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The roles of different pathways in the release of cholesterol from macrophages

Maria Pia Adorni,[1,*] Francesca Zimetti,[1,*] Jeffrey T. Billheimer,[†] Nan Wang,[§] Daniel J. Rader,[†] Michael C. Phillips,[*] and George H. Rothblat[2,*]

Gastroenterology, Hepatology, and Nutrition Division,* Children's Hospital of Philadelphia, Philadelphia, PA; Institute of Translational Medicine and Therapeutics,[†] School of Medicine, University of Pennsylvania, Philadelphia, PA; and Division of Molecular Medicine,[§] Department of Medicine, Columbia University, New York, NY

Abstract **Cholesterol efflux occurs by different pathways, including transport mediated by specific proteins. We determined the effect of enriching cells with free cholesterol (FC) on the release of FC to human serum. Loading Fu5AH cells with FC had no effect on fractional efflux, whereas enriching mouse peritoneal macrophages (MPMs) resulted in a doubling of fractional efflux. Efflux from cholesterol-normal MPM and Fu5AH cells to 15 human sera correlated well with HDL parameters. However, these relationships were reduced or lost with cholesterol-loaded MPMs. Using macrophages from scavenger receptor class B type I (SR-BI)-, ABCA1-, and ABCG1-knockout mice, together with inhibitors of SR-BI- and ABCA1-mediated efflux, we were able to quantitate efflux upon loading macrophages with excess cholesterol and to establish the contributions of the various efflux pathways in cholesterol-normal and -enriched cells. The removal of ABCA1 had essentially no effect on the total efflux when cell cholesterol levels were normal. However, in cholesterol-enriched cells, the removal of ABCA1 reduced efflux by 50%. Approximately 20% of the efflux stimulated by FC-loading MPM is attributable to ABCG1. The SR-BI contribution to efflux was small. Another pathway that is present in all cells is aqueous diffusion. Our studies demonstrate that this mechanism is one of the major contributors to efflux, particularly in cholesterol-normal cells.**—Adorni, M. P., F. Zimetti, J. T. Billheimer, N. Wang, D. J. Rader, M. C. Phillips, and G. H. Rothblat. **The roles of different pathways in the release of cholesterol from macrophages.** ***J. Lipid Res.*** **2007.** 48: **2453–2462.**

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Several epidemiological studies have shown an inverse correlation between HDL plasma level and the risk of coronary heart disease (1, 2). One of the mechanisms by which HDL is antiatherogenic is the ability to promote reverse cholesterol transport (RCT) (3, 4). In RCT, excess cholesterol is returned from the peripheral cells, such as the lipid-laden macrophages of an atherosclerotic plaque, to the liver for excretion in the bile. RCT begins with cholesterol export from macrophages to serum lipoproteins, which serve as extracellular cholesterol acceptors. It has been demonstrated that the efflux of cholesterol from cells can occur by several mechanisms, including unmediated aqueous diffusion and specific receptor-mediated processes. Among the latter is the unidirectional flux of cholesterol promoted by a transporter belonging to the ATP binding cassette superfamily, specifically ABCA1, which mediates the efflux of cholesterol to lipid-free or lipid-poor apolipoproteins (5–7). Recently, the half-transporter ABCG1 was identified as a protein involved in the efflux of cholesterol, mainly to lipid-rich acceptor particles (8, 9). In addition, scavenger receptor class B type I (SR-BI) facilitates the bidirectional flux of free cholesterol (FC) between cells and lipoprotein (10, 11). The flux of FC between lipoproteins and cells expressing SR-BI is closely linked to the phospholipid (PL) content and composition of the lipoprotein (12, 13).

The aim of this investigation arose from the observation that fractional efflux of cholesterol from Fu5AH hepatoma cells did not change when these cells were enriched in FC. Thus, the net loss of cholesterol from cholesterol-loaded Fu5AH cells was greater than that from cholesterol-normal cells simply because the cellular pool size of cholesterol was greater. This suggested that the pathway(s) mediating cholesterol efflux in normal and loaded Fu5AH cells was similar. However, when similar studies were conducted

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Abbreviations: AcLDL, acetylated low density lipoprotein; apoA-I, apolipoprotein A-I; BLT-1, 2-hexyl-1-cyclopentanone thiosemicarbazone; DPBS, phosphate-buffered saline with calcium and magnesium; FC, free cholesterol; KO, knockout; MPM, mouse peritoneal macrophage; PL, phospholipid; RCT, reverse cholesterol transport; SR-BI, scavenger receptor class B type I; WT, wild-type.

[1] M. P. Adorni and F. Zimetti contributed equally to this work.
[2] To whom correspondence should be addressed.
e-mail: rothblat@email.chop.edu

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using cholesterol-normal and FC-enriched mouse peritoneal macrophages (MPMs), we observed an enhancement in fractional efflux upon cholesterol loading. In addition, the mass flux from enriched cells was greater than could be explained by simple cholesterol pool size expansion. In contrast, the increase in fractional efflux observed when MPMs were cholesterol-enriched indicated that such treatment produced a change in the array of possible efflux mechanisms and that this change was linked to cell cholesterol accumulation.

In the present study, we compared the properties of unloaded and cholesterol-loaded Fu5AH and MPM cells in terms of the fractional release of cholesterol, using as cholesterol acceptors either 15 individual sera from normolipidemic human subjects or a pool of human serum. For both cell types, we established the correlations between the percentage efflux and HDL composition, and we determined whether a stimulation of any of the efflux mechanisms produced after cholesterol loading influenced these correlations. Finally, we used two different approaches to determine the contributions of the different efflux pathways to efflux from cholesterol-normal and cholesterol-enriched MPMs. First, we compared efflux to human serum from MPMs obtained from wild-type (WT) and genetically manipulated mice in which SR-BI, ABCA1, and ABCG1 were knocked out. Using these same cells, we also pretreated the MPMs with either probucol to inhibit ABCA1-mediated efflux or 2-hexyl-1-cyclopentanone thiosemicarbazone (BLT-1) to inhibit SR-BI-mediated efflux (14–16). These studies demonstrate that efflux from Fu5AH cells and unloaded MPMs correlates well with HDL-cholesterol, HDL-PL, and HDL-apolipoprotein A-I (apoA-I), consistent with significant contributions by SR-BI and aqueous diffusion. However, these correlations are lost, or greatly reduced, when MPMs are enriched with FC. We also demonstrate that, even though to different extents, aqueous diffusion, ABCA1 and ABCG1 plays a role in the stimulation of efflux to human serum obtained with cholesterol-enriched MPMs, whereas SR-BI has only a minor role in this process. In addition, we observed that in cholesterol-normal MPMs, aqueous diffusion of cholesterol from the plasma membrane is the major pathway for cholesterol release to serum.

