THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 48, Issue of November 30, pp. 44976–44983, 2001

Printed in U.S.A.

Acid Sphingomyelinase-deficient Macrophages Have Defective Cholesterol Trafficking and Efflux*

Received for publication, July 10, 2001, and in revised form, September 24, 2001
Published, JBC Papers in Press, September 28, 2001, DOI 10.1074/jbc.M106455200

Andrew R. Leventhal‡, Wengen Chen‡, Alan R. Tall‡, and Ira Tabas‡§¶

From the ‡Departments of Medicine and §Anatomy & Cell Biology, Columbia University, New York, New York 10032

Cholesterol efflux from macrophage foam cells, a key step in reverse cholesterol transport, requires trafficking of cholesterol from intracellular sites to the plasma membrane. Sphingomyelin is a cholesterol-binding molecule that transiently exists with cholesterol in endosomes and lysosomes but is rapidly hydrolyzed by lysosomal sphingomyelinase (L-SMase), a product of the acid sphingomyelinase (ASM) gene. We therefore hypothesized that sphingomyelin hydrolysis by L-SMase enables cholesterol efflux by preventing cholesterol sequestration by sphingomyelin. Macrophages from wild-type and ASM knockout mice were incubated with [^{3}H]cholesteryl ester-labeled acetyl-LDL and then exposed to apolipoprotein A-I or high density lipoprotein. In both cases, [^{3}H]cholesterol efflux was decreased substantially in the ASM knockout macrophages. Similar results were shown for ASM knockout macrophages labeled long-term with [^{3}H]cholesterol added directly to medium, but not for those labeled for a short period, suggesting defective efflux from intracellular stores but not from the plasma membrane. Cholesterol trafficking to acyl-coenzyme A:cholesterol acyltransferase (ACAT) was also defective in ASM knockout macrophages. Using filipin to probe cholesterol in macrophages incubated with acetyl-LDL, we found there was modest staining in the plasma membrane of wild-type macrophages but bright, perinuclear fluorescence in ASM knockout macrophages. Last, when wild-type macrophages were incubated with excess sphingomyelin to "saturate" L-SMase, [^{3}H]cholesterol efflux was decreased. Thus, sphingomyelin accumulation due to L-SMase deficiency leads to defective cholesterol trafficking and efflux, which we propose is due to sequestration of cholesterol by sphingomyelin and possibly other mechanisms. This model may explain the low plasma high density lipoprotein found in ASM-deficient humans and may implicate L-SMase deficiency and/or sphingomyelin enrichment of lipoproteins as novel atherosclerosis risk factors.

Cholesteryl ester (CE)[1]-loaded macrophages, or foam cells, are prominent features of atherosclerotic lesions and play important roles in lesion progression (1, 2). During atherogenesis, intimal macrophages internalize atherogenic lipoproteins, including modified forms of LDL, that have been retained in the arterial subendothelium (1, 3, 4). This event directly leads to esterification of cellular cholesterol by acyl-coenzyme A:cholesterol *O*-acyltransferase (ACAT), resulting in "foam cell" formation (3, 5). Foam cell formation can be prevented or reversed by the process known as cellular cholesterol efflux (6). Cholesterol efflux is the initial step of reverse cholesterol transport, a process whereby excess cholesterol in peripheral cells is delivered to the liver for excretion (6). Thus, the elucidation of cellular molecules and pathways that facilitate and regulate cholesterol efflux is a major goal of research in the area of atherosclerosis.

Atherogenic lipoproteins internalized by macrophages deliver their stores of cholesterol, which are mostly in the form of CE, to late endosomes and/or lysosomes (3). Here, lysosomal acid lipase hydrolyzes the CE to free cholesterol, which is then transported by poorly defined mechanisms to various sites in the cell (3). A major site of transport is the plasma membrane, and from there the cholesterol can be effluxed to extracellular acceptors, such as apoA-I and HDL, or transported to ACAT in the endoplasmic reticulum for re-esterification (3, 6). Efflux to apoA-I involves the initial formation of phospholipid-apoA-I particles by ABCA1-mediated phospholipid efflux, followed by cholesterol efflux to these phospholipid-rich particles (7, 8). Cholesterol efflux to HDL can be mediated by scavenger receptor type B1 (SR-B1) in those cell types that have relatively high levels of this receptor, such as human monocyte-derived macrophages, but another mechanism must be involved in cells that have very low expression of SR-B1, such as mouse peritoneal macrophages[2] (6, 9, 10).

Given that all pathways of cholesterol efflux require cholesterol transport to the plasma membrane, the identification of molecules mediating or regulating this transport process is an important goal. Thus far, only the molecules npc1, npc2 (HE1), and possibly lysobisphosphatidic acid have been shown to play a role in cholesterol transport to the plasma membrane, and the molecular mechanisms are poorly understood (11–15). We reasoned that another molecule, lysosomal sphingomyelinase (L-SMase), may also be involved in cholesterol transport from lysosomes to the plasma membrane. L-SMase, a product of the

* This work was supported by Specialized Center of Research in Atherosclerosis Grant HL-56984 (to A. R. T. and I. T.) from the NHLBI, National Institutes of Health and a research grant from Berlex Biosciences (to I. T.). The Columbia University Confocal Microscope Facility used for this study was established by National Institutes of Health Shared Instrument Grants S10 RR10506 and S10 RR13701 and the Lieber Foundation. The operation of the Facility is supported in part by National Institutes of Health Grant P30 CA13696 as part of the Herbert Irving Comprehensive Cancer Center at Columbia University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Medicine, Columbia University, 630 West 168th St., New York, NY 10032. Tel.: 212-305-9430; Fax: 212-305-4834; E-mail: iat1@columbia.edu.

[1] The abbreviations used are: CE, cholesteryl ester; ACAT, acyl-CoA: cholesterol acyltransferase; apoA-I, apolipoprotein A-I; ASM, acid sphingomyelinase; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HDL, high-density lipoprotein; LDL, low-density lipoprotein; L-SMase, lysosomal sphingomyelinase; NPC, Niemann-Pick C; PBS, phosphate-buffered saline; PS, phosphatidylserine; SMase, sphingomyelinase; SR-BI, scavenger receptor BI.

[2] Y. Sun and A. R. Tall, unpublished data.

acid sphingomyelinase (ASM) gene, hydrolyzes sphingomyelin in late endosomes and lysosomes (16). Because SM avidly binds cholesterol (17, 18), we hypothesized that sphingomyelin hydrolysis by L-SMase enables cholesterol transport by preventing cholesterol sequestration by sphingomyelin. Of interest, humans with ASM deficiency (types A and B Niemann-Pick disease) have low plasma HDL levels (19, 20), which could result from defective cholesterol efflux (*cf.* Ref. 21).

In this context, we show herein that macrophages from ASM knockout mice, which lack L-SMase (22, 23), have a defect in cholesterol efflux to both apoA-I and HDL, a decrease in the esterification of cellular cholesterol, and an accumulation of cholesterol in perinuclear vesicles. Moreover, a defect in cholesterol efflux was also observed in wild-type macrophages that internalized a large amount of sphingomyelin. These data support the hypothesis that intracellular accumulation of sphingomyelin due to L-SMase deficiency or to internalization of excess sphingomyelin leads to cholesterol sequestration and defective cholesterol trafficking and efflux.

EXPERIMENTAL PROCEDURES

Materials—The Falcon tissue culture plasticware used in these studies was purchased from Fisher Scientific Co. Tissue culture media and other tissue culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). Alexa 543-labeled dextran-10,000 was purchased from Molecular Probes, Inc. (Eugene, OR). All radiochemicals were purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). All other chemicals and reagents were from Sigma, and all organic solvents were from Fisher Scientific Co.

