Pharmacological Suppression of Hepcidin Increases Macrophage Cholesterol Efflux and Reduces Foam Cell Formation and Atherosclerosis


Objective—We recently reported that lowering of macrophage free intracellular iron increases expression of cholesterol efflux transporters ABCA1 and ABCG1 by reducing generation of reactive oxygen species. In this study, we explored whether reducing macrophage intracellular iron levels via pharmacological suppression of hepcidin can increase macrophage-specific expression of cholesterol efflux transporters and reduce atherosclerosis.

Methods and Results—To suppress hepcidin, increase expression of the iron exporter ferroportin, and reduce macrophage intracellular iron, we used a small molecule inhibitor of bone morphogenetic protein (BMP) signaling, LDN 193189 (LDN). LDN (10 mg/kg IP b.i.d.) was administered to mice, and its effects on atherosclerosis, intracellular iron, oxidative stress, lipid efflux, and foam cell formation were measured in plaques and peritoneal macrophages. Long-term LDN administration to apolipoprotein E−/− mice increased ABCA1 immunoreactivity within intraplaque macrophages by 3.7-fold (n = 8; P = 0.03), reduced Oil Red O-positive lipid area by 50% (n = 8; P = 0.02), and decreased total plaque area by 43% (n = 8; P = 0.001). LDN suppressed liver hepcidin transcription and increased macrophage ferroportin, lowering intracellular iron and hydrogen peroxide production. LDN treatment increased macrophage ABCA1 and ABCG1 expression, significantly raised cholesterol efflux to ApoA-1, and decreased foam cell formation. All preceding LDN-induced effects on cholesterol efflux were reversed by exogenous hepcidin administration, suggesting modulation of intracellular iron levels within macrophages as the mechanism by which LDN triggers these effects.

Conclusion—These data suggest that pharmacological manipulation of iron homeostasis may be a promising target to increase macrophage reverse cholesterol transport and limit atherosclerosis. (Arterioscler Thromb Vasc Biol. 2012;32: 00-00.)

Key Words: ABC transporter ■ hemoglobin ■ macrophages ■ pharmacology ■ reactive oxygen species

Although elegant genetic studies lend important insights into disease mechanisms, their potential for clinical translation is limited because selective gene manipulation within humans is not currently a viable option. To make use of the therapeutic potential of increasing macrophage cholesterol efflux for the prevention of atherosclerosis in humans, new pharmacological means to increase macrophage expression of lipid efflux transporters are needed.

We recently described within areas of intraplaque hemorrhage in post mortem human atherosclerotic plaques a specific subtype of macrophages that we termed M(Hb). These macrophages resist foam cell formation both in vivo and in response to exogenous cholesterol loading, have increased expression of ABCA1 and ABCG1, and have antioxidative characteristics. We showed that the antioxidative properties
of M(Hb) are causal in increased expression of ABCA1 and ABCG1 and originate from a reduction in intracellular free iron available for electron donation for reactive oxygen species formation. M(Hb) cells have reduced intracellular free iron because of increased expression of a free iron exporter, ferroportin (FPN). Our data suggest that reducing intracellular free iron levels within macrophages by increasing expression of macrophage FPN may be a promising strategy to increase expression of cholesterol efflux transporters.

FPN is the only known mammalian free iron exporter expressed by macrophages, and it is systemically degraded through ubiquitination after binding to a hepatic hormone, hepcidin. The promoter elements of hepcidin are activated by SMAD 1/5/8 transcription factors, which are in turn activated through bone morphogenetic protein (BMP) signaling. We used a novel small molecule inhibitor of BMP signaling, LDN 193189 (LDN), which prevents the activation of SMAD 1/5/8 to suppress hepatic hepcidin production and increase expression of FPN within macrophages. We explored the effects of this strategy on mouse atherosclerosis, macrophage intracellular iron levels, oxidative stress, lipid efflux, and foam cell formation. Our findings reveal that suppressing hepcidin by inhibiting BMP signaling through LDN significantly increases expression of ABCA1 and ABCG1 and lipid efflux by macrophages, which is associated with reduced foam cell formation and atherosclerosis in the apolipoprotein E (ApoE)/-/- mouse model.

