neither SR-BI nor ABCA1. (*Arterioscler Thromb Vasc Biol.* 2006;26:541-547.)

**Key Words:** ABCA1 ■ cholesterol efflux ■ fibroblasts ■ macrophage ■ SR-BI

The process of removing excess cholesterol from peripheral tissues has been termed reverse cholesterol transport (RCT), and cellular cholesterol efflux is the first step in RCT.<sup>1,2</sup> A number of different pathways have been identified by which unesterified/free cholesterol (FC) is removed on incubation with serum lipoproteins or apolipoproteins.<sup>3</sup> A passive process depending on aqueous diffusion occurs in all cell systems and is driven by cholesterol concentration gradients.<sup>4</sup> A number of active pathways that are linked to the presence of specific efflux proteins have been identified. These proteins include scavenger receptor class B type I (SR-BI)<sup>5,6</sup> and a range of ATP-binding cassette (ABC) transporters, ABCA1,<sup>7,8</sup> ABCG1, and ABCG4.<sup>9,10</sup> SR-BI preferentially mediates the flux of FC to larger phospholipid-rich high-density lipoproteins (HDL),<sup>11</sup> whereas ABCA1 prefers small pre $\beta$ -HDL and lipid-poor apolipoproteins (apo) such as apoA-I, apoE, and apoA-IV.<sup>12</sup>

Many studies on efflux have used purified acceptor particles such as HDL or lipid-free apolipoproteins. Fewer studies have used whole serum. When whole serum is used, individual variation in factors such as lipoprotein profile and

composition determine the efficiency of cholesterol efflux. Previous studies show that changing the concentration, size, and compositions of lipoproteins and, in particular HDL, changes the efflux potential of serum.<sup>13,14</sup>

To date there has been no way of directly comparing the contribution of the various efflux pathways when cells are exposed to whole serum. Early studies investigating SR-BI-mediated and ABCA1-mediated cellular cholesterol efflux have used different cell systems. Hence, it has been difficult to obtain a direct comparison of the relative contributions of different efflux pathways when cells are exposed to whole serum that comprises a mixture of cholesterol acceptors.

In this study, we have quantitated efflux from sublines of a transformed human embryonic fibroblast cell line, WI38VA13, that express ABCA1, SR-BI, both, or no efflux proteins. Comparative efflux values were obtained when these cells were exposed to lipid-free apoA-I, reconstituted HDL (rHDL), or human serum. In addition, we have used the SR-BI inhibitor BLT-1<sup>15,16</sup> and the ABCA1 inhibitor Probucol<sup>17</sup> to determine the contribution of the efflux pathways when the fibroblasts were incubated with serum. The inhibitors were also

Original received August 23, 2005; final version accepted December 23, 2005.

From GI and Nutrition (M.D., H.L.C., G.H.R.), The Children's Hospital of Philadelphia, Philadelphia, Pa; the School of Medicine (W.J.), University of Pennsylvania, Philadelphia, Pa; and the Department of Pharmacological and Biological Sciences and Applied Chemistries (I.Z., E.F.), University of Parma, Italy.

Correspondence to George H. Rothblat, Suite 1102 Abramson Building, The Children's Hospital of Philadelphia, 3615 Civic Center Blvd, Philadelphia, PA 19104. E-mail rothblat@email.chop.edu

© 2006 American Heart Association, Inc.

*Arterioscler Thromb Vasc Biol.* is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000203515.25574.19

used on cholesterol-enriched mouse macrophages to determine the contribution of efflux pathways to serum.

## Methods

### Probucol and BLT-1 Solutions

BLT-1 (2-hexyl-1-cyclopentanone thiosemicarbazone) was purchased from ChemBridge (San Diego, Calif) and Probucol from Sigma (St. Louis, Mo). Both were dissolved in 100% DMSO to form a 10 mmol/L stock solution. The stock solution was diluted to a 10  $\mu$ mol/L BLT-1, 0.2% bovine serum albumin (BSA) MEM-hepes solution, and 20  $\mu$ mol/L Probucol, 0.2% BSA MEM-hepes solution. When preparing a solution with both BLT-1 and Probucol, BLT-1 was diluted to a concentration of 10  $\mu$ mol/L BLT-1 plus 20  $\mu$ mol/L Probucol with 0.2% BSA in MEM-hepes. Control preincubation solution consisted of 0.2% BSA MEM-hepes. All solutions contained the same amount of DMSO.

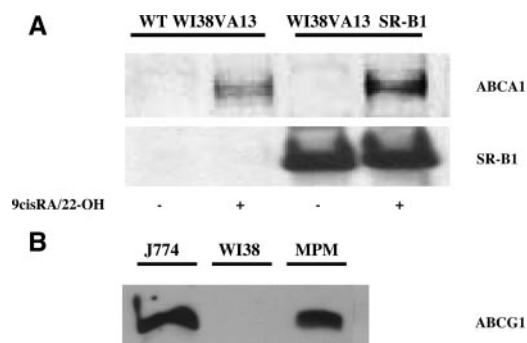
Please see <http://atvb.ahajournals.org> and figure legends for more methodological details.

## Results

One of the goals of this study was to develop a cell culture system that would allow the quantitation of individual cholesterol efflux pathways when cells are exposed to the mixture of lipoproteins and apolipoproteins present in serum. For this purpose we selected WI38VA13 human embryonic lung fibroblasts transformed by SV40 virus.<sup>18</sup>

### Demonstration of ABCA1 and SR-BI in WI38VA13 Fibroblasts

To establish that WI38VA13 cells express the appropriate efflux proteins, cell lysates were made for Western blot analysis. Untreated wild-type (WT) cells have no discernable SR-BI or ABCA1 (Figure 1A). However, when WT cells were treated with 9cisRA/22-OH, a band of 210 kDa corresponding to ABCA1 was observed (Figure 1A). WI38VA13 SR-BI<sup>19</sup> showed no ABCA1 band but exhibited expression of SR-BI (Figure 1A). When this subline was treated with 9cisRA/22-OH, both ABCA1 and SR-BI were seen (Figure 1A). In contrast to J774 macrophages, untreated WI38VA13 cells showed no detectable expression of ABCG1 (Figure 1B). Even when treated with 9cisRA/22-OH, ABCG1 could not be detected (unpublished data, 2005).



