Macrophage Phenotype in Mice Deficient in Both Macrophage-Colony–Stimulating Factor (Op) and Apolipoprotein E

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Abstract—Mice deficient in both macrophage-colony–stimulating factor (M-CSF, op) and apolipoprotein E (apoE) have elevated cholesterol levels but are protected from atherosclerosis. To assess the contribution of macrophage (Mφ) phenotypic heterogeneity and scavenger receptor (SR-A) expression to this seeming paradox, we characterized the Mφ phenotype by immunohistochemistry in these animals. Lesion size was determined in animals fed a chow or Western-type diet, and lipoprotein clearance studies were performed in vivo. Op0/E0 mice have fourfold smaller aortic root lesions than op2/E0 animals despite 2.5-fold higher total plasma cholesterol levels. Mφs in atherosclerotic lesions of op2/E0 mice constitute a predominantly recruited and M-CSF–dependent population. In addition, Mφs in different locations in plaques show phenotypic heterogeneity. SR-A expression in op0/E0 mice is reduced in proportion to the decrease in Mφ numbers, and M-CSF is thus not an essential requirement for SR-A expression in vivo. M-CSF–deficient mice degrade injected AcLDL, showing an adequate level of SR-A activity present in vivo. In contrast, β-VLDL clearance in op0/E0 mice is decreased, implicating monocytes/Mφs in its catabolism. There is prominent lipid accumulation in op2/E0 Kupffer cells and hepatocytes but not in M-CSF–independent Kupffer Mφs from op0/E0 mice. SR-A, while abundantly expressed on both Kupffer cells and sinusoidal endothelial cells in op2/E0 mice, remains mainly on sinusoidal endothelial cells in op2/E0 mice. This may explain preservation of SR-A activity in these animals. Our findings clearly illustrate the importance of both M-CSF and M-CSF–dependent monocytes/Mφs in maintaining cholesterol homeostasis and in atherogenesis. (Arterioscler Thromb Vasc Biol. 1998;18:631-640.)

Key Words: macrophages ■ macrophage-colony-stimulating factor ■ apolipoprotein E ■ atherosclerosis

The arterial fatty streak is an early and reversible precursor to advanced fibroproliferative atherosclerotic lesions.1 These early lesions contain mostly monocyte-derived Mφs that have taken up modified lipoproteins to become lipid-enriched foam cells. In addition to Mφ foam cells, human lesions also contain T cells and smooth muscle–derived foam cells,1 and an essential role of monocyte-derived Mφs in atherosclerosis remains unproven.

ApoE-deficient mice are an attractive model system in which to evaluate this question. These mice are hypercholesterolemic and develop atherosclerosis spontaneously on a low-fat chow diet and in an accelerated fashion on a high-fat Western-type diet.2 Atherosclerotic lesions in apoE-deficient mice occur throughout the aortic tree, progress with age from early fatty streaks to complex fibrous lesions with necrotic cores, and are found at the same sites of predilection as human lesions.3,4

M-CSF and its effects on Mφ development and function play a key role in atherogenesis. M-CSF injections reduce cholesterol levels in rabbits, primates, and humans5–7 and decrease atherosclerosis and carrageenan-induced granuloma lipid accumulation in Watanabe hyperlipidemic rabbits.7,8 The Mφ-specific membrane molecule macrophage scavenger receptor (or SR-A) has been proposed to have an important in vivo role in atherogenesis through its involvement in foam cell formation.9 Recent evidence supports a proatherogenic role for SR-A in that SR-A–deficient mice crossed onto an apoE-deficient background were relatively protected from atherosclerosis.10 M-CSF potently and selectively increases SR-A expression, stability, and endocytic and adhesion functions in murine Mφs in vitro.11

The osteopetrotic (op/op) mouse has a spontaneously derived recessive mutation in the gene encoding M-CSF and the phenotype has been well characterized.12–14 The op defect in the M-CSF gene is a frameshift mutation leading to the complete absence of M-CSF activity in the serum and tissues.15 Osteopetrotic mice lack osteoclasts, resulting in impaired bone remodeling and skeletal deformities, the most...
severe being a deficiency in tooth eruption, so that young op/op mice require a soft diet to survive. Hematologically, op/op mice have normal hematocrits and granulocyte counts but markedly decreased blood monocytes and peritoneal Mϕs. The op/op mouse thus provides an opportunity to examine the contribution of M-CSF to specific Mϕ populations in vivo, with particular regard to levels of SR-A expression.

