Macrophage Sphingomyelin Synthase 2 Deficiency Decreases Atherosclerosis in Mice

Jing Liu, Chongmin Huan, Mahua Chakraborty, Hongqi Zhang, Da Lu, Ming-Shang Kuo, Guoqing Cao, Xian-Cheng Jiang

Rationale: Sphingomyelin synthase (SMS)2 contributes to de novo sphingomyelin (SM) biosynthesis and plasma membrane SM levels. SMS2 deficiency in macrophages diminishes nuclear factor κB and mitogen-activated protein kinase activation induced by inflammatory stimuli.

Objective: The effects of SMS2 deficiency on the development of atherosclerosis are investigated.

Methods and Results: We measured cholesterol efflux from macrophages of wild-type (WT) and SMS2 knockout (KO) mice. We transplanted SMS2 KO mouse bone marrow into low-density lipoprotein (LDL) receptor (LDLr) knockout mice (SMS2−/−→LDLr−/−), creating a mouse model of SMS2 deficiency in the macrophages. We found that SMS2 deficiency caused significant induction of cholesterol efflux in vitro and in vivo. Moreover, we found that SMS2 KO mice had less interleukin-6 and tumor necrosis factor α in the circulation before and after endotoxin stimulation, compared with controls. More importantly, after 3 months on a western-type diet, SMS2−/−→LDLr−/− mice showed decreased atherosclerotic lesions in the aortic arch, root (57%, P<0.001), and the entire aorta (42%, P<0.01), compared with WT→LDLr−/− mice. Analysis of plaque morphology revealed that SMS2−/−→LDLr−/− mice had significantly less necrotic core area (71%, P<0.001), less macrophage content (37%, P<0.01), and more collagen content (35%, P<0.05) in atherosclerotic lesions. We also found that SMS2−/−→LDLr−/− mice had significantly lower free cholesterol and cholesteryl ester levels in the brachiocephalic artery than WT→LDLr−/− mice (33 and 52%, P<0.01 and P<0.001, respectively).

Conclusions: SMS2 deficiency in the macrophages reduces atherosclerosis in mice. Macrophage SMS2 is thus a potential therapeutic target for treatment of this disease. (Circ Res. 2009;105:295-303.)

Key Words: macrophage sphingomyelin synthase deficiency □ sphingomyelin biosynthesis □ cholesterol efflux □ inflammation □ atherosclerosis

Foam cell formation caused by excessive accumulation of cholesterol in the macrophages is a pathological hallmark of atherosclerosis,1 which is also known to be an inflammatory disease.2 The accumulation of macrophage-derived foam cells in the vessel wall is always accompanied by the production of a wide range of chemokines and cytokines that regulate the turnover and differentiation of immigrating and resident cells and subsequent plaque development.2 Thus, promoting cholesterol efflux from cholesterol-laden macrophages, as well as diminishing their inflammatory response, can both be significant antiatherogenic approaches.

The interaction between sphingomyelin (SM), cholesterol, and glycosphingolipid drives the formation of plasma membrane rafts,3 and in some cells, caveolae.4,5 SM is synthesized by sphingomyelin synthase (SMS), which transfers the phosphorylcholine moiety from phosphatidylcholine (PC) onto ceramide.6 Two SMS genes, SMS1 and SMS2, have been cloned, and their subcellular localization characterized.7,8 SMS1 is found in the trans-Golgi apparatus, whereas SMS2 is predominantly located in the plasma membranes.7,9 Our laboratory and others have shown that SMS1 and SMS2 expression levels correlate positively with those of SM in the lipid rafts.10–12 Furthermore, SMS1 has been implicated in the regulation of lipid raft SM levels, as well as raft functions such as FAS receptor clustering,10 endocytosis, and apoptosis.11

SM-enriched lipid rafts may play important roles in cholesterol efflux. ATP-binding cassette transporter (ABC)A1, ABCG1, and scavenger receptor (SR)-B1 are located in the plasma membranes, and exist either directly in rafts (SR-B1),13 or in association with the redistribution of lipids in plasma membranes (ABCA1 and ABCG1).14 It is therefore conceivable that changes in plasma membrane SM within the SMS2-null macrophages will influence the functions of these...
proteins and alter cholesterol efflux, thus influencing the development of atherosclerosis. Indeed, enhanced apolipoprotein (apo)A–I–dependent cholesterol efflux by ABCA1 from SM-deficient Chinese hamster ovary (CHO) cells has been reported.15

Manipulation of plasma membrane SM levels can also alter the structure of lipid rafts and modify inflammatory responses. It has been reported that sphingomyelinase treatment causes the clustering of several receptors, including tumor necrosis factor (TNF) receptor,16 toll-like receptors (TLRs),17 and interleukin (IL)-1 receptor,18 thus influencing downstream signaling pathways. Nuclear factor (NF)-κB19 and mitogen-activated protein (MAP) kinases20 are the key regulators of inflammation. We found that NF-κB and MAP kinase activation is attenuated in macrophages from SMS2 knockout (KO) mice in response to LPS stimulation.21 In line with these observations, we found that SMS2 deficiency substantially diminished the abundance of TLR4-MD2 complex levels on the surface of macrophages following LPS stimulation.21

