Foamy Monocytes Form Early and Contribute to Nascent Atherosclerosis in Mice With Hypercholesterolemia

Lu Xu, Xiaoyuan Dai Perrard, Jerry L. Perrard, Donglin Yang, Xinhua Xiao, Ba-Bie Teng, Scott I. Simon, Christie M. Ballantyne, Huaizhu Wu

Objective—To examine infiltration of blood foamy monocytes, containing intracellular lipid droplets, into early atherosclerotic lesions and its contribution to development of nascent atherosclerosis.

Approach and Results—In apoE−/− mice fed Western high-fat diet (WD), >10% of circulating monocytes became foamy monocytes at 3 days on WD and >20% of monocytes at 1 week. Foamy monocytes also formed early in blood of Ldlr−/− Apobec1−/− (LDb) mice on WD. Based on CD11c and CD36, mouse monocytes were categorized as CD11cCD36+, CD11cCD36−, and CD11cCD36+. The majority of foamy monocytes were CD11cCD36+, whereas most nonfoamy monocytes were CD11cCD36− or CD11cCD36− in apoE−/− mice on WD. In wild-type mice, CD11cCD36− and CD11cCD36+, but few CD11cCD36−, monocytes took up cholesteryl ester–rich very low-density lipoproteins (CE-VLDLs) isolated from apoE−/− mice on WD, and CE-VLDL uptake accelerated CD11cCD36− to CD11cCD36+ monocyte differentiation. Ablation of CD36 decreased monocyte uptake of CE-VLDLs. Intravenous injection of DiI-CE-VLDLs in apoE−/− mice on WD specifically labeled CD11cCD36+ foamy monocytes, which infiltrated into nascent atherosclerotic lesions and became CD11c+ cells that were selectively localized in atherosclerotic lesions. CD11c deficiency reduced foamy monocyte infiltration into atherosclerotic lesions. Specific and consistent depletion of foamy monocytes (for 3 weeks) by daily intravenous injections of low-dose clodronate reduced development of nascent atherosclerosis.

Conclusions—Foamy monocytes, which form early in blood of mice with hypercholesterolemia, infiltrate into early atherosclerotic lesions in a CD11c-dependent manner and play crucial roles in nascent atherosclerosis development. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.305609.)

Key Words: atherosclerosis • diet, high-fat • inflammation • lipoproteins • monocytes

Atherosclerosis is a chronic inflammatory process characterized by accumulation of foam cells—macrophages/dendritic cells (DCs) with intracellular lipid deposition—in arterial walls.1-3 Infiltration of monocytes from blood into arterial walls, where monocytes differentiate into macrophages/DCs that take up modified lipoproteins and become foam cells, is an important step for atherogenesis.1,3,4 Although this process usually takes decades in humans, in genetic disorders that result in severe hypercholesterolemia, the process is dramatically accelerated, and morbidity in children can result from extensive atherosclerotic disease.5 This provides impetus to understand better the role of circulating lipoproteins in the initiation of the inflammatory axis of atherogenesis.

In our previous study, we reported that apoE−/− mice on Western high-fat diet (WD), the commonly used mouse model of atherosclerosis,6-8 had foamy monocytes—monocytes with intracellular lipid droplets—in blood.3 Foamy monocytes accounted for ≈40% to 50% of total monocytes in blood of apoE−/− mice after WD for 12 weeks. The vast majority (>80%) of foamy monocytes were positive for CD11c, a β2 integrin, whereas most nonfoamy monocytes in these mice were CD11c−.8 Existence of foamy monocytes in blood was confirmed by other studies in both mice and humans with hyperlipidemia.8-12 Nevertheless, it remains unknown when and how foamy monocytes are formed in blood of mice fed WD. Furthermore, the evidence for direct contributions of foamy monocytes to atherosclerosis, particularly nascent...
Atherosclerosis in which recent studies showed that monocyte recruitment played a significant role, is still lacking. In this study, we observed that foamy monocytes appeared in blood early after initiation of WD in apoE–/– mice and LDLb mice and that CD36 played an important role in monocyte uptake of cholesteryl ester (CE)-rich very low-density lipoproteins (CE-VLDLs), the most abundant lipoproteins from apoE–/– mice on WD. By intravenously injecting Dil-conjugated CE-VLDLs (DiI-CE-VLDLs), we selectively labeled foamy monocytes in apoE–/– mice on WD and found that they infiltrated into nascent atherosclerotic lesions in a CD11c-dependent manner. By daily intravenous injection of low-dose clodrosome, we specifically depleted foamy monocytes in apoE–/– mice on WD and found that depletion over 3 weeks effectively reduced development of nascent atherosclerosis. These studies reveal that foamy monocytes formed early in the circulation contribute to the development of nascent atherosclerosis with severe hypercholesterolemia.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

Early Appearance of Foamy Monocytes in Blood of apoE–/– Mice and LDLb Mice Fed WD

Using flow cytometric analysis, we first examined foamy monocytes in blood of apoE–/– mice fed WD, a commonly used mouse model of atherosclerosis. Foamy monocytes were detected within the first week of WD and increased continuously through week 5 (Figure 1A). Foamy monocytes, which stained positive for Nile Red (Figure 1B) and also for oil red O, constituted >10% of total monocytes at 3 days, ≈20% of monocytes at 1 week and ≈30% of monocytes at 3 weeks after WD (Figure 1A). Appearance of foamy monocytes was accompanied by an increase in blood cholesterol (Figure I in the online-only Data Supplement). Consistent with our previous report, CD11c+ monocytes accounted for the majority of foamy monocytes in apoE–/– mice on WD (Figure 1C). A concomitant decrease in the percentage of nonfoamy CD11c+ monocytes was observed in apoE–/– mice on WD, and the fraction of blood monocytes that were CD11c+ was maintained at ≈40% over 10 weeks in apoE–/– or WT mice, regardless of diet (Figure 1C).

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**Nonstandard Abbreviations and Acronyms**

<table>
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<tr>
<td>CE</td>
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<td>CE-VLDLs</td>
<td>cholesteryl ester–rich very low-density lipoproteins</td>
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<td>DCs</td>
<td>dendritic cells</td>
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<td>HCD</td>
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**Figure 1.** Early appearance of foamy monocytes in blood of apoE–/– mice on Western high-fat diet (WD). A. Percentages of foamy monocytes in total monocytes in blood of apoE–/– mice at various time points (from 3 days to 10 weeks) after starting WD analyzed by flow cytometry. n=6 to 7 mice/group. B. Representative flow cytometric examples of monocytes in wild-type (WT) or apoE–/– mice on WD (for 3 days) from >5 independent experiments with 3 to 7 samples/group in each experiment. C. Percentages of CD11c+ monocytes in foamy, nonfoamy, and total monocytes in blood of apoE–/– and WT mice on WD or normal diet (ND). n=6 to 7 mice/group. *P<0.05 vs WT WD and P<0.01 vs WT ND and apoE–/– ND; **P<0.01, ***P<0.001 vs WT ND, WT WD and apoE–/– ND; #P<0.05 vs WT ND and WD WD. SSC indicates side scatter.
It is well known that in WT mice, cholesterol is mainly transported by high-density lipoprotein, whereas in apoE–/– mice on WD, cholesterol is mainly transported by VLDLs. We next examined monocytes in blood of LDb mice, which are deficient in Ldlr and Apobec1 and have lipoprotein profiles that mimic human hypercholesterolemia, with elevated LDL-cholesterol. Similarly, LDb mice displayed early appearance of foamy monocytes, which constituted ≈30% of total monocytes at 2 weeks after starting WD (Figure II in the online-only Data Supplement). In both models, foamy monocytes emerged early and expressed CD11c, whereas most nonfoamy monocytes were CD11c– (Figure II in the online-only Data Supplement).

As described previously, we defined and quantified foamy monocytes by markedly increased side scatter (up to the granulocyte region), which correlated with inclusion of numerous lipid droplets. Monocytes with fewer or no droplets excluded from the granulocyte region were defined as nonfoamy monocytes. Of note, although we did not observe dramatic increases in foamy monocytes by this definition in blood of apoE–/– mice on normal diet (ND) when compared with WT mice, the side scatter value of monocytes was higher in 28-week-old apoE–/– mice than WT mice on ND (Figure IIIA in the online-only Data Supplement), suggesting that monocytes in blood of apoE–/– mice on ND may also include more lipid. Consistent with these observations, monocytes from humans after a high-fat high-cholesterol diet included lipid droplets positive for oil red O staining but exhibited a minor change in side scatter.