Immunohistochemical studies in op/op mice have shown that other Mϕ populations apart from osteoclasts are critically dependent on M-CSF in vivo. These M-CSF–dependent populations include peritoneal Mϕs, splenic marginal zone metallophilic, and lymph node subcapsular sinus Mϕs. Liver Mϕs (Kupffer cells) are reduced but still readily identifiable; other Mϕ populations, including those within the thymic cortex, splenic red pulp, lymph node medulla, intestinal lamina propria, lung (alveolar Mϕs), and brain (microglia), as well as dendritic cells, remain present in substantial numbers and appear for the most part M-CSF independent.

Op/op mice were recently crossed onto the apoE-deficient background to determine the role of monocyte-derived Mϕs (and M-CSF) in atherogenesis in vivo. The double-mutant mice had an almost threefold increase in plasma cholesterol compared with apoE-deficient controls. Despite this severe hypercholesterolemia, proximal aorta atherosclerosis in these mice was significantly decreased. These findings were confirmed in a recent study describing a genetically more homogeneous population. Using a rabbit anti-mouse Mϕ polyclonal antiserum for immunohistochemistry, prominent and homogeneous Mϕ staining was revealed in small raised foam cell lesions from both op2/E0 and op0/E0 mice. This pan-Mϕ staining would not reveal any Mϕ functional heterogeneity in lesions. Interestingly, the reduced monocyte number in the op0/E0 mouse was shown statistically not to affect lesion formation independently, and a crucial factor may rather be the antiatherogenic functional modulation of those remaining M-CSF–independent Mϕs. Diminished SR-A expression, for example, may protect against foam cell formation and atherosclerosis in the doubly mutant mice.

To extend this work further, we characterized the development of atherosclerosis in apoE-deficient mice (E0; E for apoE followed by the number of wild-type alleles to describe the mouse genotype), as well as mice doubly mutant for the apoE gene and M-CSF genes (heterozygous and homozygous) (op1/E0; op0/E0: op2, op1, or op0; op for osteopetrotic followed by the number of wild-type alleles). Lesion size was measured in animals fed a chow diet for 1 year and in animals fed a Western-type diet for 12 weeks after weaning. We also examined tissues from 16-week-old mice fed a chow diet. Mϕ subpopulations in the op2/E0, op1/E0, and op0/E0 double-mutant crosses were characterized by immunohistochemistry of Mϕ membrane molecules with special emphasis on SR-A expression. In addition, lipoprotein clearance studies were performed in op2/E0 and op0/E0 mice in vivo with AcLDL and β-VLDL to determine their functional phenotype.

Methods

Mice and Diet

The creation of the apoE-deficient mice used in this study has been described. The C57BL/6 × 129 apoE-deficient female mice were bred to op heterozygous (op1) male mice (The Jackson Laboratory) on the C57BL/6 × C3H background. Heterozygous E1 progeny were screened for the op mutation by a polymerase chain reaction assay as described, and the op1/E0 mice among them were interbred to generate op1/E0 animals that served as the parental genotype for all animals in the study. Offspring of the parental op1/E0 were op0/E0, op1/E0, and op2/E0 littersmates and served as the subjects in the study. The mice were weaned at 4 weeks and maintained either on a chow diet (4.5% fat and 0.02% cholesterol by weight) or a Teklad adjustable Western-type diet (42% fat, 0.15% cholesterol, and 19.5% casein without sodium cholate by weight). Diet and water were provided ad libitum.

Immunohistochemistry

The animals were killed at 16 weeks and saline perfused. Organs (heart, thoracic and abdominal aorta, lungs, liver, spleen, gut, kidneys, and skin) were removed from op0/E0 (n = 4), op1/E0 (n = 4), and op2/E0 (n = 4) littermates. Tissues were washed in PBS, placed in Tissue-Tek OCT compound (BDH-Merck), and snap-frozen in isopentane cooled by dry ice. Frozen sections were cut on a Leica cryostat (5 μm thick), collected onto 1.5% gelatinized slides, air dried for 1 hour, and stored at −20°C for later use. Sections were fixed for 10 minutes in 2% paraformaldehyde in HEPES-buffered isotonic saline before staining.