EXPERIMENTAL PROCEDURES

Materials

Tissue culture plastic ware was obtained from Falcon (Lincoln, NJ) and from Corning, Inc. (Corning, NY). Cell culture media, PBS, and phosphate-buffered saline with calcium and magnesium (DPBS) were purchased from Mediatech Cellgro (Herndon, VA). FBS, calf serum, gentamycin, DMSO, DNase I, sodium cholate, and heparin came from Sigma-Aldrich (St. Louis, MO). BSA was obtained from Celliance (Toronto, Canada). [1,2-^{3}H]cholesterol was from Perkin-Elmer Analytical Sciences (Boston, MA). The ACAT inhibitor CP113,818 was kindly provided by Pfizer Pharmaceuticals (Groton, CT). The Amplex Red Cholesterol Assay Kit to measure cell cholesterol was purchased from Invitrogen (Eugene, OR).

Probucol and BLT-1 solutions

BLT-1 was purchased from ChemBridge (San Diego, CA) and probucol from Sigma-Aldrich. Both were dissolved in 100% DMSO to form a 10 mM stock solution. The stock solution was diluted to a 1 μM BLT-1, 0.2% BSA MEM-HEPES solution and a 20 μM 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 1 μM BLT-1 plus 20 μM 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.

Serum and lipoproteins

Human LDL used to enrich the hepatoma cell line Fu5AH was obtained by sequential ultracentrifugation as described previously (d = 1.019–1.063 g/ml) (17). LDL was chemically modified to obtain acetylated low density lipoprotein (AcLDL) using acetic anhydride as indicated by Basu et al. (18), and the modification was verified by agarose gel electrophoresis. Human serum was collected from 15 normolipidemic individuals with approved consent and used either individually or pooled. Plasma HDL-cholesterol and PL levels were measured enzymatically on a Cobas Fara II (Roche Diagnostic Systems, Inc.) using Sigma-Aldrich reagents. Plasma apoA-I levels were quantified using an immunoturbidometric assay (Wako Pure Chemical Industries) on the Cobas Fara.

Animals and cell culture

ABCA1 knockout (KO) mice were created on the DBA1 lac/J background and were purchased from the Jackson Laboratory (Bar Harbor, ME). SR-BI homozygous mice, on a C57BL/6/S129 background, were purchased from the Jackson Laboratory and bred to obtain SR-BI KO mice. WT B6C3F1 mice were purchased from Taconic Farms (Germantown, NY). ABCG1 KO mice, on a C57BL/6 background, were from the Division of Molecular Medicine, Columbia University (New York, NY). All mice were fed a chow diet. The macrophages were harvested from the peritoneal cavity of mice as described previously (19). Briefly, mice were injected intraperitoneally with 10% thioglycollate (Difco, Detroit, MI). Three days later, the mice were euthanized and macrophages were collected by lavage of the peritoneum with DPBS-heparin. Cells were seeded on 12-well plates at a density of 1×10^6 cells/well in RPMI plus 10% FBS. Rat Fu5AH hepatoma cells were cultured in 5% calf serum containing MEM. Confluent cells were trypsinized and plated at a density of 6×10^5 cells/well on 12-well plates.

Cholesterol efflux studies

After plating, cells were labeled for 24 h with 3 μCi/ml [^{3}H]cholesterol in the presence of 2.5% FBS. When used, loading of cells with cholesterol occurred during the labeling period by the addition of 50 μg/ml AcLDL to the medium. Cell monolayers were then equilibrated for 2 h in 0.2% BSA-containing medium alone or with BLT-1, or probucol, or the combination of both probucol and BLT-1. Subsequently, efflux of cholesterol was induced by incubation for 8 h with either pooled human serum or serum from each individual diluted to 2.5%. Fractional FC efflux was obtained by measuring the release of radiolabeled cholesterol into the medium, as described previously (20). All of the experiments were performed in the presence of 2 μg/ml of the ACAT inhibitor CP113,818 to prevent cellular accumulation of cholesteryl ester. Preliminary studies indicated that the addition of the ACAT inhibitor did not alter the efflux data over the time course of the experiment (data not

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shown). In all studies, a set of monolayers was incubated with protein-free medium to obtain values for "background" efflux. The efflux was always low (1.5 ± 0.1%/8 h), and this value was subtracted from the serum-mediated efflux. Because secreted apoE can act as a cholesterol acceptor, the low level of background efflux indicates that the secretion of apoE did not contribute to the total efflux we determined.

Cholesterol mass determination

Cell monolayers before incubation with the acceptors (time zero cells) were washed with PBS and lysed by the addition of 1% sodium cholate solution supplemented with 50 U/ml DNase. After shaking the plates overnight, 125 μl of a reaction buffer solution was added as described previously (19). Total cholesterol content was measured on cell lysates using the Amplex Red Cholesterol Assay Kit according to the manufacturer's instructions. A 100 μl aliquot of the cell lysates was taken to measure the protein content by a modified Lowry method (19). Total cell [^{3}H]cholesterol was measured by scintillation counting.