**Figure 1.** Presence of ABCA1, SR-BI, and ABCG1 protein in various cell lines. Cell lysates were prepared and protein expression determined by SDS-PAGE and Western blotting as described in Methods. ABCA1 and SR-BI protein expression were determined in WI38VA13 sublines (A) and ABCG1 protein expression was determined in untreated J774 and WT WI38VA13 cells using MPM treated with TO-901317 and rosiglitazone as a positive control (B).

### Cholesterol Efflux to Human Serum Supplemented With HDL<sub>3</sub> or Lipid-Free ApoA-I

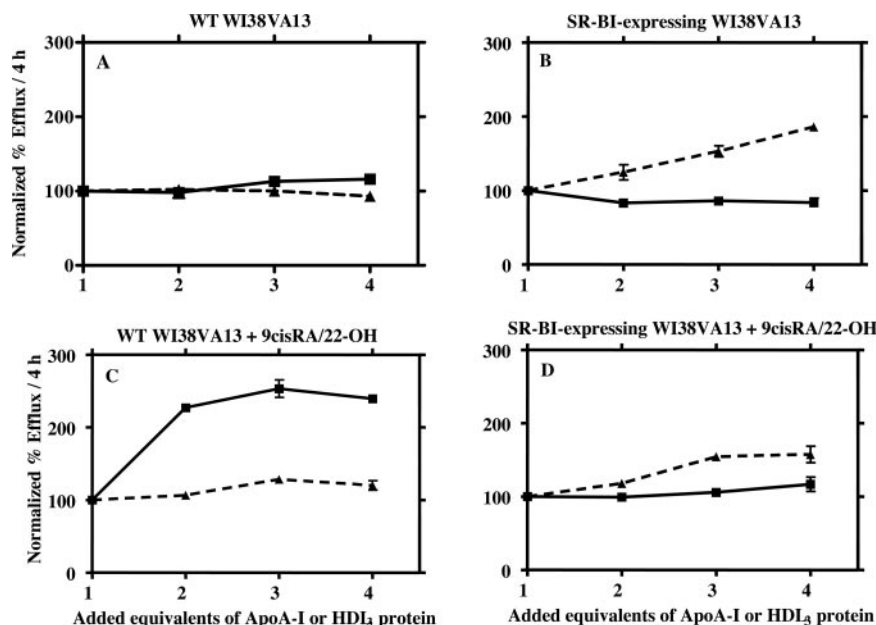
To validate that cholesterol efflux from WI38VA13 cells responded to changes in either HDL or apoA-I concentration, we measured efflux using a pool of human serum supplemented with increasing concentrations of HDL<sub>3</sub> or lipid-free apoA-I. When WT cells, containing neither ABCA1 nor SR-BI, were incubated with serum supplemented with either HDL<sub>3</sub> or apoA-I, there was only a small increase in efflux at higher HDL<sub>3</sub> concentrations (Figure 2A). This result is consistent with lack of both ABCA1 and SR-BI in the cells. When untreated WI38VA13 expressing SR-BI were incubated with serum plus apoA-I, or HDL<sub>3</sub>, efflux increased with increasing HDL<sub>3</sub>, whereas that of serum plus apoA-I remained unchanged (Figure 2B), as expected with cells expressing SR-BI. When cells were stimulated with 9cisRA/22-OH, efflux to serum plus apoA-I increased significantly with concentration of apoA-I, whereas supplementation with HDL<sub>3</sub> had no significant effect (Figure 2C). In WI38VA13 expressing SR-BI and treated with 9cisRA/22-OH, there was an increase in efflux to serum containing increased levels of HDL<sub>3</sub> (Figure 2D). This increase was reduced when compared with that obtained with expression of only SR-BI (compare Figure 2B to 2D). The elevation observed with apoA-I supplementation was considerably reduced when compared with efflux obtained when only ABCA1 was expressed (compare Figure 2D to 2C).

### Cellular Cholesterol Efflux to rHDL, Lipid-Free ApoA-I, and Serum in WI38VA13 Sublines

By using 4 different WI38VA13 cells systems (ie, no efflux proteins, ABCA1, SR-BI, both ABCA1 and SR-BI) we were able to compare efficiency of two proteins known to mediate cholesterol efflux when exposed to either lipid-free apoA-I (Figure 3A), rHDL particles containing apoA-I and phosphatidylcholine (Figure 3B) or 2.5% serum (Figure 3C). When apoA-I was the extracellular acceptor, efflux from WT-expressing and SR-BI-expressing cells was low (WT=0.51%±0.03; SR-BI=0.65%±0.03 per 4 hours). Expression of ABCA1 enhanced this efflux to 2.4%±0.16 every 4 hours and this value was reduced to 2.0±0.09 every 4 hours ( $P=0.03$ ) when both proteins were present.

Even in the absence of both proteins, efflux to rHDL was 5-fold greater than obtained with lipid-free apoA-I (Figure 3A and 3B). The most dramatic difference between apoA-I and rHDL occurred when SR-BI was present, either alone or with ABCA1. With SR-BI expression, efflux increased to 14.6%±0.7 every 4 hours (Figure 3B). Thus, with WT cells or a cell having SR-BI, efflux to a particle containing both apoA-I and phospholipid was much higher than with apolipoprotein alone.

