Myeloid Cell–Specific ABCA1 Deletion Protects Mice From Bacterial Infection

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Rationale: ATP-binding cassette transporter A1 (ABCA1) plays a critical role in eliminating excess free cholesterol from tissues by effluxing cellular free cholesterol and phospholipids to lipid-poor apolipoprotein AI. Macrophage ABCA1 also dampens proinflammatory myeloid differentiation primary-response protein 88–dependent toll-like receptor signaling by reducing cellular membrane free cholesterol and lipid raft content, indicating a role of ABCA1 in innate immunity. However, whether ABCA1 expression has a role in regulating macrophage function in vivo is unknown.

Objective: We investigated whether macrophage ABCA1 expression impacts host defense function, including microbial killing and chemotaxis.

Methods and Results: Myeloid cell–specific ABCA1 knockout (MSKO) vs wild-type mice were infected with Listeria monocytogenes (Lm) for 36 hours or 72 hours before euthanasia. Lm-induced monocytosis was similar for wild-type and MSKO mice; however, MSKO mice were more resistant to Lm infection, with significantly less body weight loss, less Lm burden in liver and spleen, and less hepatic damage 3 days postinfection. In addition, Lm-infected MSKO mouse livers had: (1) greater monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 expression; (2) more monocyte/macrophage infiltration; (3) less neutral lipid accumulation; and (4) diminished expression of lipogenic genes. MSKO macrophages showed enhanced chemotaxis toward chemokines in vitro and increased migration from peritoneum in response to lipopolysaccharide in vivo. Lm infection of wild-type macrophages markedly reduced expression of ABCA1 protein, as well as other cholesterol export proteins (such as ATP-binding cassette transporter G1 and apolipoprotein E).

Conclusions: Myeloid-specific ABCA1 deletion favors host response to and clearance of Lm. Macrophage Lm infection reduces expression of cholesterol export proteins, suggesting that diminished cholesterol efflux enhances innate immune function of macrophages. (Circ Res 2012;111:1398-1409.)

Key Words: ATP-binding cassette transporter A1 ▫ bacterial killing ▫ chemotaxis ▫ macrophage

ATP-binding cassette transporter A1 (ABCA1) is a plasma membrane protein that functions to eliminate excess free cholesterol (FC) from tissues by effluxing cellular FC and phospholipids to lipid-free apolipoprotein AI, forming nascent high-density lipoprotein particles. ABCA1 plays a critical role in the movement of cholesterol from peripheral tissues to the liver in a process known as reverse cholesterol transport. Mutations that inactivate the human ABCA1 gene result in Tangier disease, which is characterized by extremely low-plasma high-density lipoprotein cholesterol concentrations, mildly elevated plasma triglyceride levels, and accumulation of cholesterol in macrophages. ABCA1 protein is widely expressed in most cells in the body, and its expression is regulated by transcriptional activation and protein degradation. Generation of cell-specific Abca1 knockout mice has helped define the role of cell-specific ABCA1 expression in whole-body high-density lipoprotein biogenesis, as well as several unanticipated roles for the transporter. For example, hepatocyte and intestinal epithelial cell ABCA1 contribute 70% to 80% and 20% to 30% of the plasma high-density lipoprotein pool, respectively. Pancreatic β-cell ABCA1 plays a role in insulin secretion and brain ABCA1 regulates neuronal structure and function.

Macrophages protect the host against exogenous and endogenous dangers by killing invading microbes and phagocytosing apoptotic or dead cells, thereby acting as one of the primary immune cell types involved in innate immunity. To explore the specific role of ABCA1 in macrophages, we generated myeloid cell–specific ABCA1 knockout (MSKO) mice. Using this unique mouse model, we demonstrated that...
macrophages from MSKO mice have a significant increase in FC and are more proinflammatory in vivo and in vitro in response to lipopolysaccharide (LPS) via toll-like receptor (TLR) 4 compared with wild-type (WT) mice. This response was mediated through a myeloid differentiation primary-response protein 88 (MyD88)-dependent pathway and was independent of alterations in plasma lipid concentrations. The hypersensitivity of MSKO macrophages to LPS was most likely because of increased lipid raft content, presumably caused by increased intracellular FC accumulation. Yvan-Charvet et al. observed a similar inflammatory phenotype in Abca1−/− Abcg1−/− macrophages compared with WT mice. More recently, we demonstrated that macrophage ABCA1 deficiency leads to selective lipid raft FC accumulation without alteration of phospholipid composition. Macrophage ABCA1 deficiency also results in more TLRs residing in lipid rafts, resulting in enhanced TLR activation. Taken together, these data suggest that ABC transporters downregulate TLR signaling by reducing FC enrichment in lipid rafts and the trafficking of TLRs into rafts.

Despite the downregulation of TLR signaling by ABCA1, little is known about whether ABCA1 expression impacts macrophage function, such as microbial killing. In the present study, we describe the innate immune response of MSKO mice during infection with the Gram-positive facultative intracellular bacterium *Listeria monocytogenes* (Lm), a widely used model of intracellular bacterial infection known to require virtually all aspects of the innate and adaptive immune responses for effective control. Here, we document that myeloid cell–specific deletion of ABCA1 leads to a greater resistance to Lm infection in mice and enhanced chemotaxis, relative to WT control mice.

### Methods

**Detailed Methods** are provided in the Online Supplement.

**Animals**

WT and MSKO (homozygous) mice were generated as described previously. Mice were backcrossed to a C57BL/6 background for 6 generations before use in the studies. All animal procedures were approved by the Wake Forest School of Medicine Animal Care and Use Committee.

**Cell Culture**

Thioglycollate-elicited peritoneal macrophages (PMs) and bone marrow–derived macrophages (BMDM) from WT and MSKO littermate mice were cultured as previously described.