Determination of the contributions of efflux pathways

Quantitation of the different efflux pathways to total efflux was determined using an inhibitor-based protocol as described previously (15) and also by measuring the difference in efflux between WT and KO MPMs. Parallel monolayers of cholesterol-normal and cholesterol-enriched WT and KO MPMs were labeled for 24 h with [^{3}H]cholesterol added to either 2.5% FBS (cholesterol-normal cells) or 2.5% FBS plus AcLDL (50 μg/ml; cholesterol-enriched cells) followed by a 2 h equilibration period in the presence of 0.2% BSA. During this 2 h period, some monolayers were exposed to probucol (20 μM) or BLT-1 (1 μM) or to the combination of both probucol and BLT-1. All monolayers were then incubated for 8 h with a pool of human serum added at 2.5%. The contribution of ABCA1 to efflux was determined by the reduction in efflux upon exposure to probucol (15). The reduction in total efflux produced by exposure to BLT-1 was taken as the contribution of SR-BI (15). The level of residual efflux after treatment with both inhibitors was a reflection of ABCG1 and/or aqueous diffusion. Neither inhibitor reduces aqueous diffusion or ABCG1, as demonstrated using control and ABCG1-expressing BHK cells (21) (unpublished observation). The contribution of aqueous diffusion was established as the uninhibitable efflux present in cholesterol-normal WT MPMs or KO MPMs, and this value was assumed to be the same in cholesterol-enriched cells. The efflux remaining after correcting for the contributions of ABCA1, SR-BI, and aqueous diffusion was assumed to be that contributed by ABCG1. However, it is possible that this calculated value reflects the contribution to cholesterol efflux of pathways that have not yet been identified.

Statistical analysis

The experiments were conducted in triplicate, and data are expressed as means ± SD. Linear correlation coefficients were used to describe relations between cholesterol efflux and various serum parameters. All statistical analysis were performed with the GraphPad Prism program (San Diego, CA).

RESULTS

Efflux from cholesterol-normal and -enriched Fu5AH cells

In some cell systems, enrichment of cells with excess cholesterol does not change cholesterol fractional efflux (**Fig. 1**) (22). Cholesterol enrichment of Fu5AH cells was achieved by incubation of cells with 100 μg/ml LDL. Efflux was measured upon the addition of 15 sera from normolipidemic individuals at 2.5% (Fig. 1). In LDL-treated cells, we observed an initial cholesterol pool expansion of ~50% compared with untreated cells. Nonetheless, after 8 h, the average percentage cholesterol efflux from unloaded cells was 15.6 ± 0.9%, similar to that measured after enrichment of cells (15.7 ± 0.7%). This result indicated that the major efflux mechanism in the hepatoma cells remained unchanged regardless of the initial cell cholesterol content.

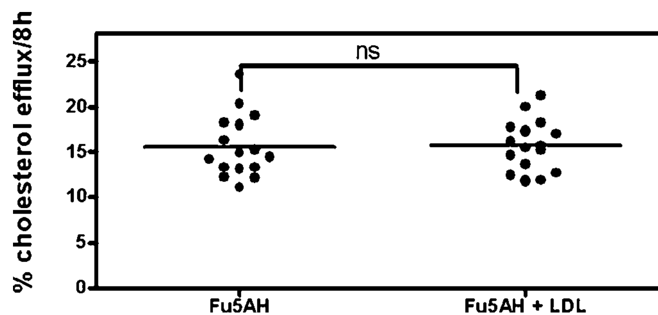

Fig. 1. Scattergram of cholesterol efflux from unloaded and LDL-loaded Fu5AH cells to 15 human sera. Fu5AH cells were radiolabeled for 24 h with 3 μCi/ml [^{3}H]cholesterol in DMEM + 2.5% calf serum and in the presence or absence of 100 μg/ml human LDL. Cells were then equilibrated for 2 h with DMEM + 0.2% BSA. Efflux was promoted by incubating cells with each individual human serum at 2.5% for 8 h. The results are expressed as means ± SD (n = 3). Initial cell cholesterol content was 13.7 ± 0.5 μg cholesterol/mg protein in normal cells and 20.3 ± 3.8 μg cholesterol/mg protein in enriched cells.

Relationship between cholesterol efflux from Fu5AH cells to serum and serum HDL parameters

We observed relationships between the percentage efflux of cholesterol from Fu5AH cells and HDL-related parameters in the 15 human serum specimens (**Fig. 2**). The fractional efflux of cholesterol from these cells ranged from 19.1 ± 0.5% to 30.9 ± 0.9%. As we observed previously (13, 23, 24) with these cells, a strong linear correlation was obtained between efflux and HDL-cholesterol, apoA-I, and HDL-PL (Fig. 2A–C). Because Fu5AH cells have high levels of SR-BI expression, the correlations found between efflux and the HDL parameters were not surprising (13, 24).

Efflux of cholesterol from cholesterol-normal and -enriched MPMs

The same 15 sera used in the Fu5AH experiments were then used as acceptors to measure their ability in promoting efflux from either normal or cholesterol-enriched MPMs. Loading of MPMs was accomplished by incubation with 50 μg/ml AcLDL added to the cells during the 24 h labeling period, after which efflux to sera was for 8 h. The average percentage of cholesterol efflux promoted by the 15 individual sera from unloaded MPMs was 14.1 ± 0.2%.

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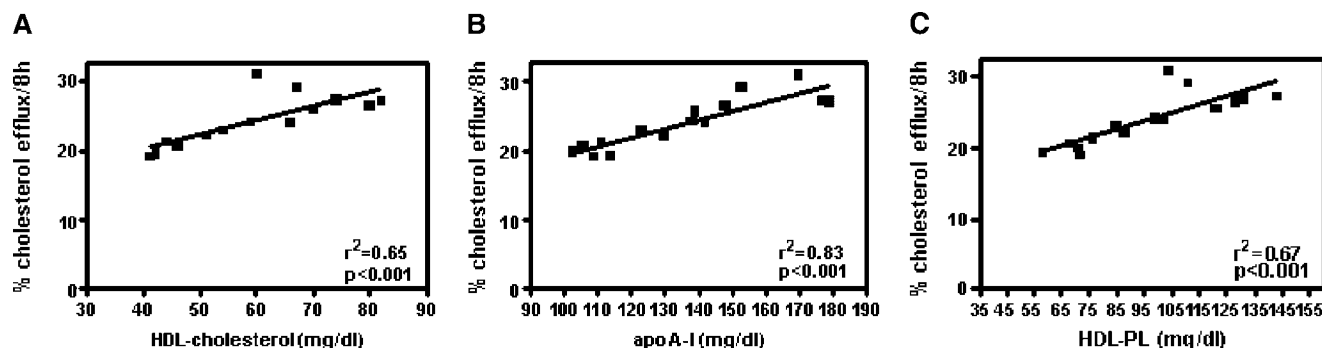

Fig. 2. Correlations between cholesterol efflux from Fu5AH cells and serum HDL parameters. Fu5AH cells were treated as described for Fig. 1. The linear relationships were between fractional efflux from Fu5AH cells and HDL-cholesterol (A), HDL-apolipoprotein A-I (apoA-I) (B), or HDL-phospholipid (PL) (C), of the sera used as acceptors. Initial cell cholesterol content was 11.8 ± 0.7 μg cholesterol/mg protein.