The mAbs used in this study are listed in Table 1, along with their specificity, isotype, and appropriate references. Fixed sections were washed in PBS containing 0.1% vol/vol Triton X-100 and treated with 2% normal rabbit serum for 30 minutes. Sections were incubated for 90 minutes with primary antibody (10 μg/mL purified, or neat tissue hybridoma supernatant), PBS, or isotype-matched control mAb. Endogenous peroxidase activity was blocked by incubation of sections with 0.01 mol/L glucose, 0.001 mol/L sodium azide, 40 U glucose oxidase in 100 mL phosphate buffer for 15 minutes at 37°C. Affinity-purified, mouse-adsorbed, biotinylated second Ab (Vector Labs) was used at 1% for 45 minutes followed by avidin-biotin-peroxidase complex (ABC elite, Vector) according to the supplier’s recommendation. The presence of antigen was revealed by incubation with 0.5 mg/mL diaminobenzidine (Polysciences, Inc) and 0.024% H2O2 in 10 mmol/L PBS imidazole, pH 7.4. Counterstaining was with cresyl fast violet acetate, and stained cells were dehydrated and mounted in DPX (BDH-Merck). Representative photographs were taken using a blue filter (Kodak, Wratten, gelatin filter No. 47) which intensifies the brown precipitate. Serial sections adjacent to sections in which immunostained morphology was recorded were stained with oil red O for the detection of lipids.

In Vivo Lipoprotein Turnover Study

AcLDL was prepared from human LDL as previously described. β-VLDL was floated by ultracentrifugation of pooled apoE-deficient mouse plasma overlayed with PBS. Lipoprotein (100 μL) was labeled by overnight incubation at 37°C with 10 μL of [1H]cholesteryl oleyl ether (Amersham), dried under nitrogen, and 1 μL of recombinant cholesterol ester transfer protein (generously provided by Alan Tall, Columbia University). Labeled lipoproteins were separated from...
unincorporated label by gel filtration. Lipoproteins were injected into the femoral vein of sodium pentobarbital–anesthetized mice, and blood was removed at the indicated times from the retroorbital plexus. For each mouse, the remaining plasma radioactivity was normalized to the radioactivity in the initial bleeding. This method of normalization was used, instead of normalization to the calculated injected radioactivity, because preliminary experiments revealed that this method was more reproducible due to difficulty in quantitatively injecting the labeled sample without any loss.

### Atherosclerosis, Cholesterol, and Monocyte Differential Assays

These assays were performed as previously described.18

### Results

#### Development of Atherosclerosis in Op2/E0 and Op0/E0 Mice

We confirmed and extended the initial finding of decreased atherosclerosis in chow-fed op0/E0 mice analyzed at 16 weeks.18 We further analyzed atherosclerosis in 1-year-old chow-fed op0/E0 and op2/E0 littersmates using an en face method along the entire aorta.34 Two males and one female of each genotype were analyzed. Oil red O–staining lesions were also abundantly expressed in the lesion. CD11b is a molecule that has both endocytic and adhesive functions, whereas SR-A expression accompanies monocyte to Mφ transition.

#### Characterization of Mφ Phenotypes in Atherosclerotic Lesions in Op2/E0, Op1/E0, and Op0/E0 Mice

Sixteen-week-old mice fed a chow diet were killed and examined. Every op1/E0 and op2/E0 mouse analyzed had atheromatous lesions in the aortic root, situated on the valve cusps and in the areas between valve cusps. These lesions comprised a spectrum of both simple raised foam cell type and larger, more developed fibroproliferative plaques. In Fig 2, immunohistochemistry from representative advanced lesions (as shown by oil red O staining) in an op2/E0 mouse is shown. Recruited Mφs within the lesions prominently expressed the Mφ–specific late endosomal membrane glycoprotein macrophage,35 This mostly intracellular membrane molecule (homologous to human CD6836) was present throughout the lesion, including its base, shoulders, and necrotic core.

CD11b (CR3) and SR-A, examples of Mφ membrane molecules that have both endocytic and adhesive functions, were also abundantly expressed in the lesion. CD11b is generally considered a marker of recently recruited Mφs,37 whereas SR-A expression accompanies monocyte to Mφ transition.

