Increased Cholesterol Deposition, Expression of Scavenger Receptors, and Response to Chemotactic Factors in Abca1-Deficient Macrophages

Omar L. Francone, Lori Royer, Germaine Boucher, Mehrdad Haghpassand, Ann Freeman, Dominique Brees, Robert J. Aiello

Objective—Studies in bone marrow transplanted from ATP-transporter cassette A1 (ABCA1)–deficient mice into normal mice provides direct evidence that the absence of leukocyte ABCA1 exerts a marked proatherogenic effect independent of changes in plasma lipids, suggesting that ABCA1 plays a key role in the regulation of cholesterol homeostasis and function of macrophages. Therefore, we examined whether the absence of ABCA1 affects the morphology, properties, and functional activities of macrophages that could be related to the development of atherosclerosis.

Methods and Results—We conducted a series of experiments in macrophages isolated from Abca1-deficient and wild-type mice and compared several of their properties that are thought to be related to the development of atherosclerosis. Macrophages isolated from Abca1-deficient mice have an increase in cholesterol content, expression of scavenger receptors, and secretion of chemokines, growth factors, and cytokines, resulting in an increased ability to respond to a variety of chemotactic factors.

Conclusion—Our studies indicate that the absence of ABCA1 leads to significant changes in the morphology, properties, and functional activities of macrophages. These changes, together with the proinflammatory condition present in ABCA1-deficient mice and increased reactivity of macrophages to chemotactic factors, play a key role in the development and progression of atherosclerosis.

Key Words: ABCA1 ■ cholesterol homeostasis ■ scavenger receptors ■ inflammation ■ atherosclerosis

Monocytes, the primary inflammatory cell in atherosclerotic lesions, are recruited into the subendothelial space, where they differentiate into mature macrophages and internalize modified lipoproteins and transform into foam cells. The trafficking of monocytes to the atherosclerotic lesion is a highly orchestrated inflammatory process involving cell–cell interactions between monocytes and endothelial cells. Activation of the endothelium is central to the response of the injury theory and is characterized by increases in the adhesion molecules vascular cell adhesion molecule, intercellular adhesion molecule (ICAM), E-selectin, and monocytic surface receptors, with locally produced chemokines such as monocyte chemoattractant protein-1 (MCP-1). A diverse number of stimuli, including turbulence, toxins, and most notably dyslipidemia, are known to activate the endothelium. Monocytes also require modification to produce a specific pattern of integrin and chemokine receptors, which promote adhesion and their subsequent transendothelial migration. The mechanism(s) whereby dyslipidemia and other atherosclerotic risk factors influence monocyte phenotype, migration, adhesion, and differentiation is less clear.

Peripheral monocytes obtained from hypercholesterolemic patients demonstrate increased adhesion to the endothelium. The decrease in plasma cholesterol levels observed with the administration of 3-hydroxy-3-methylglutaryl–coenzyme A reductase inhibitors is associated with changes in monocyte adhesiveness. Lovastatin affects the β2 integrin family of proteins by preventing the formation of CD11b/CD18 heterodimers on monocytes. In addition, fluvastatin inhibits the expression of lymphocyte function–associated antigen (CD11a/CD18) and ICAM-1 on human monocytic cell lines. It was also demonstrated that fluvastatin induced the formation of a more mature population of CD16 monocytes and caused a significant decrease in the expression of the lipopolysaccharide (LPS) receptor CD14 when compared with hypercholesterolemic controls. Additional reports suggest that plasma lipoproteins differentially control monocyte function, and that monocytes from hypercholesterolemic subjects are hyper-responsive to chemotactic stimuli, suggesting a close relationship between plasma lipids and monocyte function.

The mechanism(s) involved in the regulation of monocyte function has gained significant attention in the last few years, with the discovery of multiple transport systems involved in...
the control and trafficking of cholesterol among intracellular compartments as well as from the plasma membrane to extracellular acceptors. Genetic variation in these transporters leads to changes in their functional activities that are the cause of, or contributor to, a wide variety of human disorders with Mendelian inheritance.