With the AcLDL-treated MPMs, the fractional release increased significantly, resulting in an average efflux of 19.3 ± 0.3% (**Fig. 3A**). Figure 3B shows the same efflux results but expressed in mass terms as μg cholesterol released/mg protein. The average cholesterol mass that was released to the 15 sera from cholesterol-normal MPMs was 4.6 ± 0.1 μg/mg protein. This value was increased by ~2-fold upon cholesterol enrichment of the cells. If the increase in cholesterol mass released was a consequence only of an expanded cell cholesterol pool (~40%), the mass of cholesterol released would have been expected to be 6.6 ± 0.1 μg/mg protein, significantly lower than the 9.0 ± 0.1 μg/mg protein that was actually observed. This result suggested that the increased efflux observed after cholesterol loading was only partially related to a larger cholesterol pool, but it also reflected the induction of an additional efflux mechanism, presumably linked to the increased expression of a protein(s) mediating cholesterol efflux.

Relationship between cholesterol efflux from MPMs to serum and HDL-related parameters

As done previously with Fu5AH cells (Fig. 2), we established the relationships between cholesterol efflux from MPMs to the HDL-related parameters of each serum. As shown in **Fig. 4**, after exposure of unloaded MPMs to the 15 samples for 8 h, efflux of cholesterol linearly correlated with HDL-cholesterol (Fig. 4A), apoA-I (Fig. 4B), and HDL-PL (Fig. 4C). Furthermore, the r^2 calculated for each of the correlations was similar to that obtained with Fu5AH cells. However, after enriching MPMs with cholesterol, the relationships between efflux and HDL parameters were either lost (HDL-cholesterol; Fig. 4D) or greatly reduced, as shown for apoA-I and HDL-PL (Fig. 4E, F, respectively).

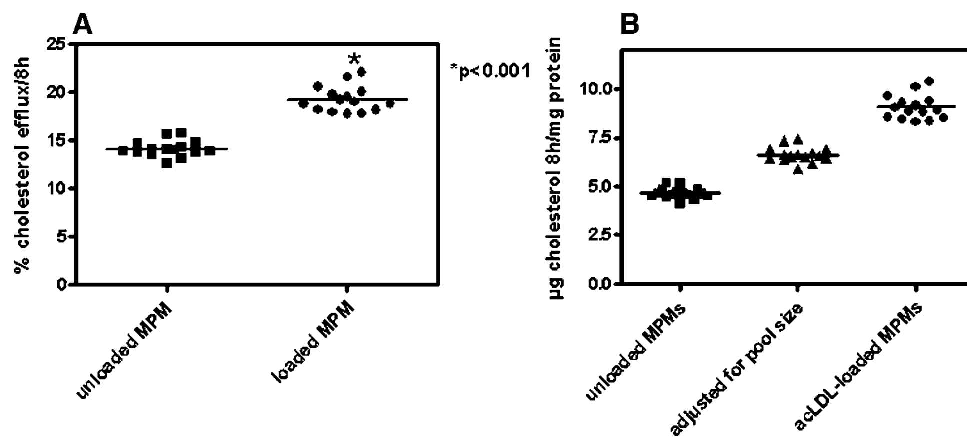

Fig. 3. A: Scattergram of cholesterol efflux from unloaded and acetylated low density lipoprotein (AcLDL)-loaded mouse peritoneal macrophages (MPMs) to 15 human sera. MPMs were radiolabeled for 24 h with 3 μCi/ml [^{3}H]cholesterol in RPMI + 2.5% FBS and in the presence or absence of 50 μg/ml AcLDL. Cells were then equilibrated for 2 h in RPMI + 0.2% BSA. Efflux was promoted by incubating cells with each individual human serum at 2.5% for 8 h. The results are expressed as means ± SD (n = 3). B: The same efflux results are converted to μg cholesterol released/mg protein by multiplying the percentage efflux by the initial cell cholesterol content. Initial cell cholesterol content was 32.8 ± 4.5 μg cholesterol/mg protein in normal cells and 46.9 ± 6.3 μg cholesterol/mg protein in enriched cells. Data represent averages from four separate experiments, each conducted in triplicate.

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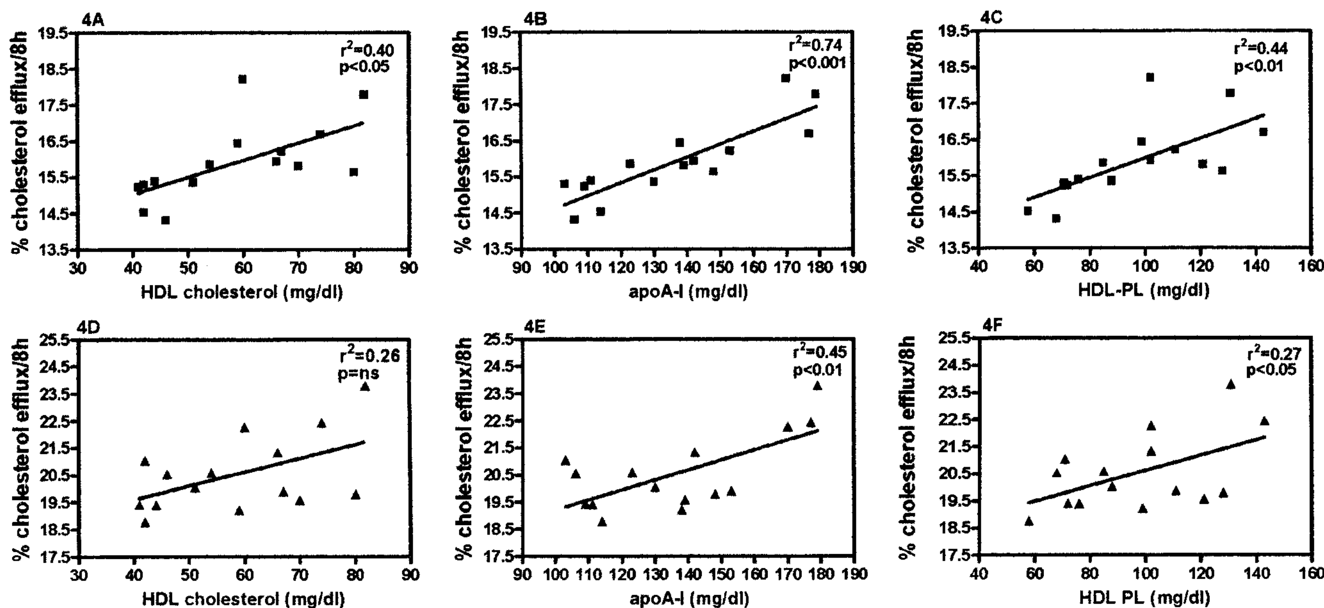