**Methods**

**Chemicals**

LDN (4-[6-(4-piperazin-1-ylphenyl)pyrazolo[1,5-a]pyrimidin-3-yl]quinoline) was synthesized as previously described. The vehicle was 2% (wt/vol) (2-hydroxypropyl)-β-cyclodextrin in PBS, pH 7.4. Control animals received vehicle alone.

**Animals and Experimental Protocols**

The Institutional Animal Care and Use Committee at Emory University approved all animal protocols. All animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male C57BL/6J mice, 12 to 14 weeks old, and male ApoE-knockout (ApoE/-/-) mice on a C57BL/6J background, 10 to 12 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME).

**Atherosclerosis**

To investigate the effect of long-term hepcidin suppression on atherosclerosis, we placed ApoE/-/- mice into 2 groups (n=8 per group): control animals receiving vehicle IP b.i.d. for 10 weeks and LDN-treated animals receiving LDN 10 mg/kg IP b.i.d. for 10 weeks. After starting LDN or control, all mice were placed on an ad libitum chow diet (Harlan Teklad, Madison, WI) for 4 days, and on day 5 animals were switched to an atherogenic diet (1.25% cholesterol, 21% fat, Harlan Teklad) that was continued until euthanasia. At the end of the 10-week treatment period, the left ventricular ejection fraction was measured by the VEVO 2100 high-resolution echocardiogram system (Visual Sons, Toronto, Ontario, Canada), and blood was collected by cardiac puncture for hematocrit, serum iron, and lipid measurements. Then, mice were perfused with normal saline and fixed with 4% paraformaldehyde through the left ventricle of the heart. After fixation, the heart, aortic arch, and descending aorta were collected for analysis of atherosclerotic plaque burden and immunohistochemistry.

**LDN Efficacy Studies**

To establish the efficacy of LDN to suppress liver hepcidin, we divided C57BL/6J mice into 2 groups: control animals receiving vehicle through intraperitoneal injection every 12 hours (b.i.d.) for 4 days and LDN-treated animals receiving a previously efficacious dose of 10 mg/kg IP b.i.d. for 4 days. On day 5, blood was collected for serum iron measurements, and liver samples were taken to measure hepcidin expression.

**Peritoneal Macrophage Isolation**

Control animals received vehicle IP b.i.d. for 4 days, and LDN-treated animals received LDN 10 mg/kg IP b.i.d. for 4 days. On day 5, all mice underwent peritoneal lavage. Peritoneal cells were plated for overnight incubation with 1 set of cells from LDN-treated animals exposed to 700 nmol/L mouse hepcidin (Peptides International, Louisville, KY) to evaluate for reversal of LDN induced effects. The following day, all nonadherent cells were removed, and after washing the cells 3 times with PBS, we were able to obtain >85% macrophages as identified by a fluorescent anti-mouse FITC/80 Alexa Fluor 488 antibody (eBioscience) (Supplemental Figure I, available online at http://atvb.ahajournals.org), consistent with previous reports. Peritoneal macrophages were scraped off plates for molecular and cellular analysis.

**Foam Cell Formation**

C57BL/6J mice were placed in the following 3 groups: (1) control animals receiving vehicle IP b.i.d. for 4 days; (2) LDN-treated animals receiving LDN 10 mg/kg IP b.i.d. for 4 days; and (3) LDN plus hepcidin-treated animals receiving LDN 10 mg/kg IP b.i.d. for 4 days and 25 μg (dissolved in 100 μL PBS) of mouse hepcidin IP on day 4. On day 5, all mice underwent peritoneal lavage. The peritoneal cells were plated overnight in serum free media with 1% Nutridoma (Boehringer). On the following day, all nonadherent cells were removed. After being washed 3 times with PBS, cells were fixed in 4% paraformaldehyde and stained with Oil Red O and hematoxylin and eosin. Cytoplasmic Oil Red O staining was quantified by color density analysis using iVision software (BioVision).