Foamy Monocytes in Blood of apoE–/– Mice Fed High-Fat Low-Cholesterol Diet or High-Cholesterol Low-Fat Diet

WD, the most commonly used atherogenic diet, is high in saturated fat and cholesterol. To examine which constituent is predominant in early foamy monocyte formation, we fed apoE–/– mice 2 additional special diets, that is, high-fat (low-cholesterol) diet (HFD) and high-cholesterol (low-fat) diet (HCD; Table I in the online-only Data Supplement). As shown in Figure IIIB in the online-only Data Supplement, at 2 weeks on HCD, high numbers of foamy monocytes were detected in blood of apoE–/– mice. In contrast, HFD feeding for 2 weeks tended to increase the proportion of foamy monocytes compared with ND. However, the proportion of foamy monocytes was much lower than that induced by WD or HCD. Compared with ND, HCD, and WD, but not HFD, markedly increased plasma levels of cholesterol, and neither HCD nor HFD significantly increased triglyceride levels, whereas HCD, WD, and HFD all tended to increase plasma TBARS values in these mice (Figure IIIB in the online-only Data Supplement). These data indicate that diet-induced increases in cholesterol levels, along with enhanced lipid oxidation (also see following data on TBARS in lipoproteins), may be the major cause of foamy monocyte formation in blood of apoE–/– mice at the early stage of dietary intervention.

Figure 2. Three major monocyte subsets in mouse blood. A, A representative sample of monocyte subsets in blood of a wild-type (WT) mouse showing 3 major subsets based on CD11c and CD36, and their relationship to Ly-6C expression, from 3 independent experiments with 3 or 4 samples in each experiment. B, A representative example of monocyte reappearance and subset conversion in blood of WT mice after monocyte depletion by intravenous injection of clodrosome (0.3 mL/mouse), from 2 independent experiments with 4 samples in each experiment. C, A representative example of subset conversion of EdU-labeled monocytes in blood of WT mice after injection with EdU, from 2 independent experiments with 3 samples in each experiment. SSC indicates side scatter.
Three Major Subsets of Monocytes in Mouse Blood

Mouse monocytes are conventionally classified into 2 major subsets based on expression of Ly-6C. Staining monocytes for CD11c and CD36 identified 3 major subsets in WT mice: CD11c–CD36–, CD11c–CD36+, and CD11c+CD36+ (Figure 2A). Staining for Ly-6C showed that CD11c+CD36– monocytes were mainly Ly-6C<sup>high</sup> and CD11c–CD36+ monocytes were mainly Ly-6C<sup>low</sup>, whereas CD11c–CD36+ monocytes included both Ly-6C<sup>high</sup> and Ly-6C<sup>low</sup> monocytes (Figure 2A).

To examine the dynamics among the 3 distinct monocyte subsets, we depleted circulating monocytes in WT mice by injecting a bolus of clodrosome at 0.3 mL/mouse and tracked each subset in blood samples. Monocyte depletion was achieved within 22 hours following clodrosome injection (Figure 2B). At 42 hours, monocytes reappeared, presumably representing those released from bone marrow. Most monocytes sampled at 42 hours were CD11c–CD36–; CD11c–CD36+ monocytes were present at 66 to 90 hours, and CD11c+CD36+ were detected at ≈90 to 114 hours (Figure 2B). These data indicated that CD11c+CD36– monocytes are those newly released from bone marrow. CD11c+CD36+ monocytes emerge over time as the most mature, whereas CD11c–CD36+ monocytes represent an intermediate stage that seems to dynamically convert in the circulation.

To confirm this, we injected WT mice with a bolus of EdU, which is incorporated into genomic DNA during cell division. This allowed us to pulse-label immature monocytes undergoing proliferation in bone marrow and to track conversion of those labeled in blood as they emerged from bone marrow. At 3 hours following EdU injection, EdU<sup>+</sup> monocytes were observed in bone marrow (Figure IV in the online-only Data Supplement) but not in blood (Figure 2C). At 24 hours after EdU injection, EdU<sup>+</sup> monocytes were present in blood and represented those monocytes newly released from bone marrow. Staining the EdU<sup>+</sup> monocytes in blood revealed that the majority at 24 hours were CD11c–CD36–. EdU<sup>+</sup> CD11c–CD36– monocytes emerged within 48 hours and abundant EdU<sup>+</sup> CD11c+CD36+ monocytes appeared by 72 hours, indicating a rapid conversion of CD11c–CD36– and CD11c–CD36+ monocytes to CD11c+CD36+ monocytes. By 120 hours, most EdU<sup>+</sup> monocytes were CD11c+CD36+ monocytes, which remained in circulation for at least 2 to 3 more days (Figure 2C). These results were consistent with the depletion studies and with previous reports on the emergence of classical and nonclassical monocytes.

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**Figure 3.** Some characteristics of foamy monocytes in apoE<sup>–/–</sup> mice on Western high-fat diet (WD). A, Representative examples of monocyte subsets (left panel) and relative ratios of CD11c+CD36+ to CD11c–CD36+ monocytes (right panel) in apoE<sup>–/–</sup> mice on normal diet (ND) or WD. n=4 to 6 mice/group. B, Representative staining for CD11c and CD36 of foamy and nonfoamy monocytes in apoE<sup>–/–</sup> mice on WD (4 weeks) from >5 independent experiments with at least 4 samples in each experiment. C, Comparisons of CD11c and CD36 mean fluorescence intensity (MFI) levels on foamy and nonfoamy CD11c+CD36+ monocytes in apoE<sup>–/–</sup> mice on WD or ND. n=5 to 9 mice/group. D, mRNA levels of tumor necrosis factor-α (TNFα) and interleukin (IL)-1β in foamy and nonfoamy monocytes from apoE<sup>–/–</sup> mice on WD. n=3 samples, each of which were pooled blood from 5 mice. *P<0.05, **P<0.001 vs ND group (A) or nonfoamy monocytes (C and D); #P<0.05, ##P<0.01 vs ND group (C).
Characteristics of Foamy Monocytes in apoE−/− Mice on WD

Similar to those in WT mice, 3 distinct subsets of monocytes were detected in blood of apoE−/− mice. In contrast to WT or apoE−/− mice on ND, however, apoE−/− mice on WD exhibited a gradual increase in the ratio of CD11c+CD36+ to CD11c–CD36+ subsets (Figure 3A). In apoE−/− mice on WD, the vast majority of foamy monocytes were CD11c+CD36+, whereas most nonfoamy monocytes were either CD11c–CD36– or CD11c–CD36+ (Figure 3B). CD36+CD11c+ monocytes also expressed elevated levels of CD36 and CD11c in foamy compared with nonfoamy monocytes (Figure 3C). In addition, CD11c+ (foamy) monocytes expressed higher levels of tumor necrosis factor α and interleukin (IL)-1β than did CD11c– (nonfoamy) monocytes from apoE−/− mice on WD (Figure 3D). Taken together, these data indicate that foamy monocytes upregulate surface expression of CD11c, CD36, and proinflammatory markers over days of maturation in the circulation of apoE−/− on WD.

Monocyte Uptake of CE-VLDLs and Differentiation of CD11c− to CD11c+ Monocytes

To investigate the process by which uptake of atherogenic lipoproteins directs an inflammatory monocyte phenotype, we isolated CE-VLDLs from apoE−/− mice on WD and labeled them with DiI. CE-VLDLs are the most abundant atherogenic lipoproteins in these mice and are similar to human LDLs in lipid composition.16 The level of oxidation for the isolated CE-VLDLs was confirmed as 12±3 TBARS value, indicating low to medium oxidation. A bolus of DiI-CE-VLDLs was injected intravenously into WT mice on ND. At 3 hours after injection, ≈40% monocytes became DiI+, indicating CE-VLDL uptake (Figure 4A). Notably, the majority of DiI+ monocytes were CD36+, whereas DiI+ monocytes included both CD11c+ and CD11c– monocytes (Figure 4A), indicating that CD11c+CD36+ and CD11c–CD36+, but few CD11c–CD36–, monocytes took up CE-VLDLs. At 24 hours after DiI-CE-VLDL injection, most DiI+ monocytes became CD11c+ (Figure 4A). Given that injection of human native LDL did not induce significant CD11c– to CD11c+ monocyte differentiation over this period of time,8 we conclude that uptake of CE-VLDLs accelerated CD11c–CD36– to CD11c+CD36+ monocyte differentiation. Consistently, repetitive daily injection of CE-VLDLs (3×) increased CD11c–CD36– to CD11c+CD36+ monocyte ratios in WT mice (Figure 4B). In contrast to monocyte uptake of CE-VLDLs, few monocytes took up DiI-high-density lipoprotein after injection (Figure V in the online-only Data Supplement). Furthermore, injection of an

Figure 4. Monocyte uptake of cholesteryl ester–rich very low-density lipoproteins (CE-VLDLs) and subset conversion in wild-type (WT) and CD36−/− mice. A, Representative flow cytometric analyses of monocytes that took up DiI-CE-VLDLs (becoming DiI+), from >5 independent experiments with at least 3 samples in each experiment. A bolus of DiI-CE-VLDLs was intravenously injected into WT mice on normal diet (ND). At 3 and 24 hours, blood was taken for flow cytometric analysis. Gated total leukocytes (for CD204) or DiI+ monocytes (for CD36 and CD11c) are presented. B, Relative ratios of CD11c+CD36+ to CD11c–CD36+ monocytes in WT mice on ND after receiving repetitive daily injection of CE-VLDLs or PBS (control) for 3 days. n=4 mice/group. C, Representative flow cytometric analysis of monocyte uptake of DiI-CE-VLDLs, and quantification of DiI– monocytes in total monocytes and DiI mean fluorescence intensity (MFI) levels on DiI+ monocytes, in WT and CD36−/− mice after receiving intravenous injection of a bolus of DiI-CE-VLDLs. n=11 to 12 mice/group. *P<0.05 vs preinjection (B) or WT controls (C).
equivalent amount of free DiI did not result in appearance of DiI+ cells in mouse blood (Figure V in the online-only Data Supplement), confirming that DiI+ monocytes after DiI-CE-VLDL injection were the result of monocyte uptake of DiI-CE-VLDLs rather than uptake of free DiI.

