Functional Role of CD11c⁺ Monocytes in Atherogenesis Associated With Hypercholesterolemia

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Background—Monocyte activation and migration into the arterial wall are key events in atherogenesis associated with hypercholesterolemia. CD11c/CD18, a β₂ integrin expressed on human monocytes and a subset of mouse monocytes, has been shown to play a distinct role in human monocyte adhesion on endothelial cells, but the regulation of CD11c in hypercholesterolemia and its role in atherogenesis are unknown.

Methods and Results—Mice genetically deficient in CD11c were generated and crossbred with apolipoprotein E (apoE)⁻/⁻ mice to generate CD11c⁻/⁻/apoE⁻/⁻ mice. Using flow cytometry, we examined CD11c on blood leukocytes in apoE⁻/⁻ hypercholesterolemic mice and found that compared with wild-type and apoE⁻/⁻ mice on a normal diet, apoE⁻/⁻ mice on a Western high-fat diet had increased CD11c⁺ monocytes. Circulating CD11c⁺ monocytes from apoE⁻/⁻ mice fed a high-fat diet exhibited cytoplasmic lipid vacuoles and expressed higher levels of CD11b and CD29. Deficiency of CD11c decreased firm arrest of mouse monocytes on vascular cell adhesion molecule-1 and E-selectin in a shear flow assay, reduced monocyte/macrophage accumulation in atherosclerotic lesions, and decreased atherosclerosis development in apoE⁻/⁻ mice on a high-fat diet.

Conclusions—CD11c, which increases on blood monocytes during hypercholesterolemia, plays an important role in monocyte recruitment and atherosclerosis development in an apoE⁻/⁻ mouse model of hypercholesterolemia. (Circulation. 2009;119:2708-2717.)

Key Words: atherosclerosis ■ cell adhesion molecules ■ leukocytes

Atherosclerosis associated with hypercholesterolemia is a complex inflammatory process, characterized pathologically by recruitment of monocytc leukocytes in the arterial wall and lipid accumulation in monocytc leukocytes.¹ Monocyte recruitment is a multistep process mediated by adhesion molecules, beginning with rolling, which is mediated by short-lived bonds between E-selectin on endothelial cells (ECs) and sialylated ligands such as P-selectin glycoprotein ligand-1 on monocytes, followed by firm arrest facilitated through interactions between activated β₁ and β₂ integrins on monocytes with vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on ECs. Firmly arrested monocytes subsequently undergo transmigration through other adhesion molecules.²⁻³ Therefore, adhesion molecules participating in monocyte-EC interactions play a critical role in atherogenesis.⁴ EC activation induced by hypercholesterolemia increases expression of VCAM-1, ICAM-1, and E-selectin, thereby contributing to atherogenesis.⁴⁻⁵ However, the effect of hypercholesterolemia on monocyte activation and its contribution to atherogenesis are less defined.

Clinical Perspective on p 2717

The β₂ integrins, which include CD11a/CD18, CD11b/CD18, CD11c/CD18, and CD11d/CD18,⁶ contribute to atherogenesis as evidenced by a significant reduction in atherosclerosis development in CD18⁻/⁻ mice, which lack all 4 CD11/CD18 integrins.⁴ CD11b has been used as an activation marker for monocytes/macrophages and increases in hyperlipidemia⁸ but was not essential for atherosclerosis development in low-density lipoprotein (LDL) receptor⁻/⁻ mice.⁹

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CD11c/CD18 is found on monocytes/macrophages, granulocytes, and subsets of dendritic cells (DCs) in humans and on DCs and subsets of monocytes/macrophages in mice. CD11c binds a variety of ligands including ICAM-1, ICAM-2, fibrinogen, collagen, iC3b, and lipopolysaccharide, and was recently demonstrated in vitro to contribute to human monocyte arrest on ECs by cooperating 3CD11c with CD49d/CD29 (very late antigen-4 [VLA-4]) in binding phages. However, the relationship between hypercholesterolemia and monocyte CD11c expression and a potential role of CD11c in atherogenesis remain to be determined.

We hypothesized that upregulation of CD11c is important in atherogenesis and examined the functionality of CD11c on mouse blood monocytes: CD11c in athameron mice.12,13 CD11c binds a variety of ligands including ICAM-1, ICAM-2, fibrinogen, collagen, iC3b, and lipopolysaccharide and was recently demonstrated in vitro to contribute to human monocyte arrest on ECs by cooperating with CD49d/CD29 (very late antigen-4 [VLA-4]) in binding to VCAM-1, which participates in atherogenesis.5 CD11c has been used as an activation marker for monocytes/macrophages. However, the relationship between hypercholesterolemia and monocyte CD11c expression and a potential role of CD11c in atherogenesis remain to be determined.