**Cholesterol Loading and Efflux Assays**

C57BL/6J mice were placed into the following 3 groups: (1) control animals receiving vehicle IP b.i.d. for 4 days; (2) LDN-treated animals receiving LDN 10 mg/kg IP b.i.d. for 4 days; and (3) LDN plus hepcidin-treated animals receiving LDN 10 mg/kg IP b.i.d. for 4 days and 25 μg (dissolved in 100 μL PBS) hepcidin IP once a day on days 3 and 4. On day 5, peritoneal cells were removed and loaded with 30 μg/mL of oxLDL for 48 hours. For cholesterol uptake studies, cells were then analyzed for cholesterol content by enzymatic assay as described below. For efflux studies, after this incubation period, cells were washed twice in PBS and ApoAI-mediated cholesterol efflux studies were immediately performed by adding fresh RPMI medium without Nutridoma with or without 20 μg/mL of ApoAI (BioVision) for 24 hours. At the end of this incubation, intracellular lipids were extracted in hexane/isopropanol and dried under nitrogen and free cholesterol, and total cholesterol and phospholipids were measured by enzymatic assays (Calbiochem). Esterified cholesterol was measured as the difference between total and free cholesterol. Cellular proteins were collected by digestion in NaOH and measured by Bradford assay (Bio-Rad). The percentage change of intracellular cholesterol amounts in the presence of ApoAI relative to ApoAI-free medium was expressed according to the following equation: percent decrease in cellular cholesterol=[{(cellularcholesterol)RPMI−(cellularcholesterol)ApoAI]/[cellular cholesterol]RPMI}×100.
RNA Isolation and Quantitative Polymerase Chain Reaction
Total cellular RNA was isolated from macrophages using Trizol (Invitrogen, Carlsbad, CA) and was reverse transcribed to create cDNA. cDNA was quantified by quantitative polymerase chain reaction on a StepOne Plus (Applied Biosystems). The primer sequences for all studied genes are listed in Supplemental Table I. Messenger RNA levels are normalized to GAPDH.

Western Blot
Western blot was conducted for ABCA1 (Abcam, Cambridge, MA) and FPN (Alpha Diagnostics, San Antonio, TX) as previously described.8 Protein samples were normalized to α-tubulin (Cell Signaling Technology, Danvers, MA). Densitometry was performed as previously described.15

Measurement of Macrophage Intracellular Free Iron by Calcein Fluorescence
Intracellular macrophage iron was measured by using calcein fluorescence, an assay that quenches calcein fluorescence by free iron, as described previously.16 In brief, macrophages were stained with 0.5 μmol/L calcein (Calbiochem) and then analyzed by a BD SR II flow cytometer and FlowJo software 7.5. Cellular fluorescence was inversely related to the free iron levels.

Measurement of Macrophage Hydrogen Peroxide Production Using Amplex Red
Macrophages were incubated in a 96-well plate with 50 μL of 100 μmol/L Amplex red/hozeradish peroxidase solution (Invitrogen). After incubation at room temperature for 24 hours, protected from light, fluorescence was measured at an excitation of 530 nm and emission of 590 nm. Cell viability using propidium iodide staining and flow cytometry was conducted at the end of 24 hours on cells from each group and demonstrated no differences between groups (control 87.5 ± 1.5% viable cells [ie, PI exclusion] in control cells versus 87.3 ± 1.1% in LDN cells, P not significant, n = 4 experiments per group).

Measurements of Hematocrit, Serum Iron, and Lipids
Approximately 1.5 mL of mouse blood was collected in heparin syringes by cardiac puncture. Then, 0.5 mL of blood was placed in a separate tube for hematocrit measurement, and the remaining sample was centrifuged at 5000 rpm for 15 minutes to obtain serum. Hematocrit, serum iron, total lipid, and triglycerides were measured commercially (Antech Diagnostics, Atlanta, GA).

Atherosclerotic Lesion Analysis and Immunohistochemistry
The heart and aortic arch were removed en bloc and frozen for tissue analysis. To obtain frozen sections, tissue was embedded in OCT, frozen in liquid nitrogen, and stored at −80°C. For morphometric lesion analysis, cryosections beginning at the base of the aortic root were obtained. Consecutive cross-sections at the level of the sinotubular junction were stained with hematoxylin and eosin, Movat Pentachrome, and Oil Red O, as previously described.17 Immunohistochemistry was done using primary rat anti-mouse Mac 3 (1:200, Lifespan Technologies Inc.) using the avidin-biotin-peroxidase complex method. Specificity of the antibody was confirmed by demonstrating lack of immunostaining of macrophages from aortic lesions from low-density lipoprotein receptor−/− mice transplanted with bone marrow from ABCA1/ABCG1−/− mice (courtesy of Alan Tall, Columbia University, New York, NY) (Supplemental Figure II). Cross-sections were imaged using an Olympus microscope, and stained area was quantified by segmentation color-threshold analysis using morphometry software (IP Laboratory, Scanalytics, Rockville, MD). The lesion area in the descending aorta was analyzed as previously described.18 For analysis of liver iron, Perl iron stain was conducted on livers from 10-week-treated control and LDN-treated ApoE−/− mice.