The observation that CD36+, but not CD36+, monocytes took up CE-VLDLs prompted us to examine the role of CD36 in CE-VLDL uptake. First, using THP1 monocytes (without induction to macrophages) in vitro, we found that treatment with CE-VLDLs caused lipid accumulation indicated by increased side scatter (Figure VI in the online-only Data Supplement) and Nile red staining (data not shown). This treatment also increased CD36 levels on THP1 monocytes (Figure VI in the online-only Data Supplement), consistent with our in vivo data showing higher CD36 levels on foamy monocytes. Along with the increase in CD36, treatment of THP1 monocytes with (unlabeled) CE-VLDLs enhanced further uptake of DiI-CE-VLDLs, indicating a positive feedback between CD36 expression and lipid uptake, by these monocytes (Figure VI in the online-only Data Supplement). Next, we used CD36+ (CD36obt/obt) mice to examine a direct role of CD36 in monocyte uptake of CE-VLDLs. We injected a bolus of DiI-CE-VLDLs into CD36−/− mice and WT controls and found that CD36−/− mice exhibited ≈30% reduction in the percentage of DiI-(CE-VLDL)+ monocytes and the levels of DiI at 3 and 24 hours (Figure 4C). In contrast to the reported role of TLR4 in macrophage uptake and inflammatory response to ox-LDL, TLR4−/− mice did not show reductions in monocyte uptake of DiI-CE-VLDLs after lipoprotein injection (Figure VII in the online-only Data Supplement). These data lead to the conclusion that CD36 on both CD11c−CD36+ and CD11c+CD36+ monocytes participates in CE-VLDL uptake. Furthermore, foamy monocyte formation in apoE−/− mice on WD induced differentiation of monocytes from CD11c−CD36− to CD11c+CD36+.

Early Development of Atherosclerosis in apoE−/− Mice After WD

The observation of early appearance of foamy monocytes in blood of mice with hypercholesterolemia prompted us to examine whether foamy monocytes infiltrate into nascent plaque and contribute to nascent atherosclerosis. We focused on atherogenesis in apoE−/− mice fed WD for 3 weeks, the time when apoE−/− mice showed aortic atherosclerotic plaques (Figure VIII in the online-only Data Supplement). Flow cytometry showed that apoE−/− mice on WD had increased aortic macrophages/DCs, mainly CD11c+CD11b+ cells (Figure VIIIB in the online-only Data Supplement), consistent with the reported changes in advanced atherosclerosis in apoE−/− mice, or in early atherosclerosis in Ldlr−/− mice. Also consistent with previous reports is the observation that CD11c+ cells selectively localized in atherosclerotic lesions (Figure VIIIIC in the online-only Data Supplement).

Compared to WT controls, apoE−/− mice on WD had increased aortic F4/80+ cells, most of which were also CD11c+, appeared in mouse aorta (Figure 5B). These data indicated that the increase in CD11c+ cells in apoE−/− mice was due to uptake of DiI-CE-VLDLs and macrophage infiltration into apoE−/− mice. CD11c+ foamy monocytes infiltrated into atherosclerotic aortas and became CD11c+ cells in the lesions. Concurrently, we also observed DiI-F4/80+ cells in mouse aorta (Figure 5B), which may represent lesional resident macrophages/DCs that took up DiI-CE-VLDLs.

Infiltration of Foamy Monocytes into Nascent Atherosclerotic Lesions

Next, we tested whether CD11c+CD36+ foamy monocytes contributed to the early increases of CD11c+CD11b+ cells in atherosclerotic lesions. In contrast to the response in WT mice (Figure 4A), injection of a bolus of DiI-CE-VLDLs in apoE−/− mice on WD resulted in exclusive lipoprotein uptake by CD11c+ foamy monocytes that became DiI+, and therefore specifically labeled CD11c+ foamy monocytes (Figure 5A). DiI+CD11c+ foamy monocytes were low in F4/80 (Figure 5A), which is highly expressed on macrophages (Figure IX in the online-only Data Supplement). At day 5 post injection, DiI-F4/80+ cells, most of which were also CD11c+, appeared in mouse aorta (Figure 5B). These data indicated that DiI-F4/80+CD11c+ foamy monocytes infiltrated into atherosclerotic aortas and became CD11c+ cells in the lesions. Concordantly, we also observed DiI-F4/80+ cells in mouse aorta (Figure 5B), which may represent lesional resident macrophages/DCs that took up DiI-CE-VLDLs.

Effects of CD11c Deficiency on Foamy Monocyte Infiltration into Nascent Atherosclerotic Lesions

Deficiency of CD11c in apoE−/− mice reduced atherosclerosis. We therefore examined the role of CD11c in foamy monocyte infiltration into nascent atherosclerotic lesions using CD11c+/−/apoE−/− and CD11c+/+/apoE−/− mice on WD (for 3 weeks). CD11c deficiency in apoE−/− did not impair DiI-CE-VLDL uptake or fluorescent bead-labeling of foamy monocytes in blood (Figure X in the online-only Data Supplement). However, at 5 days after DiI-CE-VLDL injection, foamy monocytes derived from aortic suspensions and defined by DiI and bead+ cells in blood were DiI+ foamy monocytes, whereas ≈50% of DiI+ foamy monocytes remained bead− (Figure 5A).

Histological examination of aortic sinus sections revealed that DiI+bead+ cells accumulated in atherosclerotic lesions, with 12±2 bead+ cells/lesion, and also expressed CD11c (Figure 5C).

Taken together, these data indicated that CD11c+ foamy monocytes infiltrated into nascent atherosclerotic lesions and became CD11c+ cells within the lesions.
In a final set of studies, we examined whether depletion of foamy monocytes would alter the development of nascent atherosclerosis. We intravenously injected a low dose of clodrosome into apoE−/− mice daily from day 3 on WD for an additional 3 weeks and examined development of atherosclerosis after the injection. Noteworthy is the observation that the low-dose clodrosome injection consistently and specifically depleted CD11c+ foamy monocytes, with no significant alteration of the proportions of CD11c− nonfoamy monocytes, compared with PBS injection (Figure 6A).

Figure 5. Labeling of foamy monocytes and infiltration of foamy monocytes into nascent atherosclerotic lesions. A, Representative flow cytometric analysis samples showing specific labeling of foamy monocytes and phenotypes of labeled foamy monocytes in apoE−/− mice on Western high-fat diet (WD; 3 weeks) after intravenous injection of DiI-CE-VLDLs with or without fluorescent microbeads, from >5 independent experiments with at least 3 samples in each experiment. B, Representative flow cytometric analysis samples showing foamy monocytes (DiI+F4/80low) in atherosclerotic aortas with sustained expression of CD11c, from 4 independent experiments with at least 3 samples in each experiment. C, Representative histology of aortic sinus showing accumulation of microbead+ (green) cells, which were also DiI+ (red, left panel) and CD11c+ (red, right panel), in atherosclerotic lesions of apoE−/− mice on WD (3 weeks). D, Normalized frequency of infiltrated foamy monocytes in aortas of CD11c−/−/apoE−/− and CD11c+/+/apoE−/− mice on WD (3 weeks). n=5 to 7 samples/group. E, Representative histology and quantification of foamy monocyte infiltration in aortic sinus lesions of CD11c−/−/apoE−/− and CD11c+/+/apoE−/− mice on WD (3 weeks). n=15 samples/group. CE-VLDLs indicates cholesteryl ester–rich very low-density lipoproteins; and SSC, side scatter.