### In Vivo Lm Clearance Experiments

Female mice (20–30 weeks old) were infected intraperitoneally with Lm 10403S at a dose of 5 × 10⁴ *Listeria/mouse*. Mice were euthanized 36 or 72 hours postinfection. Concentrations of cytokines/chemokines in plasma or liver lysates were measured using ELISA or Bioplex assay according to the manufacturer’s instructions. Liver protein concentration was measured by bicinchoninic acid protein assay. Bacterial burden in spleen and liver was assessed by culturing serial dilutions of tissue homogenate on brain heart infusion broth agar plates for bacterial colony formation. Small portions of liver were fixed in 10% formalin for hematoxylin and eosin or immunohistochemistry staining.

### Immunohistochemistry Staining

Livers sections were incubated with the primary antibodies to CD68 (abD Serotec), Ly6B.2 (abD Serotec), cleaved caspase-3 (Cell Signaling), and monocyte chemoattractant protein-1 (MCP-1; Novus Biologicals), followed by the biotinylated secondary antibody. The staining was visualized using ABC reagent (ABC vector kit; Vector) and AEC (Dako; for CD68, Ly6B.2 and cleaved caspase-3) or DAB substrate chromogen (Dako; for MCP-1). The percentage of liver sections covered by CD68+ cells (CD68+ area percentage) was calculated to indicate the intensity of hepatic CD68+ cells.

### Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay

In Situ Cell Death Detection Kit, Fluorescein (Roche) was used to detect hepatic apoptosis in Lm-infected mouse liver sections according to the manufacturer’s instructions.

### Macrophage Migration Assay

In vitro chemotaxis of macrophages in response to MCP-1 and macrophage inflammatory protein (MIP)-1 was performed in a 48-well microtaxis chamber as described previously. The in vivo migration assay was performed as described previously with minor modifications. Briefly, WT mice were injected intraperitoneally with 1 mL of 10% thioglycollate to elicit sterile peritonitis. Three days later, the mice were similarly injected with an equal number (5 × 10⁴) of BMDM from WT (labeled with cell tracker green CMFDA) and MSKO (labeled with cell tracker red CMPTX; both from Molecular Probes) mice. Twenty hours later, 400 ng LPS was injected intraperitoneally into the mice. Three hours later, mice were euthanized, peritoneal cells were harvested, and the proportion of fluorescent-labeled macrophages in all peritoneal cells was analyzed by flow cytometry.

### Western Blotting and Real-Time Polymerase Chain Reaction

WT BMDMs were infected with Lm 10403S for 0, 6, 12, and 24 hours before harvesting protein using radioimmunoassay precipitation assay (RIPA) buffer containing proteinase inhibitor cocktails (Roche) and RNA using TRIzol reagent (Invitrogen), respectively. Protein expression was examined using Western blotting and mRNA expression was examined using real-time polymerase chain reaction (PCR).

### Flow Cytometry

After blocking the Fcγ receptor with purified anti-mouse CD16/CD32 antibody (Fcγ receptor III/II; BD Biosciences), blood, bone marrow cells, or macrophages were incubated at 4°C for 30 minutes with isotype controls or the following Abs: FITC-anti-CD11b (M1/70; BD Pharmingen); PE-anti-Ly6C (AL-21; BD Pharmingen); APC-anti-CD45 (30-F11; BD Pharmingen); PerCP-Cy5.5-anti-Ly6G (RB6-BC6; eBioscience); or APC-C-C chemokine receptor type 2 (CCR2) (475301; R&D Systems). Cell fluorescence was measured and analyzed with FlowJo software.

### Statistics

Data are presented as the mean±SD unless indicated otherwise. Differences were compared with 2-tailed Student *t* test or 1-way
Results
MSKO Mice Are More Resistant to Lm Infection
To investigate the potential involvement of macrophage ABCA1 in bacterial killing, we first challenged WT and MSKO mice with the Gram-positive intracellular bacteria Lm; mice were euthanized 3 days postinfection. WT mice infected with Lm lost 15% of their body weight (Figure 1A). Weight loss was significantly less in Lm-infected MSKO mice, suggesting a milder effect of infection. Consistent with this finding, MSKO mice had significantly better clearance of Lm compared with WT mice, shown by 3-log lower liver Lm burden and 2-log lower spleen Lm burden (Figure 1A). Livers of WT mice had numerous microabscesses consisting of a large number of neutrophils (Figure 1B, top row); these were fewer and smaller in MSKO mice (Figure 1B, bottom row). There also were multiple droplets in WT compared with MSKO livers (Figure 1C). Enzymatic lipid assays confirmed the marked lipid accumulation induced by Lm infection in WT liver, showing a significant increase in triglyceride, total cholesterol, and free cholesterol concentrations in Lm-infected WT vs MSKO or uninfected mouse liver (Figure 1D). Furthermore, this increased lipid accumulation induced by acute Lm infection was associated with decreased expression of lipogenic genes such as sterol regulatory element–binding protein-1c and stearoyl-CoA desaturase-1 and increased expression of the cholesterol esterification gene acyl-coenzyme A:choline acyltransferase-2 in infected WT liver (Online Figure I). Collectively, compared with WT mice, MSKO mice displayed a greater resistance to Lm infection with fewer bacteria in liver and spleen and less hepatic histological damage and lipid accumulation, implying that myeloid cell–specific ABCA1 deletion protected mice from acute systemic Lm infection.

Increased Hepatic Monocyte/Macrophage Infiltration in Lm-Infected MSKO Mice
Macrophages and neutrophils are 2 major innate immune cells responsible for Lm clearance at the early stage of acute Lm infection. To examine whether the differences in Lm clearance between the 2 genotypes were attributable to differences in the number of immune cells in the liver, we first performed immunohistochemical staining to visualize monocyte/macrophages (using antibody against CD68) and neutrophils (using antibody against Ly6B.2) in the livers of control (uninfected) and Lm-infected (3-day infection) mice. CD68+ cells in Lm-infected MSKO liver were greatly increased compared with WT and uninfected control mice, indicating significantly more macrophage infiltration in livers of MSKO mice (Figure 2A). Real-time PCR analysis confirmed the increased number of macrophages in livers of MSKO mice, shown by a significantly greater expression of CD68 and F4/80 (macrophage markers) in MSKO liver compared with livers of uninfect ed and WT mice. There also was massive neutrophil influx in livers of both Lm-infected WT and MSKO mice (Online Figure II). Interestingly, the distribution of neutrophils in liver differed between 2 genotypes. Neutrophils formed large foci (abscesses) in livers of infected WT mice and were almost absent outside of abscesses. However, in infected MSKO mice, neutrophils were more diffusely distributed throughout the liver (Figure 1B; Online Figure I). Because macrophages and neutrophils are central in Lm killing during early infection, the increased infiltration of monocytes/macrophages and more evenly distributed neutrophils in livers of MSKO mice may partially explain the significantly improved Lm clearance.