Genetic mutations in the ATP-transporter cassette A1 (ABCA1), a membrane protein that promotes cholesterol and phospholipid efflux from cells, causes Tangier disease and familial hypoalphalipoproteinemia. These diseases are characterized by low to complete absence of high-density lipoprotein (HDL) levels and an increased deposition of cholesteryl esters in several tissues and cells, most notably macrophages.

Previous studies in mice and humans carrying mutations in the ABCA1 gene begin to show the association between ABCA1 and atherosclerosis. More directly, bone marrow transplant experiments in wild-type mice demonstrated that deficiency in macrophage ABCA1 is associated with increases in atherosclerosis without any significant change in plasma lipids. Furthermore, minimal changes in HDL in mice expressing ABCA1 offered significant protection from atherosclerosis. In addition, studies in humans confirmed that mutations in ABCA1 lead to increased arterial thickness and severity of atherosclerosis without notable changes in lipid levels.

Together, these studies suggest that changes in macrophage properties and functional activities as a result of the absence of ABCA1 can influence the development of atherosclerosis independent of plasma-circulating levels of HDL. Therefore, we conducted a series of experiments in macrophages isolated from Abca1-deficient mice and compared several of their properties that are thought to be related to the development of atherosclerosis. These studies demonstrate that macrophages isolated from Abca1-deficient mice have an increase in cholesterol content, expression of scavenger receptors, and secretion of chemokines, growth factors, and cytokines, resulting in an increased ability to respond to a variety of chemotactic factors.

Methods

Animals and Diets

ABCA1-deficient (Abca1−/−) mice were created in DBA1 lac/J background as described previously. LDLr−/− (Ldlr−/−) mice backcrossed to C57BL6 for 9 generations were mated to Abca1−/− mice, and double heterozygous were produced Ldlr−/−/Abca1−/− (referred to as Ldlr−/−) and Ldlr−/−/Abca1−/− mice as described previously. Mice were maintained on a 12-hour light/dark cycle and fed a rodent chow diet (Purina Frolab RMH 3000 rodent diet).

Resident peritoneal macrophages were harvested using an identical protocol to that described for the isolation of elicited peritoneal macrophages, with the exception of the injection of casein.

Transmission Electron Microscopy

Peritoneal exudate cells were collected in RPMI-1640 containing 10% FBS, 2 mmol/L glutamine, and 40 μg/mL gentamicin cell culture medium. The cell suspension was spun at 1000 rpm for 5 minutes at room temperature. After centrifugation, the supernatant was decanted; the pellet was resuspended in 1 mL of Dulbecco’s PBS and transferred to a 1.5-mL Eppendorf tube. Tubes were spun at 1000 rpm for 5 minutes at room temperature and the supernatant was decanted. Cells were then suspended in fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L NaPO4 buffer, pH 7.4, and allowed to fix for 15 minutes before centrifugation. Cells were spun at 10 000 rpm for 10 minutes at 4°C, and then the pellet was placed in fresh fixative and stored overnight at 4°C before processing. After fixation, samples were postfixed in 1% Osmium tetroxide and 0.05 mol/L potassium ferrocyanide in 0.1 mol/L NaPO4 buffer for 2 hours at 4°C and dehydrated through graded alcohol and propylene oxide before embedding in Spurr’s resin. Half-micron sections were cut using a Leica Ultracut UCT ultramicrotome, stained with Toluidine Blue, and evaluated by light microscopy. The block face was trimmed to contain the area of interest and then 75- to 90-nm sections were cut and mounted on 200 mesh copper palladium grids. The sections were stained with uranyl acetate and lead citrate before the evaluation using the Hitachi 7100 transmission electron microscope. More than 100 macrophages from each genotype were examined, and the percentage of cells containing vacuoles was determined.