Statistical Analysis
Data are expressed as mean ± SE. For comparisons between 2 groups for continuous variables, a 1-way ANOVA test was performed using JMP software. P < 0.05 was considered statistically significant.

Results
Effect of LDN on ABCA1 Immunoreactivity in Plaque Macrophages, Oil Red O–Positive Lipid Area, Total Atherosclerotic Lesion Area, and Plaque Progression
To evaluate the impact of LDN on atherosclerosis, ApoE−/− mice on a high-cholesterol diet were treated with LDN for 10 weeks. Atherosclerotic plaques in the sinotubular junction from LDN- and vehicle (PBS)-treated ApoE−/− mice were localized for histological analysis. Oil Red O–positive lipid cell area was significantly reduced in LDN-treated animals by 50% (0.035 versus 0.07 mm2; n = 8; P = 0.024), and total plaque area at the sinotubular junction was reduced by 43% (0.35 versus 0.20 mm2; n = 8; P = 0.001) in the LDN group in comparison with vehicle (Figure 1A–1C). The lesion area in the descending aorta also decreased by 45% with LDN treatment (4.6% versus 2.5%; n = 8; P = 0.002; Figure 1D and 1E). ABCA1 immunoreactivity was measured within intra-plaque Mac-3 positive macrophages regions and showed a 3.7-fold increase (n = 8; P = 0.03) with LDN treatment in comparison with vehicle (Figure 1F and 1G). Moreover, atherosclerotic plaques were classified by severity, as previously described,19 and mice that received LDN had decreased number of advanced lesions with necrotic cores in comparison with vehicle (Figure 1H).

Effect of Long-Term LDN Administration on Body Weight, Lipids, Hematocrit, Cardiac Function, and Serum and Liver Iron
Ten-week LDN treatment resulted in no significant differences in comparison with controls in weight gain, total cholesterol, triglycerides, hematocrit, and cardiac ejection fraction (Table). Serum iron levels were significantly higher with LDN treatment in comparison with control (130 versus 178 μg/dL, P < 0.05), as expected given the increased expression of macrophage FPN. Despite these higher levels of iron, no differences in liver iron as detected by Perl stain were seen in control versus LDN-treated animals after 10 weeks of treatment (data not shown).

Effect of Inhibiting BMP Signaling by LDN on Liver Hepcidin Production, FPN Expression, Intracellular Iron, and Hydrogen Peroxide Production in Peritoneal Macrophages
To investigate whether manipulation of macrophage intracellular iron is an important mechanism by which LDN decreases atherosclerosis, we first confirmed the efficacy of LDN to suppress liver hepcidin as reported previously.10,20 Ten mg/kg of LDN administered IP b.i.d. for 4 days significantly suppressed liver hepcidin mRNA by >10-fold in comparison with vehicle treatment (Figure 2A). To determine
whether hepcidin suppression increased FPN, we isolated peritoneal macrophages from LDN-treated mice and compared FPN expression with that of controls by Western blot. FPN was significantly increased after LDN treatment (Figure 2B and 2C). This upregulation of FPN by LDN was ablated by exogenous hepcidin (Figure 2B and 2C), consistent with the known effect of hepcidin on FPN expression. Next, we evaluated the impact of LDN treatment on intracellular iron and hydrogen peroxide production. Peritoneal macrophages isolated after LDN treatment showed reduced intracellular iron and hydrogen peroxide production (Figure 2E and 2F). The effects of LDN on intracellular iron and hydrogen peroxide production were reversed with exogenous hepcidin (Figure 2E and 2F).