Reduced Inflammatory Responses in MSKO Mice 3 Days After Lm Infection
To further explore enhanced bacterial clearance in MSKO mice, we next assessed the expression of plasma and liver cytokines/chemokines in WT and MSKO mice 3 days after infection. Compared with WT, Lm-infected MSKO mice showed significantly increased plasma interleukin (IL)-12p40 but a trend toward decreased interferon-γ, IL-6, and tumor necrosis factor-α (TNF-α) concentrations (Figure 3A, left panel). The levels of plasma chemokines (MCP-1, MIP-2, mouse homolog of human interleukin-8, and MIP-1β) also were generally lower in MSKO vs WT mice (Figure 3A, right panel). Furthermore, IL-6 was significantly reduced in livers of Lm-infected MSKO vs WT mice, but IL-12p40 and TNF-α production was similar. MSKO mice showed a significant reduction in liver chemokines compared with WT mice. Messenger RNA levels of liver cytokine/chemokine expression analyzed by real-time PCR were consistent with protein concentrations (Online Figure II), indicating less inflammation in the local microenvironment. The reduced Lm burden and the reduced plasma and liver cytokine/chemokine profile 3 days postinfection suggest a more rapid and efficient resolution of infection and inflammation in MSKO vs WT mice.

Apoptosis Does Not Account for the Differential Macrophage Accumulation in Lm-Infected MSKO Versus WT Mice
Expression of ABCA1 is highly regulated by liver X receptor (LXR) in macrophages.19 LXR activation protects mice from Lm infection by regulating apoptosis inhibitor expressed by macrophages; (also known as SPα) to reduce macrophage apoptosis.20,21 In our study, SPα may have been induced in ABCA1-deficient macrophages because of sterol accumulation and LXR activation, resulting in the increased hepatic accumulation of monocytes/macrophages in Lm-infected MSKO vs WT mice. However, although FC was slightly but significantly elevated in MSKO vs WT macrophages, as shown previously,12 macrophage oxysterol content did not differ between the 2 genotypes (Online Figure IVA). Macrophage SPα expression also was indistinguishable between genotypes treated with or without LXR agonist (TO-901317) (Online Figure IVB). Finally, neither terminal deoxynucleotidyl transferase dUTP nick-end labeling assays nor immunohistochemical analysis of cleaved caspase-3 revealed a difference in apoptosis in immune cells in Lm-infected livers between the 2 genotypes (Online Figure IVC and IVD). Taken together, these data rule out a major role for apoptosis in the differential accumulation of hepatic monocytes/macrophages between genotypes.
Figure 1. Myeloid cell–specific ATP-binding cassette transporter A1 knockout (MSKO) mice are more resistant to Listeria monocytogenes (Lm) infection. Wild-type (WT) and MSKO mice were intraperitoneally infected with Lm (strain 10403S) for 3 days. A, Body weight loss (left) and bacterial counts in liver (middle) and spleen (right) after 3 days of Lm infection. B, Histological analysis of the livers 3 days post-Lm infection. Neutrophilic microabscesses are visualized by hematoxylineosin (HE) staining (objective magnification ×10 and ×40) and immunohistochemical staining using an antibody against Ly6B.2 (neutrophil marker, objective magnification ×40). C and D, Lipid analysis of livers 3 days after Lm infection. HE staining (objective magnification ×40) revealed massive accumulation of hepatic lipid droplets in Lm-infected WT mice relative to MSKO mice (C). Liver triglyceride (TG), free cholesterol (FC), and total cholesterol (TC) were measured by enzymatic assays (D). Data are expressed as mean±SD. *P<0.05 and values with different letters are statistically different (P<0.05).
WT and MSKO Mice Had Similar Inflammatory Monocyte Egression From Bone Marrow to Circulation

Infection of mice with Lm rapidly induces CCR2-dependent emigration of Ly6C^{hi}CD11b^{+} monocytes from the bone marrow to the circulation, which is essential for host defense against pathogens. To determine whether the increased numbers of hepatic monocytes/macrophages in Lm-infected MSKO mice resulted from enhanced egress of Ly6C^{hi} monocytes from bone marrow to the circulation, we infected mice with Lm for 36 hours and characterized the expression of the monocyte markers CD11b and Ly6C in blood and bone marrow leukocytes (defined as CD45^{+} cells) as described by Serbina and Pamer. As shown in Figure 4A, compared with uninfected mice, Lm infection induced a significantly greater percentage of Ly6C^{hi}CD11b^{+} cells in the bloodstream. There also was a significantly reduced percentage of Ly6C^{hi}CD11b^{+} cells in the bone marrow of Lm-infected mice vs controls (data not shown). In addition, we observed a marked increase in the percentage of Ly6G^{+} cells (neutrophils) in blood circulation 36 hours after Lm infection compared with uninfected mice (Figure 4B). However, the frequency distribution of circulating Ly6C^{hi}CD11b^{+} cells and Ly6G^{+} cells did not differ between the 2 genotypes, suggesting indistinguishable egress of innate immune cells from the bone marrow into the bloodstream in response to acute Lm infection. Together, these data suggest that the greater accumulation of hepatic innate immune cells in MSKO mice 3 days postinfection likely was not because of increased bone marrow egress of myeloid cells into blood.