For further evaluation of the relationship between macrophage SMS2 deficiency and atherosclerosis, we transplanted SMS2-deficient mouse bone marrow into low-density lipoprotein (LDL) receptor (LDLr)-deficient mice (SMS2−/−→LDLr−/−), creating a model of SMS2 deficiency and LDLr expression exclusively in the macrophages. As a control, we also transplanted wild-type (WT) mouse bone marrow into LDLr-deficient mice (WT→LDLr−/−), creating a model of LDLr expression exclusively in the macrophages. We investigated the development of atherosclerosis in these animals.

**Methods**

**Mice and Diets**
LDLr-deficient (LDLr−/−) mice (8-week-old females) of a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, Me). SMS2 KO mice,22 originally of a 129 background, were backcrossed with C57BL/6 mice for 4 generations. The animals (WT and KO) used in this study were littermates. All were fed a Chow diet (Research Diets, Inc). Bone marrow transplantation was performed, and after 8 weeks all mice were switched to a western-type diet (0.15% cholesterol, 20% saturated fat) for 3 months. Experiments involving animals were conducted with the approval of State University of New York Downstate Medical Center Institutional Animal Care and Use Committee.

**Bone Marrow Transplantation to Replace Peripheral Macrophages**
Bone marrow cells were harvested from the tibias of donor mice (SMS2−/− and WT), as previously described.22 A total of 20 LDLr−/− mice (age 8 weeks) were lethally irradiated with 1000 rads (10 Gy). Ten of these animals were transplanted with SMS2−/− mouse bone marrow (5×106 cells), and the other 10 with WT bone marrow, via the femoral vein, all within 3 hours of irradiation. We monitored the process of cell replacement by polymerase chain reaction (PCR), using genomic DNA from mouse white blood cells as a template. The primers used for SMS2 KO mouse screening were: forward, 5’-AGTGACAAGCTGAGCACAG-3’ and reverse, 5’-GGCCATTGAAACAGATGGAT-3’. The primers for WT mouse screening were: forward, 5’-GGCCATTGAAACAGATGGAT-3’ and reverse, 5’-GACGGTTGTCAAGTGGATAGG-3’.

**Lipid Analyses by Light Chromatography/Tandem Mass Spectrometry**
SM, PC, and ceramide levels were measured by light chromatography/tandem mass spectrometry (LC/MS/MS), as previously described.21

**mRNA Analyses**
RNA was isolated from macrophages, using TRizol (Invitrogen). The primers used for mouse SMS2 RT-PCR were: forward, 5’-CAAAACTTTGAAAGCTCATTGGA-3’; and reverse, 5’-GGTGCGGCTTGTTGAAGTGTG-3’. The primers used for mouse SMS1 RT-PCR were: forward, 5’-GATGCCAGACCGCAGACAGAGAG-3’; and reverse, 5’-ACTAGCTTCTCCCGCCTGTTC-3’. 18S rRNA was used as an internal control. The forward and reverse primer sequences for 18S rRNA were 5’-AGTCCGAGGCGCTCTCAAA-3’ and 5’-GATCCGAGGCGCTCTCAAA-3’, respectively.

**SMS Activity Assay**
Macrophages were homogenized in a buffer containing 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 5% sucrose, and a cocktail of protease inhibitors (Sigma). The homogenate was centrifuged at 5000 rpm for 10 minutes, and the supernatant mixed in assay buffer containing 50 mmol/L Tris-HCl (pH 7.4), 25 mmol/L KCl, Ca2+-NB-2-ceramide (0.1 μg/μL), and PC (0.01 μg/μL). The mixture was incubated at 37°C for 2 hours. Lipids were extracted in chloroform: methanol (2:1), dried under N2 gas and separated by thin layer chromatography. Band intensity was quantified by Image-Pro Plus version 4.5 software (Media Cybernetics Inc).

**Lysenin Treatment and Cell Mortality Measurement**
Macrophages were washed twice in PBS and incubated with lysenin, 50 ng/mL, for 1 hour. Cell viability was measured using WST-1 cell

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**Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette transporter</td>
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<tr>
<td>apo</td>
<td>apolipoprotein</td>
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<tr>
<td>BCA</td>
<td>brachiocephalic artery</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>KO</td>
<td>gene knockout</td>
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<tr>
<td>LC/MS/MS</td>
<td>liquid chromatography/tandem mass spectrometry</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LDLr</td>
<td>low-density lipoprotein receptor</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
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<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SM</td>
<td>sphingomyelin</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<td>SMS</td>
<td>sphingomyelin synthase</td>
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<tr>
<td>SR-B1</td>
<td>scavenger receptor B1</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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proliferation reagent according to the instructions of the manufacturer (Roche).