Effect of LDN on ABCA1 and ABCG1 Expression, Cholesterol Efflux, and Foam Cell Formation

To determine whether the antioxidative effects produced by lowering intracellular iron in macrophages by LDN impact lipid efflux, we measured the expression of ABCA1 and ABCG1. Peritoneal macrophages isolated after 4 days of LDN treatment showed significant 2-fold and 7.3-fold increases in mRNA of ABCA1 and ABCG1, respectively, in 2B and 2C). This upregulation of FPN by LDN was ablated by exogenous hepcidin (Figure 2B and 2C), consistent with the known effect of hepcidin on FPN expression. Next, we evaluated the impact of LDN treatment on intracellular iron and hydrogen peroxide production. Peritoneal macrophages isolated after LDN treatment showed reduced intracellular iron and hydrogen peroxide production (Figure 2E and 2F). The effects of LDN on intracellular iron and hydrogen peroxide production were reversed with exogenous hepcidin (Figure 2E and 2F).

Table. Weight Gain, Serum Total Cholesterol Levels, Serum Triglyceride Levels, Hematocrit, Serum Iron, and Cardiac Ejection Measurements From Apolipoprotein E−/− Mice Treated With Phosphate-Buffered Saline (Control) or LDN 193189 (LDN) for 10 Wk

<table>
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<th>Control (n=8)</th>
<th>LDN (n=8)</th>
<th>P Value</th>
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<td>Weight gain, g</td>
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<td>1.57 (±1.0)</td>
<td>NS</td>
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<td>Total cholesterol, mg/dL</td>
<td>1110 (±88)</td>
<td>1085 (±152)</td>
<td>NS</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>128 (±22)</td>
<td>109 (±29)</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit, g/dL</td>
<td>38.4 (±2.4)</td>
<td>38.7 (±2.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum iron, μg/dL</td>
<td>130 (±6)</td>
<td>178 (±22)</td>
<td>0.02</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>68.1 (±1.6)</td>
<td>60.6 (±5.6)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant.
comparison with vehicle (Figure 2G). Correspondingly, ABCA1 protein levels were increased with LDN treatment and returned to near baseline with exogenous overnight hepcidin treatment (Figure 2H and 2I), indicating the importance of FPN in the mechanism of ABC transporter upregulation. Overnight in vitro incubation of LDN (at a dose previously shown to inhibit BMP signaling\(^21\)) with isolated peritoneal macrophages had no effect on the expression of ABCA1 and ABCG1 (Supplemental Figure IIIA), further supporting an iron-related mechanism of ABC transporter expression by LDN.

To evaluate whether increased expression of ABCA1 and ABCG1 influence lipid efflux, functional experiments were performed to determine the influence of LDN treatment on macrophage cholesterol efflux to ApoAI. We incubated peritoneal macrophages from control and LDN-treated mice with oxLDL (30 \(\mu\)g/mL) for 48 hours to induce cholesterol accumulation. We subsequently exposed them to ApoAI (20 \(\mu\)g/mL) for 24 hours to induce cholesterol efflux and then determined cholesterol levels by enzymatic assay. ApoAI treatment significantly increased cholesterol efflux in macrophages from LDN-treated animals as compared with control, as indicated by significantly decreased levels of total, free, and esterified cholesterol levels (Figure 3A). This increase in lipid efflux by LDN was ablated by concurrent hepcidin treatment (Figure 3A). Lastly, to observe the influence of LDN on morphological foam cell formation, we stained lipid loaded macrophages isolated after LDN versus control treatment with Oil Red O. LDN-treated macrophages had a significant reduction in foam cell formation as evident by reduced cytoplasmic Oil Red O staining in comparison with control (Figure IIIB). These nonfoam cell foaming effects of LDN are consistent with its ability to upregulate ABCA1 and ABCG1, which are known to promote lipid efflux.