Increased Chemokine Expression in Liver in MSKO Mice at 36 hours After Lm Infection

Because the profile of plasma and liver cytokines/chemokines 3 days postinfection suggested better-resolved inflammation in MSKO mice, we measured plasma and liver cytokine/chemokine expression at an earlier time point (ie, 36 hours) after Lm infection. Lm-infected mice had increased plasma concentrations of cytokines (IL-6 and IL-12p40) and chemokines (MCP-1, MIP-2) compared with uninfected mice (Figure 5A), but there was no difference between genotypes, unlike results obtained 3 days after infection (Figure 3). However, Lm-infected MSKO mice had significantly greater hepatic expression of MCP-1 and MIP-2 and a trend toward increased MIP-1α expression (Figure 5B). Hepatic expression of cytokines (IL-6, TNF-α, and IL-12p40) and other chemokines (MIP-1α and mouse homolog of human...
interleukin-8) was indistinguishable between the 2 genotypes (data not shown). MCP-1 was mainly expressed in perivascular-infiltrated mononuclear leukocytes (Figure 5C). Of the 6 liver sections from each genotype, only 1 WT mouse liver had MCP-1−positive perivascular inflammatory infiltrates, whereas 4 of 6 MSKO mouse livers showed massive perivascular leukocyte infiltration with positive MCP-1 staining (Figure 5C). Because MCP-1 and MIP-2 are potent chemoattractants for macrophages and neutrophils, respectively, increased hepatic MCP-1 and MIP-2 expression in Lm-infected MSKO mice during the early stages of infection may have promoted Lm clearance by rapid recruitment of myeloid cells into tissues involved in Lm clearance, such as the liver.

**MSKO Macrophages Have Enhanced Chemotaxis**

Whether ABCA1 is involved in the regulation of macrophage chemotaxis is controversial. We first examined the chemotactic response of MSKO and WT macrophages to MCP-1 and MIP-1α using a 2-chamber chemotaxis assay. BMDM from MSKO mice showed significantly increased migration toward MCP-1 and MIP-1α gradients compared with WT mice (Figure 6A). This difference suggests that ABCA1 deficiency enhances macrophage migration in response to chemotactic factors, consistent with previous findings by Francone et al. Similar data were obtained with thioglycollate-elicited PMs (Online Figure V). This enhanced chemotaxis to MCP-1 in MSKO macrophages was not associated with a difference in the CCR2 surface expression (data not shown). Furthermore, the increased migration capacity of MSKO vs WT macrophages also was observed in vivo, with more rapid egress of MSKO macrophages out of the peritoneum in response to LPS injection (Figure 6B). Thus, the increased hepatic monocyte/macrophage infiltration in Lm-infected MSKO mice results both from enhanced chemokine expression in locally inflamed tissues and from accelerated chemotaxis of ABCA1-deficient macrophages.

**Lm Infection Downregulates Lipid Export Gene Expression in WT Macrophages**

Gram-negative bacterial (Escherichia coli) or viral infection downregulates LXR target gene expression, such as ABCA1 and apolipoprotein E (apoE), via activation of transcription factor interferon regulatory factor-3, but the physiological role of this downregulation is unclear. Lm upregulates expression of the LXR target gene SPα, which inhibits macrophage apoptosis. To investigate whether Lm also regulates ABCA1 and other lipid metabolism-related LXR target gene expression, we infected WT macrophages with Lm for 6, 12, and 24 hours. Lm infection strongly inhibited the protein expression of ABCA1, ABCG1, and apoE, especially at 24 hours postinfection (Figure 7A). Lm infection also downregulated mRNA expression of LXR target genes such as ABCG1, apoE, phospholipid transfer protein, and sterol regulatory element–binding protein-1c, as measured by real-time PCR (Figure 7B). mRNA levels of ABCA1 were unchanged by Lm infection and ABCG1 mRNA expression was much less reduced compared with protein reduction induced by Lm, indicating that inhibition of these lipid export proteins induced by Lm infection most likely occurs at the posttranscriptional level. Interestingly, Lm infection significantly induced LXRα expression without alteration of LXRβ (Figure 7C), consistent with the findings of Joseph et al. As expected, Lm infection induced expression of multiple inflammatory genes (Figure 7D). Because macrophages...
lacking ABCA1 clear and eliminate Lm more efficiently (Figure 1A), these results suggest that downregulation of lipid export gene expression by Lm infection facilitates macrophage clearance of pathogens.

Discussion

Macrophages play a critical role in innate immunity by killing invading pathogens and eliminating apoptotic cells. Macrophage ABCA1 expression dampens MyD88-dependent TLR signaling by facilitating FC efflux and reducing plasma membrane lipid rafts. However, whether ABCA1 expression also impacts macrophage function, such as bacterial killing or chemotaxis, is less clear. In this study, we demonstrate that myeloid cell–specific ABCA1 deficiency renders mice more resistant to Lm infection and macrophage Lm infection downregulates cholesterol export proteins. We also show that macrophage ABCA1 deletion accelerates macrophage migration toward chemoattractants, which may partially explain the enhanced bacterial killing in MSKO mice. Collectively, our data suggest that deletion of macrophage ABCA1 enhances macrophage function.

The gram-positive facultative intracellular bacterium Lm is a widely used model of intracellular bacterial infection. Bacteria are first internalized into a vacuole, also known as a phagosome. In the vacuoles, Lm secretes the pore-forming toxin Listeriolysin O and phospholipase C, which lyse phagosomal membranes and allow Lm to escape into cytosol. In the cytosol, the bacteria rapidly replicate and recruit and polymerize host cell actin. The polymerization of actin at one pole of the cell produces energy to propel bacterial entry into neighboring cells. Lm infection stimulates macrophage MyD88-dependent secretion of IL-12 and TNF-α, which stimulates natural killer cells to generate interferon-γ, enhancing macrophage bactericidal action. Mice lacking MyD88, IL-12, or interferon-γ are more susceptible to Lm infection by macrophages, indicating the important role of MyD88-dependent cytokine production in Lm clearance.