### Table 1. Monoclonal Antibodies Used in This Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Expression</th>
<th>Isotype</th>
<th>Reference</th>
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<tr>
<td>F4/80</td>
<td>F4/80</td>
<td>Mature resident Mφ</td>
<td>Rat IgG2b</td>
<td>20</td>
</tr>
<tr>
<td>FA-11</td>
<td>Macrophilin (CD68) on Mφ</td>
<td>Pan-Mφ, endosomal compartments</td>
<td>Rat IgG2a</td>
<td>21</td>
</tr>
<tr>
<td>5C6</td>
<td>CD11b subunit of CR3</td>
<td>Recruited Mφ, monocytes, PMNs</td>
<td>Rat IgG2b</td>
<td>22</td>
</tr>
<tr>
<td>2F8</td>
<td>SR-A (SR-AI and II)</td>
<td>Differentiated Mφ</td>
<td>Rat IgG2b</td>
<td>23</td>
</tr>
<tr>
<td>3D6</td>
<td>Sialoadhesin on Mφ</td>
<td>Stromal Mφ</td>
<td>Rat IgG2a</td>
<td>24</td>
</tr>
<tr>
<td>8D2</td>
<td>CD44</td>
<td>Mature resident Mφ, lymphocytes and fibroblasts</td>
<td>Rat IgG2b</td>
<td>Doyle and Gordon, unpublished</td>
</tr>
<tr>
<td>2.4G2</td>
<td>FcγRII (CD32)</td>
<td>Pan-Mφ, monocytes</td>
<td>Rat IgG2b</td>
<td>25</td>
</tr>
<tr>
<td>TIB120</td>
<td>MHC class II, IA/IE</td>
<td>Activated Mφ</td>
<td>Rat IgG2b</td>
<td>26</td>
</tr>
<tr>
<td>NLDC145</td>
<td>NLDC145</td>
<td>Interdigitating (dendritic cells)</td>
<td>Rat IgG2a</td>
<td>27</td>
</tr>
<tr>
<td>GK 1.5</td>
<td>CD4</td>
<td>CD4+ T cells</td>
<td>Rat IgG2b</td>
<td>28</td>
</tr>
<tr>
<td>53.6.72</td>
<td>CD8</td>
<td>CD8+ T cells</td>
<td>Rat IgG2a</td>
<td>29</td>
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</table>

### Figure 1. Atherosclerotic lesion areas in op0/E0 and op2/E0 mice. Mice were fed a high-fat Western-type diet and killed at 12 weeks of age. Mean lesion area in the aortic root was determined in 5 op0/E0 males and 15 op2/E0 males, *P<.01 compared with op2/E0 males by a two-tailed t test. Mean lesion area was also determined in 13 op0/E0 females and 10 op2/E0 females, **P<.001 compared with op2/E0 females by a two-tailed t test.
Mφs in the atherosclerotic lesions also expressed FcγRII (CD32). Oxidized lipoprotein epitopes are present in the lesions of E0 mice, and sera from these mice contain high titers of autoantibodies to oxidized lipoproteins. This molecule may therefore be functionally relevant in IgG-mediated phagocytosis by lesional Mφs. As dendritic cells (NLDC145) and T lymphocytes (CD4 and CD8) (not shown) were virtually absent, prominent MHC class II staining indicates the presence mainly of activated lesional Mφs. The reported predominant Th1 phenotype of T lymphocytes in the atherosclerotic plaque may implicate IFN-γ-dependent upregulation of MHC class II expression on these Mφs.

In contrast to these markers of a recruited Mφ population, F4/80 and sialoadhesin expression levels in the lesions were unimpressive and consistent with their status as resident and stromal Mφ markers, respectively. Lesions in op1/E0 animals showed a similar Mφ presence and phenotype as op2/E0 mice; and a gene dosage effect of M-CSF deficiency could not be demonstrated (data not shown). The distribution of Mφ membrane molecules within op2/E0 atherosclerotic lesions is tabulated in Table 2.

All the lesions in op0/E0 animals were at the aortic root on the valve cusps. The modulation in E0 atherogenesis brought about by the op/op mutation was readily apparent (Fig 3). Oil red O staining revealed that M-CSF deficiency resulted in fewer and smaller lesions in op0/E0 mice; these were of mostly early foam cell type and had not progressed to more advanced stages and were primarily located on valve cusps. When compared with op2/E0 mice, the decrease in atherosclerotic lesions was paralleled by an overall reduction in Mφs present in lesions. It was not possible to ascertain whether SR-A expression was disproportionately reduced in op0/E0 lesional Mφs in comparison to expression levels of macrosialin, CD11b (CR3), FcγRII, MHC class II, or CD44.

The Mφs characterizing the aortic root lesions in op2/E0 mice are therefore predominantly recruited and M-CSF dependent. Some Mφs still remain as an integral component of the smaller lesions present in op0/E0 mice, and these represent an M-CSF–independent population. While the M-CSF deficiency in op0/E0 animals affected Mφ functional heterogeneity within atherosclerotic lesions, this is unlikely due to a selective event and may rather reflect heterogeneity among Mφ populations in the whole animal in general.