Macrophages—Littermate wild-type and homozygous ASM knockout mice were obtained by breeding ASM heterozygous knockout mice, which were provided by Dr. Edward Schuchman, Mt. Sinai School of Medicine (22, 23). Macrophages were harvested from the peritoneum of these mice 3 days after the intraperitoneal injection of 40 μg of concanavalin A in 0.5 ml of PBS and then cultured as described previously (24).

Lipoproteins and Liposomes—LDL (*d*, 1.020–1.063 g/ml), HDL_2 (*d*, 1.063–1.125 g/ml), HDL_3 (*d*, 1.125–1.21 g/ml) from fresh human plasma were isolated by preparative ultracentrifugation as described (25). Acetyl-LDL was prepared by reaction with acetic anhydride (26) and labeled with [^{3}H]CE as described (27). Briefly, label was transferred from [^{3}H]cholesteryl oleate-containing liposomes to HDL_3 by CE transfer protein, followed by CE transfer protein-mediated transfer of the [^{3}H]CE from HDL_3 to acetyl-LDL, which were then separated from each other by density ultracentrifugation. The final specific activity of the labeled acetyl-LDL was 17 cpm/ng of protein. [^{3}H]Cholesterol-containing liposomes were prepared by first mixing 100 μCi of [^{3}H]cholesterol and 2.8 mg of phosphatidylserine (PS) in chloroform, in the absence or presence of 1.25 mg of sphingomyelin. The chloroform was evaporated under nitrogen, and the dried lipids were sonicated in 3 ml of PBS at 4 °C under argon using 15 4-min bursts at setting number 2 on a Branson 450 sonicator equipped with a tapered microtip. [^{14}C]Sphingomyelin-containing liposomes were made in the same manner except 1.2 μCi of [^{14}C]sphingomyelin was used instead of [^{3}H]cholesterol. For certain experiments in which we needed to fluorescently label PS-sphingomyelin liposomes, the PBS contained 0.5 mg/ml Alexa 543-labeled dextran-10,000; after sonication, the solution was dialyzed using dialysis tubing with a 100,000-MW pore size.

Labeling of Cells with [^{3}H]Cholesterol—Cell monolayers were labeled by one of three methods. For labeling by acetyl-LDL or PS liposomes, cells were incubated for 4 h in DMEM, 0.2% BSA containing 10 μg/ml [^{3}H]CE-acetyl-LDL or [^{3}H]cholesterol-labeled phosphatidylserine-liposomes such that the final PS concentration was 93 μg/ml. For long-term labeling of cellular cholesterol, macrophages were grown for 24 h in DMEM, 10% FBS containing 0.5 μCi/ml [^{3}H]cholesterol, then equilibrated for 12 h in DMEM, 0.2% BSA. The [^{3}H]cholesterol-containing medium was made by adding the labeled cholesterol, which was in ethanol, dropwise (0.5 μl/ml) into DMEM, 10% FBS while stirring at 37 °C. After a 30-min incubation at 37 °C, the medium was passed through a 0.45-μm filter. For short-term labeling experiments, macrophages were incubated for 15 min at room temperature in DMEM containing methyl-β-cyclodextrin-[^{3}H]cholesterol complex. To prepare this solution, methyl-β-cyclodextrin and unlabeled cholesterol (8:1 molar ratio) were dissolved in DMEM and sonicated using a Fisher Scientific Sonic Dismembranator-60 at level 5 for 5 min. [^{3}H]Cholesterol (1 μCi/ml) was then added, and the solution was incubated overnight in a shaking water bath at 37 °C. The solution was then filtered through a 0.45-μm filter.

Cholesterol Efflux Assay—After labeling the cells, the medium was changed to DMEM, 0.2% BSA containing 20 μg/ml apoA-I, 15 μg/ml HDL_2, or 10 μg/ml HDL_3. At the indicated time points, 100 μl of media was removed and spun for 5 min at 14,000 rpm in a microcentrifuge to remove cellular debris, and the radioactivity in this fraction of media was quantified by liquid scintillation counting. After the last time point, the remainder of the media was removed, and the cells were dissolved in 1 ml of 0.1 N NaOH at room temperature for 5 h. A 100-μl aliquot of the cell lysate was counted, and the percent efflux was calculated as [(media cpm) ÷ (cell + media cpm)] × 100.[3] Note that there was no statistical difference in cellular counts/min between wild-type and ASM-deficient macrophages using any of the three [^{3}H]cholesterol labeling methods. To obtain the value for acceptor-stimulated efflux, the percent efflux in the absence of acceptor (*i.e.* DMEM, 0.2% BSA without apoA-I or HDL) was subtracted from the percent efflux in the presence of acceptor; the basal efflux values were <10% of those in the presence of acceptor.

Whole Cell Cholesterol Esterification Assay—In the first method, macrophages were incubated in DMEM, 0.2% BSA containing 0.1 mM [^{14}C]oleate complexed with albumin and 3 μg/ml acetyl-LDL. In the second method, cells were labeled with [^{3}H]cholesterol by long-term incubation with [^{3}H]cholesterol-labeled medium as described above, followed by incubation with 3 μg/ml unlabeled acetyl-LDL or 5 μM 25-hydroxycholesterol for up to 12 h. At the indicated time points, the cells were washed two times with cold PBS, and the cell monolayers were extracted twice with 0.5 ml of hexane/isopropyl alcohol (3:2, v/v) for 30 min at room temperature. Whole cell cholesterol esterification activity was assayed by determining the cellular content of cholesteryl [^{14}C]oleate or [^{3}H]cholesteryl ester by thin-layer chromatography (28). The cell monolayers were dissolved in 1 ml of 0.1 N NaOH, and aliquots were assayed for protein by the method of Lowry *et al.* (29).

In Vitro ACAT Assay—Macrophages in 100-mm dishes were incubated for 24 h in DMEM, 10% lipoprotein-deficient serum and then scraped in 2.5 ml of ice-cold 20 mM potassium phosphate buffer, 2 mM dithiothreitol, pH 7.4. This suspension was sonicated for five 3-s bursts on level 10 of a Fisher Scientific Sonic Dismembranator-60. Aliquots of the cell lysates were removed for protein determination, and then 130-μl of the cell lysates were added to capped glass test tubes that contained 20 μl of 0.5 mg/ml cholesterol-rich PS liposomes in PBS, which were prepared as described previously (30), or 20 μl of PBS alone. After a 15-min incubation at 37 °C, 30 μl of 40 mg/ml fatty acid-free BSA in 100 mM potassium phosphate buffer, 2 mM dithiothreitol, pH 7.4, and 20 μl of 25 μM [^{14}C]oleoyl-CoA (40 μCi/μmol) were added. After a 15-min incubation at 37 °C, the reaction was stopped by adding 2 ml of 2:1 chloroform:methanol containing 250 μg of unlabeled CE internal standard. The mixture was vortexed and allowed to sit at room temperature for 30 min. Four hundred μl of 0.88% KCl were added to each tube, and the tubes were then centrifuged at 1000 rpm for 10 min. The lower organic phase was collected and separated by thin layer chromatography, and the CE spot was scraped and counted.