Contribution of Foamy Monocytes to the Development of Nascent Atherosclerosis

In a final set of studies, we examined whether depletion of foamy monocytes would alter the development of nascent atherosclerosis. We intravenously injected a low dose of clodrosome into apoE−/− mice daily from day 3 on WD for an additional 3 weeks and examined development of atherosclerosis after the injection. Noteworthy is the observation that the low-dose clodrosome injection consistently and specifically depleted CD11c+ foamy monocytes, with no significant alteration of the proportions of CD11c− nonfoamy monocytes, compared with PBS injection (Figure 6A).
reasoned that foamy monocytes have enhanced capacity to phagocytose clodrosome and that this leads to specific depletion of foamy monocytes. To confirm this, we injected a bolus of DiI-clodrosome into apoE–/– mice on WD and found that at 3 hours after injection of a low dose of DiI-clodrosome, ≈90% of foamy monocytes became DiI+, indicating uptake of DiI-clodrosome, whereas <10% of nonfoamy monocytes became DiI+. In contrast, injection of a larger dose (0.2 mL/mouse) of DiI-clodrosome caused ≈50% of nonfoamy monocytes to take up DiI-clodrosome and >90% of foamy
monocytes to take up clodrosome (Figure XII in the online-only Data Supplement).

Notably, specific depletion of foamy monocytes (for 3 weeks) resulted in significantly lower proportions of aortic CD11b+CD11c- cells compared with controls (Figure 6B). Analysis of aortic sinus lesions showed that mice with foamy monocyte depletion had smaller lesions, as assessed by oil red O staining (Figure 6C), and less CD11c (and CD11b), as assessed by immunofluorescent staining (Figure 6D).

To exclude the possibility that the clodrosome-induced changes in atherosclerosis were caused by direct depletion of lesional macrophages/DCs, we injected a larger bolus (0.2 mL/mouse) of clodrosome into apoE-/- mice on WD (3 weeks). At 22 hours post injection, most monocytes in blood were depleted. However, the proportions of CD11b+CD11c- cells in aortic cell suspensions did not change (Figure XIII in the online-only Data Supplement), indicating that clodrosome did not alter numbers of resident macrophages/DCs in aortas, consistent with previous studies.14,23 Therefore, the lower proportions of aortic CD11b+CD11c- cells and smaller atherosclerotic lesions in mice treated repetitively with low-dose clodrosome injections were most likely caused by decreased infiltration of circulating foamy monocytes into atherosclerotic lesions.

Discussion

Infiltration of monocytes from the circulation into atherosclerosis-prone vessels is a crucial early step in atherogenesis.1,3,14 Genetic disorders in humans with severe increases in apoB-containing lipoproteins in the blood, such as familial hypercholesterolemia with increased LDLs and type III hyperlipoproteinemia with increased VLDLs (retnans), lead to premature atherosclerosis and xanthoma at early ages.25 Based on our previous report,8 we postulate that uptake of apoB-containing CE-rich lipoproteins and foamy monocyte formation in blood plays a critical role in this process. Indeed, humans with familial hypercholesterolemia had foamy leukocytes in blood.26 In this study, we report that foamy monocytes formed early in blood of both WD-fed apoE-/- mice, in which CE is transported by VLDLs, and LDLb mice, in which CE is transported by LDLs.27 Further examination revealed upregulation of CD11c and CD36 on these monocytes and a direct correlation between monocyte uptake of CE-rich lipoproteins and upregulation of CD11c and CD36 with increased scavenger function of CD36. We also demonstrated that foamy monocytes trafficked to atherosclerotic aortas and became CD11c macrophages/DCs within nascent atherosclerotic lesions. The frequency of infiltrated foamy monocytes in these lesions was decreased by ≈2-fold in the absence of CD11c. We conclude that CD11c may serve as a biomarker of monocyte inflammatory state in the circulation and function to enhance the capacity of monocytes to infiltrate nascent lesions and differentiate into CD11c macrophages/DCs.

Monocytes are a heterogeneous population in the circulation, and surface receptors are used to discriminate between several subsets.26,27 Based on CD11c expression, we previously classified mouse monocytes into CD11c+ and CD11c- subsets.14 In this study, detection of CD36 and CD11c expression provided discrimination of 3 distinct subsets. CD11c+CD36- and CD11c-CD36+ monocytes took up CE-VLDLs isolated from WD-fed apoE-/- mice, and this resulted in upregulation of CD11c in the latter subset. This result is consistent with the observation that apoE-/- mice on WD, with elevated levels of CE-VLDLs, registered a 2-fold increase in the ratio of CD11c+CD36+ to CD11c CD36+ monocytes. Our further investigation was in part motivated by previous reports of a crucial role for CD36 in macrophage uptake of modified LDL, inflammasome activation and atherogenesis.28 We now demonstrate an important role for CD36 in CE-VLDL uptake by circulating monocytes, supported by the observations that CE-VLDL uptake was prevalent in CD36+, but not CD36-, monocytes, and that the absence of CD36 reduced monocyte uptake of CE-VLDLs. The findings that CD36 levels on foamy monocytes exceeded those on nonfoamy monocytes and that treatment of THP1 monocytes with CE-VLDLs increased CD36 levels suggest that lipid accumulation itself signals a feedforward mechanism for CD36 upregulation that promotes foam cell formation. This may also explain why foamy monocytes, but few nonfoamy monocytes, in apoE-/- mice on WD took up DiI-CE-VLDLs. It is possible that elevated levels of endogenous CE-VLDLs in apoE-/- mice on WD predispose CD36+ monocytes to take up CE-VLDLs preferentially and become foamy. Consistently, CD36+ monocytes accounted for a minor portion of nonfoamy monocytes in these mice. Our current observations in mice are in agreement with previous human studies showing increased expression of CD36 and CD11c on CD16+ monocytes in patients with familial hypercholesterolemia compared with healthy controls, increased ox-LDL uptake via CD36 by CD16+ monocytes from hypercholesterolemic subjects,29 and high CD36 expression, preferential lipid accumulation and avid ox-LDL uptake in CD14+/CD16+ monocytes of patients with chronic kidney disease.30

The role of monocyte heterogeneity in human versus mouse atherogenesis has been controversial. Human studies have shown increased CD16+ monocytes, particularly CD14+/CD16+ intermediate monocytes, in hyperlipidemia and association of CD16+ monocytes with atherosclerotic cardiovascular disease.27,30,31 In contrast, initial studies in mouse models of hypercholesterolemia have implicated a preferential increase of Ly-6Chigh monocytes, analogous to human CD16+ monocytes, in blood, and the participation of Ly-6Chigh monocytes in atherogenesis.6,7 However, more recent studies revealed similar ratios of Ly-6Cint and Ly-6Chigh monocytes in hypercholesterolemic mice,23 contributions or infiltration of both subsets to atherosclerosis,9,33 and higher correlation of Ly-6Chigh monocyte number with lesion size.24 Furthermore, recent studies indicated that reduced atherosclerosis was correlated with decreased recruitment of Ly-6Cint monocytes.34-36 These disparate results may be related to the fact that most previous studies focused on advanced atherosclerosis.6,7,34,35 In which macrophage proliferation plays a dominant role.34 Monocyte recruitment plays a more important role in early atherogenesis.1,14 Our data are consistent with early monocyte activation and differentiation in blood, which correlates with enhanced recruitment in early atherosclerosis. Indeed, our current study revealed early emergence of foamy monocytes in blood of mice with severe hypercholesterolemia and upregulation of CD11c on this population. Moreover, we report that CD11c+CD36+ foamy monocytes infiltrated into early atherosclerotic lesions and maintained high expression of CD11c within the lesions. We have previously reported that...
CD11c represents a functional integrin on foamy monocytes in human and mouse circulation and is necessary for shear-resistant adhesion to VCAM-1 upregulated on inflamed arterial endothelium.\textsuperscript{8,11} Induction of the high-affinity ligand-binding state of CD11c on monocytes correlates with lipid uptake and enhances monocyte recruitment to VCAM-1 through coalescence on the plasma membrane and subsequent activation of the αβ\textsubscript{3}−integrin (VLA-4).\textsuperscript{37} Significantly, we currently showed that deletion of CD11c reduced foamy monocyte infiltration into early atherosclerotic lesions, and the specific depletion of foamy monocytes decreased CD11c\textsuperscript{+} cells in atherosclerotic aortas and reduced development of early atherosclerosis. These data support a crucial role of foamy monocytes and increased expression and function of CD11c on monocytes in activation of recruitment to sites of early atherosclerosis. Also noteworthy is the observation that CD16\textsuperscript{+} monocytes from humans with familial hypercholesterolemia, with elevated levels of CD11c, showed enhanced adhesion to activated endothelium.\textsuperscript{29}

Studies from Cybulsky’s group indicated that macrophages/DCs also proliferated in early atherosclerosis and that Ly-6C\textsuperscript{high} monocytes infiltrated into early atherosclerotic lesions. Notably, most of the cells they observed in early lesions expressed CD11c.\textsuperscript{19,38} Our findings are consistent with these reports and another study\textsuperscript{1} showing preferential increases in CD11c\textsuperscript{+}/CD11b\textsuperscript{+} cells in advanced atherosclerotic aortas. Furthermore, CD11c\textsuperscript{+} cells seem to be selectively localized in atherosclerotic lesions (but rarely in adventitia where there are CD11b/CD11c\textsuperscript{−} cells)\textsuperscript{2,6,8,19,22,38} and are the major cell type undergoing proliferation in early atherosclerotic lesions.\textsuperscript{19} Cell proliferation may contribute to the increases in CD11c\textsuperscript{+} cells in atherosclerotic lesions.\textsuperscript{19,38} Here, we demonstrated that infiltration of CD11c\textsuperscript{+} foamy monocytes contributes to the increase in CD11c\textsuperscript{+} cells in the lesions and development of early atherosclerosis. Consistent with our findings is the report of Tacke et al\textsuperscript{8} showing that Ly-6C\textsuperscript{+} monocytes, most of which were CD11c\textsuperscript{+}, were more prone to become CD11c\textsuperscript{+} cells in atherosclerotic lesions after infiltration.