**Myocardial Interstitial Mφs in Op2/E0 and Op0/E0 Mice**

Numerous Mφs were present in the op2/E0 myocardial interstitium, as was evident from immunostaining of the Mφ-specific molecules macrosialin and SR-A, as well as

**TABLE 2. Distribution of Mφ Membrane Molecules in op2/E0 Atheromatous Lesions**

<table>
<thead>
<tr>
<th>Location</th>
<th>Macrosialin</th>
<th>CR3</th>
<th>SR-A</th>
<th>FcγRII (CD32)</th>
<th>CD44</th>
<th>MHC Class II</th>
<th>F4/80</th>
<th>Sialoadhesin</th>
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<tbody>
<tr>
<td>Base</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Shoulder</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>Necrotic core</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>−</td>
<td>+/−</td>
<td>−</td>
</tr>
<tr>
<td>Fibrous cap</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+++</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
</tr>
</tbody>
</table>

The distribution of Mφ membrane molecules within op2/E0 atherosclerotic lesions tabulated with + to +++ indicating the graded presence and − the absence of antigen expression.
CD44, MHC class II, CR3, and FcγRII molecules (data not shown). These \(M\)fs have the characteristics of a recruited and not resident population, as suggested by the low expression of F4/80 and sialoadhesin. In contrast, expression of \(M\)f membrane molecules, including SR-A, was markedly reduced in op0/E0 animals (data not shown). This would suggest a decrease in the interstitial myocardial \(M\)f population as a whole in op0/E0 mice, indicating its M-CSF–dependent nature.

**In Vivo Turnover of Lipoproteins in Op2/E0 and Op0/E0 Mice**

Sixteen-week-old op0/E0 mice fed a chow diet were previously shown to have smaller atherosclerotic lesions than their op1/E0 or op2/E0 littermates, despite having 2.5-fold increased levels of plasma cholesterol.\(^{18}\) The increased cholesterol is primarily due to an increased level of cholesteryl ester–enriched \(\beta\)-VLDL.\(^{18,39}\) Since M-CSF has been reported to induce SR-A mRNA,\(^{11}\) the hypothesis that the increased plasma cholesterol in the op0/E0 mice is associated with decreased SR-A activity was tested. Op0/E0 and op2/E0 mice were injected IV with \(^{[3]H}\)AcLDL, and plasma radioactivity was determined from retroorbital bleedings and normalized to the initial bleeding immediately after injection. Each point represents the mean of the fraction of radioactivity remaining in the plasma compared with the initial point. B, \(\beta\)-VLDL turnover. Op2/E0 and op0/E0 mice (4 each) were injected with \(^{[3]H}\)\(\beta\)-VLDL and bled after 2, 5, 15, 30, 60, 120, 180, 240, and 300 minutes. Each point represents the mean of the fraction of radioactivity remaining in the plasma compared with the initial 2-minute time point.

**Mf Populations in Noncardiovascular Organs of Op2/E0 and Op0/E0 Mice**

The liver in op2/E0 mice appeared macroscopically enlarged and fatty and oil red O staining confirmed marked foamy...
lipid accumulation in both Kupffer cells and hepatocytes (Fig 5). Immunohistochemical analysis of hepatic MΦs from op2/E0 and op0/E0 mice is shown in Fig 6. The Kupffer cell population expressed macrosialin, SR-A, and CD44 (not shown) prominently. SR-A staining of sinusoidal endothelium was more apparent than previously described, most likely due to differences in tissue preparation and immunostaining techniques used in this study. The prominent expression of SR-A by sinusoidal ECs (and its possible functional relevance in clearing atherogenic lipoproteins) contrasts markedly with its complete absence from ECs elsewhere, including myocardial endothelium. The phenotypic status of Kupffer cells as a mature resident MΦ population was confirmed by the lack of CD11b (CR3) expression (not shown) and low levels of sialoadhesin staining.