Filipin Staining of Free Cholesterol and Fluorescence Microscopy—For filipin staining, the method of Blanchette-Mackie *et al.* (31) was employed. Briefly, a 0.05 mg/ml filipin solution was made from a 5 mg/ml stock in Me_2SO by dilution with 10% FBS in PBS. Macrophages were cultured on polylysine-coated coverslip bottom dishes (32) and preincubated for 24 h in DMEM, 10% LPDS. After the incubation described in the legend to Fig. 5, the cells were washed with PBS and fixed with 3% paraformaldehyde in PBS for 1 h at room temperature. The macrophages were subsequently rinsed three times with PBS and incubated with 1.5 mg of glycine/ml of PBS for 10 min at room temperature. Next, the cells were incubated with the 0.05 mg/ml filipin solution for 2 h at room temperature. The cells were then washed three times with PBS and viewed with a Zeiss Axiovert S100 epifluorescence microscope using a UV filter set (340–380-nm excitation, 70-nm dichroic, 430-nm long pass filter). For the double-label filipin/Alexa 543-dextran study displayed in Fig. 6, dual-photon microscopy was con-

[3] In the experiments with apoA-I, the percent of [^{3}H]cholesterol in the medium represents net efflux, because the medium contains no unlabeled cholesterol at the beginning of the experiment. In the experiments with HDL, however, this method used in this study does not distinguish between net cholesterol efflux and exchange of cholesterol between the cells and HDL.

ducted using an LSM 510 nonlinear optics (NLO) Zeiss dual photon confocal microscope equipped with a 100X/1.3 NA Plan-Neofluor objective lens. For Alexa-546, the pinhole was adjusted to produce an optical section of 1.0 μm. A helium-neon laser (543 nm) was used for excitation and a 560-nm long-pass was used as an emission filter. For filipin, the pinhole was completely open, and a Coherent titanium-sapphire laser tuned to 800 nm was used for excitation and a 390–465-nm band pass was used as an emission filter. Pilot studies indicated that this configuration allowed good resolution of both Alexa-546 and filipin fluorescence without cross-over fluorescence.

Statistics—Results are given as mean ± S.E. (n = 3); absent error bars in the figures signify S.E. values smaller than the graphic symbols. For the data in Fig. 7*A*, the unpaired, two-tailed t test was used to determine statistically significance.

RESULTS

Efflux of Acetyl-LDL-derived [^{3}H]Cholesterol and Cellular Cholesterol Labeled by Long-term Incubation with [^{3}H]Cholesterol-containing Medium Is Defective in ASM Knockout Macrophages—To assess the role of macrophage L-SMase in the efflux of acetyl-LDL-derived cholesterol, peritoneal macrophages from wild-type and ASM knockout mice were incubated with [^{3}H]CE-labeled acetyl-LDL for 4 h and then chased in serum-free medium containing lipid-free apoA-I, which mediates cholesterol efflux through the ABC1 pathway, and HDL_3, which involves mostly other pathways (33). [^{3}H]Cholesterol derived from [^{3}H]CE-acetyl-LDL traffics through late endosomes and lysosomes, which are known sites of L-SMase activity and SM accumulation in ASM-deficient cells (16). As shown in Fig. 1, cholesterol efflux to both apoA-I (*panel A*) and HDL_3 (*panel B*) was reduced by ~60–70% in the ASM knockout macrophages. For example, efflux to apoA-I at 12 h was 4.3% in ASM knockout cells *versus* 13.4% in wild-type cells (Fig. 1*A*).[3]

Cellular cholesterol can also be labeled by long-term incubation with [^{3}H]cholesterol added directly to the medium. In this case, the plasma membrane is labeled first, followed by equilibration with intracellular stores that probably include recycling endosomes and the trans-Golgi network (34, 35). To assess the efflux or exchange of cellular cholesterol pools labeled in this manner, wild-type and ASM knockout macrophages were labeled for 24 h in the presence of 0.5 μCi/ml [^{3}H]cholesterol. After a 12-h equilibration period, the cells were incubated with HDL, and [^{3}H]cholesterol in the medium was measured. As shown in Fig. 2*A*, there was an ~50% decrease in the percent [^{3}H]cholesterol in the medium of ASM knockout macrophages under these conditions.

One interpretation of the data in Fig. 2*A* is that ASM knockout macrophages have a defect in the efflux or exchange of plasma membrane cholesterol. To address this issue, wild-type and ASM knockout macrophages were incubated for 15 min at room temperature with [^{3}H]cholesterol-charged methyl-β-cyclodextrin, and then chased for 1 or 3 h in medium without label but containing HDL. According to Lange *et al.* (34), the 15-min labeling procedure labels mostly plasma membrane cholesterol, which then eventually equilibrates with intracellular pools. As shown in Fig. 2*B*, medium [^{3}H]cholesterol at the early time point was the same in the two cell types, while after a 3-h chase it was less in the ASM knockout macrophages. These data suggest that transfer of cholesterol directly from the plasma membrane to HDL is not defective in ASM knockout macrophages but that efflux from the intracellular site(s) that accumulates nonlipoprotein cholesterol is affected.

In view of these data, we considered the possibility that a decrease in expression of ABCA1 or SR-B1 could explain the defect in efflux. However, quantitative polymerase chain reaction showed no difference in ABCA1 mRNA expression between wild-type and ASM knockout macrophages (data not shown). Although there could be differences in ABCA1 localization or activity, a defect in cholesterol efflux from ASM knockout

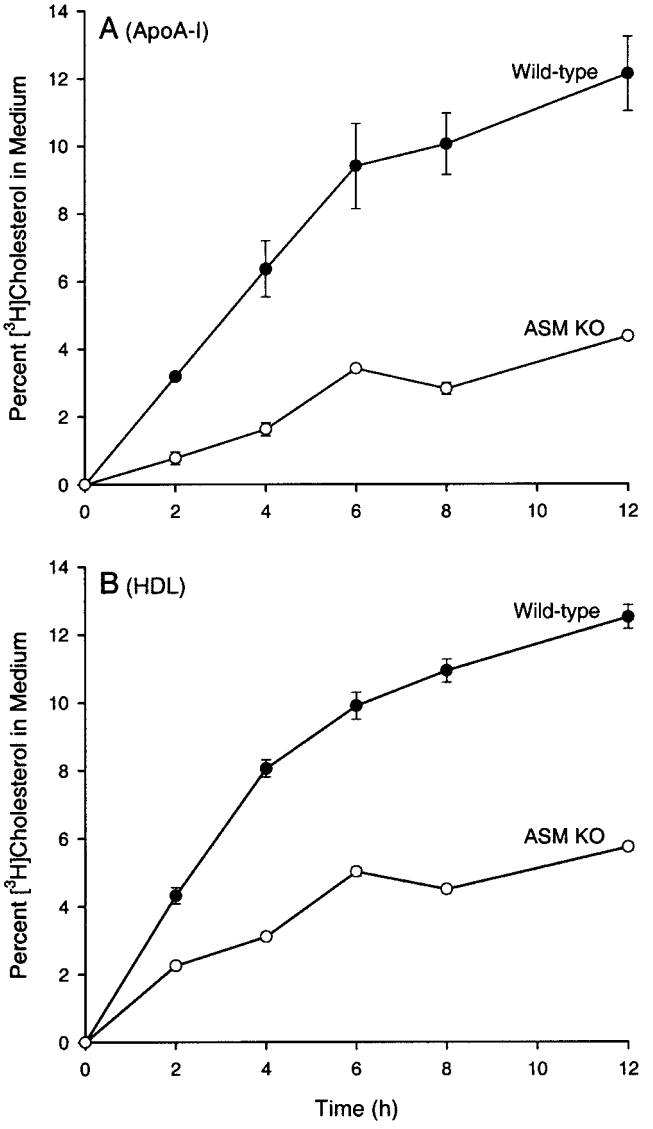

FIG. 1. **Efflux of acetyl-LDL-derived [^{3}H]cholesterol from wild-type and ASM knockout macrophages.** Monolayers of peritoneal macrophages from wild-type (*closed circles*) or ASM knockout (*open circles*) mice were incubated with 10 μg/ml [^{3}H]CE-labeled acetyl-LDL in DMEM, 0.2% BSA for 4 h. The cells were then rinsed and incubated with fresh medium containing either 20 μg/ml human apoA-I (*A*) or 10 μg/ml human HDL_3 (*B*) for the indicated times. ^{3}H-Labeled cpm in the media and cells were measured to calculate percent [^{3}H]cholesterol in the medium.

macrophages was shown using HDL_2 (Fig. 2*B* and data not shown), which mediates efflux by an ABCA1-independent mechanism (7). SR-B1 almost certainly is not involved, because its expression is very low in mouse peritoneal macrophages,[2] and an SR-B1 neutralizing antibody does not block cholesterol efflux from these cells to HDL.[4] Given these data, we focused our efforts on the hypothesis that decreased efflux in ASM knockout macrophages was caused by defective intracellular cholesterol trafficking.