Flow Cytometry

Next, 5×105 peritoneal exudate cells were incubated with BD Fc block for 30 minutes to block nonspecific antibody binding to Fc receptors. Cells were then incubated for 30 minutes on ice in the dark with the following antibodies: monoclonal anti-murine CD36 (Cascade Biologies), rabbit anti–scavenger receptor class B type 1 (SR-BI; extracellular domain NB-400-113; Novus Biologicals), rat anti-murine IgG2b F4/80 (Serotec), goat anti-murine LOX-1 (R & D Systems), or 2F8 rat anti-mouse scavenger receptor class A (SR-A; Research Diagnostics Inc). Cells were then washed 3× with PBS containing 2% heat-inactivated FBS and 0.02% Na azide (fluorescence-activated cell sorter [FACS] buffer) and resuspended in FACS buffer containing the appropriate secondary antibody and incubated in the dark for 1 hour at room temperature. CD36 was determined using fluorescein isothiocyanate (FITC)–conjugated rat anti-murine IgA (BD Biosciences Pharmingen); SR-BI was detected by using phycoerythrin-conjugated Fab anti-rabbit IgG (Jackson ImmunoResearch); F4/80 and SR-A were detected using donkey anti-murine IgG2b F4/80 (Serotec), goat anti-murine LOX-1 (R & D Systems), or 2F8 rat anti-mouse scavenger receptor class A (SR-A; Research Diagnostics Inc). Cells were then washed 3× with PBS containing 2% heat-inactivated FBS and 0.02% Na azide (fluorescence-activated cell sorter [FACS] buffer) and resuspended in FACS buffer containing the appropriate secondary antibody and incubated in the dark for 1 hour at room temperature. CD36 was determined using fluorescein isothiocyanate (FITC)–conjugated rat anti-murine IgA (BD Biosciences Pharmingen); SR-BI was detected by using phycoerythrin-conjugated Fab anti-rabbit IgG (Jackson ImmunoResearch); F4/80 and SR-A were detected using donkey anti-rat FITC (Jackson ImmunoResearch); and LOX-1 was detected by using donkey anti-goat FITC conjugated from Jackson ImmunoResearch.

After washing extensively with FACS buffer, tubes were spun for 5 minutes at 1200 rpm and the supernatant decanted. Cells were then resuspended in BD lysis buffer (BD Biosciences Pharmingen) and incubated at room temperature for 10 minutes. After incubation, tubes were spun at 1200 rpm for 5 minutes and pellets resuspended in FACS buffer. Subsequently, cells were analyzed on a BD FACS
Peritoneal chemotaxis was assessed by incubating eosinophils with films of various concentrations of chemoattractant.

Results
Increased cholesterol deposition and lipid filled vesicles in Abca1-deficient macrophages. The effect of ABCA1 deficiency in the morphology of macrophages was examined by electron microscopy. Elicited peritoneal cells were isolated from wild-type, Abca1−/−, Ldlr−/−, and Ldlr−/−/Abca1−/− mice fed a chow diet, and macrophages were examined as detailed in Methods. Approximately 75% of the cells harvested in these conditions have the visual characteristics of macrophages by electron microscopy. As shown in Figure 1, Abca1-deficient macrophages were characterized by the presence of lipid-filled vacuoles in their cytoplasm. In certain cells, the entire cytoplasm was filled with lipid vacuoles, giving the appearance of foamy macrophages. The presence of lipid-filled vacuoles was confirmed by staining freshly isolated cells with oil red O (data not shown). Lipid vesicles were observed in Abca1-deficient macrophages isolated from normal lipidemic (DBA1 lac/J background) or hypercholesterolemic (Ldlr-deficient background) Abca1-deficient mice (Figure 1), suggesting that the circulating level of plasma cholesterol is not a prerequisite for the formation and accumulation of lipid vacuoles.

Ultracut UCT ultramicrotome, stained with Toluidine Blue, and evaluated by light microscopy. Sections containing areas of interest were cut into 75- to 90-nm sections, mounted on copper palladium grids, stained, and evaluated in a Hitachi 7100 transmission electron microscope. A, Wild-type. B, Abca1−/−. C, Ldlr−/−. D, Ldlr−/−/Abca1−/−. Arrows show lipid vesicles. Bars=5 μm.