Fig. 4. Correlations of cholesterol efflux from unloaded or cholesterol-loaded MPMs and serum HDL parameters. MPMs were treated as described for Fig. 3. The relationships shown were between fractional efflux from unloaded MPMs and HDL-cholesterol (A), apoA-I (B), or HDL-PL (C) of the sera and between fractional efflux from AcLDL-loaded MPMs and HDL-cholesterol (D), apoA-I (E), or HDL-PL (F) of the sera used as acceptors. Initial cholesterol content was 32.8 ± 4.5 μg cholesterol/mg protein in normal cells and 46.9 ± 6.3 μg cholesterol/mg protein in enriched cells. Data represent averages from four separate experiments, each conducted in triplicate.

Relationship between cholesterol efflux from MPMs and cholesterol efflux from Fu5AH cells

To compare data obtained with Fu5AH cells and macrophages, we plotted the percentage cholesterol efflux from cholesterol-normal MPMs against cholesterol efflux from Fu5AH cells. As shown in **Fig. 5A**, the strong linear correlation between fractional efflux was consistent with the model in which the two types of cells behaved similarly in terms of the promotion of cholesterol efflux to serum. This relationship was lost after loading MPMs with AcLDL (Fig. 5B), possibly reflecting the induction of additional efflux pathways when MPMs were enriched with cholesterol.

Cholesterol efflux to serum from SR-BI KO MPMs

We first evaluated the role of SR-BI in the serum-dependent stimulation of fractional cholesterol release from macrophages grown in AcLDL. We performed efflux experiments using MPMs from both WT and SR-BI KO

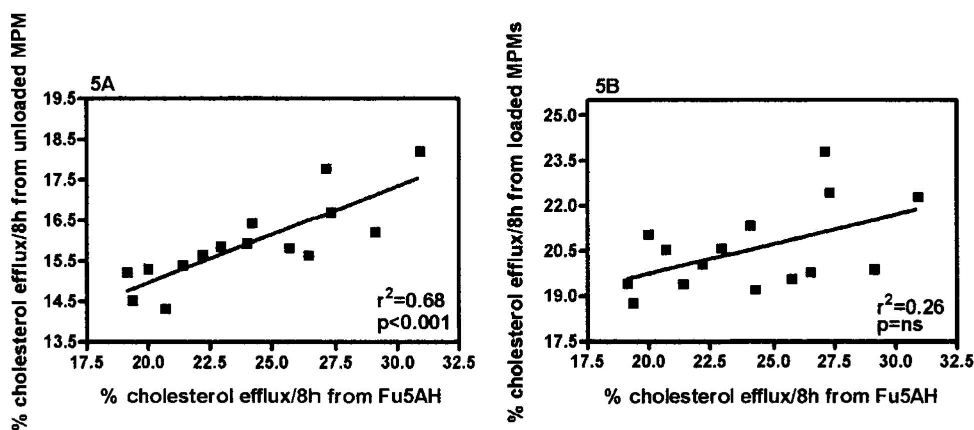

Fig. 5. Correlations between cholesterol efflux from Fu5AH cells and cholesterol efflux from unloaded (A) or AcLDL-loaded (B) MPMs. MPMs were radiolabeled for 24 h with 3 μCi/ml [^{3}H]cholesterol in RPMI + 2.5% FBS and in the presence or absence of AcLDL (50 μg/ml). Initial cholesterol content was 32.8 ± 4.5 μg cholesterol/mg protein in normal cells and 46.9 ± 6.3 μg cholesterol/mg protein in enriched cells. Fu5AH cells were radiolabeled for 24 h with 3 μCi/ml [^{3}H]cholesterol in DMEM + 2.5% calf serum. Cells were then equilibrated for 2 h in 0.2% BSA-containing medium. Efflux was promoted by incubating the cells with each individual human serum at 2.5% for 8 h. Data represent averages from four separate experiments, each conducted in triplicate.

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mice, exposed for 8 h to a pool of human serum added at 2.5%. The percentage efflux from unloaded MPMs to the serum was similar for both WT and KO mouse-derived cells (11.0 ± 0.3% and 11.5 ± 1.5%, respectively). Loading of macrophages with AcLDL caused a doubling in fractional cholesterol release. This happened with approximately the same magnitude in WT and SR-BI KO cells (23.1 ± 0.3% and 28.6 ± 0.8) (**Fig. 6**). These observations clearly indicated that the SR-BI receptor did not contribute to the stimulation of efflux we observed after cholesterol enrichment of MPMs. With cholesterol-normal cells, either WT or SR-BI KO, the major contributor to efflux was aqueous diffusion. Upon cholesterol enrichment, the greatest contribution to efflux occurred via the ABCA1 pathway, with a significant contribution from ABCG1 (Fig. 6).

Cholesterol efflux to serum from ABCA1 KO MPMs

In studies similar to those described above for SR-BI, we measured cholesterol efflux from unloaded and loaded MPMs harvested from either WT or ABCA1 KO mice and exposed for 8 h to the pool of human serum at 2.5% (**Fig. 7**). The objective of the experiment was to establish the contribution of the ABCA1 receptor to the stimulation of cholesterol efflux from cholesterol-enriched MPMs and to estimate the contributions of other pathways to total efflux in control and ABCA1 KO cells. In cholesterol-normal cells, either WT or KO, ~80% of the efflux was via the aqueous diffusion pathway. Loading WT macrophages with AcLDL increased cholesterol efflux from 12.9 ± 0.3% to 30.9 ± 0.6%. This increase could be attributed to the upregulation of both ABCA1 and ABCG1. In the ABCA1 KO cells, the loading with FC stimulated the efflux from 11.5 ± 0.4% to 15.3 ± 0.8%. The reduction in total efflux from cholesterol-enriched ABCA1 KO cells compared with cholesterol-enriched WT MPMs can be attributed primarily to the elimination of the ABCA1 component of efflux (Fig. 7).