Trafficking of Cholesterol to ACAT Is Defective in ASM Knockout Macrophages—To determine if other cellular cholesterol trafficking pathways were altered in ASM knockout macrophages, we assessed the ability of the cells to esterify cholesterol, which requires cholesterol transport to ACAT in the endoplasmic reticulum (3). First, wild-type and ASM

[4] W. Chen and A. R. Tall, unpublished data.

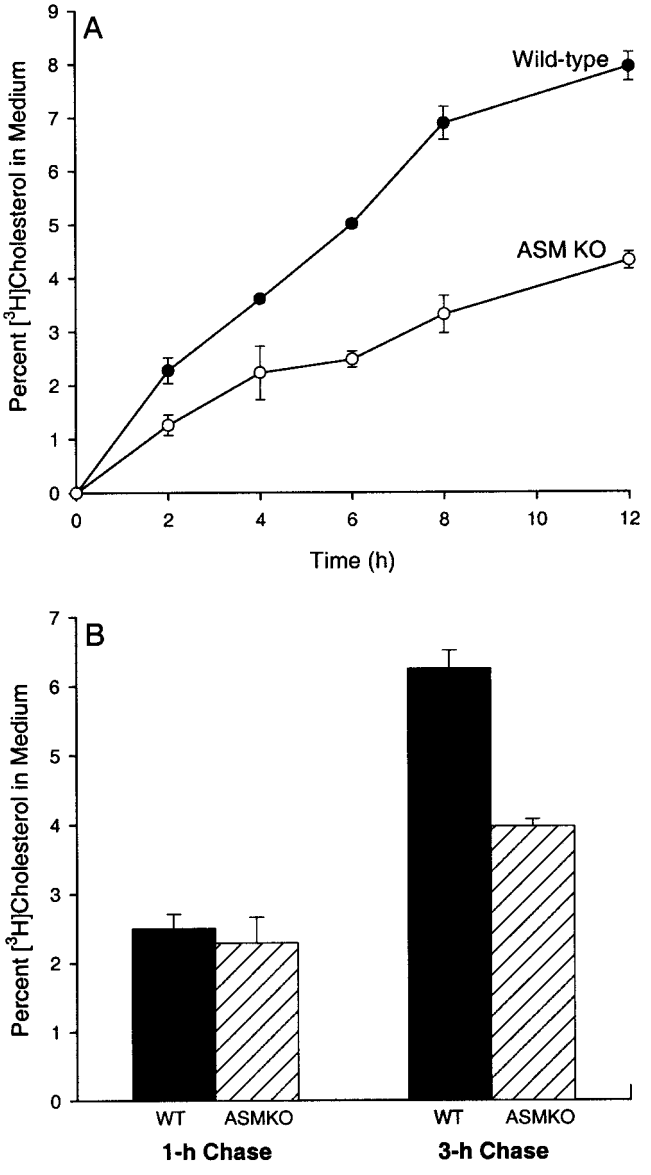

FIG. 2. **Efflux of cellular cholesterol labeled by long-term incubation of macrophages with [^{3}H]cholesterol-containing medium.** *A*, macrophages from wild-type (*closed circles*) or ASM knockout (*open circles*) mice were incubated for 24 h in DMEM, 10% FBS containing 0.5 μCi/ml [^{3}H]cholesterol. The cells were then incubated for an additional 12 h in DMEM, 0.2% BSA without label, and then finally incubated in the same medium containing 10 μg/ml HDL_3 for the indicated times. Percent [^{3}H]cholesterol in the medium was then determined. *B*, macrophages from wild-type (*solid bars*) or ASM knockout (*hatched bars*) mice were incubated for 15 min at room temperature in DMEM, 0.2% BSA containing 1 μCi/ml [^{3}H]cholesterol-charged methyl-β-cyclodextrin. The cells were then rinsed and incubated with fresh medium containing 15 μg/ml HDL_2 for either 1 or 3 h, and then percent [^{3}H]cholesterol in the medium was determined.

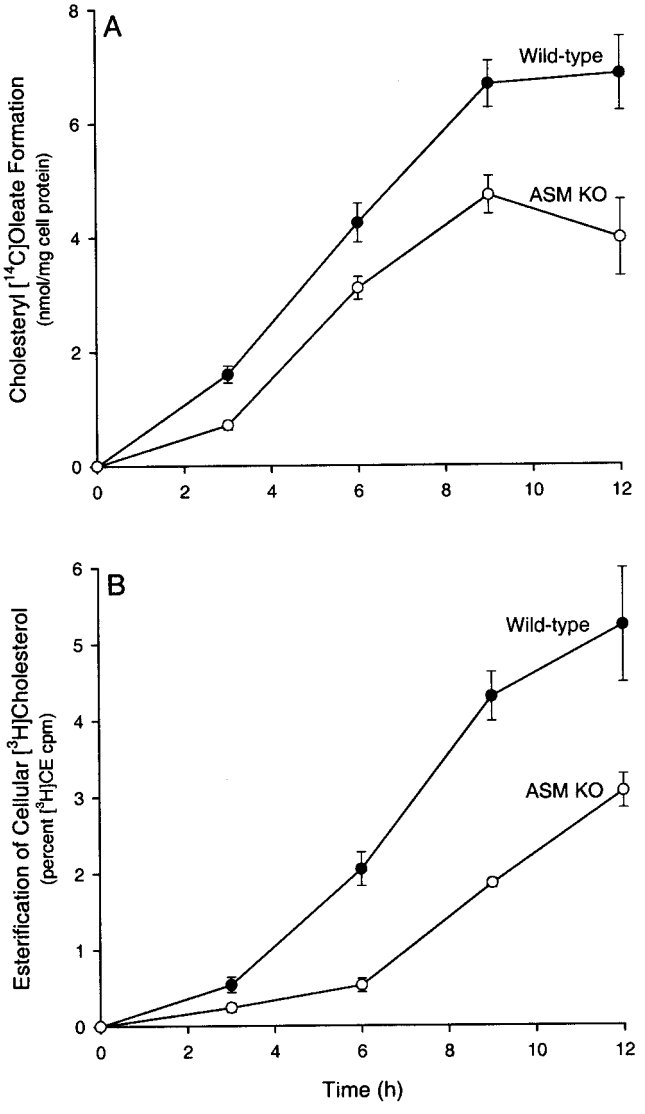

FIG. 3. **Cholesterol esterification in wild-type and ASM knockout macrophages incubated with acetyl-LDL.** *A*, macrophages from wild-type (*closed circles*) or ASM knockout (*open circles*) mice were incubated for the indicated times in DMEM, 0.2% BSA containing 3 μg/ml acetyl-LDL and 0.1 mM [^{14}C]oleate. Cellular lipids were then extracted and assayed for cholesteryl [^{14}C]oleate. *B*, the macrophages were incubated for 24 h in DMEM, 10% FBS containing 0.5 μCi/ml [^{3}H]cholesterol. The cells were then incubated for an additional 12 h in DMEM, 0.2% BSA without label, and then finally incubated in the same medium containing 3 μg/ml acetyl-LDL for the indicated times. Cellular lipids were extracted and assayed for [^{3}H]cholesteryl ester.

knockout macrophages were incubated with acetyl-LDL and [^{14}C]oleate, and the formation of cholesteryl [^{14}C]oleate was assayed. Under these conditions, acetyl-LDL-derived cholesterol mixes with cellular cholesterol in the plasma membrane, and when a threshold level of cholesterol is reached, this cholesterol is transported to ACAT and esterified (36, 37). As shown in Fig. 3*A*, ASM knockout macrophages demonstrated a 30–50% decrease in CE formation. Next, the macrophage cholesterol pools were labeled by long-term incubation with [^{3}H]cholesterol-labeled medium (above), and the formation of [^{3}H]cholesteryl ester in response to subsequent incubation with acetyl-LDL was assayed (Fig. 3*B*). Using this experimental protocol, there was even a greater defect in cholesterol esterification in the ASM knockout cells.