Figure 1. Presence of lipid-filled vesicles in Abca1-deficient macrophages. Peritoneal exudate cells were harvested from wild-type, Abca1−/−, Ldlr−/−, and Ldlr−/−/Abca1−/− mice, fixed, dehydrated, and embedded in Spurr’s resin as described in Methods. Half-micron sections were obtained using a Leica Ultracut UCT ultramicrotome, stained with Toluidine Blue, and evaluated by light microscopy. Sections containing areas of interest were cut into 75- to 90-nm sections, mounted on copper palladium grids, stained, and evaluated in a Hitachi 7100 transmission electron microscope. A, Wild-type. B, Abca1−/−. C, Ldlr−/−. D, Ldlr−/−/Abca1−/−. Arrows show lipid vesicles. Bars=5 μm.
Abca1 Deficiency Affects Expression of Scavenger Receptors in Macrophages

The intracellular levels of cholesteryl esters observed in Abca1-deficient macrophages and the increased deposition in cells incubated in the presence of acetylated or oxidized LDL suggest that the uptake of cholesterol is increased in Abca1-deficient macrophages. To test this hypothesis, we determined the expression level of scavenger receptors SR-A, CD-36, LOX-1, and SR-BI in peritoneal macrophages harvested from Ldlr−/−/Abca1−/− and Ldlr−/− control littersmates. No difference in the number of cells present in the peritoneal cavity was noted (2.6×10⁶ and 2.4×10⁶ cells/mL for Ldlr−/−/Abca1−/− and Ldlr−/− mice, respectively; n=6). In addition, forward and right light scattering shows no gross differences in either size or granularity among the cell populations present in either the Abca1-deficient mice or their control littermates (data not shown). The population of cells expressing F4/80, a surface marker present in mature macrophages, was gated for further analysis. This cell population represented >80% of the cells harvested from the peritoneal cavity. The expression of scavenger receptors associated with the binding and internalization of acetylated and oxidized LDL (SR-A, CD-36, and LOX-1) is significantly increased in Abca1-deficient macrophages (Table 1). Most notably, the fluorescent intensities for SR-A and LOX-1 receptors are increased 2.4-fold in Abca1-deficient macrophages (n=6; P<0.05). SR-BI expression, a scavenger receptor involved in the internalization of HDL cholesteryl ester and efflux of cholesterol, is essentially identical in both genotypes.

Abca1 Deficiency Affects Macrophage TNF-α Secretion and Chemotaxis

Macrophages are a primary source of inflammatory cytokines within the atherosclerotic lesions. Through modulation of such functions as leukocyte recruitment, autocrine/paracrine induction of cytokines, synthesis, and synthesis/degradation of vascular extracellular matrix constituents, cytokines can influence lesion initiation and progression. Therefore, to ascertain the capacity of Abca1-deficient macrophages to produce cytokines in response to an inflammatory stimulus, elicited peritoneal cells were incubated for 18 hours in RPMI-1640 containing 10% FBS, 2 mmol/L glutamine, 40 μg/mL gentamicin sulfate alone, or containing 1 ng/mL LPS. The accumulation of TNF-α in growth media was determined by ELISA. One nanogram per milliliter LPS was sufficient to evoke the maximum response. As shown in Figure 3, LPS stimulated the secretion of TNF-α by macrophages isolated from both genotypes. However, Abca1-deficient macrophages secreted 1.8-fold more TNF-α than macrophages isolated from Ldlr−/− mice (n=3; P<0.05), suggesting a hypersensitivity of Abca1-deficient macrophages to inflammatory stimulus mediated through the Toll-like receptor pathway. As expected, unstimulated macrophages secreted very low levels of TNF-α. A similar experiment was conducted using nonelicited resident macrophages harvested from Abca1-deficient mice and control littermates with an identical outcome, indicating that the hypersensitivity observed in elicited macrophages is attributable to the absence of Abca1 and unrelated to the injection of casein (data not shown).