Figure 4. Listeria monocytogenes (Lm) infection induced blood monocytosis and neutrophilia in mice. Wild-type (WT; n=6) and myeloid cell–specific ATP-binding cassette transporter A1 knockout (MSKO; n=9) mice were infected intraperitoneally with Lm (strain 10403S) for 36 hours. A, Lm infection resulted in increased Ly6C<sup>hi</sup> (Ly6Chi) CD11b<sup>+</sup> monocytes in peripheral blood relative to uninfected mice. Left panel shows representative fluorescence-activated cell sorting (FACS) plots of blood cells gated on CD45<sup>+</sup> leukocytes and stained for CD11b and Ly6C; right panel shows quantified data. B, Lm infection resulted in increased Ly6G<sup>+</sup> cells in peripheral blood. Left panels show representative FACS plots of blood cells gated on CD45<sup>+</sup> leukocytes and stained for Ly6G; right panels show quantified data. Data are expressed as mean±SD. **P<0.01.
Figure 5. Cytokine/chemokine expression in mouse plasma and liver 36 hours after *Listeria monocytogenes* (Lm) infection. Wild-type (WT; n=6) and myeloid cell−specific ATP-binding cassette transporter A1 knockout (MSKO; n=9) mice were intraperitoneally infected with Lm (strain 10403S) for 36 hours. Plasma (A) and liver (B) cytokine/chemokine concentrations were quantified by Bioplex assay. Liver protein concentration was measured by bicinchoninic acid protein assay. C, Monocyte chemoattractant protein-1 (MCP-1)−positive cells in liver were visualized by immunohistochemical staining using antibody against MCP-1 (objective magnification ×10 and ×40). Data are expressed as mean±SEM. *P<0.05. MIP-2 indicates macrophage inflammatory protein-2; IL, interleukin.
Because our previous studies established that ABCA1-deficient macrophages are hypersensitive to MyD88-dependent TLR stimulation, we hypothesized that MSKO mice may have enhanced Lm clearance with increased proinflammatory cytokine production because of exaggerated MyD88-dependent TLR response to Lm infection. In support of this hypothesis, we observed significantly better clearance of Lm in MSKO mice. However, the cytokine profile in mice at 36 hours or 3 days postinfection showed no differences in MSKO mice, suggesting that MyD88-dependent cytokine production induced by Lm infection does not play a significant role in the increased Lm clearance in MSKO mice. Alternatively, increased efficiency of Lm clearance in MSKO mice may have allowed a more rapid return of plasma cytokines to basal levels.

Compared with MSKO mice, WT mice infected with Lm showed marked hepatic triglyceride and cholesterol accumulation. In rodents, infection and inflammation stimulate adipose tissue lipolysis and increase de novo hepatic fatty acid and cholesterol synthesis, coupled with suppression of fatty acid oxidation, decreased low-density lipoprotein clearance, and conversion of cholesterol to bile acids. In our study, the more severe Lm infection in WT vs MSKO mice may have led to greater adipose tissue lipolysis, resulting in increased hepatic lipogenesis (fatty acid, triglyceride, and cholesterol) and lipid accretion. However, the marked hepatic lipid accumulation in Lm-infected mice resulted in downregulation of de novo lipogenic genes (eg, 3-hydroxyl-3-methyl-glutaryl-CoA reductase synthase, sterol regulatory element–binding protein-1c, stearoyl-CoA desaturase-1, and acetyl-CoA carboxylase 1) and increased acyl-coenzyme A:cholesterol acyltransferase-2 to reduce FC accumulation in liver by conversion of FC to cholesteryl ester (Online Figure I). The more efficient clearance of Lm in MSKO vs WT mice reduced the inflammatory state of the liver and completely abrogated the increase in hepatic neutral lipid content (Figure 1C and 1D).

Macrophages and neutrophils are the 2 major innate immune cells responsible for Lm killing during the early phase of infection. Our results demonstrate that 3 days postinfection, MSKO mice had significantly more monocytes/macrophages and neutrophils in the liver than WT mice, indicating an improved local microenvironment for bacterial clearance. We hypothesized that increased macrophage/macrophages in Lm-infected MSKO liver might result from: (1) decreased hepatic immune cell apoptosis; (2) increased migration of Ly6Chigh monocytes from the bone marrow; (3) increased hepatic chemokine production; or (4) increased chemotaxis of MSKO macrophages to the liver. Our data indicate that the increased macrophage and neutrophil accumulation in the livers of MSKO mice is not because of the decreased apoptosis of the hepatic leukocytes or enhanced recruitment of Ly6C<sup>high</sup> inflammatory monocytes from the bone marrow; (3) increased hepatic chemokine production; or (4) increased chemotaxis of MSKO macrophages to the liver. Our data indicate that the increased macrophage and neutrophil accumulation in the livers of MSKO mice is not because of the decreased apoptosis of the hepatic leukocytes or enhanced recruitment of Ly6C<sup>high</sup> inflammatory monocytes from the bone marrow to the bloodstream. We observed a significant elevation in hepatic MCP-1 and MIP-2 production and a marked increase in positive staining for MCP-1 in the perivascular leukocyte infiltrates in MSKO vs WT mice at 36 hours post-Lm infection. MIP-2 is a potent neutrophil chemoattractant. Signaling via its receptor CCR2, MCP-1 is a potent monocyte/macrophage chemoattractant and is readily detected in liver and spleen of Lm-infected mice. Mice lacking the CCR2 receptor are highly susceptible to Lm infection. Thus, increased hepatic MCP-1 and MIP-2 expression and increased MCP-1–producing leukocyte infiltration in MSKO mice may partially explain the more rapid recruitment of monocytes/macrophages and neutrophils to the foci of infection, leading to a more efficient pathogen clearance. Macrophage MCP-1
Figure 7. *Listeria monocytogenes* (Lm) infection inhibits cellular lipid export gene expression. Bone marrow-derived macrophages from wild-type (WT) mice were infected with Lm for 0, 6, 12, and 24 hours before analysis. A, Protein expression in control and Lm-infected macrophages was analyzed by Western blots. β-actin was used as a loading control. The intensity of each target protein band was normalized to β-actin and the intensity of 1 uninfected sample was set to 1; intensity of each protein band relative to the uninfected sample is shown under the gel bands.