A similar experiment to that in Fig. 3*B* was conducted, but 25-hydroxycholesterol was used as the stimulator of ACAT instead of acetyl-LDL (36). In this scenario, the formation of [^{3}H]cholesteryl ester was almost totally abolished in the ASM knockout macrophages (Fig. 4*A*). We next determined if these data could be explained by decreased active ACAT enzyme in the ASM knockout macrophages. Wild-type and ASM knockout macrophages were incubated for 24 h in the absence of lipoproteins, and then lysates from these cells were assayed for ACAT activity *in vitro* in the absence or presence of exogenous cholesterol (Fig. 4*B*). In the absence of exogenous cholesterol, ACAT activity was low and similar in both cell types; in the presence of exogenous cholesterol, ACAT activity was higher than that in the absence of cholesterol and, surprisingly, somewhat greater in the lysates of ASM knockout mice. Although we

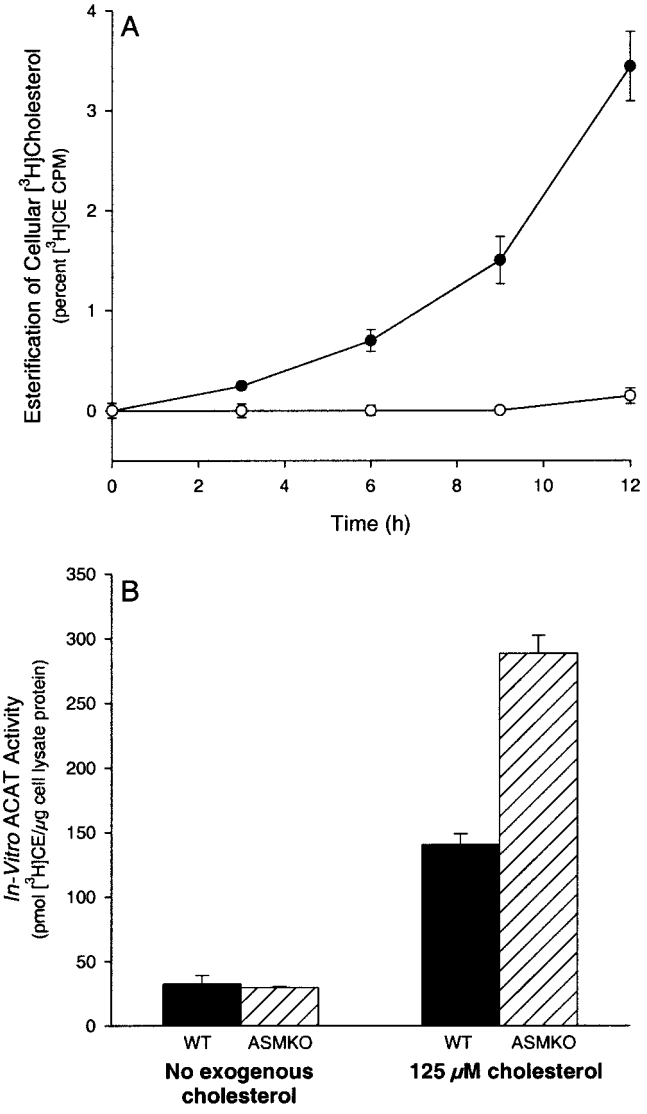

FIG. 4. **25-Hydroxycholesterol-induced cholesterol esterification and *in vitro* ACAT activity in wild-type and ASM knockout macrophages.** *A*, macrophages from wild-type (*closed circles*) or ASM knockout (*open circles*) mice were incubated for 24 h in DMEM, 10% FBS containing 0.5 μCi/ml [^{3}H]cholesterol. The cells were then incubated for an additional 12 h in DMEM, 0.2% BSA without label, and then finally incubated in the same medium containing 5 μg/ml 25-hydroxychoelsterol for the indicated times. Cellular lipids were extracted and assayed for [^{3}H]cholesteryl ester. *B*, lysates of macrophages from wild-type (*solid bars*) or ASM knockout (*hatched bars*) mice were assayed for ACAT activity in the absence or presence of 125 μM cholesterol.

do not know the cause of this increase, the data strongly suggest that the defect in cholesterol esterification in ASM knockout macrophages (Fig. 3) reflects a defect in cholesterol trafficking to ACAT rather than decreased active ACAT enzyme.

ASM Knockout Macrophages Accumulate Free Cholesterol in Perinuclear Sites—To directly assess the intracellular distribution of free cholesterol, wild-type and ASM knockout macrophages were incubated with acetyl-LDL (4-h incubation followed by 4-h chase) and then stained with filipin, a fluorescent free cholesterol-binding molecule. The filipin-staining pattern in wild-type macrophages was modest in intensity, although greater than in cells not exposed to acetyl-LDL, and the plasma membrane was the principal site of staining (Fig. 5*A*). In striking contrast, ASM knockout macrophages showed bright, perinuclear, punctate fluorescence (Fig. 5*B*). These data directly

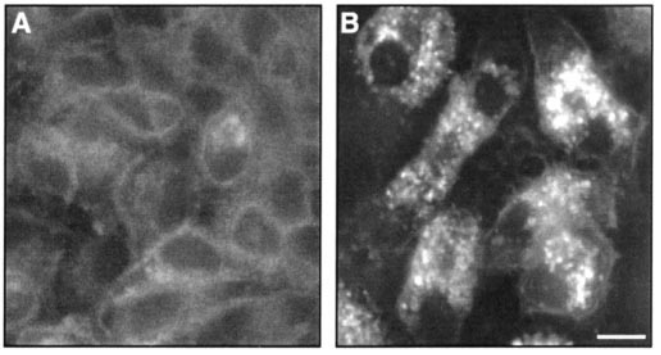

FIG. 5. **Free cholesterol distribution in wild-type and ASM knockout macrophages.** Macrophages from wild-type (*A*) or ASM knockout (*B*) mice were incubated with DMEM, 0.2% BSA containing 100 μg of acetyl-LDL/ml for 4 h and then chased in medium without lipoproteins for an additional 4 h. The cells were then fixed, stained with filipin, and visualized by fluorescence microscopy. *Bar*, 5 μm.

demonstrate intracellular sequestration of cholesterol in ASM knockout macrophages.

We next determined if the free cholesterol that accumulates in ASM-deficient macrophages is localized solely or mostly in late endosomes and lysosomes. Macrophages from ASM knockout mice were incubated with acetyl-LDL and Alexa 546-labeled dextran for 4 h, and then chased in medium alone for an additional 4 h. The dextran is taken up by fluid-phase pinocytosis and, after a 4-h chase, labels late endosomes and lysosomes; for example, in macrophages and other cells, the staining pattern with dextran is essentially identical to that with the lysosomal marker LAMP1 (38). After fixing and staining with filipin to detect the cholesterol, the cells were viewed by dual-photon confocal fluorescence microscopy. An example of one ASM-deficient macrophage is shown in Fig. 6, *A–C*, and a cluster of macrophages is shown in Fig. 6, *D–F*. *Panels A* and *D* show the filipin staining pattern (pseudocolored *green*), *panels B* and *E* the Alexa-dextran pattern, and *panels C* and *F* the merged images. As in Fig. 5*B*, free cholesterol accumulated in a perinuclear distribution (*A* and *D*). Alexa-dextran also accumulated in a perinuclear pattern (*B* and *E*), which is consistent with a late endosome/lysosome pattern (38). Although there was some co-localization of cholesterol and Alexa-dextran (*yellow* vesicles in *panels C* and *F*), there were also a number of vesicles that were distinct, as indicated by the pure *red* and pure *green* vesicles in *panels C* and *F*. Thus, the cholesterol that accumulates in ASM-deficient macrophages accumulates both in dextran-containing late endosomes/lysosomes and in other perinuclear sites (see "Discussion").