Few studies have been conducted using whole serum that contains a mixture of potential acceptors. The data shown in Figure 3C illustrate the efflux observed when WI38VA13 sublines were exposed to pooled human serum at a concentration of 2.5%. Expression of ABCA1 enhanced efflux by 63%, whereas expression of SR-BI resulted in a 288% increase in efflux (Figure 3C). As was noted earlier (Figure 2D), upregulation of ABCA1 in cells expressing SR-BI



**Figure 2.** WI38VA13 sublines selectively expressing different efflux proteins incubated with 1% human serum that was supplemented with either apoA-I or HDL. 1% whole human serum supplemented with either apoA-I or HDL. 1% whole human serum supplemented with apoA-I (solid line) or HDL<sub>3</sub> (dashed line) were incubated for 4 hours at 37°C with [<sup>3</sup>H]cholesterol-labeled WI38VA13 sublines expressing different combinations of proteins. 1% pooled human serum was supplemented with either apoA-I or HDL so that only the concentration of these components was increased and the other plasma factors remained constant. HDL and apoA-I were added at protein amounts equivalent to 2-, 3-, and 4-times that found 1% serum (9.6, 19.2, 28.8, and 38.4 μg/mL for HDL protein and 10, 20, 30, and 40 μg/mL for apoA-I, respectively). Unsupplemented serum is 1 on the x-axis; % efflux is relative to the efflux seen with unsupplemented serum that was set equal to 100%. This actual efflux value to 1% serum was 0.98% for WT WI38VA13 (A), 4.54% for SR-BI-expressing WI38VA13 (B), 2.24% for WT WI38VA13 treated with 9cisRA/22-OH (C), and 4.35% for SR-BI-expressing WI38VA13 treated with 9cisRA/22-OH (D). All data points are the mean ± SD of 3 separate values.

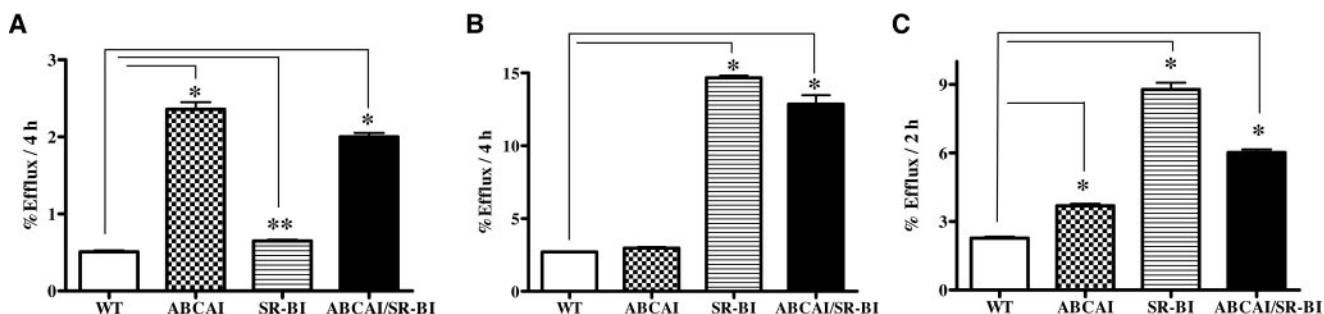
reduced efflux to the human serum when compared with SR-BI expression alone (Figure 3C).

### Inhibition of ABCA1- and SR-BI-Mediated Efflux by BLT-1 and Probucol

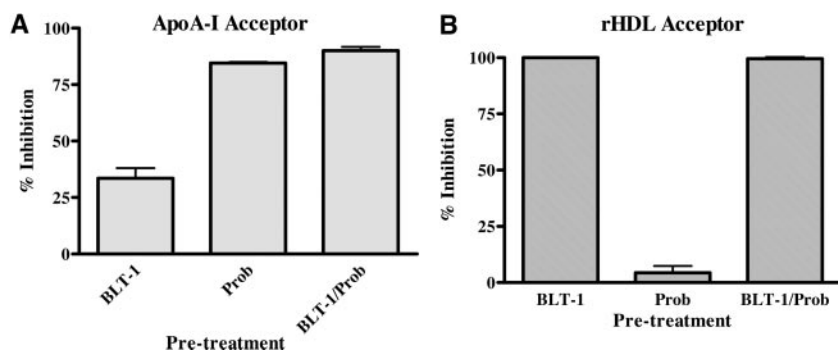
To gain quantitative data on the contribution of efflux pathways, we used 2 inhibitors previously shown to block either ABCA1-mediated or SR-BI-mediated efflux. Probucol is a effective inhibitor of the ABCA1 pathway<sup>17</sup> and is very specific for inhibiting ABCA1 with no effect on SR-BI-mediated efflux.<sup>17</sup> BLT-1 is a low-molecular-weight compound that Nieland et al<sup>15,16</sup> have shown to be an inhibitor of SR-BI selective uptake and FC flux. To examine specificity and efficiency of these inhibitors, we pretreated cells for 2 hours before initiation of efflux. Cells upregulated for

ABCA1 were incubated with apoA-I, whereas cells expressing SR-BI were incubated with rHDL. Treatment with BLT-1 was not completely specific for SR-BI-expressing cells because it depressed efflux to apoA-I from ABCA1 expressing cells by ≈30% (Figure 4A). Pretreatment with Probucol was effective in reducing ABCA1-mediated efflux with ≈85% inhibition. The combination of both inhibitors resulted in a small increase in inhibition above that seen with Probucol alone.