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immunophenotyped by staining with PE–anti-mouse Gr-1 and AF647–anti-mouse CD204 mAbs.

**Analysis of Atherosclerotic Lesions**

Atherosclerotic lesions in thoracoabdominal aortas were assessed by Sudan IV staining.15 Macrophages in atherosclerotic lesions were assessed after immunohistochemistry staining for Mac-3 on cross cryosections of aortic sinus.16

**Statistical Analysis**

GraphPad Prism 4 was used for statistical analyses. Values are presented as mean ± SEM. Student t tests (for comparison between 2 groups) or 1-way ANOVA (for comparisons of ≥3 groups) followed by Tukey post hoc pairwise tests were used for statistical analyses.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Generation of Mice Deficient in CD11c**

Embryonic stem cells with a targeted event had a 4.0-kb HindIII fragment compared with the 9.4-kb fragment in wild-type (WT) allele (Figure 1D). The expected targeted allele of 4.0 kb was identified by the 3′-flanking probe on Southern blots of DNA from mutant mice (Figure 1E).

CD11c mutant mice were fertile. After 12 generations of backcrossing onto C57BL/6, CD11c−/− mice did not demonstrate any gross abnormalities. CD11c was undetectable on blood leukocytes and splenocytes from CD11c−/− mice as examined by FACS (data not shown).

**Effect of Hypercholesterolemia on CD11c Expression on Mouse Blood Monocytes**

We defined mouse monocytes as CD204+ leukocytes, which were also positive for CD115 (Figure 2A), another widely used monocyte marker.13 On the basis of CD11c expression, we classified mouse monocytes as CD11c+/CD204+ and CD11c−/CD204− subsets (Figure 2B), which were examined in 3 groups of mice with various plasma levels of cholesterol—WT on ND (plasma cholesterol, 42.6 ± 3.9 mg/dL), apoE−/− on ND (plasma cholesterol, 258.0 ± 37.0 mg/dL), and apoE−/− on HFD (for 12 weeks; plasma cholesterol, 1095 ± 131.6 mg/dL)—and found to differ significantly (P < 0.001; Figure 2C). Compared with WT or apoE−/− on ND, apoE−/− mice on HFD had significantly increased proportion of CD11c+/CD204− monocytes in total leukocytes (Figure 2C). CD11c−/CD204+ monocytes were also increased in apoE−/− mice on HFD, whereas apoE−/− mice on ND did not show significant changes in the proportion of CD11c+/CD204+ and CD11c−/CD204+ monocytes compared with WT (Figure 2C).

**Characteristics of CD11c+ Monocytes in Hypercholesterolemic Mice**

Scatter pattern (forward scatter [cell size] versus side scatter [granularity]) of total mouse blood leukocytes, as examined by FACS, showed 3 regions: R1 (high granularity/medium cell size) is the granulocyte region, R2 (low granularity/large cell size) is the monocyte region, and R3 (low granularity/small cell size) is the lymphocyte region (Figure 3A and 3B). As expected, R2/monocytes but not R1/monocytes contained monocytes (CD204+) in WT (Figure 3A) and apoE−/− mice on ND (data not shown). In contrast, blood from apoE−/− mice on HFD contained monocytes in both R1 and R2 (Figure 3B). However, relative proportions of the CD11c+ monocyte subset in total monocytes differed significantly among R1 and R2 of apoE−/− mice on HFD and WT (P < 0.001; Figure 3C). The CD11c+ subset accounted for 23% of monocytes in R2 of apoE−/− mice on HFD, which was lower than in WT (Figure 3C) or apoE−/− mice on ND (data not shown), and 84% of monocytes in R1, which was higher than the proportion in R2 of apoE−/− mice on HFD or in WT (Figure 3B and 3C). CD11c+ monocytes in R1 and R2 of apoE−/− mice on HFD were CD11b+, Gr-1low, CD49d+ (Figure 3B), F4/80+, Mac-3-, and CD205- (Figure 1 in the online-only Data Supplement), confirming their characteristics as monocytes but not CD205+ DCs or granulocytes, which are Gr-1high. However, CD11c+ monocytes in R1 and R2 of apoE−/− mice on HFD and in WT showed different levels of CD11b and CD29 (P = 0.0080 for CD11b, Figure 3D; P = 0.012 for CD29, Figure 3E), with higher CD11b and CD29 levels on CD11c+ monocytes in R1 of apoE−/− mice on HFD (Figure 3D and 3E). Therefore, compared with those in WT or apoE−/− mice on ND, CD11c+ monocytes in apoE−/− on HFD were not only increased in number but also displayed increased granularity with increased expression of CD11b and CD29, which are associated with cell activation.
Morphological examination revealed that most CD11c⁺ cells from blood of apoE⁻/⁻ mice on HFD showed typical morphology of monocytes and included a large number of intracellular vacuoles, which contained abundant lipid as examined by Oil Red O staining and confirmed by electron microscopy (Figure 3F). These lipid vacuoles were not found in CD11c⁻ cells from blood of WT (Figure 3F). Further examination showed that CD11c⁺/CD204⁻ (and CD11c⁻/CD204⁻) cells in both R1 and R2 of apoE⁻/⁻ on HFD displayed morphology of monocytes (Figure 3G). CD11c⁺ monocytes in R1 appeared to have more intracellular lipid vacuoles than the monocytes in R2 (Figure 3G), suggesting that the increased granularity of the CD11c⁺ monocytes in R1 may be due to lipid accumulation within these cells. Therefore, in addition to the “foam cells” observed in atherosclerotic plaques, we found lipid-laden “foamy” CD11c⁺ monocytes in blood of apoE⁻/⁻ on HFD.