Delivery of Excess SM to Cells Can Decrease Cholesterol Efflux in Wild-type Macrophages—The data in this report support the hypothesis that the accumulation of SM in late endosomes, lysosomes, and probably other sites as a result of L-SMase deficiency leads to a defect in cholesterol efflux. According to this hypothesis, it might be possible to "saturate" L-SMase activity in wild-type cells by delivering excess SM, thus resulting in a decrease in cholesterol efflux. To test this prediction, wild-type macrophages were incubated for 4 h with PS liposomes that had tracer amounts of [^{3}H]cholesterol and either no sphingomyelin or sphingomyelin (sphingomyelin:PS, 1:2 molar ratio). Note that PS-containing liposomes, like acetyl-LDL, are endocytosed by macrophages through the type A scavenger receptor pathway and possibly through a newly identified PS receptor (39, 40). The cells were then rinsed and incubated for 12 h with fresh medium containing HDL, after which the percent [^{3}H]cholesterol in the medium was determined. Using parallel experiments in which the sphingomyelin-PS liposomes contained tracer [^{14}C]SM, we calculated that

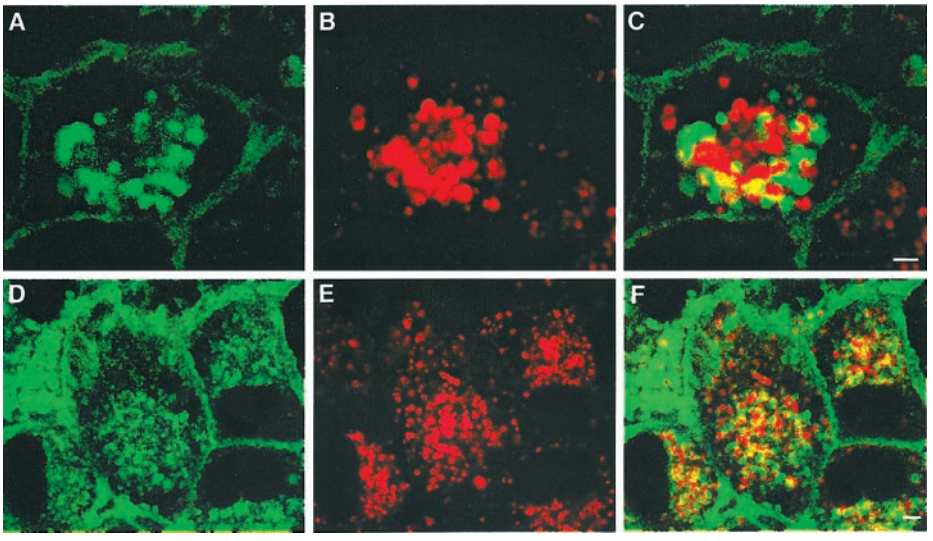

FIG. 6. **Free cholesterol and late endosome/late lysosome distribution in wild-type and ASM knockout macrophages.** Macrophages from ASM knockout were incubated with medium containing 100 μg of acetyl-LDL/ml and 0.5 mg/ml Alexa 546-labeled dextran-10,000 for 4 h and then chased in medium without lipoproteins or dextran for an additional 4 h. The cells were then fixed, stained with filipin, and visualized by dual-photon confocal fluorescence microscopy. An example of one ASM-deficient macrophage is shown in *A-C*, and a cluster of macrophages is shown in *D-F*. *Panels A* and *D* show the filipin staining pattern (pseudocolored *green*), *panels B* and *E* the Alexa-dextran pattern, and *panels C* and *F* the merged images. *Bar*, 1 μm.

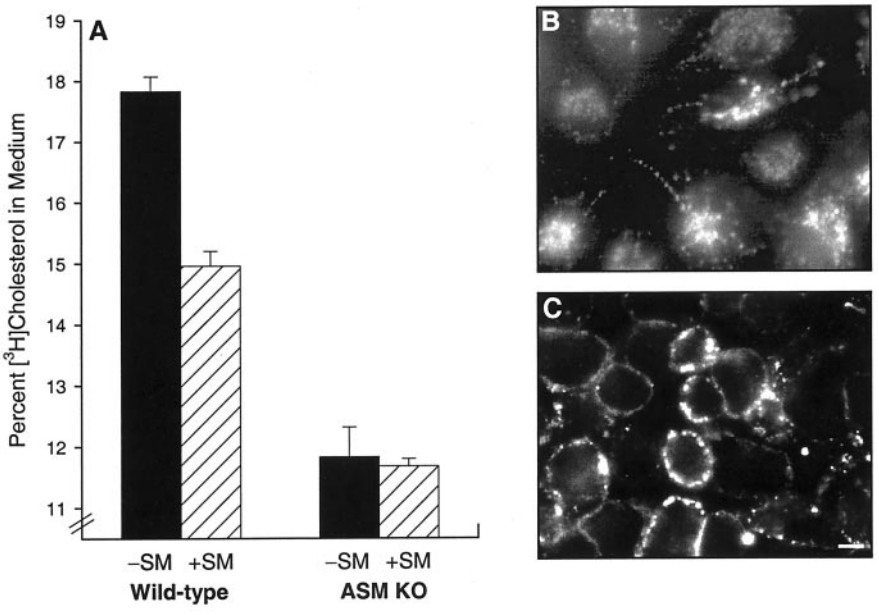

FIG. 7. **Effect of liposomal sphingomyelin content on the efflux of liposome-derived cholesterol from wild-type and ASM knockout macrophages.** *A*, macrophages from wild-type (*solid bars*) or ASM knockout (*hatched bars*) mice were incubated for 4 h with DMEM, 0.2% BSA containing PS liposomes that had tracer amounts of [^{3}H]cholesterol and either no sphingomyelin (*−SM*) or sphingomyelin (sphingomyelin:PS, 1:2 molar ratio; *+SM*). In all incubations, the final PS concentration was 93 μg/ml, and the incubation volume was 0.5 ml. The cells were then rinsed and incubated for 12 h with fresh medium containing 15 μg/ml HDL_2, after which the percent [^{3}H]cholesterol in the medium was determined. The difference in efflux between *−SM* and *+SM* liposomes in the wild-type macrophages were statistically significant ($p = 0.001$). *B*, to verify that the liposomes were internalized, macrophages were incubated with sphingomyelin/PS liposomes labeled with Alexa 543-dextran-10,000 for 4 h and then chased for an additional 3 h in medium without liposomes. *C*, for comparison of the image in *B* with a cell-surface pattern, macrophages were incubated with the Alexa-labeled liposomes for 1 h at 4 °C. *Bar*, 5 μm.

this protocol led to the delivery of 6.6 ± 0.8 μg of sphingomyelin/mg of cell protein. As expected for receptor-mediated endocytic targeting to late endosomes and lysosomes, the vesicles were internalized and delivered to perinuclear organelles (Fig. 7*B*); for comparison, Fig. 7*C* shows a typical cell-surface distribution of macrophages incubated with vesicles at 4 °C.