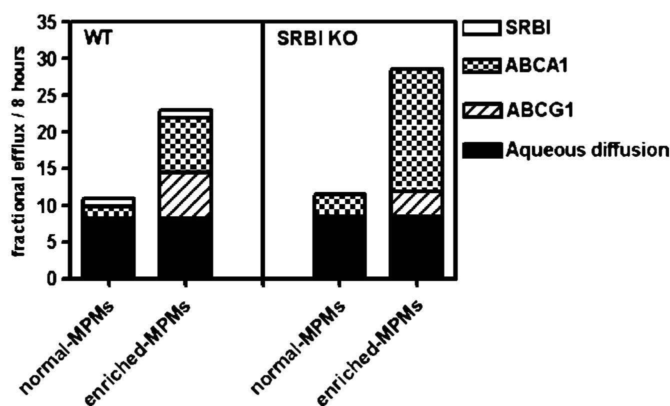

Fig. 6. Cholesterol efflux to human serum from unloaded and AcLDL-loaded MPMs from either wild-type (WT) or scavenger receptor class B type I (SR-BI) knockout (KO) mice. MPMs were radiolabeled for 24 h with 3 μCi/ml [^{3}H]cholesterol in RPMI + 2.5% FBS and in the presence or absence of AcLDL (50 μg/ml). Cells were then pretreated for 2 h with RPMI + 0.2% BSA, 2-hexyl-1-cyclopentanone thiosemicarbazone (BLT-1; 1 μmol/l), probucol (20 μmol/l), or both BLT-1 (1 μmol/l) and probucol (20 μmol/l). Efflux was promoted by incubating the cells with a pool of human serum at 2.5% for 8 h. The results are expressed as means ± SD (n = 3). Initial cholesterol content was 37.6 ± 1.7 and 39.7 ± 1.8 μg cholesterol/mg protein in normal cells (WT and KO, respectively) and 45.9 ± 1.0 and 55.4 ± 1.4 μg cholesterol/mg protein in enriched cells (WT and KO, respectively). Mice used for this study were on a C57BL/6/S129 background.

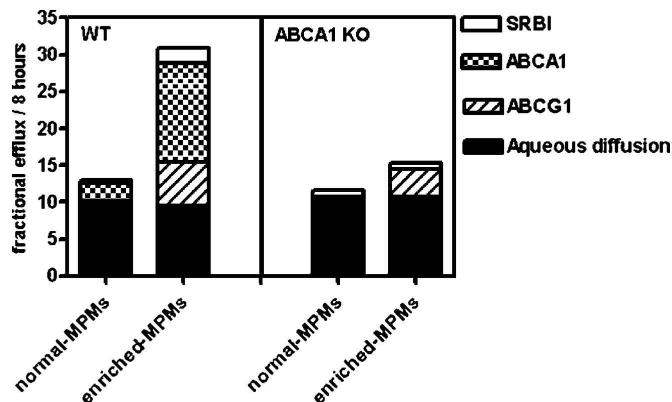

Fig. 7. Cholesterol efflux to human serum from unloaded and AcLDL-loaded MPMs from either WT or ABCA1 KO mice. MPMs were treated as described for Fig. 6. Initial cholesterol content was 36.8 ± 20.9 and 37.6 ± 1.2 μg cholesterol/mg protein in normal cells (WT and KO, respectively) and 50.7 ± 0.7 and 53.9 ± 1.9 μg cholesterol/mg protein in enriched cells (WT and KO, respectively). The results are expressed as means ± SD (n = 3). Mice used in this study were on a DBA1 lac/Jd background.

Cholesterol efflux to serum from ABCG1 KO MPMs

To assess the role of ABCG1 in the stimulated fractional release of cholesterol after loading of MPMs with AcLDL, we performed efflux experiments on macrophages derived from either WT and ABCG1 KO mice (**Fig. 8**). The fractional release of cholesterol to serum increased from

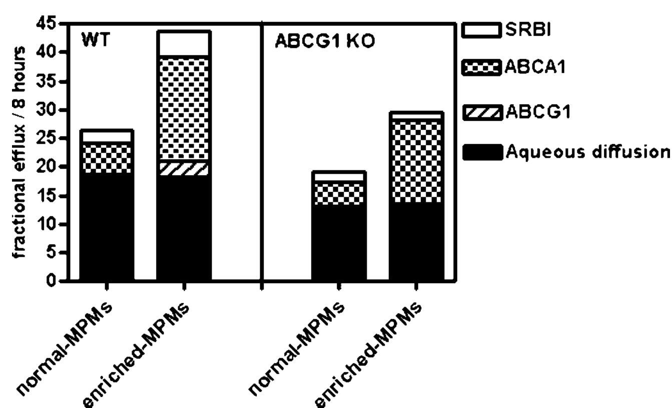

Fig. 8. Cholesterol efflux to human serum from unloaded and AcLDL-loaded MPMs from either WT or ABCG1 KO mice. MPMs were treated as described for Fig. 6. Initial cholesterol content was 25.7 ± 0.4 and 27.8 ± 2.7 μg cholesterol/mg protein in normal cells (WT and KO, respectively) and 42.7 ± 3.7 and 34.2 ± 2.1 μg cholesterol/mg protein in enriched cells (WT and KO, respectively). The results are expressed as means ± SD (n = 3). Mice used in this study were on a C57BL/6 background.

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26.3 ± 0.8% to 43.9 ± 2.1% in WT macrophages. Elimination of ABCG1 reduced the percentage stimulation of cholesterol efflux in cholesterol-enriched cells compared with unloaded cells (from 19.1 ± 0.6% to 29.5 ± 0.6%). Thus, eliminating ABCG1 reduced the stimulation of efflux occurring upon cholesterol enrichment, but the impact of the removal of ABCG1 was less dramatic than that observed with ABCA1 KO macrophages (Fig. 7).