In contrast, the livers from op0/E0 mice appeared nonfatty despite the extremely high levels of plasma cholesterol. Hepatocytes showed little evidence of lipid accumulation, but Kupffer cells (reduced in number) were still oil red O positive and remained capable of lipid uptake (Fig 5). The M-CSF–dependent reduction in Kupffer cells was further demonstrated by the decrease in macrosialin and SR-A staining (Fig 6), and foamy MΦs were sparsely grouped throughout the hepatic architecture. SR-A expression remained well preserved on the sinusoidal EC population, which did not appear foamy. As these cells lack the M-CSF receptor (c-fms), it is not surprising that M-CSF deficiency did not affect SR-A expression on sinusoidal ECs in vivo. Hepatic SR-A expression in op0/E0 animals was thus reduced, but not disproportionately so in comparison with other Kupffer cell markers.

In the small intestine lamina propria, MΦs from op2/E0 or op0/E0 did not accumulate lipid (by oil red O staining) or appear foamy (Fig 7). The M-CSF–independent nature of this differentiated MΦ population was shown by the abundant expression of the antigens macrosialin, CD11b (Fig 7), FcγRII, F4/80, and sialoadhesin (not shown) in both op2/E0 and op0/E0 animals; and the unchanged MΦ population size in op0/E0 mice. Interestingly, SR-A expression in op0/E0 mice appeared selectively decreased in this MΦ population compared with op2/E0 controls.

Analysis of the spleens revealed the combined absence of CD11b, sialoadhesin, and SR-A staining in marginal zones of op0/E0 mice (not shown). This finding confirms the op0 status of the doubly mutant animals as splenic marginal zone metallophil MΦs are absent in op0 mice. Absence of SR-A expression in this M-CSF–dependent marginal zone metallophil population indicated loss of a specific MΦ subpopulation rather than a selective decrease in antigen.

Discussion

An unexpected finding from the previous study of Smith and colleagues was the significant inverse correlation between plasma cholesterol levels and aortic root lesion area in chow diet–fed op2/E0, op1/E0, and op0/E0 populations; implicating M-CSF gene dosage as the underlying cause for both phenomena. We confirmed and extended this finding in the current study. Twelve-week-old Western diet–fed op0/E0 mice had 4-fold smaller aortic root lesions than their op2/E0
littermates, and similarly, 1-year-old chow-fed op0/E0 mice had ≈10-fold less aortic lesion surface area than their op2/E0 controls as determined by an en face assay. These decreases in the extent of atherosclerotic lesion development occur despite the markedly increased total plasma cholesterol levels in the op0/E0 mice. Recently, in both a dietary and apoE-knockout model, M-CSF deficiency similarly resulted in significantly reduced atherosclerosis.59 M-CSF therefore affects susceptibility to atherosclerosis-prone op2/E0 mice, despite a fourfold increase in plasma cholesterol in op0/E0 animals. The increased lipid uptake in op2/E0 Kupffer cells and hepatocytes contrasted markedly with a low level of uptake by Kupffer Mφs in op0/E0 animals. The expression of oxidized lipoprotein, has been proposed for this molecule.63 CR3-positive cells within the lesions were all Mφs, and not neutrophils, indicating monocyte-specific recruitment and rapid turnover within a chronic inflammatory focus.

The op0/E0 lesions were smaller and contained fewer M-CSF–independent Mφs. These cells, similar to the Mφs in op2/E0 lesions, represent a recruited population, as shown by an overall reduction in F4/80 and sialoadhesin levels. SR-A expression in op0/E0 mice is present but reduced in proportion to the decrease in Mφ numbers. This observation would suggest that M-CSF is not an essential requirement for SR-A expression in vivo.

Lesional Mφs in different locations in plaques showed phenotypic heterogeneity by immunohistochemical staining. SR-A was present throughout the lesion except for the necrotic core, while CR3 expression in the murine advanced lesion (op2/E0) was not only confined to the deeper layers but also occurred in the superficial layers and necrotic core. Almost all the Mφs within lesions in op2/E0 and op0/E0 mice expressed macrophilin and MHC class II antigens. This finding agrees with the reported pattern of CD68 and the HLA-DR expression within human atherosclerotic lesions from the aorta and coronary and carotid arteries.45 Mφs within atherosclerotic lesions may also be heterogeneous with regard to expression of SR-A isoform (type I or II). The 2F8 mAb recognizes both types of murine SR-A, and this issue was thus not definitively addressed. Interestingly, type II SR-A seems the predominant murine form expressed in vivo,23,30 whereas no differential expression of human SR-A isoforms could be detected by immunostaining in Mφ from various organs and atherosclerotic foam cells.49

Lesional Mφ foam cell functional heterogeneity may be influenced by (1) the duration of residence within the lesion; (2) the local microenvironment of stimulatory and inhibitory growth factor and cytokine signals; and (3) regional location within the lesion. For example, Mφs at the shoulder of lesions express stromelysin activity selectively,46 while within the necrotic core of advanced plaques Mφ cell death may be either programmed (apoptosis)47 or result from toxic accumulation of modified lipoprotein derivatives and nitric oxide or local depletion of M-CSF.46 There is, however, little evidence that gene expression by lesional Mφs in vivo changes cellular behavior or influences the initiation or progression of atherosclerosis.