As shown in the first pair of *bars* in Fig. 7*A*, there was a statistically significant decrease in efflux of [^{3}H]cholesterol delivered in the sphingomyelin-containing liposomes compared with [^{3}H]cholesterol delivered in liposomes without SM. As expected, [^{3}H]cholesterol efflux from ASM knockout macrophages was decreased to an even greater degree (*second pair* of *bars* in Fig. 7*A*). Interestingly, the presence of excess sphingomyelin did not further decrease efflux in these cells, presumably because intracellular SM levels were already very high due to L-SMase deficiency in the face of exogenous SM (from prior incubation in serum-containing medium) and endogenous SM (41). These data are consistent with a model in which the activity of L-SMase in normal macrophages can be partially "saturated" by the endocytosis of SM, leading to intracellular SM accumulation and defective cholesterol trafficking.

DISCUSSION

The results of this study have implications ranging from the basic cellular biology of lipid trafficking to the important physiologic area of macrophage cholesterol efflux in atherosclerosis. The mechanisms and regulation of cholesterol trafficking from lysosomes to the plasma membrane are poorly understood. Data from mutant cells indicate that the proteins npc1 and npc2 (HE1) have functions along this pathway (11–15), and antibody experiments may suggest a role for the lipid lysobisphosphatidic acid (15). The mechanism of action of these

molecules, however, is not known. The idea that the sphingomyelin content of lysosomes could be an important regulatory factor is based upon the ability of this lipid to bind cholesterol (17, 18). Indeed, Aviram and colleagues (42) suggested that the ability of oxysterols in oxidized LDL to inhibit L-SMase in murine and human macrophages may account for the accumulation of lysosomal FC under these conditions, although molecular genetic proof was not provided to support their hypothesis. In contrast, cholesterol esterification and trafficking to the plasma membrane were reported as being normal in several lines of fibroblasts from humans with types A and B Niemann-Pick disease (43, 44). It is possible that the human fibroblasts data could have been influenced by residual L-SMase activity in these cells (16) or by inherent differences in cholesterol trafficking between fibroblasts and macrophages.

Our working hypothesis states that defective cholesterol trafficking in ASM knockout macrophages is due to sequestration of cholesterol by sphingomyelin. This model can readily explain the acetyl-LDL-cholesterol trafficking data, because acetyl-LDL-derived cholesterol traffics through lysosomes, which is a known site of SM accumulation in ASM-deficient cells (16). With regard to those experiments in which the macrophages were labeled by long-term incubation with [^{3}H]cholesterol-containing medium, it is possible that this method also labels lysosomal pools of cholesterol. However, when a similar method was used in Chinese hamster ovary cells to incorporate the fluorescent sterol dehydroergosterol or cholesterol itself, followed by filipin labeling, the major sites of accumulation were the endosomal recycling compartment and the trans-Golgi network (35). If this were the case in macrophages, it would indicate defective trafficking and efflux of *non*-lysosomal cholesterol and therefore might imply that ASM deficiency leads to SM accumulation in the endosomal recycling compartment, trans-Golgi network, or other nonlysosomal sites (*cf.* Refs. 15, 34, and 45). This idea might also provide an explanation for our finding that not all of the acetyl-LDL-derived free cholesterol that accumulates in ASM-deficient macrophages is in dextran-containing late endosomes and lysosomes (Fig. 6). Detailed sphingomyelin localization studies in ASM knockout macrophages will be required to sort out these possibilities.

One must also consider the possibility that direct sequestration of cholesterol by SM is not the only mechanism behind defective cholesterol trafficking in ASM knockout macrophages. In this context, there is evidence that initial accumulation of unesterified cholesterol in lysosomes or late endosomes can lead to *secondary* defects in vesicular trafficking. For example, the defective trafficking of lactosyl ceramide in ASM-deficient fibroblasts and the defects in late endosomal tubulovesicular trafficking observed in NPC cells can be corrected by cellular cholesterol depletion (46, 47), and defects in the trafficking of sucrose can be induced in normal cells by cellular cholesterol enrichment (48). Thus, it is possible that abnormal SM accumulation in ASM knockout macrophages causes an initial accumulation of FC in lysosomes or some other site, but that at least some of the trafficking and efflux defects observed in our studies are due to membrane vesiculation defects secondary to this initial cholesterol accumulation.

The cholesterol trafficking defect described in this report is similar to that which occurs in fibroblasts from patients with Niemann-Pick C (NPC) disease (11–14). Moreover, recent work from our groups has shown that macrophages from NPC mice have a defect in cholesterol trafficking (49). Although the molecular etiologies are distinct, important interrelationships between the cholesterol trafficking defects in ASM deficiency and Niemann-Pick C disease may exist. For example, Reagan *et al.* (50) made the interesting observation that lysosomal cholesterol accumulation in Chinese hamster ovary cells induced by either the NPC mutation or progesterone causes a decrease in the enzymatic activity of L-SMase. Therefore, in view of our data, it is possible that the cholesterol trafficking defects in NPC cells and progesterone-treated cells may be amplified by secondary inhibition of L-SMase.

The findings in this report may have important implications for foam cell biology. Macrophages in advanced lesions are known to accumulate large amount of free cholesterol, much of which appears to be in lysosomes (51–55). On the one hand, it is possible that exposure of these macrophages to oxidized LDL or oxysterols, by inhibiting L-SMase (42), or SM-rich lipoproteins, by "saturating" L-SMase (see Fig. 6), may contribute to this event. Regarding this latter possibility, Jiang *et al.* (56) recently found that a high plasma SM level is an independent risk factor for coronary artery disease in humans. On the other hand, the accumulation of lysosomal cholesterol, even if caused by another process, might be expected to secondarily inhibit L-SMase (50), which could further exacerbate the accumulation of lysosomal cholesterol and inhibit cholesterol efflux.

The findings in this study raise additional questions related to lipoprotein abnormalities and atherosclerotic risk in humans with ASM deficiency (types A and B Niemann-Pick disease). These subjects have markedly low plasma HDL levels (19, 20). Given that low plasma HDL can result from defective cellular cholesterol efflux (21), our current data may provide a mechanism that contributes to this lipoprotein abnormality. Regarding atherosclerotic risk, one must focus on type B Niemann-Pick patients, who survive to adulthood due to low levels of residual ASM activity, and type A or type B obligate heterozygotes, who are reported to be "normal" (16). In considering the potential atherogenic effects of L-SMase deficiency in these subjects, it is interesting to consider that the ASM gene also gives rise to secretory SMase (57). Because secretory SMase promotes the subendothelial aggregation and retention of lipoproteins, leading to enhanced foam cell formation, S-SMase deficiency, unlike L-SMase deficiency, may decrease cholesterol accumulation in lesional macrophages (57). Therefore, while the deficiency of S-SMase in these subjects might be protective, defective L-SMase activity, by inhibiting cholesterol efflux from lesional macrophages and possibly by leading to low HDL levels, may promote atherosclerotic vascular disease.

Acknowledgments—We thank Dr. Edward Schuchman for providing the ASM heterozygote knockout mice that were used to generate the wild-type and ASM homozygous knockout mice used in this study, Dr. Anthony Johns (Berlex Biosciences) for performing the ABCA1 quantitative polymerase chain reaction assay, and Theresa Swayne for assistance with the fluorescence microscopy studies.