Finally, we need to point out that our current study focused on the role of foamy monocytes, but not specifically Ly-6C\textsuperscript{high} monocytes, in nascent atherosclerosis. Deficiency of NR4A1 in apoE\textsuperscript{−/−} or Ldlr\textsuperscript{−/−} mice decreased abundance of Ly-6C\textsuperscript{high} monocytes, but increased\textsuperscript{39} or did not change\textsuperscript{40} the development of atherosclerosis. However, loss of NR4A1 increased lipid accumulation in circulating monocytes, also supporting a role of lipid accumulation within monocytes in atherosclerosis.\textsuperscript{39}

A limitation of our current study is that we used apoE\textsuperscript{−/−} mice on WD as the animal model of atherosclerosis. Although this mouse model has been widely used,\textsuperscript{2,6,7} these mice have severe hypercholesterolemia. Foamy monocytes in these mice may have higher lipid content than foamy monocytes in human subjects after high-fat high-cholesterol diet.\textsuperscript{10–12} Nevertheless, because of the prevalence of foamy monocytes and easier identification of the early stage of atherogenesis in these mice, we think that the data presented with this mouse model are relevant to human studies showing the presence of foamy monocytes in the circulation after high-fat high-cholesterol diet or with severe hypercholesterolemia.\textsuperscript{10–12,25}

In summary, we reveal the dynamics of inflammatory changes in monocytes in apoE\textsuperscript{−/−} mice fed a WD, an established model of hypercholesterolemia and atherosclerosis. Blood monocytes take up CE-VLDLs and become foamy, and this elicits subsequent phenotypic changes, including upregulation of CD11c, CD36, and proinflammatory cytokines. Foamy monocytes infiltrate into early atherosclerotic lesions and become CD11c\textsuperscript{+} cells. We show that CD11c specifically expressed on foamy monocytes supports their selective trafficking to atherosclerosis-prone lesions in aorta and differentiation into CD11c\textsuperscript{+} macrophages/DCs. Evidence from other reports\textsuperscript{49,50} supports their proliferation within the lesions. These processes may represent the earliest events and play a major role in nascent atherosclerosis.

Acknowledgments

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Disclosures

None.

References

Hypercholesterolemia is a major risk factor for atherosclerosis. Monocyte infiltration from the circulation into the arterial wall is a crucial early step in atherogenesis. However, effects of atherogenic lipoproteins on circulating monocytes and their consequences on early atherosclerosis remain largely unknown. We previously reported foamy monocytes, containing intracellular lipid droplets, in the circulation of mice with hypercholesterolemia. We now report early emergence of foamy monocytes, with upregulation of CD14+ and CD36, in blood of mice with severe hypercholesterolemia, and revealed an important role of CD36 in monocyte uptake of atherogenic lipoproteins. Specific labeling or depletion of circulating foamy monocytes demonstrated that foamy monocytes infiltrated into nascent atherosclerotic lesions in a CD14-dependent manner and contributed to early atherosclerosis. Therefore, our current studies add novel information to the current concept of development of early atherosclerosis with severe hypercholesterolemia.

Significance
Foamy Monocytes Form Early and Contribute to Nascent Atherosclerosis in Mice With Hypercholesterolemia
Lu Xu, Xiaoyuan Dai Perrard, Jerry L. Perrard, Donglin Yang, Xinhua Xiao, Ba-Bie Teng, Scott I. Simon, Christie M. Ballantyne and Huaizhu Wu
SUPPLEMENTAL MATERIAL

Materials and Methods

Animals and Diet
Male apoE<sup>−/−</sup> mice, LDb (Ldlr<sup>−/−</sup> Apobec1<sup>−/−</sup>) mice, TLR4<sup>−/−</sup> mice (all on a C57BL/6 background) and C57BL/6 wild-type (WT) mice, CD11c<sup>+</sup>/apoE<sup>−/−</sup> and CD11c<sup>+</sup>/apoE<sup>−/−</sup> littermates, CD36<sup>ob/ob</sup> mice<sup>3</sup> (deficient in functional CD36 and on a C57BL/6 background; obtained through Mutant Mouse Regional Resource Centers) and CD36<sup>+/+</sup> WT littermate controls were used. ApoE<sup>−/−</sup> mice, TLR4<sup>−/−</sup> mice and C57BL/6 mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME) and the mouse colonies were maintained in a pathogen-free animal facility of Baylor College of Medicine. Mice were either maintained on normal chow diet (ND, PicoLab Rodent Chow 5010; LabDiet, St. Louis, MO) throughout, or switched to western high-fat diet (WD) (21% milkfat [w/w], 0.2% cholesterol [w/w]; Dyets Inc., Bethlehem, PA) at the age of 8 weeks and maintained on WD for up to 10 weeks, or switched to a high-fat low-cholesterol diet (HFD) (21% milkfat [w/w], 0.05% cholesterol [w/w]; Dyets Inc.) or low-fat high-cholesterol diet (HCD) (5% milkfat [w/w], 0.2% cholesterol [w/w]; Dyets Inc.) at the age of 8 weeks and maintained on these diets for 2 weeks. A total of 346 male mice, including 116 “WT,” 170 apoE<sup>−/−</sup>, 5 LDb, 12 CD36<sup>ob/ob</sup>, 3 TLR4<sup>−/−</sup>, 20 CD11c<sup>+</sup>/apoE<sup>−/−</sup> and 20 CD11c<sup>−/−</sup>/apoE<sup>−/−</sup>, were used. All mice were housed in a pathogen-free facility that maintained a 12-hour light/12-hour dark cycle. Blood was drawn via retro-orbital puncture, and plasma total cholesterol and triglyceride levels were measured using enzymatic procedures with Wako products (Wako Diagnostics, Richmond, VA). All animal studies were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

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The following monoclonal antibodies (mAbs) to mouse antigens, with appropriate isotype negative controls, were used: CD45 (FITC-conjugated), CD11c (FITC-, PE- or PerCP-Cy5.5-conjugated), CD11b (FITC-conjugated), Ly-6C (FITC- or APC-conjugated) (BD Biosciences, San Jose, CA); CD115 (PE-conjugated), F4/80 (FITC- or PerCP-Cy5.5-conjugated) (eBioscience, San Diego, CA); CD204 (FITC-conjugated), and CD36 (FITC-conjugated) (AbD Serotec Inc., Raleigh, NC).

For FACS analysis of blood, blood was drawn through retro-orbital puncture. After dilution with equal volume of PBS supplemented with 0.5% BSA, whole blood was incubated with various combinations of fluorescence-conjugated mAbs as described in Results or with appropriate isotype negative controls for 20 min. Then, the samples were washed once with PBS, incubated with BD FACS Lysing Solution (BD Biosciences) for 10 min to lyse red blood cells, and washed another time with PBS. In some cases, after lysing red blood cells, the samples were stained with Nile red using Lipid Droplets Fluorescence Assay Kit (Cayman Chemical Company, Ann Arbor, MI). Finally, the stained cells were resuspended in 1% paraformaldehyde in PBS. Data were collected with a BD FACScan or LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR). Total leukocytes were gated based on scatter pattern (forward scatter [FSC] vs. side scatter [SSC]) and data were expressed as percentages of positive cells in total leukocytes or monocytes as indicated in Results. In some cases, data were analyzed in specific gated regions as described previously<sup>2</sup> and in Results. Based on the overlapping expression of CD115 and CD204,<sup>2</sup> and to minimize the numbers of
fluorophore-conjugated antibodies used, we defined mouse total monocytes as PE-CD115$^+$ or FITC-CD204$^+$ leukocytes, depending on the commercial availability of fluorophore choices for other markers simultaneously examined. To examine the 3 major monocyte subsets in mice (see Results), we used the combination of PE-anti-CD115 + FITC-anti-CD36 + PerCP-Cy5.5 anti-CD11c antibodies. To examine Ly-6C expression on the 3 monocyte subsets, we used the combination of PE-anti-CD115 + FITC-anti-CD36 + PerCP-Cy5.5 anti-CD11c + APC-anti-Ly-6C antibodies.