Figure 2. LDN 193189 (LDN) inhibits systemic iron metabolism and affects macrophage intracellular iron, reactive oxygen species, and ATP-binding cassette transporter expression. A, Expression of liver hepcidin in mice after treatment with PBS (control) or LDN (n=7 per group). B, Western blots showing ferroportin (FPN) in mouse peritoneal macrophages after treatment with PBS (control), LDN, or LDN + hepcidin. \(\alpha\)-Tubulin is shown as an internal control. C, Relative densitometry of FPN/\(\alpha\)-tubulin Western blots in mouse peritoneal macrophages after treatment with PBS (control), LDN, or LDN + hepcidin (n=3 per group). D, Serum iron measurements in mice treated with PBS (control) or LDN (n=7 per group). E, Calcein fluorescence histograms of peritoneal macrophages from mice treated with PBS (control, black line), LDN (orange line), or LDN + hepcidin (blue line). Calcein fluorescence is inversely proportional to intracellular iron. This is a representative image from 4 separate experiments. F, Hydrogen peroxide production, as quantitated by Amplex Red in peritoneal macrophages from mice treated with PBS (control), LDN, or LDN + hepcidin (n=4 per group). G, ABCA1 and ABCG1 expression in peritoneal macrophages from mice treated with PBS (control), LDN, or LDN + hepcidin (n=4 per group). H, Western blots showing ABCA1 in mouse peritoneal macrophages after treatment with PBS (control), LDN, or LDN + hepcidin. \(\alpha\)-Tubulin is shown as an internal control. I, Relative densitometry of FPN/\(\alpha\)-tubulin Western blots in mouse peritoneal macrophages after treatment with PBS (control), LDN, or LDN + hepcidin (n=4 per group).
LDN were reversed with concurrent exogenous hepcidin (Figure 3C–3E). These data suggest that the effects of LDN on macrophage ABC transporter expression and cholesterol efflux are mediated via its effects on macrophage intracellular iron.

To further investigate whether the lack of foam cell formation observed with LDN treatment was due increased cholesterol efflux rather than to limited lipid uptake, scavenger receptor expression and lipid uptake were examined in the peritoneal macrophages of LDN-treated mice. Quantitative polymerase chain reaction data for both class A (I and II) and class B scavenger receptor showed no changes in expression of these receptors (Supplemental Figure IIIB) compared with control. In addition, macrophages from control and LDN-treated animals were loaded in vitro with oxLDL (30 μg/mL) for 48 hours and cholesterol content determined by enzymatic assay. Both total and free cholesterol content increased to a similar degree in macrophages from control and LDN animals exposed to oxLDL as compared with unexposed cells (Supplemental Figure IIIC). However, this was not the case for esterified cholesterol, which increased more than 500% in control cells receiving oxLDL but only 44% in cells from LDN-treated animals (Supplemental Figure IIIC). These data indicate that although some uptake mechanisms still seem to be active in macrophages from LDN-treated animals, those involving production of esterified cholesterol, the hallmark of foam cells, are not as active in macrophages from LDN-treated animals as in control cells.

**Discussion**

Atherosclerosis is primarily an inflammatory disease driven by uptake of oxLDL into macrophages, transforming them into foam cells. Current clinical strategies have focused on lipid lowering, primarily using statins as a means to prevent...
atherosclerosis progression. However, even in this patient population, measures of cholesterol efflux potential, such as high-density lipoprotein, remain independent predictors of cardiovascular risk.22 Despite abundant experimental data using pharmacological or genetic means to demonstrate that increasing macrophage cholesterol efflux proteins ABCA1 and ABCG1 might be another promising strategy to prevent atherosclerosis progression,23,24 a limited number of agents to increase macrophage cholesterol efflux are in clinical development. Our previous work suggests that modulating intracellular iron levels within macrophages by increasing macrophage FPN may be a promising strategy to increase expression of cholesterol efflux transporters.8 Here we demonstrate for the first time how pharmacological alternation of systemic iron metabolism using a compound that suppresses liver hepcidin can alter macrophage cholesterol efflux transporters and reduce atherosclerotic lesion progression.

In this study, we showed that suppression of liver hepcidin by a BMP signaling inhibitor, LDN, leads to increased expression of FPN and to reduced intracellular iron and oxidative stress within peritoneal macrophages. These molecular alterations are associated with increased expression of the ABC transporters ABCA1 and ABCG1. Macrophages from LDN-treated mice demonstrated increased lipid efflux and reduced foam cell formation. Additional mechanistic data to support a causal role for manipulation of intracellular iron by LDN in ABC transporter expression and foam cell formation were found using hepcidin, a liver peptide known to degrade FPN. Exogenous hepcidin was able to reverse expression of macrophage ABCA1, cholesterol efflux, and the effects of LDN on foam cell formation, suggesting the importance of FPN in these LDN-mediated effects. This conceptual framework is depicted in Figure 4. In addition, in vitro incubation of LDN with isolated peritoneal macrophages had no effect on the expression of ABCA1 and ABCG1. Collectively, these data suggest that hepcidin must be suppressed in vivo to increase the expression of ABCA1 and ABCG1 in macrophages.