B–D, mRNA expression of lipid export genes (B), liver X receptor (LXR) α, LXRβ (C), and inflammatory genes (D) was measured by real-time polymerase chain reaction and was normalized to GAPDH. Data are expressed as mean±SD of 2 independent experiments. Values with different letters are statistically different (P<0.05). ABCA1 indicates ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; apoE, apolipoprotein E; TNF-α, tumor necrosis factor-α; IL, interleukin.
production can be induced by WT Lm, but not by heat-killed or Listeriolysin O–deficient Lm, even though macrophage TNF-α or IL-12p40 production is similar.30,32 These results suggest that Lm-induced MCP-1 production requires cytosolic invasion by bacteria and is independent of classic TLR-MyD88 signaling, and likely is mediated via unknown cytosolic receptors. If so, then enhanced hepatic production of MCP-1, and perhaps MIP-2, in Lm-infected MSKO mice may result from both upregulated MyD88-dependent TLR signaling and MyD88-independent cytosolic receptor signaling.

ABCA1 has been implicated in macrophage chemotaxis, although results have been conflicting.16,24 Using resident PMs, Francone et al.16 observed a significant increase in chemotactic migration of Abca1−/−LDLr−/− vs LDLr−/− macrophages, suggesting that ABCA1 expression limits macrophage migration toward chemokines. In contrast, using thioglycollate-elicited PMs, Pagler et al.24 found no difference in macrophage migration toward chemokines between ABCA1-deficient and WT macrophages. Instead, they found that Abca1−/−Abcg1−/− vs WT macrophages had increased plasma membrane FC and defective redistribution of sterol to the outer leaflet, which resulted in increased Rac1 signaling and impaired chemotaxis. Using both elicited PMs or BMDMs, we consistently observed a significant increase in chemotaxis of MSKO macrophages in response to MCP-1 and MIP-1α, consistent with Francone’s finding. Meanwhile, our in vivo migration assay further supported enhanced chemotaxis of MSKO vs WT macrophages. The enhanced chemotaxis in ABCA1-deficient macrophages provides another potential explanation for increased monocytic/macrophage infiltration in Lm-infected MSKO mice, although the underlying mechanism is unknown and still under investigation.

Expression of ABCA1, especially in macrophages, is highly regulated by LXR,17 a nuclear receptor with an established role in lipid metabolism. Interestingly, one function of LXR is to regulate apoptosis inhibitor of macrophages (also known as SPα) to reduce macrophage apoptosis in response to Lm infection.20,21 Gram-negative bacteria, or bacterial products such as LPS, downregulate LXR targeted gene expression related to lipid metabolism,25 although the physiological role of this downregulation remains unknown. In our current study, we observed that Lm infection, like Gram-negative bacterial infection, markedly reduced expression of ABCA1, ABCG1, apoE, and other LXR-responsive genes, mainly at the posttranscriptional level. Notably, macrophages lacking ABCA1 and ABCG1 are proinflammatory.13–14 This, thus, acute bacterial infection not only activates LXR to upregulate SPα expression to prevent macrophages from apoptosis but also lowers cholesterol transporter gene expression to mount a more robust inflammatory response that aids host defense against infections. However, infection also may worsen chronic inflammation-related diseases, such as atherosclerosis, by exaggerating foam cell formation because of the downregulation of cellular lipid export proteins.

In summary, we have shown that ABCA1 deficiency in myeloid cells protects the host against bacterial infection by accelerating macrophage chemotaxis and increasing chemokine expression in local infected tissue. Bacterial infection also downregulates macrophage LXR-induced cholesterol export proteins. Thus, ABCA1 not only plays a key role in lipid metabolism but also plays a key role in innate immunity, suggesting a more diverse functional role for this transporter than originally envisioned. Our study also suggests that diminished myeloid cholesterol efflux enhances macrophage function and facilitates innate immunity against acute pathogen infection.

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Disclosures
None.

References
Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages.


What Is Known?

• ATP-binding cassette transporter AI (ABCA1) is a plasma membrane protein that transports cellular free cholesterol and phospholipids to lipid-free apolipoprotein A1, forming nascent high-density lipoprotein particles and eliminating excess free cholesterol from tissues.

• ABCA1 attenuates macrophage inflammation by downregulating toll-like receptor signaling via reducing free cholesterol enrichment in membrane lipid rafts and the trafficking of toll-like receptors into rafts.

What New Information Does This Article Contribute?

• Myeloid cell-specific Abca1 knockout (MSKO) mice are more resistant to acute infection with intracellular bacteria Listeria monocytogenes (Lm) compared with wild-type mice.

• MSKO mice infected with Lm have enhanced macrophage chemotaxis and increased hepatic chemokine expression, resulting in more rapid and efficient clearance and killing of Lm.

• Lm infection reduces expression of macrophage cholesterol export proteins, suggesting that diminished myeloid cholesterol efflux enhances macrophage innate immune function.

Macrophages are one of the primary cell types involved in innate immunity and chronic inflammatory diseases, such as atherosclerosis, type 2 diabetes mellitus, and metabolic syndrome. The membrane lipid transporter ABCA1 plays a key role in removing excess cholesterol from peripheral tissues for transport to liver for excretion. In macrophages, ABCA1 also attenuates inflammatory signaling by decreasing plasma membrane free cholesterol and lipid raft content. We evaluated the hypothesis that specific deletion of ABCA1 in myeloid cells (macrophages and neutrophils) protects mice from acute bacterial infection. We show that MSKO mice infected with Lm have less bacterial burden in liver and spleen concomitant with increased macrophage and neutrophil infiltration. Increased myeloid cell infiltration results from elevated tissue chemokine expression that is augmented by bacterial infection. As well as enhanced migration of macrophages toward chemokine gradients in MSKO mice compared with wild-type controls. Bacterial infection markedly reduced expression of cellular lipid export proteins (e.g., ABCA1, ABCG1, and apolipoprotein E), mainly at the post-transcriptional level. Our data suggest that acute bacterial infection likely lowers cellular cholesterol export protein expression to mount a more robust inflammatory response that aids host defense against infections. However, acute infection or chronic low-grade inflammatory diseases, such as atherosclerosis, may exacerbate macrophage foam cell formation by downregulating cholesterol export proteins, worsening outcome.