The chemotactic response of Abca1-deficient and control macrophages to MCP-1, MIP1α, and FMLP-1 was also examined. For this purpose, nonelicited resident peritoneal macrophages were isolated from wild-type, Ldlr−/−, and Ldlr−/−/Abca1−/− mice, and their chemotactic activity was tested in a 2-chamber chemotaxis assay. As shown in Figure 4, Abca1-deficient macrophages showed a dose-dependent increase in the response to MCP-1 and MIP-1α at concentra-
tions ranging from 0.1 to 100 nmol/L. In addition, the response to the chemotactic peptide FMLP is also increased in Abca1-deficient macrophages, suggesting that Abca1–/– mice have a general increased ability to respond to a variety of chemotactic factors.

LPS-Induced Septic Shock Is Exacerbated in Abca1-Deficient Mice

Circulating plasma levels of JE/MCP-1, interleukin-1β (IL-1β), and macrophage colony–stimulating factor (m-CSF) are significantly increased in Abca1-deficient mice (Table 2). The increase in the plasma levels of JE/MCP-1, IL-1β, and m-CSF occurred without concomitant changes in the number of circulating white blood cells (5200±900 and 5600±2200 cells/μL in Ldlr+/+ and Ldlr+/–/Abca1–/– mice, respectively; n=6). The number of neutrophils, lymphocytes, monocytes, eosinophils, and basophils remains the same between both genotypes (results not shown), suggesting a hyper-responsiveness and proinflammatory state in mice deficient in Abca1. To determine whether Abca1-deficient mice are more responsive to endotoxemia, Ldlr+/+ and Ldlr+/–/Abca1–/– mice were injected with LPS in their peritoneal cavity. LPS released from the outer membrane of gram-negative bacteria triggers leukocytes to synthesize and release a cascade of inflammatory mediators that ultimately can lead to tissue injury and multiple organ failure.22 Multiple cells from the immune system are involved in this process. The hyper-responsiveness of Abca1-deficient mice to inflammation was demonstrated when Abca1-deficient mice were administered intraperitoneal injections of LPS at various doses. Plasma levels of JE/MCP-1 rose in response to LPS at all doses tested and in both genotypes. In Ldlr+/+ mice, plasma JE/MCP1 concentrations were 4246±1177, 18 088±4676, and 118 028±33 917 ng/mL when the animals received 0.03, 0.3, and 3.0 mg/kg of LPS, respectively. In Ldlr+/–/Abca1–/– mice, on average, the plasma level of JE/MCP-1 is 8×

### Table 1. Abca1 Deficiency Affects Expression of Scavenger Receptors in Macrophages

<table>
<thead>
<tr>
<th>Mice</th>
<th>Cells×10⁶</th>
<th>F4/80</th>
<th>SR-A</th>
<th>LOX-1</th>
<th>CD36</th>
<th>SR-Bi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ldlr+/+</td>
<td>2.6</td>
<td>164±49</td>
<td>75±17</td>
<td>13±3</td>
<td>111±11</td>
<td>73±13</td>
</tr>
<tr>
<td>Ldlr+/–/Abca1–/–</td>
<td>2.4</td>
<td>199±37</td>
<td>174±33*</td>
<td>31±10*</td>
<td>167±25*</td>
<td>89±15</td>
</tr>
</tbody>
</table>

Peritoneal cells were harvested from Ldlr+/+ and Ldlr+/–/Abca1–/– mice 4 days after casein injection. Peritoneal cell counts and mean fluorescent intensity of scavenger receptors SR-A, LOX-1, CD36, and SR-Bi were determined as described in Methods. Values are expressed as mean fluorescent intensity±SD (n=4).

*P<0.01.

Figure 3. Increased TNF-α secretion by Abca1-deficient macrophages in response to an inflammatory stimulus. Peritoneal macrophages harvested from Ldlr+/+ and Ldlr+/–/Abca1–/– mice were incubated for 18 hours in the presence or absence of LPS as described in Methods. The accumulation of TNF-α in the culture medium was determined by ELISA as described in Methods. Values are shown as mean±SD (n=3). *P<0.05.