For FACS analysis of mouse aortas, mice were sacrificed and perfused with PBS containing 20 U/ml of heparin, and whole aortas, from 2 mm distal to the heart to just beyond the renal artery, were dissected from mice and cleared of all adipose tissue under a dissection microscope. The dissected aortas were weighed to control the total amount of collected aortic tissues, and then were minced and digested with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I, 60 U/ml DNase I, and 450 U/ml collagenase type I (Sigma-Aldrich) in PBS containing 20 mM HEPES at 37°C for 1 hour. Cell suspension was obtained by pressing the aorta through a 70-μm strainer. After being washed with PBS twice, cells were incubated with Mouse BD Fc Block (BD Biosciences) at 4°C for 5 min, then incubated with mAbs or negative isotype controls and viability dye (eBioscience) for 20 min at 4°C, then washed with PBS twice more. Data were collected with a BD LSRII flow cytometer and analyzed using FlowJo software. Cells were first gated based on FSC and SSC pattern, then viable cells were analyzed for CD45 expression. Data are presented as percentages of targeted cell populations in viable CD45$^+$ cells of aortic cell suspensions. For this assay, each sample included aortas pooled from 2–3 mice of the same strain.

**Monocyte uptake of cholesterol ester–rich very-low-density lipoproteins (CE-VLDLs)**
CE-VLDLs were isolated from plasma of apoE$^{-/-}$ mice on WD by ultracentrifugation and labeled with DiI by incubation at room temperature for 30 min with subsequent overnight dialysis in PBS. CE-VLDL oxidation was estimated by malondialdehyde levels (TBARS value) as measured using TBARS assay kit (Cayman Chemical, Ann Arbor, MI). DiI-CE-VLDLs were stored at 4°C and used within 2 weeks. To examine monocyte uptake of CE-VLDLs, a bolus of DiI-CE-VLDLs, at 75 μg cholesterol/g body weight, was injected intravenously (and in some cases, intraperitoneally) into mice. At 3 and 24 hours postinjection, blood was collected via retroorbital puncture, and monocyte uptake of CE-VLDLs and phenotypic changes were examined by flow cytometry. As a control, a bolus of free DiI was injected intravenously into mice, and monocytes were examined by flow cytometry at 3 and 24 hours postinjection.

**Monocyte isolation**
Blood was collected by cardiac puncture from apoE$^{-/-}$ mice on WD under deep anesthesia. Mononuclear cells (MNCs) were first isolated from whole blood using Histopaque 1083 (Sigma-Aldrich, St. Louis, MO). Total monocytes were then purified from MNCs using a customized EasySep Mouse Monocyte Enrichment Kit (Stemcell Technologies, Vancouver, BC). CD11c–conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to separate total monocytes into CD11c$^+$ foamy monocytes and CD11c$^-$ nonfoamy monocytes. CD11c$^+$ and CD11c$^-$ monocytes were dissolved in Trizol reagent (Life Technologies, Carlsbad, CA) for RNA isolation.
**EdU injection**

Mice were injected intraperitoneally with a bolus of EdU (Life Technologies) at 4 µg/g body weight. Blood was collected through retro-orbital puncture at various time points as indicated in Results and stained for EdU using Click-iT® EdU Flow Cytometry Assay Kit (Life Technologies). EdU+ monocytes, which represented newly released monocytes from bone marrow, were phenotyped for CD11c and CD36 expression by flow cytometry.

**Tissue culture with THP1 monocytes**

THP1 monocytes (ATCC, Manassas, VA) were cultured and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol and penicillin/streptomycin. For experiments, THP1 monocytes were switched to RPMI-1640 medium supplemented with 0.05 mM 2-mercaptoethanol and penicillin/streptomycin, and maintained in this medium for 14 hours (overnight). Then, the cells were treated with CE-VLDLs (isolated from apoE–/– mice on WD), at 300 mg/dl cholesterol, or PBS (control) in serum-free medium for 48 hours. After this, THP1 monocytes were examined by flow cytometry for lipid accumulation indicated by SSC intensity and Nile red staining, and for CD36 expression after staining with an FITC-anti-human CD36 antibody (BD Biosciences) or a FITC-isotype control. To examine effects of CE-VLDL treatment on monocyte further uptake of Dil-CE-VLDL, THP1 monocytes were pretreated with (unlabeled) CE-VLDLs or PBS for 48 hours as described above. Following this, the cells were washed twice with PBS and then incubated with Dil-CE-VLDLs in serum-free medium for an additional 4 hours. Monocyte uptake of Dil-CE-VLDLs was examined by flow cytometry.

**Monocyte depletion**

To deplete total monocytes, a bolus of clodrosome (Encapsula NanoSciences LLC, Nashville, TN) was intravenously injected into mice at 0.3 ml/mouse. At the time points indicated in Results, blood was collected, and appearance and phenotypes of newly released monocytes were examined by flow cytometry.

In separate experiments, after WD for 3 days, apoE–/– mice received daily intravenous injections of clodrosome at 0.1 ml/mouse after 1:5 dilution in PBS for an additional 3 weeks, during which mice were maintained on WD. A control group of apoE–/– mice was injected with PBS at 0.1 ml/mouse and maintained on WD. At 24 hours after the final injection of clodrosome, mice were sacrificed. Hearts and whole aortas were collected for analysis of atherosclerosis development and macrophage/DC contents in atherosclerotic lesions/aortas as described in the following sections.

To examine monocyte uptake of clodrosome, a bolus of Dil-clodrosome (Encapsula NanoSciences LLC), at the doses indicated in Results, was injected intravenously into apoE–/– mice that had been on WD for 3 weeks. At 3 hours postinjection, blood was collected via retro-orbital puncture and stained with FITC-anti-mouse CD204 antibody. Monocyte uptake of Dil-clodrosome was examined by flow cytometry.

**Foamy monocyte infiltration into atherosclerotic lesions**

A bolus of Dil-CE-VLDLs and/or 0.5 µm Fluoresbrite FITC-dyed (YG) plain microspheres (Polysciences Inc., Warrington, PA) (1:5 dilution with PBS8, 9 was injected intravenously into apoE–/– or CD11c–/–apoE–/– mice and their CD11c+/–apoE–/– littermate controls at day 16 on WD. At 3, 24 and 120 hours after injection, blood was collected through retro-orbital puncture, and
monocyte labeling and phenotypes were examined by flow cytometry. This procedure allowed us to label foamy monocytes specifically, with few nonfoamy monocytes labeled, in these mice (see Results). At 120 hours postinjection, mice were sacrificed, and whole aortas were dissected and digested as described above. Foamy monocytes that infiltrated into atherosclerotic aortas were identified as DilF4/80low cells in viable CD45+ cells of aortic cell suspension as examined by flow cytometry. For comparison between CD11c−/apoE−/− and CD11c+/apoE−/− mice, percentages of infiltrated foamy monocytes in aortic viable CD45+ cells were normalized by the average proportions of Dil+ foamy monocytes in blood of the same mouse at the time points indicated.