**Cholesterol Efflux From Macrophages**

Mouse peritoneal macrophages were labeled with [3H]cholesterol carried by acetylated LDL. After labeling, cells were washed with PBS, equilibrated with DMEM, 0.2% BSA for 1 hour, and incubated with 10 μg/mL purified human apoA-I or high-density lipoprotein (HDL) in 0.5 mL of DMEM, 0.2% BSA. The medium was collected at 8 hours and centrifuged at 6000 g for 10 minutes to remove cell debris and cholesterol crystals. Radioactivity in an aliquot of supernatant was determined by liquid scintillation counting. The cells were finally lysed in 0.5 mL of 0.1 mol/L sodium hydroxide, 0.1% sodium dodecyl sulfate (SDS), and the radioactivity in an aliquot was determined. Cholesterol efflux was expressed as the percentage of the radioactivity released from the cells into the medium relative to the total radioactivity in cells and medium.

**Western Blot for Macrophage ABCA1, ABCG1, and SR-B1**

Macrophages were lysed in 200 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, and 1% (vol/vol) protease inhibitor cocktail (Sigma). Cell debris was cleared by centrifugation at 8200g for 10 minutes. Lysates were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The blots were probed with antibodies against ABCA1 (Novus), ABCG1 (Abcam), and SR-B1 (Novus). β-Actin was used as a loading control. Blots were developed by a chemiluminescence detection system (SuperSignal West detection kit, Pierce). The maximum intensity of each band was measured by Image–Pro Plus version 4.5 software (Media Cybernetics Inc).

**In Vivo Macrophage Cholesterol Efflux Measurement**

In vivo macrophage cholesterol efflux was measured as described in Methods. A, Cholesterol efflux toward apoA-I. B, Cholesterol efflux toward HDL. Values are means±SD (N=5). *P<0.01.

**Mouse Atherosclerotic Lesion Measurement**

The aorta was dissected and the arch photographed, as previously reported.23 Aortic lesion en face assay was performed as previously described.24 For morphometric lesion analysis, sections were stained with Harris’ hematoxylin/eosin. Total intimal lesion area and acellular/anuclear areas (negative for hematoxylin-positive nuclei) per cross section were quantified by taking the average of 6 sections spaced 30 μm apart, beginning at the base of the aortic root. Histomorphologic analysis of collagen was performed with Masson’s trichrome stain (Richard-Allan Scientific, Kalamazoo, Mich). Images were viewed and captured with a Nikon Labophot 2 microscope equipped with a SPOT RT3 color video camera attached to a computerized imaging system with Image–Pro Plus version 4.5 software (Media Cybernetics Inc).

**Immunostaining of Macrophage and Smooth Muscle Cell in Plaques**

Sequential sections 10 μm thick was stained with macrophage-specific antibody (A1A31240, Accurate Chemical and Scientific Corp) and anti-smooth muscle cell (anti-SMC) actin antibody (1A4, Zymed). Primary antibodies were incubated for 1 hour at room temperature in 3% serum matched to the species of the secondary antibodies. Biotinylated secondary antibodies were incubated for 30 minutes, followed by 45 minutes of horseradish peroxidase-conjugated streptavidin and visualization with diaminobenzidine. Nuclei were counterstained with hemalaun. The mean area of staining per section per animal from 7 sections were determined for each animal. Staining areas were quantified with Image–Pro Plus 4.5 software.

**Cell Surface ABCA1 and SR-B1 Analysis by FACS**

Acetyl-LDL–treated macrophages were scraped off the plates and resuspended in PBS to make single-cell suspension. Cells were treated with Fc receptor block (mAb 2-4G2; BD Pharmingen) and

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** SMS2 deficiency significantly increased macrophage cholesterol efflux in vitro. Cholesterol efflux was measured as described in Methods. A, Cholesterol efflux toward apoA-I. B, Cholesterol efflux toward HDL. Values are means±SD (N=5). *P<0.01.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** SMS2 deficiency significantly increased macrophage cholesterol efflux in vivo. SMS2 KO or WT bone marrow–derived macrophages were loaded with cholesterol by incubation with acetylated LDL and [3H]cholesterol. Labeled macrophages were injected intraperitoneally into WT mice. Plasma was collected at 12, 24, and 48 hours. Feces were collected at 24 and 48 hours. All were analyzed for tracer counts. Values are means±SD (N=5). *P<0.01. A, [3H]cholesterol in plasma; B, [3H]cholesterol in feces.
stained with antibodies to SR-B1 (Novus) or ABCA1 (Novus) with 1:50 dilution. After wash, cells were stained with goat anti-rabbit second antibody conjugated with green fluorescence (Invitrogen) with 1:100 dilutions. After wash, cells were suspended in PBS containing 1 μg/mL propidium iodide. Cells were analyzed on a FACScan with CellQuest (Benton Dickinson). Dead cells were excluded from the analysis according to propidium iodide staining.