![Figure 3](http://example.com/figure3.png)

Figure 4. Increased chemotaxis to MCP-1, MIP-1α, and FMLP in Abca1-deficient macrophages. Nonelicited peritoneal macrophages were isolated from wild-type, Ldlr+/+, and Ldlr+/–/Abca1–/– mice, and their chemotactic response to MCP-1 (A), MIP-1α (B), and FMLP (C) was tested in a 2-chamber chemotaxis assay as described in Methods. Values are mean±SD.

![Figure 4](http://example.com/figure4.png)
higher ($P<0.05$; $n=3$) in Abca1-deficient mice regardless of the dose of LPS used (31 131 ± 10 903, 161 579 ± 6971, and 712 377 ± 29 797 ng/mL at doses corresponding to 0.03, 0.3, and 3.0 mg/kg, respectively). To determine whether this hypersensitivity also includes other inflammatory mediators, Abca1-deficient and control littersmates were injected intraperitoneally with 3.0 mg/kg LPS, and euthanized 3 hours after injection. Cytokines, chemokines, and growth factors were determined as described in Methods and expressed as ng/mL of plasma. Data are shown as mean ± SD ($n=6$ mice per group).

Plasma cholesterol levels were 463.2 ± 50.3 and 192.5 ± 24 mg/dL ($n=6$) for Ldlr$^{-/-}$ and Ldlr$^{-/-}$/Abca1$^{-/-}$, respectively.

HDL cholesterol was 49.7 ± 8.2 and 192.5 ± 38 mg/dL ($n=6$) for Ldlr$^{-/-}$ and Ldlr$^{-/-}$/Abca1$^{-/-}$, respectively.

IFN-γ indicates interferon-γ; GM-CSF, granulocyte-macrophage colony-stimulating factor.

Discussion

The recruitment and infiltration of monocytes in the arterial intima is essential to the development of atherosclerosis. Fatty streaks, which are the earliest forms of visible atherosclerotic lesions, consist almost entirely of macrophage-derived foam cells. The current literature supports the hypothesis that monocytes are initially attracted to lesion-prone sites by cell adhesion molecules, which are expressed on activated endothelial cells. P-selectin mediates the initial interaction and subsequent adhesion to the endothelium. Integrins enable the attachment of monocytes to the endothelium, whereas the presence of chemoattractant leads to monocyte migration into the subendothelial space.21-24

The accumulation of monocytes to susceptible areas is a dynamic process controlled by site-specific mechanisms responsible for the influx and efflux of cells into lesion-prone areas. These processes are influenced by local but also systemic factors controlling the fate and tissue targeting of monocytes. Atherosclerosis is a chronic inflammatory process with great complexity, and the rate at which the disease progresses depends on a variety of factors, including hypercholesterolemia.25 Studies in a large number of animal models and humans demonstrated that high levels of plasma cholesterol accelerate the differentiation as well as the influx of monocytes to the arterial wall.1-6 More recently, the discovery of membrane transporters involved in the regulation of cellular cholesterol homeostasis in macrophages further supports the hypothesis that intracellular cholesterol levels in macrophages play a pivotal role in the initial steps leading to the development of atherosclerosis.

ABCA1, a membrane transporter that promotes cholesterol efflux from cells to apolipoprotein acceptors,26 appears to play a significant role in this process, as demonstrated in bone marrow transplant studies using apolipoprotein E (apoE) and LDL receptor–deficient mice.16,27 In these studies, bone marrow transplanted from Abca1-deficient mice into normal mice demonstrated that the absence of leukocyte ABCA1 exerted a marked proatherogenic effect. This increase in atherosclerosis occurred in the absence of any changes in plasma cholesterol and HDL levels. These studies also suggest that the absence of ABCA1 leads to a series of changes in monocytes and macrophages, resulting in an accelerated influx into the vessel wall, influencing the progression of atherosclerosis. Therefore, we conducted a series of experiments in which we used either resident or peritoneal macrophages harvested from Ldlr$^{-/-}$ and Ldlr$^{-/-}$/Abca1$^{-/-}$ mice as a model to assess whether the absence of ABCA1 leads to changes in the morphology and functional properties of macrophages. These studies were conducted using LDL receptor–deficient mice to ensure that the changes in cellular cholesterol homeostasis, if any, were not dependent on the presence of LDL receptors. Our studies indicate that Abca1-deficient mice have increased circulating levels of chemokines, cytokines, and growth factors, which is most evident after the injection of LPS. Abca1-deficient macrophages have greater cellular cholesteryl ester content, express higher