Myocardial interstitial Mφs also represented a recruited and M-CSF–dependent cell population. This is interesting because the myocardial interstitial Mφ population in wild-type C57BL/6 mice fed a normal chow diet appeared less prominent in number and mostly resident in nature.49 The recruitment of interstitial Mφs into the myocardium of atherosclerosis-prone op2/E0 mice may be secondary to myocardial muscle injury, although no areas of focal necrosis or fibrosis due to myocardial infarction could be detected.

An intriguing finding in the current study was the prominent lipid accumulation in the livers of op2/E0 but not op0/E0 mice, despite a fourfold increase in plasma cholesterol in op0/E0 animals. The increased lipid uptake in op2/E0 Kupffer cells and hepatocytes contrasted markedly with a low level of uptake by Kupffer Mφs in op0/E0 animals. The...
op0/E0 Kupffer cells represent a remnant M-CSF–independent Mφ population that exhibits both fewer Mφs and a nonselective decrease of Mφ markers, including SR-A.

The increased plasma cholesterol in the op0/E0 mice was previously shown to be due primarily to increased levels of cholesterol-enriched β-VLDL.39 This could be due to either an increased production rate or a decreased fractional catabolic rate. In the present study, we performed in vivo turnover studies to address this issue and to determine whether this effect might be mediated to SR-A. Both op0/E0 and op2/E0 mice degraded AcLDL equivalently and rapidly with a half-life of ≈1.5 minutes. However, after these studies were completed, Suzuki et al10 demonstrated that even in mice that have been made deficient in SR-A, the turnover in AcLDL is not impaired in in vivo turnover experiments. Thus, other molecules functionally related to SR-A, such as MARCO,50 macrosialin,41,51 CD36,52 and SR-B153 may provide sufficient uptake pathways for AcLDL and similar ligands. Due to the redundancy in AcLDL receptors, it is not possible to demonstrate SR-A defects in vivo using AcLDL as a ligand. Therefore, despite our observations that the hepatic distribution of SR-A was different in op2/E0 and op0/E0 mice, with the former expressing SR-A on both Kupffer and sinusoidal cells and the latter expressing predominantly on sinusoidal ECs, no change in AcLDL turnover was detected. Although M-CSF upregulates SR-A expression on Mφs in vitro, this study demonstrates that M-CSF is not essential for SR-A expression on Mφs and sinusoidal endothelium in vivo.

In contrast to the AcLDL turnover, the β-VLDL turnover studies revealed a difference in the two types of mice. Op2/E0 mice cleared the tracer with a half-life of 2 hours, as opposed to a 5-hour half-life in the M-CSF–deficient op0/E0 animals. The turnover of β-VLDL in both types of mice appeared to have two kinetic phases, as is commonly observed in many lipoprotein turnover studies, with the more rapid phase accounting for ≈20% of the tracer disappearance in the first 30 minutes. This phase was similar in both types of mice and might be due to equilibration of the tracer in the extravascular compartment. The turnover of the β-VLDL tracer then begins to diverge after this rapid phase, yielding a ≈2.5-fold increased β-VLDL fractional catabolic rate for the op2/E0 mice compared to the M-CSF–deficient op0/E0 mice. In a steady state situation, the β-VLDL production rate must equal the absolute β-VLDL degradation rate, and while the production rate is independent of the β-VLDL pool size (zero order reaction), the turnover rate is a product of the β-VLDL fractional catabolic rate and the β-VLDL pool size (first-order reaction). Thus, the β-VLDL production rate equals the β-VLDL fractional catabolic rate times β-VLDL pool size. Since the chow-fed op0/E0 mice have a β-VLDL pool size 2.5-fold larger and a β-VLDL fractional catabolic rate 2.5-fold smaller than the op2/E0 mice, we can conclude that the β-VLDL production rate need not be different to account for the observed difference in β-VLDL pool size. Therefore, although the absolute mass of β-VLDL cleared per hour is the same in the two different types of mice, the steady state levels of β-VLDL are higher in the op0/E0 mice due to the decreased fractional catabolic rate. A similar result is obtained in LDL receptor deficiency states, in which the absolute mass clearance of LDL per hour, via receptor-independent pathways, is normal or even elevated due to the higher steady state levels of LDL caused by a decrease in its catabolic rate.54 We do not know what receptor is responsible for the uptake of β-VLDL in vivo, but the current study indicates that this receptor’s activity or level is induced by M-CSF and most likely resides on monocytes/Mφs. Recently, a specific and saturable receptor activity for β-VLDL derived from apoE-deficient mice was characterized in a murine Mφ cell line.55 Binding of β-VLDL to this receptor is not competed for by LDL or by AcLDL but is competed for by normal VLDL.