A similar series of studies was performed using SR-BI-expressing WI38VA13 incubated with rHDL as the extracellular acceptor (Figure 4B). Pre-exposure to BLT-1 was very effective in reducing efflux (100% inhibition), whereas Probucol produced essentially no inhibition. No observable cell toxicity by the inhibitors was seen.



**Figure 3.** Cholesterol efflux in the WI38VA13 sublines expressing different proteins to lipid-free apoA-I, rHDL, or 2.5% human serum. Sublines selectively expressing no proteins (WT), ABCA1 only (ABCA1), SR-BI only (SR-BI), or both ABCA1 and SR-BI (ABCA1/SR-BI) were labeled with [<sup>3</sup>H]cholesterol as described in Methods. Sublines were incubated for 4 hours with apoA-I (10 μg/mL) (A), rHDL (50 μg phospholipid/mL; 20 μg protein/mL) (B), or 2.5% whole human serum (C). \**P*<0.0001; \*\**P*=0.005.



**Figure 4.** The effectiveness of BLT-1 and Probucol on cholesterol efflux to lipid-free apoA-I and rHDL. Radiolabeled WI38VA13 fibroblasts, expressing either ABCA1 or SR-BI, were pretreated for 2 hours with media, BLT-1 (10  $\mu\text{mol/L}$ ), Probucol (20  $\mu\text{mol/L}$ ), or both BLT-1 (10  $\mu\text{mol/L}$ ) and Probucol (20  $\mu\text{mol/L}$ ) before an efflux period of 2 hours with apoA-I (10  $\mu\text{g/mL}$ ) (A) or rHDL (50  $\mu\text{g}$  phospholipid/mL) (B). Inhibition was calculated as cholesterol efflux from cells treated with the respective inhibitors, as percentage of efflux from cells incubated without inhibitors.

### Quantitation of ABCA1-Mediated and SR-BI-Mediated Efflux to Serum Using BLT-1 and Probucol

Having established that Probucol and BLT-1 were both effective inhibitors of cell cholesterol efflux, we used these compounds to determine the contribution of ABCA1, SR-BI, and other pathways to efflux from fibroblasts exposed to human serum.

Using BLT-1 and Probucol pretreatment of the fibroblasts, the calculated efflux to 2.5% human serum was determined and compared with control cells not treated with inhibitors (Figure 5). The difference between efflux obtained with cells treated with an inhibitor, compared with similar untreated cells represents the contribution of the specific membrane protein to efflux. For example, in a representative experiment the efflux from SR-BI-expressing cells to serum was 6.3% per 2 hours, whereas the efflux with similar cells pretreated with BLT-1 was 2.1% every 2 hours. These values were very similar to those of cells pretreated with both inhibitors. Thus, contribution of SR-BI was 4.2% every 2 hours, or 67% of total efflux (Figure 5A). Probucol had no effect on efflux, indicating that ABCA1 was not contributing to total efflux from these SR-BI expressing cells, confirming the lack of ABCA1.

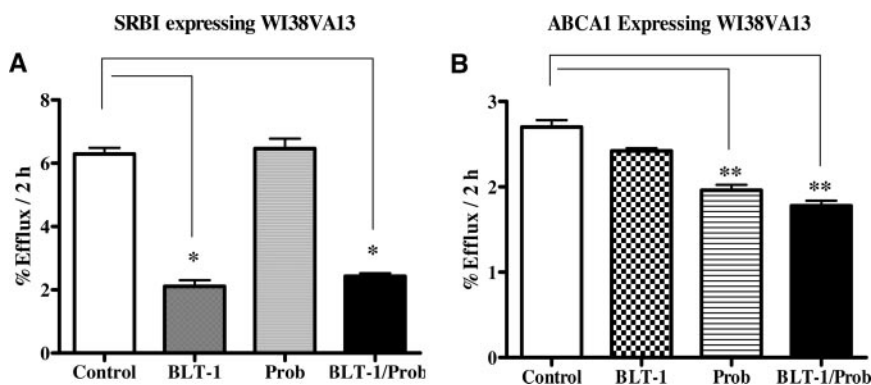
Using a similar approach, we determined the contribution of ABCA1 to efflux from ABCA1-expressing fibroblasts when incubated with serum (Figure 5B). In this case ABCA1 contributed 26% of total efflux as demonstrated by the

reduction of total efflux of 2.7% every 2 hours to 2.0% every 2 hours after exposure to Probucol. A similar reduction was obtained with the combination of inhibitors. The 11% reduction obtained on BLT-1 treatment is consistent with partial reduction previously observed (Figure 4A).

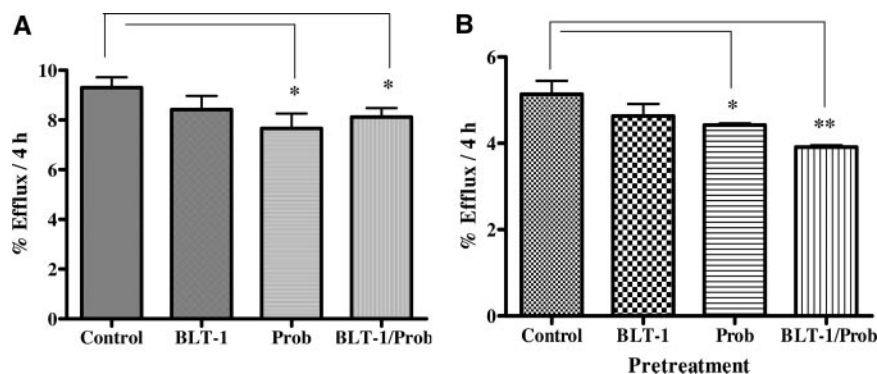
Figure 5A and 5B show considerable efflux occurs from both types of cells even when they are pretreated with a combination of inhibitors. With SR-BI this “background” or “uninhibitable” efflux accounts for  $\approx 40\%$  of total efflux to 2.5% serum. For ABCA1-expressing fibroblasts, uninhibitable efflux accounts for  $\approx 65\%$  of total efflux. It should be noted that although total efflux values from cells expressing either protein were very different (compare control values in Figure 5A and 5B), the uninhibitable values were similar (compare BLT-1/Probucol in Figure 5A and 5B). Thus, considerable cholesterol efflux is occurring by pathway(s) not involving either ABCA1 or SR-BI.