### Table 2. Plasma Levels of Chemokines, Cytokines, and Growth Factors in Ldlr$^{-/-}$ and Ldlr$^{-/-}$/Abca1$^{-/-}$ Mice

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Ldlr$^{-/-}$</th>
<th>Ldlr$^{-/-}$/Abca1$^{-/-}$</th>
<th>Ldlr$^{-/-}$/LPS</th>
<th>Ldlr$^{-/-}$/Abca1$^{-/-}$ + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>JE/MCP-1</td>
<td>40 ± 17</td>
<td>79 ± 17*</td>
<td>48 709 ± 9018</td>
<td>254 083 ± 65 065**</td>
</tr>
<tr>
<td>Cytokines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>8 ± 5</td>
<td>15 ± 11*</td>
<td>701 ± 164</td>
<td>5547 ± 1828**</td>
</tr>
<tr>
<td>IL-1β</td>
<td>2 ± 1</td>
<td>20 ± 6*</td>
<td>131 ± 39</td>
<td>396 ± 86**</td>
</tr>
<tr>
<td>IL-6</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>18 200 ± 8274</td>
<td>224 320 ± 9116**</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>23 ± 7</td>
<td>189 ± 41**</td>
</tr>
<tr>
<td>Growth factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-CSF</td>
<td>1170 ± 145</td>
<td>2240 ± 311*</td>
<td>10 481 ± 1161</td>
<td>16 294 ± 899**</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>56 ± 31</td>
<td>294 ± 69**</td>
</tr>
</tbody>
</table>

Mice were injected in the peritoneal cavity with a solution containing either saline or 3 mg/kg LPS and euthanized 3 hours after injection. Cytokines, chemokines, and growth factors were determined as described in Methods and expressed as ng/mL of plasma.
levels of scavenger receptors, and secrete more TNF-α in response to LPS. Abca1−/− macrophages also exhibit a greater ability to respond to a variety of chemotactic factors. The results from these studies emphasize the crucial role that Abca1 plays in determining cellular cholesterol homeostasis and the subsequent changes in macrophage function. Although changes in the content of cholesteryl esters in Abca1-deficient macrophages has been documented previously,\textsuperscript{19} this study is the first to provide new insights into the changes in macrophage function and properties as a result of the inactivation of ABCA1. These data provide support and credence to the hypothesis relating cholesterol efflux, cellular cholesterol content, and activation of macrophages into a proinflammatory state.

Macrophages internalize cholesterol-rich lipoproteins and cell debris by receptor and phagocytic pathways that are not repressed when cells acquire excess cholesterol,\textsuperscript{28} making these cells dependent on cholesterol-removal mechanisms to prevent excess deposition of sterols. Although extremely important for macrophage cholesterol homeostasis and function, cholesterol removal mechanisms operating in macrophages such as those involving the ABCA1 and ABCG1 transporters, do not contribute to plasma HDL cholesterol levels.\textsuperscript{17,29} Furthermore, their contribution to the overall cholesterol transport in the body is essentially undetected by conventional methods. However, these studies emphasize the importance of the ABCA1-dependent cholesterol efflux and reverse cholesterol transport pathways in modulating macrophage function. Changes in cholesterol homeostasis and functional properties are probably manifested in circulating monocytes as a result of ABCA1 deficiency, as documented by studying subpopulations of monocytes in hypercholesterolemic individuals.\textsuperscript{6} The development of more sophisticated and sensitive techniques will enable us to conduct similar experiments in circulating leukocyte populations in animal models and identify valuable biomarkers and predictors of disease development and progression.