Brachiocephalic Artery Total Cholesterol and Cholesteryl Ester Measurement

Total cholesterol, cholesteryl ester, SM, PC, and ceramide levels in brachiocephalic artery (BCA) were measured according to a method previously reported by us.12,25

Statistical Analysis

Each experiment was conducted at least 3 times. Data were typically expressed as means±SD. Differences between groups were tested by the Mann–Whitney U test (nonparametric test). Probability values of <0.05 were considered significant.

Results

SMS2-Deficient Macrophages Produce More Cholesterol Efflux

Because plasma membrane SM levels regulate a spectrum of important genes involved in cholesterol efflux and signal transduction, we speculated that the SMS2, located in the plasma membranes, might be important in regulating macrophage cholesterol efflux. We, therefore, measured efflux from peritoneal macrophages of WT and SMS2-deficient mice. The deficiency of SMS2 caused a significant induction in cholesterol efflux, compared with controls, using either apoA-I or HDL as the cholesterol acceptor (75 and 65%, P<0.01, respectively; Figure 1A and 1B).

Promoting reverse cholesterol transport is considered to be an antiatherogenic process, one that might be altered through macrophage SMS2 deficiency. We used an approach reported by Rader’s group23 to investigate the effect of SMS2 deficiency on macrophage cholesterol efflux in vivo. Bone marrow–derived macrophages from both WT and SMS2 KO mice were loaded with [3H]cholesterol by incubation with acetylated LDL, and then injected intraperitoneally into WT animals. We found that SMS2 KO macrophages produce significantly more [3H]cholesterol efflux into the circulation at 24- and 48-hour time points (Figure 2A). Furthermore, feces from KO mice also accumulated more [3H]cholesterol 24 and 48 hours after injection (Figure 2B), compared with that of controls.

To elucidate the possible mechanisms of cholesterol efflux induction in SMS2-deficient macrophages, we performed

![Figure 3](http://circres.ahajournals.org/)

Figure 3. SMS2 deficiency significantly increased macrophage ABCA1, ABCG1, and SR-B1 protein levels. Macrophage lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with ABCA1, ABCG1, and SR-B1 antibodies. GAPDH was used as a loading control. A, ABCA1, ABCG1, and SR-B1 fluorogram, which is representative of 3 independent experiments. B, Quantitative display of macrophage ABCA1, ABCG1, and SR-B1. Values are means±SD (N=4). *P<0.01. C and D, cell surface ABCA1 and SR-B1 analysis by FACS. This is representative of 3 independent experiments.
Western blot measurement of ABCA1, ABCG1, and SR-B1. SMS2 deficiency significantly increased ABCA1 (190±27%, \( P<0.01 \)), ABCG1 (139±28%, \( P<0.01 \)), and SR-B1 (211±50%, \( P<0.01 \)) in macrophages, compared with controls (Figure 3A and 3B). This suggests that all 3 molecules might contribute to higher cholesterol efflux from SMS2-null macrophages in vitro and in vivo. We also investigated ABCA1 and SR-B1 levels on macrophage plasma membrane. FACS analysis showed that, after acetyl-LDL stimulation, ABCA1 and SR-B1 levels on macrophage plasma membrane are higher in SMS2 KO macrophages than those in SM2 KO macrophages (Figure 3C), suggesting alteration of SM levels on plasma membrane influences both proteins, thus influencing cholesterol efflux.

SMS2 KO Mice Have Significantly Lower Inflammatory Cytokines in the Circulation

We have reported previously that SMS2 deficiency substantially diminished the abundance of TLR4-MD2 complex levels on the surface of macrophages after LPS stimulation. Likewise, NF-κB and MAP kinase activation was attenuated in macrophages from SMS2 KO mice.21 In line with these findings, we have discovered in this study that plasma IL-6 and TNFα levels in SMS2 KO mice were significantly decreased before and after LPS stimulation (Table 1).

### Table 1. Plasma IL-6 and TNFα Measurement in Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>IL-6 (pg/mL)</th>
<th>TNFα (pg/mL)</th>
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<tr>
<td>WT</td>
<td>39±2</td>
<td>1±1</td>
</tr>
<tr>
<td>SMS2KO</td>
<td>15±5**</td>
<td>6±3*</td>
</tr>
<tr>
<td>WT (LPS)</td>
<td>1818±59</td>
<td>85±11</td>
</tr>
<tr>
<td>SMS2KO (LPS)</td>
<td>1376±99**</td>
<td>48±5**</td>
</tr>
<tr>
<td>WT→LDLr KO</td>
<td>41±3</td>
<td>9±2</td>
</tr>
<tr>
<td>SMS2KO→LDLr KO</td>
<td>16±17**</td>
<td>5±1*</td>
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Values are means±SD (N=5), *\( P<0.05 \); **\( P<0.01 \).