To establish that time of the efflux period did not influence interpretation of data, efflux from cells expressing both ABCA1 and SR-BI was measured over a 6-hour period. The differences in  $t_{1/2}$  values were consistent with inhibition data collected at other time points (Figure I, available online at <http://atvb.ahajournals.org>).

To establish the relationships between serum concentration and components that contributed to cholesterol efflux (ie, Probucol-inhibited ABCA1, BLT-1-inhibited SR-BI, and uninhibitable), we incubated WI38VA13 cells with increasing concentrations of serum (Figure II, available online at <http://atvb.ahajournals.org>).



**Figure 5.** Comparison of the inhibition of cholesterol efflux by BLT-1 and Probucol in SR-BI-expressing and ABCA1-expressing cells incubated with 2.5% human serum. A, Radiolabeled SR-BI-expressing WI38VA13 were pretreated with media alone, BLT-1 (10  $\mu\text{mol/L}$ ), Probucol (20  $\mu\text{mol/L}$ ) or combination of 10  $\mu\text{mol/L}$  BLT-1 plus 20  $\mu\text{mol/L}$  Probucol before incubation with 2.5% human serum for 2 hours at 37°C. SR-BI-mediated efflux is calculated as difference in efflux between untreated control and BLT-1-treated cells. B, ABCA1-mediated efflux is calculated as efflux difference between untreated control cells and Probucol-treated cells. \* $P=0.0001$ ; \*\* $P=0.002$ . All data are the mean  $\pm$  SD,  $n=3$ .



**Figure 6.** Cholesterol efflux from MPM and J774 incubated with human serum. MPM that have been cholesterol-enriched using acLDL were pretreated with efflux inhibitors as described. After pretreatment cells were incubated for 4 hours with 2.5% human and cholesterol efflux determined (A). Efflux to human serum from J774 that was cholesterol-enriched using acLDL was pretreated with efflux inhibitors. Cholesterol efflux over 4 hours was determined when cells were incubated with 2.5% human (B). \* $P < 0.02$ ; \*\* $P = 0.002$ . All data are the mean  $\pm$  SD,  $n = 3$ .

atvb.ahajournals.org). SR-BI-mediated efflux increased linearly (Figure IIA). There was a linear increase in the uninhibited efflux that contributed 30% to 40% of total efflux. With ABCA1-expressing cells, total efflux was approximately one-third that of SR-BI-expressing cells (Figure IIB). The uninhibited contribution to total efflux was very large, ranging from 67% to 81% as serum concentration increased. ABCA1 contribution to total efflux was small and unchanged by serum concentration. With both SR-BI-expressing and ABCA1-expressing cells, the level of uninhibited efflux ranged from 2% every 2 hours at 2.5% serum to 4% every 2 hours at 7.5% serum. Thus, the inhibitor resistant component of efflux was serum concentration dependent and unaffected by the presence of either SR-BI or ABCA1.

### Quantitation of ABCA1-Mediated and SR-BI-Mediated Efflux to Serum in the J774 and MPM

Efflux inhibitors were used on cholesterol-enriched J774 and MPM to quantitate contributions of different efflux mechanism. Conditions were similar to those used for fibroblasts with the exception that the ACAT inhibitor was not used and the cells were cholesterol-enriched because we wanted the macrophages to model foam cells. In preliminary experiments, Probucol inhibited 80% of ABCA1-mediated efflux to apoA-I. This was determined as the reduction by Probucol of the difference in efflux between 9cisRA/22-OH-treated cells and untreated cells. The selectivity of BLT-1 for SR-BI in macrophages was demonstrated by an experiment using MPM from SR-BI knockout mice in which BLT-1 had no effect on efflux to rHDL. Based on MPM inhibitor results, the contribution of ABCA1 and SR-BI to total efflux to serum was minor ( $18 \pm 6.5\%$  and  $6 \pm 5.9\%$ , respectively). The uninhibited efflux was the main contributor to total efflux with  $87 \pm 3.9\%$  over 4 hours (Figure 6A). With cholesterol-enriched J774 (Figure 6B), the contributions of SR-BI-mediated and ABCA1-mediated efflux to total efflux were also small ( $10 \pm 5.4\%$  and  $14 \pm 0.8\%$ , respectively). As with MPM, in J774 the uninhibited efflux also comprised the majority of the efflux ( $76 \pm 0.7\%$ ) (Figure 6B).