The absence of ABCA1 has a profound impact on the response to an inflammatory stimulus, as demonstrated by the greater increase in plasma levels of chemokines, cytokines, and growth factors observed in Abca1-deficient mice injected with LPS. This hypersensitivity might involve many cell types and could be partially attributed to the low levels of HDL present in Ldr−/−/Abca1−/− mice. This is consistent with several studies showing that lipoproteins bind endothoxin,\textsuperscript{30} and that transgenic mice with elevated HDL levels are protected from LPS-induced toxicity.\textsuperscript{31} Nevertheless, the hyper-response observed in Ldr−/−/Abca1−/− mice occurred over a wide range of doses and at concentrations below the saturation capacity of lipoproteins to bind LPS.\textsuperscript{32} In addition, the incubation of Abca1-deficient macrophages with LPS demonstrated the increased capacity of Abca1-deficient macrophages to produce cytokines in response to an inflammatory stimulus independently of plasma lipoprotein levels. Additional studies using mice with targeted inactivation of ABCA1 in macrophages and normal HDL levels\textsuperscript{17} will be needed to determine the contribution of macrophage ABCA1 on the response to an inflammatory stimulus.

The relationship between changes in cellular cholesterol levels and the activation of macrophages into a proinflammatory state is unclear. The increase in macrophage cholesterol content, increased expression of scavenger receptors, increased secretion of TNF-α, and response to MIP1α, MCP-1, and FMLP, as observed in Abca1-deficient macrophages, suggests a coordinate regulation between cellular cholesterol homeostasis and macrophage activation/differentiation. It is widely believed that HDL protects against the development of atherosclerosis by acting as an acceptor for cholesterol and thus removing excess cholesterol from cells present in the artery wall, particularly macrophages. However, it is also plausible that HDL exerts its protective role in the plasma compartment through changes in intracellular cholesterol levels in circulating monocytes, resulting in a monocyte subpopulation that is less proinflammatory. Many lines of evidence present in the literature support this hypothesis. Several years ago, Bell and Gerrity\textsuperscript{33} demonstrated increased lipid levels in circulating blood monocytes under conditions of hyperlipidemia in swine. Also, Rothe et al\textsuperscript{6} demonstrated the presence of an inflammatory phenotype in circulating peripheral blood monocytes that closely correlate to cellular lipid content and lipoprotein risk factors. The pool size of CD14\textsuperscript{+} and CD16\textsuperscript{+} monocytes, which are characterized by high expression of β\textsubscript{1} and β\textsubscript{2} integrins and a lack of production of the anti-inflammatory cytokine IL-10, positively correlates to plasma cholesterol, triglyceride levels, and apoE4 phenotype and is inversely correlated to the level of HDL cholesterol. Additional evidence also suggests a close relationship between HDL metabolism, monocyte function, and cholesterol trafficking to HDL. During acute infection and inflammation, an acute-phase reaction develops to protect the host from further injury. Serum amyloid A replaces apoA1 on the HDL surface, leading to changes in HDL composition and its ability to efflux cholesterol from monocytes promoting chemotaxis.\textsuperscript{34} Moreover, the relationship between CCR2 expression, chemotaxis, and cellular cholesterol levels has been shown in hypercholesterolemic patients and animal models.\textsuperscript{35} It is currently being elucidated how physiological levels of HDL and its various species affect the subpopulations of circulating monocytes, chemotaxis, and the development of atherosclerosis. Our studies indicate that the absence of ABCA1 leads to significant changes in the morphology, properties, and functional activities of macrophages. These changes, together with the proinflammatory condition present in Abca1−/− mice and increased reactivity of macrophages to a variety of chemotactic factors, are likely to play a key role in the development and progression of atherosclerosis.

Acknowledgments
We thank Tom Sand for his assistance with the gas chromatography analysis of macrophage samples, Carol Donovan for her expertise and assistance in the flow cytometry analysis of peritoneal macrophages, and William Reagan for his efforts in determining leukocyte counts.

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Arterioscler Thromb Vasc Biol. published online April 14, 2005; Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231 Copyright © 2005 American Heart Association, Inc. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

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