Mouse hearts were also harvested, embedded in OCT (Triangle Biomedical Sciences, Durham, NC) and stored at −80°C. To examine infiltration of foamy monocytes into aortic sinus atherosclerotic lesions, serial 5-μm transverse cryosections of aortic sinus that showed all 3 aortic valves were prepared. In each mouse, ~20 consecutive sections starting from the first section to show all 3 aortic valves were prepared and numbered 1–20. Slides were stained with DAPI (Molecular Probes Inc., Eugene, OR): after equilibrating briefly with PBS, the samples were incubated with DAPI for 5 min and then rinsed several times in PBS and dried before mounting with VectaMount AQ Aqueous Mounting Medium (Vector Laboratories Inc., Burlingame, CA). The samples were viewed using an EVOS fl Fluorescence Microscope (Life Technologies) with appropriate filters. For quantification and comparison of foamy monocyte infiltration into aortic sinus lesions, 5 aortic sinus sections (numbers 1, 5, 10, 15 and 20) from each mouse were used. The Dil bead+ cells, which represented infiltrated monocytes, were counted in each lesion section. Data are presented as average numbers of infiltrated monocytes per lesion section normalized by the proportions of Dil bead+ foamy monocytes in blood of each mouse.8,9

Analysis of atherosclerotic lesions in aortic sinus
To examine and quantify atherosclerotic lesions in aortic sinus and macrophages/DCs in the lesions, serial transverse cryosections (~20 sections/mouse) of aortic sinus that included all 3 aortic valves were made. Five sections (numbers 1, 5, 10, 15 and 20) from each mouse were stained with oil red O as described previously.2 Another five sections (numbers 2, 6, 11, 16 and 19) from each mouse were stained for CD11b and CD11c.2 Briefly, after blocking with 1% BSA for 30 min, sections were stained with PE-conjugated anti-mouse CD11c and/or FITC-conjugated anti-mouse CD11b mAbs by coincubation overnight at 4°C. After washing, the sections were stained for nuclei with DAPI and viewed with an EVOS fl Fluorescence Microscope. In a separate experiment, macrophage content was examined in aortic sinus atherosclerotic lesions by immunohistochemistry staining for Mac-3.2 The size of atherosclerotic lesions, indicated by oil red O staining, the quantitation of CD11c+ macrophages/DCs, indicated by staining for CD11c, and the quantitation of Mac-3+ macrophages, indicated by staining for Mac-3, were calculated in each section using Image J software (NIH, Bethesda, MD) and the mean lesion size or areas positive for CD11c or Mac-3 per section are presented.

Quantitative reverse transcriptase polymerase chain reaction
Total RNA was extracted using Trizol reagent from isolated monocytes or whole aortas, which were collected from mice and stored in RINalater solution (Life Technologies). The relative mRNA quantities of target molecules were examined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using predesigned primers and probes from Applied
Biosystems and normalized to that of 18S ribosomal RNA.\textsuperscript{7}

**Statistics**
GraphPad Prism 5.04 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analyses. Values are presented as mean±SEM. For statistical analyses, Mann–Whitney tests (for comparison between 2 groups) or Kruskal–Wallis tests (for comparisons of 3 or more groups) followed by Dunn’s multiple pairwise comparison test were used. Differences were considered significant for P values ≤0.05.
References


SUPPLEMENTAL MATERIAL

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CE-VLDLs were isolated from plasma of apoE−/− mice on WD by ultracentrifugation and labeled with DiI by incubation at room temperature for 30 min with subsequent overnight dialysis in PBS. CE-VLDL oxidation was estimated by malondialdehyde levels (TBARS value) as measured using TBARS assay kit (Cayman Chemical, Ann Arbor, MI). DiI-CE-VLDLs were stored at 4°C and used within 2 weeks. To examine monocyte uptake of CE-VLDLs, a bolus of DiI-CE-VLDLs, at 75 μg cholesterol/g body weight, was injected intravenously (and in some cases, intraperitoneally) into mice. At 3 and 24 hours postinjection, blood was collected via retro-orbital puncture, and monocyte uptake of CE-VLDLs and phenotypic changes were examined by flow cytometry. As a control, a bolus of free DiI was injected intravenously into mice, and monocytes were examined by flow cytometry at 3 and 24 hours postinjection.

**Monocyte isolation**

Blood was collected by cardiac puncture from apoE−/− mice on WD under deep anesthesia. Mononuclear cells (MNCs) were first isolated from whole blood using Histopaque 1083 (Sigma-Aldrich, St. Louis, MO). Total monocytes were then purified from MNCs using a customized EasySep Mouse Monocyte Enrichment Kit (Stemcell Technologies, Vancouver, BC). CD11c-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to separate total monocytes into CD11c+ foamy monocytes and CD11c− nonfoamy monocytes. CD11c+ and CD11c− monocytes were dissolved in Trizol reagent (Life Technologies, Carlsbad, CA) for RNA isolation.
**EdU injection**

Mice were injected intraperitoneally with a bolus of EdU (Life Technologies) at 4 µg/g body weight. Blood was collected through retro-orbital puncture at various time points as indicated in Results and stained for EdU using Click-iT® EdU Flow Cytometry Assay Kit (Life Technologies). EdU+ monocytes, which represented newly released monocytes from bone marrow, were phenotyped for CD11c and CD36 expression by flow cytometry.

**Tissue culture with THP1 monocytes**

THP1 monocytes (ATCC, Manassas, VA) were cultured and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol and penicillin/streptomycin. For experiments, THP1 monocytes were switched to RPMI-1640 medium supplemented with 0.05 mM 2-mercaptoethanol and penicillin/streptomycin, and maintained in this medium for 14 hours (overnight). Then, the cells were treated with CE-VLDLs (isolated from apoE−/− mice on WD), at 300 mg/dl cholesterol, or PBS (control) in serum-free medium for 48 hours. After this, THP1 monocytes were examined by flow cytometry for lipid accumulation indicated by SSC intensity and Nile red staining, and for CD36 expression after staining with an FITC-anti-human CD36 antibody (BD Biosciences) or a FITC-isotype control. To examine effects of CE-VLDL treatment on monocyte further uptake of Dil-CE-VLDL, THP1 monocytes were pretreated with (unlabeled) CE-VLDLs or PBS for 48 hours as described above. Following this, the cells were washed twice with PBS and then incubated with Dil-CE-VLDLs in serum-free medium for an additional 4 hours. Monocyte uptake of Dil-CE-VLDLs was examined by flow cytometry.

**Monocyte depletion**

To deplete total monocytes, a bolus of clodrosome (Encapsula NanoSciences LLC, Nashville, TN) was intravenously injected into mice at 0.3 ml/mouse. At the time points indicated in Results, blood was collected, and appearance and phenotypes of newly released monocytes were examined by flow cytometry.

In separate experiments, after WD for 3 days, apoE−/− mice received daily intravenous injections of clodrosome at 0.1 ml/mouse after 1:5 dilution in PBS for an additional 3 weeks, during which mice were maintained on WD. A control group of apoE−/− mice was injected with PBS at 0.1 ml/mouse and maintained on WD. At 24 hours after the final injection of clodrosome, mice were sacrificed. Hearts and whole aortas were collected for analysis of atherosclerosis development and macrophage/DC contents in atherosclerotic lesions/aortas as described in the following sections.

To examine monocyte uptake of clodrosome, a bolus of Dil-clodrosome (Encapsula NanoSciences LLC), at the doses indicated in Results, was injected intravenously into apoE−/− mice that had been on WD for 3 weeks. At 3 hours postinjection, blood was collected via retro-orbital puncture and stained with FITC-anti-mouse CD204 antibody. Monocyte uptake of Dil-clodrosome was examined by flow cytometry.

**Foamy monocyte infiltration into atherosclerotic lesions**

A bolus of Dil-CE-VLDLs and/or 0.5 µm Fluoresbrite FITC-dyed (YG) plain microspheres (Polysciences Inc., Warrington, PA) (1:5 dilution with PBS) was injected intravenously into apoE−/− or CD11c−/−apoE−/− mice and their CD11c+/+apoE−/− littermate controls at day 16 on WD. At 3, 24 and 120 hours after injection, blood was collected through retro-orbital puncture, and
monocyte labeling and phenotypes were examined by flow cytometry. This procedure allowed us to label foamy monocytes specifically, with few nonfoamy monocytes labeled, in these mice (see Results). At 120 hours postinjection, mice were sacrificed, and whole aortas were dissected and digested as described above. Foamy monocytes that infiltrated into atherosclerotic aortas were identified as DiI$^+$F4/80$^{low}$ cells in viable CD45$^+$ cells of aortic cell suspension as examined by flow cytometry. For comparison between CD11c$^+$apoE$^{-/-}$ and CD11c$^{+/+}$apoE$^{-/-}$ mice, percentages of infiltrated foamy monocytes in aortic viable CD45$^+$ cells were normalized by the average proportions of DiI$^+$ foamy monocytes in blood of the same mouse at the time points indicated.