As expected, bone marrow–derived SMS2−/− macrophages had no SMS2 expression (Figure 4B) but had normal SMS1 mRNA levels (Figure 4C). SMS2−/− or SMS2−/−→LDLr−/− peritoneal macrophages have significantly lower total SMS activity than that of controls (\( \sim 20\% \), \( P<0.05 \); Figure I in the Online Data Supplement, available at http://circres.ahajournals.org). Cellular SM levels were decreased (\( P<0.05 \); Figure 4D and Online Table I), whereas ceramide

![Figure 4. Preparation of LDLr KO mice with a macrophage-specific SMS2 deficiency. LDLr−/− animals were lethally irradiated. After 3 hours, half of them were transplanted with SMS2−/− mouse bone marrow cells (SMS2−/−→LDLr−/−), and the other half with WT cells (WT→LDLr−/−). A, Genotype determination of mouse peripheral cells. Genomic DNA was extracted from white blood cells of the same mouse, before and after irradiation. The genotypes of SMS2 were determined with PCR, as described in Methods. WT mice had a 500-bp PCR product, and KO mice had a 300-bp PCR product. B, Macrophage SMS2 mRNA levels measured by real-time PCR. C, Macrophage SMS1 mRNA levels measured by real-time PCR. D through F, Macrophage SM, ceramide, and PC measurement. G, Macrophage plasma membrane SM measurement (lysinen-mediated cell lysis assay). Values in B through G are means±SD (N=5). *\( P<0.05 \).](http://circres.ahajournals.org)
levels were increased ($P<0.05$; Figure 4E), mainly related to ceramide 16:0 increasing (Online Table II). There was no significant change in cellular PC levels (Figure 4F), but there was a significant decrease of diacylglycerol levels (Online Table III, $P<0.05$). To measure SM on plasma membrane, we took the advantage of lysenin, a recently discovered SM-specific cytotoxin. Lysenin recognizes SM only when it forms aggregates or microdomains on the plasma membrane and then lyse the cells. Lysenin-mediated cell mortality can indirectly reflect SM levels on the plasma membrane. Based on lysenin-mediated cell lysis assay, we found that the SMS2 KO/LDLr KO mice had significantly less SM in the plasma membranes, compared with controls ($P<0.01$; Figure 4G). We also measured cellular cholesterol levels and did not find significant difference (Online Figure II). Cholesteryl ester content was undetectable regardless of genotype, which is similar to a previous report.

At this point, the rest of the animals were switched to a Western diet (0.15% cholesterol, 20% saturated fat) for 3 months. We found that SMS2 KO/LDLr KO mice had significantly lower basal IL-6 and TNF-$\alpha$ levels in the circulation (Table 1), compared with WT→LDLr KO mice. These differences were highly significant ($P<0.01$ and $P<0.001$, respectively; Figure 5C and 5E). Analysis of plaque morphology revealed substantial differences between the SMS2 KO/LDLr KO and WT→LDLr KO mice. As illustrated by the hematoxylin/eosin–stained images, plaques from the SMS2 KO/LDLr KO animals had a significant decrease in necrotic core areas that were anuclear, atherosclerotic, and eosin-negative (71% $P<0.01$; Figure 5F).

Furthermore, the SMS2 KO/LDLr KO mice demonstrated substantially more collagen, as illustrated by the trichrome-stained images (Figure 6A; 35%, $P<0.05$). We performed immunohistochemistry for macrophages and SMCs. We

**Figure 5.** SMS2 KO/LDLr KO mice demonstrated significantly decreased atherosclerotic lesion size and necrotic area. A, Mice were euthanized, and the aortas dissected and photographed. This set of images is representative of 9 sets. Atherosclerotic lesions are indicated by arrows. B, Oil red O staining for whole aorta (en face assay). C, Quantitative display of en face assay. D, Hematoxylin/eosin staining for proximal aorta (root assay). NE indicates necrotic core area. E, Quantitative display of root assay. F, Quantitative display of necrotic core area. Values are means±SD (N=9). *$P<0.01$.

**Evaluation of Atherosclerosis in Macrophage-Specific SMS2 KO Mice**

To evaluate the impact of macrophage SMS2 deficiency on atherogenesis, we dissected mouse aortas and photographed them. We also measured proximal and whole aortic lesion areas. After 3 months on a western-type diet, we found that all mice (18/18) had lesions in the aortic arch. However, the SMS2 KO/LDLr KO animals had noticeably smaller lesions than the WT→LDLr KO mice (Figure 5A).