We also quantified the expression of lipid scavenger receptors and cholesterol uptake by macrophages to assess whether the lack of foam cell formation observed with LDN treatment was due to limited lipid uptake. We found no changes in the expression of lipid uptake receptors and in the quantity of total and free cholesterol after lipid loading. However, esterified cholesterol levels were significantly less in macrophages from LDN-treated animals as compared with control animals after cholesterol loading. Although not specifically investigated here, we have previously shown that the effects of lowering intracellular iron triggers ABC transporter activation through induction of liver X receptor-α, which is known to reduce cholesterol ester formation in macrophages.8,25 Thus, we cannot preclude that the effects of LDN on reducing foam cell formation and atherosclerosis could also be related to this mechanism in addition to increasing reverse cholesterol transport.

To evaluate the effect on atherosclerosis, LDN was administered to ApoE−/− mice on a high-cholesterol diet for 10 weeks. Results show a significant reduction in intraplaque Oil Red O-positive lipid area, total plaque area, and plaque severity, along with elevated ABCA1 immunoreactivity within plaque macrophage rich regions. These findings suggest that LDN increases the expression of ABCA1 in macrophages within atherosclerotic plaques, leading to limited lesion progression and plaque burden.

Further support for our proposed mechanism was made by showing that long-term LDN treatment resulted in no changes in serum total cholesterol and triglycerides in comparison with controls.

We did observe that LDN-treated ApoE−/− mice had less weight gain; however, these findings were not statistically significant and may be attributable to increased variability in the cohort. Previous investigators have reported this compound is well tolerated.12 Lastly, although mice treated with LDN had higher serum iron levels related to macrophage iron loss, we did not observe this manifest as iron overload toxicity on hematocrit, liver iron deposition, and cardiac function.

Prior findings from human coronary plaques and in vitro studies8 revealed that reduction in intracellular iron and iron-induced oxidative stress by increased FPN are molecular triggers that promote expression of ABCA1 and ABCG1 within macrophages localized to regions of intraplaque hemorrhage. In this investigation, we have translated these findings by pharmacologically increasing FPN in macrophages through suppressing hepcidin to increase their lipid efflux capacity and reduce foam cell formation.

**Figure 4.** Summarizing schematic. A, (1) Bone morphogenetic protein (BMP) binds (2) BMP receptor (BMPR) in the liver and activates specific transcription factors, (3) leading to transcription of hepcidin. (4) Hepcidin degrades ferroportin (FPN) in macrophages to cause accumulation of intracellular iron and iron-induced reactive oxygen species (ROS), which (5) inhibits expression of ABCA1 and ABCG1 and lipid efflux. B, LDN 193189 (LDN) inhibits BMP signaling to prevent activation of hepcidin transcription factors, leading to reduced hepcidin and maintenance of FPN in macrophages, thereby reducing intracellular iron and ROS and increasing the expression of ABCA1 and ABCG1 to promote lipid efflux and reduce foam cell formation.
A limitation inherent to our findings is that LDN does not directly inhibit hepcidin but yields its effect by inhibiting upstream BMP signaling. Prior investigations show an atheroprotective role of inhibiting BMP by transgenic overexpression of matrix Gla protein, an endogenous inhibitor of BMP signaling, leading to reduced vascular calcification, expression of intercellular adhesion molecule/vascular cell adhesion molecule, and inflammation. Thus, our findings pertaining to limited foam cell formation by augmentation of lipid efflux provide an additional—perhaps more direct—mechanism in the atheroprotective role of inhibiting systemic BMP signaling.

Despite the promising results shown here, it is important to note that before human therapy could be considered using this approach, significantly longer and more comprehensive toxicity studies would need to be performed to specifically define potential effects of short- and long-term treatment given its potential effects on iron related toxicities and tissue repair. Given the pivotal role of hepcidin in regulating iron homeostasis, its chronic inhibition could potentially result in an iron overload-like state. Although multiple mechanisms exist within humans to counteract iron-related toxicity, including the iron binding proteins ferritin and transferrin, it remains possible that such toxicity could be a limitation to the actual clinical adoption of such a strategy.