Mouse hearts were also harvested, embedded in OCT (Triangle Biomedical Sciences, Durham, NC) and stored at −80°C. To examine infiltration of foamy monocytes into aortic sinus atherosclerotic lesions, serial 5-μm transverse cryosections of aortic sinus that showed all 3 aortic valves were prepared. In each mouse, ~20 consecutive sections starting from the first section to show all 3 aortic valves were prepared and numbered 1–20. Slides were stained with DAPI (Molecular Probes Inc., Eugene, OR): after equilibrating briefly with PBS, the samples were incubated with DAPI for 5 min and then rinsed several times in PBS and dried before mounting with VectaMount AQ Aqueous Mounting Medium (Vector Laboratories Inc., Burlingame, CA). The samples were viewed using an EVOS fl Fluorescence Microscope (Life Technologies) with appropriate filters. For quantification and comparison of foamy monocyte infiltration into aortic sinus lesions, 5 aortic sinus sections (numbers 1, 5, 10, 15 and 20) from each mouse were used. The DiI$^+$ bead$^+$ cells, which represented infiltrated monocytes, were counted in each lesion section. Data are presented as average numbers of infiltrated monocytes per lesion section normalized by the proportions of DiI$^+$ bead$^+$ foamy monocytes in blood of each mouse.$^8,9$

**Analysis of atherosclerotic lesions in aortic sinus**

To examine and quantify atherosclerotic lesions in aortic sinus and macrophages/DCs in the lesions, serial transverse cryosections (~20 sections/mouse) of aortic sinus that included all 3 aortic valves were made. Five sections (numbers 1, 5, 10, 15 and 20) from each mouse were stained with oil red O as described previously.$^2$ Another five sections (numbers 2, 6, 11, 16 and 19) from each mouse were stained for CD11b and CD11c.$^2$ Briefly, after blocking with 1% BSA for 30 min, sections were stained with PE-conjugated anti-mouse CD11c and/or FITC-conjugated anti-mouse CD11b mAbs by coincubation overnight at 4°C. After washing, the sections were stained for nuclei with DAPI and viewed with an EVOS fl Fluorescence Microscope. In a separate experiment, macrophage content was examined in aortic sinus atherosclerotic lesions by immunohistochemistry staining for Mac-3.$^2$ The size of atherosclerotic lesions, indicated by oil red O staining, the quantitation of CD11c$^+$ macrophages/DCs, indicated by staining for CD11c, and the quantitation of Mac-3$^+$ macrophages, indicated by staining for Mac-3, were calculated in each section using Image J software (NIH, Bethesda, MD) and the mean lesion size or areas positive for CD11c or Mac-3 per section are presented.

**Quantitative reverse transcriptase polymerase chain reaction**

Total RNA was extracted using Trizol reagent from isolated monocytes or whole aortas, which were collected from mice and stored in RNAlater solution (Life Technologies). The relative mRNA quantities of target molecules were examined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using predesigned primers and probes from Applied
Biosystems and normalized to that of 18S ribosomal RNA.\textsuperscript{7}

**Statistics**
GraphPad Prism 5.04 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analyses. Values are presented as mean±SEM. For statistical analyses, Mann–Whitney tests (for comparison between 2 groups) or Kruskal–Wallis tests (for comparisons of 3 or more groups) followed by Dunn’s multiple pairwise comparison test were used. Differences were considered significant for P values ≤0.05.
Supplemental Figure I

**Supplemental Figure I.** Plasma levels of total cholesterol in apoE<sup>−/−</sup> mice at various time points on WD. n=7 mice.
Supplemental Figure II. Monocytes in blood of LDb mice on WD (2 weeks) examined by flow cytometry. Upper panels: representative flow cytometric analysis of foamy and nonfoamy monocytes in blood of LDb mice (see Figure 1B for representative WT control) on WD with staining for: I, CD11c and negative control for CD204; II, CD204 and negative control for CD11c; III, CD11c and CD204; and IV, Nile Red. Lower panel: relative ratios of CD11c+ and CD11c− foamy and nonfoamy monocytes in blood of WT and LDb mice on WD.
Supplemental Figure III

A. Monocyte SSC values in apoE−/− mice and WT mice on ND

Supplemental Figure III. Monocytes in blood of apoE−/− mice on various types of diet. A. SSC values of blood monocytes in 28-week-old apoE−/− mice and WT mice on ND as examined by flow cytometry. n=6–7 mice/group. B. Proportions of foamy monocytes and levels of cholesterol, triglyceride and TBARS in blood of apoE−/− mice on various types of special diet for 2 weeks. n=3–6 mice/group.
Supplemental Figure IV

Supplemental Figure IV. A representative flow cytometric analysis showing EdU+ cells in bone marrow of WT mice at 3 hours after EdU injection.
Supplemental Figure V. Flow cytometric analysis of blood monocytes in WT mice after intravenous injection of a bolus of Dil-HDL (upper panels) or free Dil (lower panels). Representatives of 3–4 mice in each condition with similar results are shown.
Supplemental Figure VI. Effects of CE-VLDLs on THP1 monocytes in vitro. THP1 monocytes were treated with (unlabeled) CE-VLDLs (isolated from apoE<sup>−/−</sup> mice on WD), at 300 mg/dl cholesterol, or PBS (cont) for 48 hours and examined for lipid accumulation indicated by SSC change (left panel) and for CD36 expression (middle panel). For DiI-CE-VLDL uptake (right panel), THP1 monocytes were pretreated with (unlabeled) CE-VLDLs or PBS for 48 hours followed by removal of the CE-VLDLs and incubated with Dil-CE-VLDLs for an additional 4 hours. Then monocyte uptake of Dil-CE-VLDLs was examined by flow cytometry. Data were representatives from 3 independent experiments with similar results.
Supplemental Figure VII. Uptake of Dil-CE-VLDLs by blood monocytes of WT and TLR4−/− mice after intravenous injection of a bolus of Dil-CE-VLDLs. n=3 mice/group.
Supplemental Figure VIII. Early development of atherosclerosis and increased inflammation in atherosclerotic aortas in apoE−/− mice on WD (3 weeks). A, Representatives of atherosclerotic plaques in aortic arch of an apoE−/− mouse on WD and a WT (on ND). B, CD11b+/CD11c+ cells in mouse aortas quantified by flow cytometry. n=3–4 samples/group. C, Representative immunofluorescent staining for CD11c (red) and CD11b (green) in aortic sinus sections of apoE−/− mice on WD. D, mRNA levels of CD11c and inflammatory markers in mouse aortas. n= 5–10 mice/group. *P<0.05, **P<0.01.
Supplemental Figure IX

**F4/80 expression on Dil-CE-VLDL–labeled blood monocytes and peritoneal macrophages**

**Supplemental Figure IX.** A bolus of Dil-CE-VLDLs was injected intravenously and intraperitoneally into apoE<sup>−/−</sup> mice on WD (3 weeks). At 24 hours after injection, blood monocytes and peritoneal macrophages were collected and stained for F4/80 separately or blood and peritoneal cells were mixed and then stained for F4/80. The data shown were representatives from 3 mice with similar results and showed higher F4/80 levels on peritoneal macrophages than blood monocytes. However, the Dil(-CE-VLDL) levels were not comparable between peritoneal macrophages and blood monocytes in our models given that once intravenously injected, Dil-CE-VLDLs circulated in blood and were diluted quickly, whereas after intraperitoneal injection, Dil-CE-VLDLs stayed “static” and may also have been more concentrated in the peritoneal cavity, thereby leading to “longer” interaction of peritoneal macrophages with Dil-CE-VLDLs in the peritoneal cavity, which may explain the higher Dil levels on peritoneal macrophages.
Supplemental Figure X

CD11c+/+/apoE−/− and CD11c−/−/apoE−/− mice on WD (3 weeks) were injected intravenously with Dil-CE-VLDLs and fluorescent microbeads. At 24 hours after injection, blood was collected and uptake of Dil-CE-VLDLs and fluorescent microbeads by blood monocytes was examined by flow cytometry.
Supplemental Figure XI

Atherosclerotic lesions and macrophages in CD11c<sup>+/+</sup>/apoE<sup>−/−</sup> and CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice

Supplemental Figure XI. CD11c<sup>+/+</sup>/apoE<sup>−/−</sup> and CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice were fed WD for 3 weeks. Aortic sinus atherosclerotic lesion size, as indicated by oil red O staining, and lesional macrophage content, as indicated by immunohistochemistry staining for Mac-3, were examined. Magnification of the representative images was 20X. n=3–4 mice/group.
Supplemental Figure XII. A bolus of “low-dose” (0.1 ml/mouse after 1:5 dilution) or “high-dose” (0.2 ml/mouse undiluted) Dil-clodrosome or free Dil was intravenously injected into apoE−/− mice on WD. At 3 hours after injection, blood monocytes were examined by flow cytometry after staining for CD204. Representative data are presented from 3 mice in each condition with similar results.
Supplemental Figure XIII.

Effects of a bolus injection of clodrosome on blood monocytes and aortic CD11b⁺/CD11c⁺ cells in apoE⁻/⁻ mice on WD.

Supplemental Figure XIII. A bolus (0.2 ml/mouse) of clodrosome or PBS (control) was intravenously injected into apoE⁻/⁻ mice on WD (3 weeks). At 22 hours after injection, blood monocytes and CD11b⁺/CD11c⁺ cells in aortas were examined by flow cytometry. n=3 samples/group.
**Supplemental Table I. Nutrition facts of special diets**

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*SFA: saturated fat; MUFA: monounsaturated fat; PUFA: polyunsaturated fat.

#This indicates cholesterol content from milkfat (0.256%) only for HFD, or cholesterol from milkfat plus additional sources for WD and HCD.
References