Zhu et al  ABCA1 and Macrophage Function 1409

Novelty and Significance

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Supplemental Material

Myeloid Cell Specific ABCA1 Deletion Protects Mice from Bacterial Infection

Xuewei Zhu¹, Marlena M. Westcott², Xin Bi¹, Mingxia Liu¹, Kymberly M. Gowdy³, Jeongmin Seo¹, Qiang Cao⁴, Abraham K. Gebre¹, Michael B. Fessler³, Elizabeth M. Hiltbold² and John S. Parks¹, ⁵

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Running title: ABCA1 and macrophage function

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MATERIALS AND METHODS

Animals
Wild type (WT) and myeloid cell specific ABCA1 knockout (MSKO, homozygous) mice were generated as described previously. Mice were backcrossed to C57BL/6 background for six generations before use in the studies. All animal procedures were approved by the Wake Forest School of Medicine Animal Care and Use committee.

Cell culture
Peritoneal macrophages (PMs) were harvested from WT and MSKO littermate mice 4 days after receiving an intraperitoneal injection of 1 ml 10% thioglycolate and plated in RPMI media containing 1% Nutridoma SP (NutSP) media (Roche Applied Science) as previously described. Bone marrow from WT and MSKO littermate mice was isolated and cultured in DMEM media containing 20% FBS and 30% L929 conditioned media for 5-7 days before being used in experiments as bone marrow-derived macrophages (BMDMs).

Bacteria
L. monocytogenes strains used in this study was the wt strain 10403S. For Lm infection experiments, bacteria were first grown overnight at 30°C in brain–heart infusion broth (BHI) to reach the stationary phase before washed twice in PBS and resuspended in PBS or medium at indicated concentrations based on experimental designs as described below.

In vivo Lm clearance experiments
Female mice (20-30 wk old) were infected i.p. with Lm 10403S at a dose of 5 × 10⁴ Listeria/mouse. At 36h or 72h post infection, mice were sacrificed using ketamine/xylazine. Blood was taken via cardiac puncture for plasma cytokine and chemokine measurement by ELISA or Bioplex assay. Mouse body weight was monitored before and after 3 days of Lm infection. To determine organ Listeria burden, spleen and liver were homogenized in sterile H2O at day 3 infection. Serial dilutions of homogenate were plated on brain heart infusion agar, and bacterial colony formation was assessed after overnight growth at 37°C. Small portions of liver were fixed in 10% formalin.

Histology and immunohistochemistry (IHC)
Formalin fixed liver tissues were dehydrated in ethanol and embedded in paraffin. Sections (4 μm thick) were cut and stained with hematoxylin and eosin (H&E) for evaluation of pathological changes. For IHC analysis, livers sections were deparafinized and hydrated through ethanol to H2O. Antigen retrieval was achieved using a microwave method with target retrieval solutions (Dako). Endogenous peroxidase activity was blocked in 0.3% H₂O₂ in PBS (20 min). The tissue sections were incubated with an appropriate normal serum (30 min) before incubation for 1 h at room temperature (RT) with the primary antibodies to CD68 (abD Serotec), Ly6B.2 (abD Serotec), cleaved caspase-3 (Cell signaling) and MCP-1 (Novus Biologicals). The sections were then washed and incubated for 30 min at RT with the appropriate biotinylated secondary antibody. ABC reagent was then added to the sections for 30 min (ABC vector kit; Vector) and revealed with AEC (Dako; for CD68, Ly6B.2 and cleaved caspase-3) or DAB substrate chromogen (Dako; for MCP-1). The sections were counterstained with Hematoxylin and mounted from water using an aqueous mounting medium (Vector). The staining was analyzed by Image Pro software. The percentage of liver sections covered by CD68⁺ cells (% CD68⁺ area) was calculated to indicate the intensity of hepatic CD68⁺ cells.

TUNEL assay
In Situ Cell Death Detection Kit, Fluorescein (Roche) was used to detect hepatic apoptosis in Lm infected mouse liver sections according to the manufacturer’s instruction. Lm infected mouse liver sections incubated with Label solution only (without terminal transferase) instead of the TUNEL reaction mixture was used as a negative control. Lm infected mouse liver sections pre-incubated with DNase I prior to labeling procedures was used as a positive control.

Oxysterol measurement
Oxysterol content of thioglycollate-elicited peritoneal macrophages from WT (n=3) and MSKO (n=4) mice was measured by using isotope dilution-mass spectrometry as described by Dzeletovic et al and results were normalized to cellular protein measured by Lowry assay.
**Macrophage Migration Assay**

In vitro chemotaxis assay was performed as described before with minor modification. Thioglycollate elicited PMs were incubated in RPMI-1640 containing 1% NutSP media overnight. PMs were then gently scraped from dishes and resuspended in RPMI-1640 + 1% NutSP media (chemotaxis media) before used in the chemotaxis assay. BMDMs were incubated in chemotaxis media for at least 4 hours before used in the assay. For chemotaxis assay, cells were suspended at a concentration of 2×10^6 cells/mL in chemotaxis media. Fifty microliter of cell suspension was loaded in the upper chamber of a 48-well microtaxis chamber. And 25 μl of MCP-1 (1 μM and 10 μM, Pepro Tech Inc) and MIP-1α (10 μM, Pepro Tech Inc) in chemotaxis media was added to the lower chamber. A 5-μm polycarbonate membrane separated the upper and bottom chambers. After a 2-hour incubation at 37°C, macrophages attached to the underside of the membrane were fixed and stained using the Diff-Quick stain set (Dade Behring Inc). The results are expressed as the mean number of cells that migrated in 5 high-power fields (40× objective) in 4 replicate samples. An in vivo migration assay was performed as described before with minor modification.

Briefly, WT mice were injected intraperitoneally (i.p.) with 1 ml of 10% thioglycollate to elicit sterile peritonitis. Three days later, the mice were i.p. injected with an equal number (5×10^6) of BMDMs from WT (labeled with cell tracker green CMFDA) and MSKO (labeled with cell tracker red CMPTX; both from Molecular Probes) mice. Twenty hours later, 400 ng LPS was i.p. injected into the mice. Three hrs later, mice were sacrificed, peritoneal cells were harvested with PBS, and the frequency of fluorescent-labeled macrophages in total peritoneal cells was analyzed by flow cytometry.