Recently, hepatic SR-A was overexpressed in transgenic mice to investigate the functional role of these site-specific receptors in clearing potentially atherogenic lipoproteins.56 The mouse transferrin promoter targeted expression of the bovine SR-A type I to murine liver. Overexpression of hepatic SR-A enhanced cholesterol flux, and transgenic SR-A mice on an atherogenic diet accumulated less apoB-containing lipoprotein cholesterol and secreted more biliary cholesterol as bile acids. This increased removal of modified lipoproteins would be consistent with a protective antiatherogenic role for SR-A.

Indirect evidence for SR-A as a proatherogenic molecule emerged with the report that mice lacking tumor necrosis factor receptor p55 develop accelerated atherosclerosis.57 The increase in lesion size was accompanied by a threefold elevation in SR-A activity and overexpression of SR-A in aortic sinus sections. Direct evidence was provided by the observation that targeted disruption of the SR-A gene results in a ±50% reduction in the size of atherosclerotic lesions in apoE-deficient mice.10

The explanation for the seeming paradox in the atherogenic effects of low and high SR-A expression may be the tissue location of expression and effects on cholesterol flux. In addition, the balance or competition that exists in vivo between arterial subendothelial SR-A and liver Kupffer and sinusoidal endothelial SR-A for removal of modified lipoproteins may prove of critical importance. Thus, when hepatic SR-A is overexpressed, modified lipoproteins would be less likely to bind to SR-A present on Mφs in the aortic subendothelium (antiatherogenic scenario). ApoE-deficient mice, in contrast, develop hypercholesterolemia due to decreased hepatic clearance of atherogenic lipoproteins, thereby increasing access of modified lipoproteins to subendothelial Mφs. This shifts the balance to increased SR-A binding, enhanced arterial lipid deposition, and foam cell formation (proatherogenic scenario). In op0/E0 mice, hepatic uptake and clearance of atherogenic lipoproteins may be inefficient due to reduced Kupffer cell numbers and SR-A expression, resulting in severe hypercholesterolemia. The reduction in M-CSF–dependent vascular tree Mφ numbers (subendothelial Mφs and myocardial interstitial Mφs) and SR-A expression, however, limits arterial modified lipoprotein uptake, and severe atherosclerosis fails to develop.

This hypothesis would be consistent with several studies that have demonstrated M-CSF–induced decreases in plasma cholesterol in normal rabbits and hypercholesterolemic LDL receptor–defective Watanabe heritable hyperlipidemic rab-
bits, nonhuman primates, and normocholesterolemic and hypercholesterolemic humans, as well as patients with homozygous familial hypercholesterolemia.6,7,8 M-CSF also decreased foam cell development in Mφ-rich carrageenan-induced granulomas in Watanabe heritable hyperlipidemic rabbits and decreased progression and enhanced regression of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits and decreased progression and enhanced regression of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits and decreased progression and enhanced regression of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits.6,8,9 The cholesterol-lowering effects of M-CSF have been ascribed to (1) increased biliary cholesterol excretion,6,9 (2) the upregulation of apoE secretion by Mφs, thereby enhancing Mφ cholesterol efflux and reverse cholesterol transport,6,9 and (3) increased expression of additional Mφ receptors important in cholesterol clearance, such as the apoE-binding protein LDL receptor–related protein.60,61 In conclusion, we have characterized the Mφ phenotype in op2/E0, op1/E0, and op0/E0 mice and have clearly illustrated the importance of M-CSF and M-CSF–dependent monocyte/Mφ subpopulations in maintaining cholesterol homeostasis and in the pathogenesis of atherosclerosis.

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Macrophenage Phenotype in Mice Deficient in Both Macrophage-Colony-Stimulating Factor (Op) and Apolipoprotein E

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