We also found that the SMS2 KO/LDLr KO animals had 42% less lesion area in the whole aorta (Figure 5B and 5D) and 57% less in the proximal aorta (Figure 5D and 5E), compared with WT→LDLr KO mice. These differences were highly significant ($P<0.01$ and $P<0.001$, respectively; Figure 5C and 5E). Analysis of plaque morphology revealed substantial differences between the SMS2 KO/LDLr KO and WT→LDLr KO mice. As illustrated by the hematoxylin/eosin–stained images, plaques from the SMS2 KO/LDLr KO animals had a significant decrease in necrotic core areas that were anuclear, atherosclerotic, and eosin-negative (71% $P<0.01$; Figure 5F).
We isolated the BCAs from both groups and extracted lipid from them. Using LC/MS/MS to measure the free cholesterol and cholesteryl ester levels, we found that the SMS2\(^{−/−}\)→LDLr\(^{−/−}\) mice had significantly lower free cholesterol and cholesteryl ester levels in the BCAs than that in the WT→LDLr\(^{−/−}\) mice (33% and 52%, \(P<0.01\) and \(<0.001\), respectively; Figure 6C). We also measured SM, ceramide, and PC in BCAs. We did not find changes of PC (Table 2). We did find decrease of SM in SMS2\(^{−/−}\)→LDLr\(^{−/−}\) BCAs, but such change did not reach to a statistical significance (\(P=0.063\); Table 2). Moreover, we found PC/SM ratio is significantly increase in SMS2\(^{−/−}\)→LDLr\(^{−/−}\) BCAs compared with that in WT→LDLr\(^{−/−}\) BCAs (\(P<0.05\); Table 2). We also measure ceramide levels in BCAs and we did not find changes (Table 2 and Online Table VI).

**Discussion**

As expected, SMS2 deficiency in bone marrow–derived cells led to a decrease in atherosclerosis in LDLr\(^{−/−}\) mice fed a western-type diet for 3 months. These findings appear to be explained by an increase in macrophage cholesterol efflux, in vitro and in vivo, and a decrease in inflammatory response.

We reported previously that plasma SM is an independent and positive risk factor for coronary heart disease and that SM levels may serve as a marker for atherogenic remnant lipoprotein accumulation.\(^{28,29}\) In this study, instead of study SM in the circulation, we focused on the macrophage plasma membrane SM levels. We believe that SM level reduction in the circulation could cause a global effect on atherosclerosis, whereas SM level reduction on cell membrane, such as macrophage membrane, could create a microenvironment, influencing cholesterol efflux and inflammation, thus causing antatherogenic consequences.

Macrophage plasma membrane SM levels are related to cholesterol efflux. Foam cell formation caused by excessive accumulation of cholesterol by macrophages is a pathological hallmark of atherosclerosis.\(^{1}\) Macrophages cannot limit the uptake of cholesterol, and therefore depend on cholesterol efflux pathways to prevent their transformation into foam cells. We found that SMS2-deficient macrophages had significantly less SM in the plasma membranes (Figure 4G), and more cholesterol efflux than control cells in vitro (Figure 1) and in vivo (Figure 2). This observation could have important implications in terms of vessel wall homeostasis in response to atherogenic insult. It has been reported that lysosomal sphingomyelinase is involved in cholesterol transport from lysosomes to the plasma membranes.\(^{30}\) Sphingomyelinase hydrolyzes SM in late endosomes and lysosomes.\(^{31}\) Because SM avidly binds cholesterol,\(^{32}\) sphingomyelinase deficiency inhibits macrophage cholesterol efflux through promoting cholesterol sequestration by SM.\(^{30}\) It is conceivable that...
SMS2 deficiency may have the opposite effect as sphingomyelinase deficiency, in reference to macrophage cholesterol efflux, because SMS2 deficiency decreases de novo SM biosynthesis and decreases plasma membrane SM levels (Figure 4G).21

Macrophage plasma membrane SM levels are related to the proteins associated with cholesterol efflux. In macrophages, ABCA1 exports cholesterol and phospholipid to lipid-free apolipoproteins, whereas ABCG1 exports cholesterol to phospholipid-containing acceptors.14 ABCA1-dependent cholesterol export involves an initial interaction of apoA-I with SM-rich lipid raft membrane domains.33 Such reorganization effectively expands the non–raft membrane fractions and consequently preconditions cells for cholesterol efflux.34 ABCG1 exports cholesterol to HDL and other phospholipid-containing acceptors. These include particles generated during the lipolysis of apoA-I by ABCA1, suggesting that the 2 transporters cooperate in cholesterol export.35 ABCG1 is mainly found intracellularly in the basal state, with little cell surface presentation. But on stimulation, for example by liver X receptor agonist treatment, ABCG1 redistributes to the plasma membranes and increases cholesterol mass efflux to HDL.36 SR-B1 also facilitates cholesterol efflux from macrophages.37 It is well known that SR-B1 is located in SM-rich caveolae and lipid rafts.38 Inactivation of macrophage SR-B1 promotes atherosclerotic lesion development in apoE KO mice.39 In this study, we found that SMS2 deficient macrophages upregulate all these cholesterol-efflux-related transporters and receptor (Figure 3A and 3B). Moreover, SMS2 KO macrophages, after acetyl-LDL stimulation, have more ABCA1 and SR-B1 on the cell surface than that on control macrophages (Figure 3C and 3D), suggesting that these proteins are located or associated with SM-rich microdomains (lipid rafts) on the macrophage plasma membrane and alteration of SM levels on these microdomains could influence both proteins, thus influencing cholesterol efflux.