The assumption that cholesterol loading does not change fractional efflux was consistent with the efflux data generated using Fu5AH cells (Fig. 1) and by Johnson et al. (22). This was further confirmed using cholesterol-normal and -enriched ABCG1 KO cells. When these cells were treated with probucol and BLT-1, aqueous transfer was essentially the only pathway remaining, and aqueous diffusion was similar in both cholesterol-normal and -enriched ABCG1 KO macrophages (Fig. 8). The contribution of ABCG1 could not be measured directly but was estimated as the residual efflux remaining after subtracting from total efflux the contributions of aqueous diffusion, ABCA1, and SR-BI.

DISCUSSION

The efflux of cholesterol from macrophages in the atherosclerotic plaque represents one of the earliest steps of RCT (4). The regulation of the movement of cholesterol out of macrophages is influenced by both the array of potential extracellular acceptors and the cholesterol status of the cells. Most published studies have used either isolated lipoproteins or purified apolipoproteins as cholesterol acceptors, and few have used whole serum, with its mixture of lipoproteins and apolipoproteins. In the present study, we examined the impact of enriching MPMs with cholesterol in terms of the serum lipoprotein composition and the participation of the various efflux pathways that have been identified in MPMs. To obtain mechanistic information, we specifically concentrated on the efflux component of the bidirectional flux of cholesterol that occurs when cells are exposed to cholesterol-containing lipoproteins. The mechanisms promoting cholesterol efflux are much easier to address than cholesterol influx, which involves the uptake of both FC and esterified cholesterol by a number of different mechanisms (19).

Contribution of the diffusional pathway to FC efflux

Our initial studies compared cholesterol-normal and cholesterol-enriched Fu5AH hepatoma cells and determined fractional efflux when these cells were exposed to 15 normolipidemic samples of human serum. We observed that fractional efflux to the sera did not change when the cells were enriched with cholesterol (Fig. 1). When cholesterol fractional efflux values were converted to cholesterol mass efflux, there was an increase in mass efflux; however, this increase could be accounted for simply by efflux occurring from an increased size of the cell cholesterol pool. The Fu5AH hepatoma cells are very rich in SR-BI (25), and efflux from these cells occurs by both the SR-BI pathway and a contribution by the aqueous diffusion pathway (26). Both of these pathways for efflux are sensitive to the level of HDL to which the cells are exposed, and the strong linear correlations between efflux and the HDL-related components shown in Fig. 2 are consistent with numerous reports demonstrating that efflux from these hepatoma cells is very closely linked to HDL concentration and subfraction composition (27). The similar fractional efflux values observed with cholesterol-normal and -enriched cells is consistent with a model in which the mechanism(s) for efflux does not change upon enrichment.

In contrast to the results from the hepatoma cells, cholesterol enrichment of MPMs resulted in a large increase in fractional efflux when these cells were exposed to the 15 serum samples (Fig. 3A). When the percentage efflux values are converted to cholesterol mass, there is a 200% increase in mass efflux from the enriched cells, with only ~45% of this increase attributable to cell cholesterol pool expansion. The increase in fractional efflux upon cholesterol loading indicates that the enrichment either increased the contribution to efflux of already existing pathways or upregulated additional pathways. Examination of the relationships between efflux from both cholesterol-normal and -enriched MPMs and HDL parameters further supports this interpretation. Correlations between efflux from cholesterol-normal cells to HDL-cholesterol, apoA-I, and HDL-PL were all very high (Fig. 4A–C) and similar to those obtained with Fu5AH cells (Fig. 2). However, when the same analysis was done using efflux values obtained with cholesterol-enriched MPMs, the relationships between percentage efflux and HDL parameters were lost or reduced considerably (Fig. 4D–F). This reduction can be attributed to the upregulation of ABCA1, which uses lipid-free or lipid-poor apolipoproteins as cholesterol acceptors (28–30). Thus, with cholesterol-enriched MPMs, there is a large contribution from ABCA1-mediated efflux to specific HDL lipid-free/poor subfractions such as preβ-HDL (27). It can be estimated that free apoA-I in 2.5% human serum is ~3 μg/ml, and the correlation between percentage efflux and apoA-I level will be specifically sensitive to variations among individuals in this small concentration. To further emphasize these differences, we correlated the efflux from Fu5AH cells to the efflux obtained with cholesterol-normal MPMs (Fig. 5A) and cholesterol-enriched MPMs (Fig. 5B). The relationship between the efflux values with the hepatoma versus unloaded MPMs is high, whereas the correlation with efflux from cholesterol-loaded MPMs is not significant.

To identify the protein(s) that was upregulated upon cholesterol loading of MPMs, we used macrophages derived from either WT or genetically manipulated mice, in which SR-BI, ABCA1, or ABCG1 were knocked out. To further quantitate the contributions of different efflux pathways in control and KO MPMs, we used probucol to inhibit ABCA1 and BLT-1 to block SR-BI-mediated efflux. The stimulation of efflux by cholesterol enrichment was determined with both WT and KO MPMs. As can be seen from Fig. 6, the removal of SR-BI had no effect on the fractional

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efflux from either cholesterol-normal or -enriched cells. In addition, knocking out SR-BI, ABCA1, or ABCG1 had little or no effect on efflux from cholesterol-normal cells. This demonstrated that the dominant mechanism for efflux from cholesterol-normal MPMs is aqueous diffusion, ranging from 70% to 90% of total efflux, whereas SR-BI makes little or no contribution to efflux.

Contributions of ABC transporters to FC efflux

A similar approach to that described above for SR-BI (Fig. 6) was used to determine whether ABCA1 contributed to the upregulated fractional cholesterol efflux from MPMs (Fig. 7). The percentage efflux was similar for cholesterol-normal WT and ABCA1 KO cells, but there was much less stimulation of efflux with the ABCA1 KO cells upon cholesterol loading. The removal of ABCA1 had essentially no effect on total efflux when cell cholesterol levels were normal, but removal of the contribution of ABCA1 in enriched cells reduced total efflux by 50%. These results indicate that ABCA1 has little or no impact on efflux from cholesterol-normal MPMs but mediates a large component of the efflux that is stimulated when MPMs are cholesterol loaded.