REFERENCES

1. Ross, R. (1995) *Annu. Rev. Physiol.* **57,** 791–804
2. Libby, P., and Clinton, S. K. (1993) *Curr. Opin. Lipidol.* **4,** 355–363
3. Tabas, I. (2000) *Biochim. Biophys. Acta* **1529,** 164–174
4. Williams, K. J., and Tabas, I. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15,** 551–561
5. Brown, M. S., and Goldstein, J. L. (1983) *Annu. Rev. Biochem.* **52,** 223–261
6. Tall, A. R. (1998) *Eur. Heart J.* **19,** A31-A35
7. Wang, N., Silver, D. L., Thiele, C., and Tall, A. R. (2001) *J. Biol. Chem.* **276,** 23742–23747
8. Fielding, P. E., Nagao, K., Hakamata, H., Chimini, G., and Fielding, C. J. (2000) *Biochemistry* **39,** 14113–14120
9. Ji, Y., Jian, B., Wang, N., Sun, Y., Moya, M. L., Phillips, M. C., Rothblat, G. H., Swaney, J. B., and Tall, A. R. (1997) *J. Biol. Chem.* **272,** 20982–20985
10. Rothblat, G. H., Llera-Moya, M., Atger, V., Kellner-Weibel, G., Williams, D. L., and Phillips, M. C. (1999) *J. Lipid Res.* **40,** 781–796
11. Liscum, L., and Klansek, J. J. (1998) *Curr. Opin. Lipidol.* **9,** 131–135
12. Blanchette-Mackie, E. J. (2000) *Biochim. Biophys. Acta* **1486,** 171–183
13. Ory, D. S. (2000) *Biochim. Biophys. Acta* **1529,** 331–339
14. Naureckiene, S., Sleat, D. E., Lackland, H., Fensom, A., Vanier, M. T., Wattiaux, R., Jadot, M., and Lobel, P. (2000) *Science* **290,** 2298–2301
15. Kobayashi, T., Beuchat, M. H., Lindsay, M., Frias, S., Palmiter, R. D., Sakuraba, H., Parton, R. G., and Gruenberg, J. (1999) *Nat. Cell Biol.* **1,** 113–118

16. Schuchman, E. H., and Desnick, R. J. (1995) in *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 2601–2624, McGraw-Hill, New York
17. Slotte, J. P. (1999) *Chem. Phys. Lipids* **102,** 13–27
18. Ridgway, N. D. (2000) *Biochim. Biophys. Acta* **1484,** 129–141
19. Viana, M. B., Giugliani, R., Leite, V. H., Barth, M. L., Lekhwani, C., Slade, C. M., and Fensom, A. (1990) *J. Med. Genet.* **27,** 499–504
20. Worgall, T. S., McGovern, M. M., Jiang, X. C., Berglund, L., Shea, S., Tabas, I., and Deckelbaum, R. J. (2001) *Circulation* **102,** II–30
21. Tall, A. R., and Wang, N. (2000) *J. Clin. Invest.* **106,** 1205–1207
22. Horinouchi, K., Erlich, S., Perl, D., Ferlinz, K., Bisgaier, C. L., Sandhoff, K., Desnick, R. J., Stewart, C. L., and Schuchman, E. H. (1995) *Nature Gen.* **10,** 288–293
23. Otterbach, B., and Stoffel, W. (1995) *Cell* **81,** 1053–1061
24. Tang, W., Walsh, A., and Tabas, I. (1999) *Biochim. Biophys. Acta* **1437,** 301–316
25. Havel, R. J., Eder, H., and Bragdon, J. (1955) *J. Clin. Invest.* **34,** 1345–1353
26. Basu, S. K., Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3178–3182
27. Rinninger, F., Brundert, M., Jackle, S., Kaiser, T., and Greten, H. (1995) *Biochim. Biophys. Acta* **1255,** 141–153
28. Tabas, I., Boykow, G. C., and Tall, A. R. (1987) *J. Clin. Invest.* **79,** 418–426
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
30. Tabas, I., Marathe, S., Keesler, G. A., Beatini, N., and Shiratori, Y. (1996) *J. Biol. Chem.* **271,** 22773–22781
31. Blanchette-Mackie, E. J., Dwyer, N. K., Amende, L. M., Kruth, H. S., Butler, J. D., Sokol, J., Comly, M. E., Vanier, M. T., August, J. T., Brady, R. O., and Pentchev, P. G. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8022–8026
32. Tabas, I., Lim, S., Xu, X., and Maxfield, F. R. (1990) *J. Cell Biol.* **111,** 929–940
33. Wang, N., Silver, D. L., Costet, P., and Tall, A. R. (2000) *J. Biol. Chem.* **275,** 33053–33058
34. Lange, Y., Ye, J., Rigney, M., and Steck, T. L. (2000) *J. Biol. Chem.* **275,** 17468–17475
35. Mukherjee, S., Zha, X., Tabas, I., and Maxfield, F. R. (1998) *Biophys. J.* **75,** 1915–1925
36. Tabas, I., Rosoff, W. J., and Boykow, G. C. (1988) *J. Biol. Chem.* **263,** 1266–1272
37. Xu, X., and Tabas, I. (1991) *J. Biol. Chem.* **266,** 17040–17048
38. Botelho, R. J., Hackam, D. J., Schreiber, A. D., and Grinstein, S. (2000) *J. Biol. Chem.* **275,** 15717–15727
39. Nishikawa, K., Arai, H., and Inoue, K. (1990) *J. Biol. Chem.* **265,** 5226–5231
40. Fadok, V. A., Bratton, D. L., Rose, D. M., Pearson, A., Ezekewitz, R. A., and Henson, P. M. (2000) *Nature* **405,** 85–90
41. Levade, T., Salvayre, R., Maret, A., and Douste-Blazy, L. (1988) *J. Inherit. Metab. Dis.* **11,** 151–157
42. Maor, I., Mandel, H., and Aviram, M. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15,** 1378–1387
43. Liscum, L., Ruggiero, R. M., and Faust, J. R. (1989) *J. Cell Biol.* **108,** 1625–1636
44. Pentchev, P. G., Comly, M. E., Kruth, H. S., Vanier, M. T., Wenger, D. A., Patel, S., and Brady, R. O. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 8247–8251
45. Cruz, J. C., Sugii, S., Yu, C., and Chang, T. Y. (2000) *J. Biol. Chem.* **275,** 4013–4021
46. Puri, V., Watanabe, R., Dominguez, M., Sun, X., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (1999) *Nat. Cell Biol.* **1,** 386–388
47. Zhang, M., Dwyer, N. K., Neufeld, E. B., Love, D. C., Cooney, A., Comly, M., Patel, S., Watari, H., Strauss, J. F., III, Pentchev, P. G., Hanover, J. A., and Blanchette-Mackie, E. J. (2001) *J. Biol. Chem.* **276,** 3417–3425
48. Zhang, M., Dwyer, N. K., Love, D. C., Cooney, A., Comly, M., Neufeld, E., Pentchev, P. G., Blanchette-Mackie, E. J., and Hanover, J. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4466–4471
49. Chen, W., Sun, Y., Welch, C., Gorelik, A., Leventhal, A. R., Tabas, I., and Tall, A. R. (2001) *J. Biol. Chem.* **276,** 43564–43569
50. Reagan, J. W., Jr., Hubbert, M. L., and Shelness, G. S. (2000) *J. Biol. Chem.* **275,** 38104–38110
51. Lundberg, B. (1985) *Atherosclerosis* **56,** 93–110
52. Small, D. M., Bond, M. G., Waugh, D., Prack, M., and Sawyer, J. K. (1984) *J. Clin. Invest.* **73,** 1590–1605
53. Rapp, J. H., Connor, W. E., Lin, D. S., Inahara, T., and Porter, J. M. (1983) *J. Lipid Res.* **24,** 1329–1335
54. Shio, H., Haley, N. J., and Fowler, S. (1979) *Lab. Invest.* **41,** 160–167
55. Tangirala, R. K., Mahlberg, F. H., Glick, J. M., Jerome, W. G., and Rothblat, G. H. (1993) *J. Biol. Chem.* **268,** 9653–9660
56. Jiang, X. C., Paultre, F., Pearson, T. A., Reed, R. G., Francis, C. K., Lin, M., Berglund, L., and Tall, A. R. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 2614–2618
57. Tabas, I. (1999) *Chem. Phys. Lipids* **102,** 131–139