SMS2 deficiency could alter the structure of lipid rafts and modify inflammatory responses. In our previous study, we found that SMS2 deficiency attenuates NF-κB activation. We observed that on stimulation by TNFα, the recruitment of TNF receptor (TNFR)1 receptor to lipid rafts following ligand stimulation was blocked in SMS2 knockout cells,21 suggesting a mechanism for the modulation of NF-κB activity by SMS2. This finding is in agreement with previous reports indicating that raft association of TNFR1 was found to be crucial for TNFα-mediated NF-κB activation in human fibrosarcoma cells.39 We also found that LPS-induced plasma membrane recruitment of TLR4-MD2 complex was diminished in SMS2 KO macrophages.31 SMS2 deficiency may also influence signal transduction pathways other than NF-κB activation. The activation of MAP kinases was likewise attenuated in SMS2 KO macrophages.21 Taken together, these findings strongly suggest the critical role of SM, synthesized by SMS2, in the normal function of TNFR1 and TLR4 receptors in the plasma membranes following stimulation by their respective ligands. More importantly, SMS2 deficiency decreases IL-6 and TNFα levels in the circulation (Table 1).

It was noticed that after bone marrow transplantation the SMS2−/−→LDLr−/− macrophages express LDLr. Herijgers et al have reported that the presence or absence of the LDLrs in the transplanted bone marrow does not influence atherogenicity in LDLr KO mice.40 Many researchers use this approach to study atherosclerotic lesion formation.40–42

The key findings of this study are the changes of atherosclerotic plaque size and plaque morphology in SMS2−/−→LDLr−/− mice, compared with those in WT→LDLr−/− animals. Macrophage SMS2 deficiency decreased the plaque size and the necrotic core area (Figure 5), which is composed primarily of dead macrophages,43 and increased collagen content (Figure 6A), a sign of the integrity of the aorta. We measured cholesterol and cholesteryl ester contents in BCAs and found that both are significantly decreased in the SMS2−/−→LDLr−/− mice (Figure 6C). We also found decrease of SM in SMS2−/−→LDLr−/− BCAs, but such a change did not reach to a statistical significance (P=0.063; Table 2). Moreover, we found PC/SM ratio is significantly increase in SMS2−/−→LDLr−/− BCAs compared with that in WT→LDLr−/− BCAs (P<0.05; Table 2). The significance of this finding deserves further investigation.

In conclusion, SMS2 physiologically contributed to de novo SM biosynthesis and plasma membrane SM levels. SMS2 deficiency in the macrophages caused blunted NF-κB and MAP kinase responses to inflammatory/immunologic stimuli, promoted cholesterol efflux, and reduced atherosclerosis in a mouse model. Thus, macrophage SMS2 is a potential therapeutic target for the treatment of atherosclerosis.

Acknowledgments

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Disclosures

None.

References


Macrophage Sphingomyelin Synthase 2 Deficiency Decreases Atherosclerosis in Mice
Jing Liu, Chongmin Huan, Mahua Chakraborty, Hongqi Zhang, Da Lu, Ming-Shang Kuo, Guoqing Cao and Xian-Cheng Jiang

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Supplement Table I. Sphingomyelin measurement in WT and SMS2 KO macrophages.

<table>
<thead>
<tr>
<th>Mice</th>
<th>C16:0</th>
<th>C24:1</th>
<th>C24:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>25.7±1.9</td>
<td>10.5±0.7</td>
<td>7.5±0.3</td>
</tr>
<tr>
<td>SMS2KO</td>
<td>21.2±1.0*</td>
<td>8.7±0.3*</td>
<td>4.8±0.8*</td>
</tr>
</tbody>
</table>

Values are mean±SD, n=4. *P<0.05.

Supplement Table II. Ceramide measurement in WT and SMS2 KO macrophages.

<table>
<thead>
<tr>
<th>Mice</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C20:0</th>
<th>C24:1</th>
<th>C24:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.377±0.009</td>
<td>0.073±0.001</td>
<td>0.035±0.001</td>
<td>0.399±0.008</td>
<td>0.373±0.007</td>
</tr>
<tr>
<td>SMS2KO</td>
<td>0.589±0.028*</td>
<td>0.121±0.035*</td>
<td>0.078±0.022*</td>
<td>0.366±0.018</td>
<td>0.351±0.003</td>
</tr>
</tbody>
</table>

Values are mean±SD, n=4. *P<0.05.

Supplement Table III. Diacylglycerol measurement in WT and SMS2 KO macrophages.