### Discussion

The first well-studied mechanism mediating cholesterol release from cells was aqueous diffusion. This is an unmediated process in which cholesterol desorbs into the aqueous phase and is incorporated into phospholipid-containing acceptors.<sup>4</sup>

Because this is a bidirectional process, net movement of FC between the cellular compartment and acceptor is governed by cholesterol gradients determined by cholesterol and phospholipid contents and composition of cells and acceptor.<sup>19,20</sup> It has become clear that efflux is a more complex process and there are a number of pathways in which efflux is mediated by cell proteins including SR-BI,<sup>21</sup> ABCA1,<sup>7,8,22</sup> and more recently ABCG1.<sup>9,10</sup> These proteins all play important roles in cholesterol metabolism, as is demonstrated by the extensive pathology exhibited in animals in which these proteins are either absent or overexpressed.<sup>23–26</sup>

Despite extensive studies on cholesterol flux, there have been no detailed comparisons quantitating the contribution of each of the efflux pathways using a single cell system. Additionally, almost all studies on efflux have used single, purified particles such as HDL or apoA-I. In this investigation we addressed the following questions. What are the relative efficiencies of SR-BI and ABCA1 when expressed in a cell system either alone or in combination? What are the efficiencies of purified acceptor particles under conditions in which efflux proteins expression is controlled? What are the contributions of efflux pathways when cells are exposed to mixtures of lipoproteins and apolipoproteins present in serum? To accomplish these aims, we have used human fibroblasts and mouse macrophages. Untreated, WI38VA13 cells express no SR-BI, have low expression of ABCA1 (Figure 1A), and do not appear to express ABCG1 (Figure 1B). If pretreated with LXR/RXR ligands, there is a marked expression of ABCA1 (Figure 1A). We also have a subline of WI38VA13 that express SR-BI<sup>19</sup> (Figure 1A). Treatment of SR-BI cells with 9cisRA/22-OH increases expression of ABCA1 without effecting SR-BI protein levels. Because of different antibodies used to detect ABCA1 and SR-BI, we were unable to compare the relative protein expression of efflux proteins in the sublines; however, high expression levels are present in both cases.

To establish that sublines of WI38VA13 cells responded to acceptors as would be predicted based on efflux proteins that were expressed, efflux from WT and sublines was measured with cells incubated in 1% human serum and the same serum supplemented with increasing concentrations of HDL<sub>3</sub> or apoA-I (Figure 2). The pattern of cellular efflux was consistent with the pattern of efflux protein expression. WT cells were unresponsive to supplementation with either HDL<sub>3</sub> or apoA-I. SR-BI-expressing cells were highly responsive to

enrichment of serum with HDL<sub>3</sub> but showed no increase in efflux when apoA-I was raised. This response pattern was reversed on upregulation of ABCA1. In these experiments, the serum was added at only 1% to ensure that added apoA-I would not entirely associate with serum lipoproteins and some of the added apolipoprotein would remain sufficiently lipid-free to enhance ABCA1-mediated efflux. The coexpression of both proteins reduced the stimulatory effect of supplementation with apolipoprotein or HDL<sub>3</sub>. The reduction of ABCA1-mediated efflux in cells expressing SR-BI is consistent with earlier studies demonstrating a similar phenomenon<sup>27</sup> and that SR-BI overexpression had no effect on ABCA1 mRNA; this is consistent with our observation that there was no reduction in protein expression of ABCA1 when SR-BI was coexpressed. It is evident that coexpression of the 2 proteins reduces SR-BI-mediated efflux as well.

A comparison of the extent of efflux to the different acceptors (compare Figure 3A to 3B) illustrates that rHDL is a more efficient acceptor than lipid-free apolipoprotein. This difference in efflux can perhaps be explained by a disparity in protein expression; however, this has been observed previously in other cell systems and persists over a wide range of acceptor concentrations. The difference becomes more pronounced as acceptor concentration increases because ABCA1 efflux is saturable at low apoA-I concentrations ( $\approx 10 \mu\text{g/mL}$ ),<sup>28</sup> whereas SR-BI-mediated efflux of FC does not saturate.<sup>29</sup> Although ABCA1 efflux is low compared with SR-BI, it is a unidirectional process so it represents net movement of cholesterol from donor cells. This is also the case with rHDL, but this would not apply to native HDL. With cholesterol-containing lipoproteins net cholesterol flux cannot be predicted; however, net efflux can occur under conditions where there is both influx and efflux of cholesterol.<sup>13,19</sup>

Efflux of cholesterol to serum is more complicated than with purified acceptors because serum contains a variety of potential acceptors. When serum was used as an acceptor, expression of ABCA1 resulted in a modest increase in cholesterol efflux (Figure 3C). The increase promoted by SR-BI expression was more dramatic with efflux being  $\approx 3$ -fold greater than that obtained with control fibroblasts (Figure 3C).

To quantitate the contribution of individual efflux pathways to total cholesterol efflux to serum we used BLT-1 and Probucol to specifically inhibit protein-mediated efflux. Figure 4 illustrates the high degree of specificity of these compounds. Studies have shown that Probucol rapidly and specifically inhibits ABCA1-mediated efflux.<sup>17,30</sup> The mechanism by which BLT-1 inhibits SR-BI-mediated FC efflux and CE selective uptake is not as well understood.<sup>16</sup> In our experiments, BLT-1 produced a modest inhibition of ABCA1-mediated efflux. In a study by Nieland et al,<sup>16</sup> BLT-1 did not inhibit ABCA1; however, other BLT compounds did demonstrate some cross-inhibition. The difference between the studies may be related to different cell types.

With cells expressing either protein total efflux is linearly associated with serum concentration. SR-BI-mediated efflux demonstrates such linearity (Figure IIA), whereas ABCA1-