The contribution of ABCG1 to total cholesterol efflux from MPMs was obtained in a similar manner (Fig. 8). With the ABCG1 KO cells, there was a reduction in efflux in both the cholesterol-normal and cholesterol-enriched states. Because there was essentially no ABCG1 contribution in cholesterol-normal WT cells and only ~5% efflux per 8 h, or 10% of the total efflux in enriched-WT cells, the reduction in efflux observed with the ABCG1 KO macrophages reflects a general reduction in efflux via all pathways (compare cholesterol-normal WT with cholesterol-normal KO and enriched WT with enriched KO in Fig. 8). This general reduction in all efflux mechanisms was not observed in the SR-BI KO and ABCA1 KO cells and may be linked to a model in which ABCG1 functions primarily as an intracellular transporter that enriches the plasma membrane with cholesterol that then can be removed by a variety of different acceptors (9).

Based on the above studies, we estimate that ABCA1-mediated efflux to human serum contributes ~80% of the stimulated component of total cholesterol efflux, with ABCG1-mediated efflux responsible for the remaining 20% of the stimulation of efflux. It follows that enhanced aqueous diffusion is not responsible for the stimulation in efflux from MPMs obtained upon cholesterol loading, because essentially all of this stimulation can be attributed to ABCA1 and ABCG1. ABCA1-mediated efflux is associated with lipid-poor apoA-I acceptor particles (preβ-HDL), and such particles constitute only a small fraction of the total HDL (31, 32). In the future, it will be necessary to determine the HDL subfraction distribution of serum to establish the relationships between the various HDL fractions, the expression level of ABCA1, and the stimulation of efflux obtained when MPMs are enriched with cholesterol.

The physiological significance of the estimates of efflux pathways obtained in the present study using MPMs is greatly enhanced by comparison of the in vitro results with in vivo data collected using a mouse RCT assay (33). In the in vivo protocol, labeled macrophages from KO mice were injected into the peritoneum of WT recipient animals, and the appearance of labeled cholesterol in plasma was determined after 24 h (34). The reduction in plasma radioactivity compared with WT MPMs reflects the contribution of the specific pathway to the early steps of RCT. **Figure 9** compares the estimates of pathway contributions obtained using the in vitro approach, described in this study, with estimates determined from in vivo mouse RCT assays (34). The in vivo study demonstrated that ABCA1 plays a major role in removing labeled cholesterol from macrophages injected intraperitoneally in mice. ABCG1 also made a significant contribution to in vivo RCT, whereas SR-BI had no impact (34). The results obtained using these two entirely different protocols are remarkably similar (Fig. 9).

In this study, we enriched the MPMs with FC by exposure to AcLDL in the presence of an ACAT inhibitor. The accumulation of FC in the cells was below that causing toxicity, as determined microscopically and by lactate dehydrogenase release. The differences in the level of enrichment reflect different strains of mice and different preparations of AcLDL, along with the normal variation in the metabolism of cholesterol between different preparations of MPMs. We chose to use only FC-enriched cells and not to study cells having expanded cholesteryl ester stores because the presence of cholesteryl ester complicates the determination of fractional release. Under some conditions, the hydrolysis of cholesteryl ester becomes rate-limiting. In addition, obtaining equal cholesterol specific activities in the FC and cholesteryl ester pools is not readily feasible. Our primary comparative measurement for cholesterol efflux is fractional (percentage) efflux. Changes in fractional efflux reflect changes in efflux kinetics (rate constant) associated with changes in the mechanisms of efflux (35). Although differences in the absolute level of efflux probably reflect both variation in receptor expression encountered with different preparations of MPMs and differences related to different strains of mice, a gen-

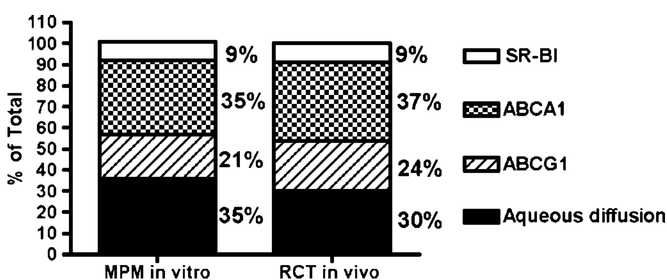

Fig. 9. Comparison of the contributions of cholesterol efflux pathways as determined from in vitro and in vivo studies. Data are from in vitro experiments (left) and mouse in vivo reverse cholesterol transport (RCT) assays (34) (right). The in vitro data are averages of nine independent studies using cholesterol-enriched WT MPMs and include cells obtained from the different strains of mice used in this study. The in vivo values were determined from the radioactivity in the plasma of recipient mice at 24 h after the injection of radiolabeled MPMs obtained from control and KO donor animals (34).

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eral efflux pattern was apparent. Thus, it can be concluded that *1*) the primary mechanism for cholesterol efflux to serum from cholesterol-normal MPMs is aqueous diffusion, with a much smaller contribution from ABCA1; *2*) SR-BI plays a relatively minor role in efflux from macrophages, either cholesterol-normal or -enriched; *3*) cholesterol enrichment of MPMs produces a large increase in the contribution of ABCA1 to total efflux; and *4*) ABCG1 does not contribute to efflux from cholesterol-normal cells but is upregulated upon cholesterol enrichment and then contributes between 10% and 25% of the total efflux to 2.5% human serum.

In the present study, we chose to use 2.5% human serum as an acceptor. This concentration was selected for two reasons: *1*) the levels of lipoproteins that are present in interstitial fluid are less than in whole serum (∼10%) (36), and the ratio of lipoproteins to cell mass is presumably much higher with cells in culture than in vivo; and *2*) many samples of human serum at concentrations >10% are toxic to mouse cells, as a result of the presence of antibodies that cross-react with antigens on mouse cells (37, 38). The relative contributions of the various pathways to cholesterol efflux are those established with 2.5% human serum. There were some changes in the contributions of the pathways when human serum concentrations ranged from 2.5% to 7.5%. The most important change was a reciprocal shift in the relative contributions of ABCA1 and ABCG1 as serum concentrations increased. Changes in the relative contribution of pathways as serum concentrations increase are to be expected, because ABCA1-mediated efflux saturates (39), whereas others, such as SR-BI and aqueous diffusion, do not demonstrate saturation kinetics (40).

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