**Flow cytometry**

Blood were collected from uninfected or Lm infected mice via cardio-puncture. Bone marrow cells were harvested from mouse femurs. Erythrocytes were lysed with ACK lysis buffer. After blocking the Fcγ receptor with purified anti-mouse CD16/CD32 antibody (Fcγ receptor III/II; BD Biosciences), blood or bone marrow cells were incubated at 4 °C for 30 min with isotype controls or the following Abs: FITC-anti-CD11b (M1/70, BD phamingen), PE-anti-Ly6C (AL-21, BD phamingen), APC-anti-CD45 (30-F11, BD phamingen) and Percp-Cy5.5-anti-Ly6G (RB6-BC6, eBioscience). Cell fluorescence was determined using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software.

**Western Blotting and Real time PCR**

In vitro Lm infection was performed as described before. Briefly, 2×10^6 BMDMs in RPMI-1640 containing 10% FBS were seeded on a 60-mm tissue culture dish. Approximately 8×10^6 wt bacteria (10403S) were used to infect BMDMs as described before. One hour post infection, the macrophages were washed with PBS and fresh medium containing 10 μg/ml gentamicin was added to kill extracellular bacteria. After 6, 12, and 24h infection, macrophages were lysed for protein using RIPA buffer containing proteinase inhibitor cocktails (Roche) or RNA using TRIzol reagent (Invitrogen), respectively. Protein expression was examined using western blotting and mRNA expression was examined using real time PCR. In a separate experiment, BMDMs from WT and MSKO mice were incubated with 10 μM TO-901317 or vehicle (DMSO) for 24h before harvesting RNA. Primers for SPα were provided by Dr. Peter Tontonoz (Howard Hughes Medical Institute, University of California, Los Angeles School of Medicine). Other primers can be found in our previous published paper or were listed in Supplemental Table I.

**Statistics**

Differences were compared with two-tailed Student’s t-test using GraphPad Prism software. P < 0.05 was considered statistically significant. Data are presented as the means ± SD unless indicated otherwise.

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**Supplemental Table I: Real time PCR primer sequences**

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Reference List


Online Figure I. Lipogenic gene expression in livers in control (uninfected) and Lm infected (3 days) mice. Gene expression was analyzed using real time PCR and normalized to GAPDH. WT: wild type; MSKO: myeloid cell-specific ABCA1 knockout.

Online Figure II. Neutrophil infiltration in livers 3 days post Lm infection. Immunohistochemistry staining was performed to visualize neutrophils in liver in uninfected and Lm infected mice using antibody against Ly6B.2 (objective magnification 10× and 40×). WT: wild type; MSKO: myeloid cell-specific ABCA1 knockout.

Online Figure III. Inflammatory cytokine and chemokine mRNA expression in livers of control (uninfected) and Lm infected (3 days) mice. Gene expression was analyzed using real time PCR and normalized to GAPDH. Values with different letters are statistically different (P<0.05). WT: wild type; MSKO: myeloid cell-specific ABCA1 knockout.

Online Figure IV. Oxysterol and SPα expression in macrophages and apoptosis in livers of Lm infected mice. (A) Oxysterol species in thioglycollate elicited PMs from WT (n=3) and MSKO (n=4) mice were measured using isotope dilution-mass spectrometry. (B) SPα mRNA expression in BMDMs from WT and MSKO mice was measured by real time PCR and normalized to GAPDH. Macrophages were treated with synthetic LXR agonist 10 μM TO-901317 or vehicle DMSO for 24h before RNA isolation. (C-D) Mice were i.p. infected with Lm for 3 days. (C) Liver sections from Lm infected mice were analyzed by TUNEL staining. Objected magnification: 10×. Lm infected mouse liver section incubated with Label solution only (without terminal transferase) instead of TUNEL reaction mixture was used as negative control. Lm infected mouse liver section pre-incubated with DNase I prior to labeling procedures was used as positive control. (D) Immunohistochemical analysis of cleaved caspase-3 revealed very few apoptotic cells (immune cells and hepatocytes) in the livers of mice. WT: wild type; MSKO: myeloid cell-specific ABCA1 knockout.

Online Figure V. ABCA1 deficient peritoneal macrophages display increased chemotaxis towards MIP-1α. The chemotactic response of thioglycollate-elicited peritoneal macrophages from WT and MSKO mice to MIP-1α was tested in a 48-well microchemotaxis chamber as described in the Methods section.
Online Figure I

Liver gene expression

Relative mRNA expression

- Uninfected WT
- Uninfected MSKO
- Lm-WT
- Lm-MSKO

Genes: HMGCoA synthase, ACAT2, SREBP1c, SCD1, ACC1, DGAT1, PGC1α

Y-axis: Relative mRNA expression

X-axis: Genes
Online Figure II

WT

- Lm (10X) 100μm  - Lm (40X) 50μm

MSKO

+ Lm (10X)  + Lm (40X)

WT

+ Lm (10X)  + Lm (40X)

MSKO
Online Figure III

- **IL-12p40**
  - mRNA relative expression
  - Control-WT, Control-MSKO, Lm-WT, Lm-MSKO

- **IFN-γ**
  - mRNA relative expression
  - Control-WT, Control-MSKO, Lm-WT, Lm-MSKO

- **TNF-α**
  - mRNA relative expression
  - Control-WT, Control-MSKO, Lm-WT, Lm-MSKO

- **MCP-1**
  - mRNA relative expression
  - Control-WT, Control-MSKO, Lm-WT, Lm-MSKO

- **KC**
  - mRNA relative expression
  - Control-WT, Control-MSKO, Lm-WT, Lm-MSKO

- **MIP-2**
  - mRNA relative expression
  - Control-WT, Control-MSKO, Lm-WT, Lm-MSKO
Online Figure IV

(A) oxysterol

(B) relative mRNA expression

(C) positive negative WT MSKO

(D) cleaved caspase-3 (10X) cleaved caspase-3 (40X)

WT MSKO