mediated efflux is largely independent of serum concentration at concentrations  $>2.5\%$  (Figure IIB). What is particularly striking about these efflux protein inhibitor studies is that with all of the fibroblast sublines, there is a significant portion of the total efflux to serum that is uninhibitable and represents background efflux. Thus, even though total efflux to serum from ABCA1-expressing cells is  $\approx 50\%$  that of SR-BI-expressing cells (Figure 5A and 5B), the fractional efflux from cells treated with the combination of inhibitors is essentially the same,  $\approx 2\%$  per 2 hours. Uninhibited, or background, efflux remains a constant fraction of total efflux over an entire incubation period of 6 hours. The contribution of this background efflux to total efflux can be very high if total efflux is low, as in cells expressing only ABCA1, in which uninhibited efflux is 70% of total and linear with increasing serum concentration (Figure IIB). Even when SR-BI expression stimulates total efflux, the uninhibitable component is a significant fraction of total ( $\approx 35\%$ ), and this fractional contribution is relatively constant over serum concentrations ranging from 2.5% to 7.5% (Figure IIA). In all studies with the fibroblasts, an ACAT inhibitor was used to insure that differences in cell cholesteryl ester content did not influence comparative efflux data between sublines because expression of SR-BI can increase cholesteryl ester content.<sup>31,32</sup> However, the mouse macrophages were used as model foam cells and thus were cholesterol-enriched in the absence of an ACAT inhibitor. When efflux inhibitors were used on cholesterol-enriched MPM and J774 the fraction of SR-BI-mediated and ABCA1-mediated efflux was relatively small, with most of total efflux reflecting uninhibited efflux (Figure 6). Previous studies<sup>33</sup> have shown that SR-BI expression decreases with cholesterol loading and this is supported by the observation that SR-BI contribution to total efflux to serum is reduced on loading in these macrophages (unpublished data, 2005). The contribution of ABCA1 to total efflux is also minor relative to the uninhibited efflux. The contribution of ABCA1 would be proportional to the concentration of lipid-poor/free apolipoprotein and this is low in serum at 2.5%. Because these cells were cholesterol-loaded, it is likely that some fraction of this uninhibited efflux is caused by ABCG1. The extent to which ABCG1 contributes to efflux from macrophages to serum has not been established.

The nature of the background/uninhibitable efflux remains to be determined. A number of characteristics of this pathway(s) are known. Background efflux requires phospholipid-containing acceptors and is directly correlated with concentration of serum in the medium. The fractional efflux contributed by this uninhibited pathway/s from the sublines was constant regardless of the type of protein expressed, or even if neither ABCA1 nor SR-BI was expressed. To eliminate the possibility that the high levels of 9cisRA were artificially inducing the uninhibited efflux, we compared 9cisRA to the synthetic ligand TO-901317. We observed that the level of uninhibited efflux was similar when the fibroblasts were upregulated with either 9cisRA or TO-901317 (Figure III, available online at <http://atvb.ahajournals.org>). In addition to ABCA1 and SR-BI, the number of proteins that are associated with cellular cholesterol efflux is growing, and

it is possible that a poorly characterized or as yet unidentified protein is responsible for the uninhibited efflux. Although proteins other than ABCA1 or SR-BI may be responsible for the uninhibited efflux a likely candidate is aqueous diffusion. This mechanism has been extensively studied and would be present in all cell types.<sup>34</sup> A significant contribution of background efflux to total efflux is not confined to fibroblasts because uninhibited efflux to serum from macrophages contributes significantly to total efflux (Figure 6). It has been reported for other cells, such as endothelial cells, in which there was low expression of ABCA1, SR-BI, and ABCG1, that cholesterol efflux to HDL<sub>3</sub> was independent of these efflux proteins.<sup>35</sup> The experimental approaches developed in this study can now be applied to other cells, and it is possible that aqueous transfer mechanism may be quantitatively as important to the efflux of cholesterol as ABCA1, SR-BI, or ABCG1.

### Acknowledgments

These studies were supported in part by National Institutes of Health grants HL-22633 and HL-63768. The authors thank Alan Tall and Mollie Ranalletta for their advice and expert technical assistance with the ABCG1 Western blots.