In conclusion, we show for the first time that the selective BMP inhibitor LDN can limit foam cell formation and atherosclerosis by reducing macrophage intracellular iron leading to enhanced ABC transporter expression and lipid efflux capacity. The molecular pathways by which iron-induced oxidative stress limits the expression of ABCA1 and ABCG1 are beyond the scope of this investigation but clearly merit further study. Nonetheless, the translational findings reported in this investigation emphasize that reducing intracellular iron in macrophages can be applied as a potential therapeutic strategy to augment reverse cholesterol transport and limit atherosclerosis.

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Disclosures
None.

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Supplemental Material

Supplemental Figures

Supplemental Figure I.
Supplemental Figure II.

ApoE−/− mice: 8 weeks of Lard

LDL−/− ABCA1−/− ABCG1−/− mice
Supplemental Figure III.

A. In-Vitro LDN

- ABCA1: Control (n=3) vs. in-vitro LDN (n=3)
- ABCG1: Control (n=3) vs. in-vitro LDN (n=3)

B. Scavenger Receptor Expression

- SR-A1: Control (n=6) vs. LDN (n=6)
- SR-A2: Control (n=6) vs. LDN (n=6)
- SR-B1: Control (n=6) vs. LDN (n=6)
- CD-36: Control (n=6) vs. LDN (n=6)

C. Percentage Increase

- Total Cholesterol: Control (N=4) vs. LDN (N=4)
- Free Cholesterol: Control (N=4) vs. LDN (N=4)
- Esterified Cholesterol: Control (N=4) vs. LDN (N=4)

p=0.02
### Supplemental Table 1

Forward and reverse primer sequences of the mouse genes investigated in this study.

<table>
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<tr>
<th>Gene</th>
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<th>Reverse Sequence</th>
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<td>NM_013454</td>
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<td>5-CAAGAATCTCCGGCTTTAGG-3</td>
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Supplemental Figure Legends

Supplemental figure I: Flow cytometry on mouse peritoneal cells using a macrophage specific antibody. A, peritoneal cells were isolated by lavage and incubated overnight. Then non-adherent cells were removed and remaining cells were incubated with (blue line) or without (black line) a macrophage specific fluorescent F4/80 antibody for 30 minutes. Shown are representative flow cytometric measurements from 4 separate experiments.

Supplemental figure II: Demonstration of negative antibody staining for the ATP-binding cassette transporter ABCA1 in LDL receptor knockout mice transplanted with bone marrow from ABCA1(-/-)/ABCG1(-/-) mice. Panels A to E, histologic sections from a homozygous ApoE (-/-) mouse fed a lard diet for 8 weeks. Panel A, positive staining for ABCA1 (reddish brown reaction product, arrow) exhibited by crypt cells in a section of small bowel. Panels B to E, serial sections from a foam cell-rich plaque localized to the sinotubular junction. Panel C, shows a collection of CD107b (Mac-3) positive macrophages near the luminal surface. Panel D, immunohistochemical detection of ABCA1 using a polyclonal antibody (Lifespan Technologies Inc.) shows strong positive staining in foamy macrophages. Panel E, negative control staining without primary antibody. Panel F, atherosclerotic lesion (sinotubular junction) from a LDL receptor knockout mouse transplanted with bone marrow from ABCA1/ABCG1(-/-) mice (unstained slides were courtesy of Dr. Alan R. Tall, Columbia University, NY). Panel G, atherosclerotic lesion consisting of mostly CD107b positive macrophages. Panel H, antibody staining against ABCA1 is essentially negative and similar to panel E. Panels B and F, hematoxylin and eosin staining.

Supplemental figure III: Effects of LDN treatment on macrophage ABC transporters (when given in-vitro), scavenger receptors, and cholesterol loading. A, peritoneal macrophages were incubated with or without 0.4um LDN in-vitro overnight and then RNA was extracted to measure relative expression of ABCA1 and ABCG to GAPDH (n=3, per group). B, scavenger receptor (SR-A1, SR-A2, SR-B1, CD-36) expression as assessed by quantitative pcr in peritoneal macrophages from mice treated with PBS (control) or LDN (n=6, per group). C, Percentage increase in total (left), free (center), and esterified (right) cholesterol in peritoneal macrophages from control and LDN treated mice after 48 hour loading with ox LDL (30ug/ml). Data are expressed as percentage increase (i.e. increase compared to macrophages from control and LDN mice not exposed to ox LDL).