<table>
<thead>
<tr>
<th>Mice</th>
<th>C32_253</th>
<th>C32_255</th>
<th>C32_281</th>
<th>C32_283</th>
<th>C34_255</th>
<th>C34_281</th>
<th>C34_283</th>
<th>C36_281</th>
<th>C36_283</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>137±12</td>
<td>182±20</td>
<td>75±6</td>
<td>477±31</td>
<td>273±28</td>
<td>117±15</td>
<td>198±27</td>
<td>159±10</td>
<td>239±12</td>
</tr>
<tr>
<td>SMS2KO</td>
<td>121±9*</td>
<td>81±5*</td>
<td>60±4*</td>
<td>290±18*</td>
<td>238±30</td>
<td>137±13</td>
<td>212±25</td>
<td>89±8*</td>
<td>229±21</td>
</tr>
</tbody>
</table>

Values are mean±SD, n=4. Carbon number and molecular weight of each diacylglycerol are indicated. *P<0.05.
Supplement Table IV. Plasma lipid measurement in WT→LDLr−/− and SMS2−/−→LDLr−/− mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Chol</th>
<th>HDL-C</th>
<th>PL</th>
<th>HDL-PL</th>
<th>SM</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT→LDLRKO</td>
<td>1197±129</td>
<td>399±37</td>
<td>937±351</td>
<td>286±39</td>
<td>68±7</td>
<td>144±18</td>
</tr>
<tr>
<td>SMS2−/−→LDLR−/−</td>
<td>1205±319</td>
<td>367±52</td>
<td>1065±244</td>
<td>297±26</td>
<td>61±9</td>
<td>124±21</td>
</tr>
</tbody>
</table>

Values are mean±SD, n=10.

Supplement Table V. Blood cell counts in WT and SMS2 KO mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>WT</th>
<th>SMS2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red Blood Cells (M/µl)</td>
<td>6.9±1.1</td>
</tr>
<tr>
<td></td>
<td>White Blood Cells (K/µl)</td>
<td>9.4±3.4</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin (g/dl)</td>
<td>15±0.6</td>
</tr>
<tr>
<td></td>
<td>Platelets (K/µl)</td>
<td>815±84</td>
</tr>
<tr>
<td></td>
<td>Neutrophils (K/µl)</td>
<td>0.28±0.16</td>
</tr>
<tr>
<td></td>
<td>Eosinophils (K/µl)</td>
<td>0.55±0.02</td>
</tr>
<tr>
<td></td>
<td>Basophils (K/µl)</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td></td>
<td>Monocytes (K/µl)</td>
<td>1.32±0.40</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes (K/µl)</td>
<td>7.32±1.81</td>
</tr>
</tbody>
</table>

Values are mean±SD, n=6. M, million; K, thousand.

Supplement Table VI. Ceramide measurement in WT→LDLr−/− and SMS2−/−→LDLr−/− mouse BCA.

<table>
<thead>
<tr>
<th>Mice</th>
<th>C16:0</th>
<th>C18:0</th>
<th>C20:0</th>
<th>C24:1</th>
<th>C24:0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/whole BCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT→LDLR−/−</td>
<td>8.8±0.7</td>
<td>2.1±0.3</td>
<td>1.2±0.2</td>
<td>16.5±1.7</td>
<td>7.9±0.5</td>
</tr>
<tr>
<td>SMS2−/−→LDLR−/−</td>
<td>7.8±0.5</td>
<td>2.2±0.2</td>
<td>1.2±0.1</td>
<td>16.2±1.5</td>
<td>7.1±1.0</td>
</tr>
</tbody>
</table>

Values are mean±SD, n=6.
Supplement figure legends

Supplement Figure I. Macrophage SMS activity assay. Macrophages were homogenized in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5% sucrose, and a cocktail of protease inhibitors (Sigma). The homogenate was centrifuged at 5000 rpm for 10 minutes and the supernatant mixed in assay buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, C6-NBD-ceramide (0.1 μg/μl), and phosphotidylcholine (0.01 μg/μl). The mixture was incubated at 37°C for 2 hours. Lipids were extracted in chloroform: methanol (2:1), dried under N2 gas, and separated by thin layer chromatography (TLC). Band intensity was quantified by Image–Pro Plus version 4.5 software (Media Cybernetics Inc.).

Supplement Figure II. Macrophage cholesterol measurement. Cellular lipids were extracted with isopropanol (including 5α-cholestane as internal standard) at room temperature overnight and analyzed for cholesterol content by gas-liquid chromatography at Department of Medicine, Columbia University.

Supplement Figure III. Smooth muscle cell immunostaining. The procedure was described in “Materials and Methods”.

Supplement Figure II.

WT SMS2KO

Cholesterol concentration (μg/mg cell protein)

WT  SMS2KO

0  25  50  75  100
Supplement Figure III. Liu et al.

WT SMS2 KO

Lesion area with smooth muscle cells (%)

WT SMS2 KO