### References

- Brewer HB. High-Density Lipoproteins: A New Potential Therapeutic Target for the Prevention of Cardiovascular Disease. *Arterioscler Thromb Vasc Biol.* 2004;24:387–391.
- Rader DJ. Regulation of reverse cholesterol transport and clinical implications. *Am J Cardiol.* 2004;92:421–491.
- Yancey PG, Bortnick AE, Kellner-Weibel G, de la Llera-Moya M, Phillips MC, Rothblat GH. Importance of different pathways of cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol.* 2003;23:712–719.
- Johnson WJ, Mahlberg FH, Rothblat GH, Phillips MC. Cholesterol transport between cells and high density lipoproteins. *Biochim Biophys Acta.* 1991;1085:273–298.
- Krieger M. Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. *J Clin Invest.* 2001; 108:793–797.
- Williams DL, Connelly MA, Temel RE, Swanakar S, Phillips MC, de la Llera-Moya M, Rothblat GH. Scavenger receptor BI and cholesterol trafficking. *Curr Opin Lipidol.* 1999;10:329–339.
- Wang N, Tall AR. Regulation and mechanisms of ATP-binding cassette transporter AI-mediated cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol.* 2003;23:1178–1184.
- Oram JF. ATP-binding cassette transporter A1 and cholesterol trafficking. *Curr Opin Lipidol.* 2002;13:373–381.
- Nakamura K, Kennedy M, Baldan A, Bojanic D, Lyons K, Edwards P. Expression and regulation of multiple murine ATP-binding cassette transporter G1 mRNAs/Isoforms that stimulate cellular cholesterol efflux to high density lipoprotein. *J Biol Chem.* 2004;279:45980–45989.
- Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoprotein. *Proc Natl Acad Sci.* 2004;101:9774–9779.
- Yancey PG, Kawashiri M, Moore R, Glick JM, Williams DL, Connelly MA, Rader DJ, Rothblat GH. In vivo modulation of HDL phospholipid has opposing effects on SR-BI- an ABCA1-mediated cholesterol efflux. *J Lipid Res.* 2004;45:337–346.
- Bortnick AE, Rothblat GH, Stoudt G, Hoppe KL, Royer LJ, McNeish J, Francone OL. The correlation of ABC1 mRNA levels with cholesterol efflux from various cell lines. *J Biol Chem.* 2000;275:28634–28640.
- Yancey PG, de la Llera-Moya M, Swarnakar S, Monzo P, Klein SM, Connelly MA, Johnson WJ, Williams DL, Rothblat GH. HDL phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor-BI (SR-BI). *J Biol Chem.* 2000;275:36596–36604.
- Davidson WS, Rodriguez VV, Lund-Katz S, Johnson WJ, Rothblat GH, Phillips MC. Effects of acceptor particle size on the efflux of cellular free cholesterol. *J Biol Chem.* 1995;270:17106–17113.
- Nieland T, Penman M, Dori L, Krieger M, Kirchhausen T. Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI. *Proc Natl Acad Sci.* 2002;99:15422–15427.
- Nieland T, Chroni A, Fitzgerald ML, Maliga Z, Zannis VI, Kirchhausen T, Krieger M. Cross-inhibition of SR-BI and ABCA1-mediated cholesterol transport by the small molecules BLT-4 and Glyburide. *J Lipid Res.* 2004; 45:1256–1265.
- Favari E, Zanotti I, Zimetti F, Ronda N, Bernini F, Rothblat GH. Probucol inhibits ABCA1-mediated cellular lipid efflux. *Arterioscler Thromb and Vascular Biol.* 2004;24:2345–2350.
- Howard BV, Kritchevsky D. The lipids of normal diploid (WI-38) and SV40-transformed human cells. *Int J Cancer.* 1969;4:393–402.
- de la Llera-Moya M, Connelly MA, Drazul D, Klein SM, Favari E, Yancey PG, Williams DL, Rothblat GH. Scavenger receptor, class B, type I (SR-BI) affects cholesterol homeostasis by magnifying cholesterol flux between cells and HDL. *J Lipid Res.* 2001;42:1969–1978.
- Johnson WJ, Bamberger MJ, Latta MJ, Rapp RA, Phillips MC, Rothblat GH. The bidirectional flux of cholesterol between cells and lipoproteins. *J Biol Chem.* 1986;261:5766–5776.
- Ji Y, Jian B, Wang N, Sun Y, de la Llera Moya M, Phillips MC, Rothblat GH, Swaney JB, Tall AR. Scavenger receptor B1 promotes high density lipoprotein-mediated cellular cholesterol efflux. *J Biol Chem.* 1997;272: 20982–20985.
- Brewer Jr HB, Santamarina-Fojo S. New insights into the role of the adenosine triphosphate-binding cassette transporters in high-density lipoprotein metabolism and reverse cholesterol transport. *Am J Cardiol.* 2003;91:3E–11E.
- Kennedy M, Barrera GC, Nakamura K, Baldan A, Tarr P, Fishbein M, Frank J, Francone O, Edwards P. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metabol.* 2005;1:121–131.
- Zhang W, Yancy PG, Su YRS, Babaev VR, Zhang Y, Fazio S, Linton MF. Inactivation of Macrophage Scavenger Receptor Class B Type I Promotes Atherosclerotic Lesion Development in Apolipoprotein E-deficient Mice. *Circulation.* 2004;108:2258–2263.
- Covey SD, Krieger M, Wang W, Penman M, Trigatti BL. Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL receptor - negative mice involves its expression in bone marrow-derived cells. *Arterioscler Thromb Vasc Biol.* 2003;23:1589–1594.
- Francone O. SR-BI a new player in an old game. *Arterioscler Thromb Vasc Biol.* 2003;23:1486–1487.
- Chen W, Silver DL, Smith JD, Tall AR. Scavenger receptor-BI inhibits ATP-binding cassette transporter 1-mediated cholesterol efflux in macrophages. *J Biol Chem.* 2000;275:30794–30800.
- Vedhachalam C, Liu L, Nickel M, Dhanasekaran P, Anantharamaiah GM, Lund-Katz S, Rothblat GH, Phillips MC. Influence of apoA-I structure on the ABCA1-mediated efflux of cellular lipids. *J Biol Chem.* 2004;279: 49931–49939.
- Thuahnai ST, Lund-Katz S, Dhanasekaran P, de la Llera-Moya M, Connelly MA, Williams DL, Rothblat GH, Phillips MC. Scavenger receptor class B type I-mediated cholesterol ester-selective uptake and efflux of unesterified cholesterol: Influence of high density lipoprotein size and structure. *J Biol Chem.* 2004;279:12448–12455.
- Wu C-A, Tsujita M, Hayashi M, Yokoyama S. Probucol inactivates ABCA1 in the plasma membrane with respect to its mediation of apolipoprotein binding and high density lipoprotein assembly and to its proteolytic degradation. *J Biol Chem.* 2004;279:30168–30174.
- Stangl H, Cao G, Wyne KL, Hobbs HH. Scavenger receptor, class B, type-I-dependent stimulation of cholesterol esterification by high density lipoproteins, low density lipoproteins, and nonlipoprotein cholesterol. *J Biol Chem.* 1998;273:31002–31008.
- Kellner-Weibel G, de la Llera-Moya M, Connelly MA, Stoudt G, Christian AE, Haynes MP, Williams DL, Rothblat GH. Expression of scavenger receptor BI in COS-7 cells alters cholesterol content and distribution. *Biochemistry.* 2000;39:221–229.
- Yu L, Cao G, Repa J, Stangl H. Sterol regulation of scavenger receptor class B type I in macrophages. *J Lipid Res.* 2004;45:889–899.
- Phillips MC, Johnson WJ, Rothblat GH. Mechanism and consequence of cellular cholesterol exchange and transfer. *Biochim Biophys Acta.* 1987; 906:223–276.
- O'Connell BJ, Denis M, Genest J. Cellular physiology of cholesterol efflux in vascular endothelial cells. *Circulation.* 2004;110:2881–2888.