**Regulation of CD11c on Mouse Leukocytes by ox-LDL**

In vitro study showed that compared with native LDL, ox-LDL (20 μg/mL) significantly increased the proportion of CD11c⁺ monocytes in MNCs from apoE⁻/⁻ mice on ND after coincubation for 24 hours (Figure 4A).
To examine the effect of ox-LDL on CD11c expression in vivo, Dil–ox-LDL was injected intravenously into apoE<sup>−/−</sup> mice on ND, and changes in CD11c expression on monocytes that bound ox-LDL were traced by detection of Dil with CD11c by FACS. Dil–ox-LDL injection resulted in obvious Dil signal in leukocytes (Figure 4B), indicating binding and/or uptake of the exogenous ox-LDL by the leukocytes. As expected, only CD204<sup>+</sup> monocytes (up to 70%) exhibited Dil(ox-LDL)<sup>+</sup> at 1 hour and 24 hours after injection (Figure 4B, top row). CD11c staining indicated that both CD11c<sup>+</sup> and CD11c<sup>−</sup> monocytes exhibited Dil<sup>+</sup> at 1 hour. At 24 hours, however, the vast majority of Dil<sup>+</sup> cells were CD11c<sup>+</sup> (Figure 4B, middle row), with a significant increase in the proportion of CD11c<sup>+</sup> cells in total Dil<sup>+</sup> cells compared with 1 hour (Figure 4C), indicating that the interaction between ox-LDL and monocytes drove CD11c<sup>−</sup>-to-CD11c<sup>+</sup> monocyte differentiation. In contrast, injection of Dil–native LDL did not change the ratio of CD11c<sup>−</sup>/Dil<sup>+</sup> to CD11c<sup>+</sup>/Dil<sup>+</sup> cells at 24 hours compared with 1 hour after injection (Figure 4B, bottom row; Figure 4C).

Figure 4. ox-LDL increases CD11c expression on mouse MNCs. A, Proportion of CD11c<sup>+</sup> monocytes in mouse MNCs after incubation with native LDL or ox-LDL (20 µg/mL) for 24 hours in vitro (n=8 per group). B, Representative FACS of monocytes in apoE<sup>−/−</sup> mice on ND at different time points after intravenous injection of Dil–ox-LDL or Dil–native LDL with phosphate-buffered saline as negative control. C, Relative ratio of CD11c<sup>−</sup>/Dil<sup>+</sup> to CD11c<sup>+</sup>/Dil<sup>+</sup> cells in blood (total Dil<sup>+</sup> cells assumed to be 100%) of apoE<sup>−/−</sup> mice at 1 hour and 24 hours after Dil–ox-LDL or Dil–native LDL injection. n=5 for Dil–ox-LDL; n=3 for Dil–native LDL; *P=0.0018 vs 1-hour Dil–ox-LDL.
Role of CD11c in MNC Adhesion on VCAM-1/E-Selectin Under Shear Flow

To examine the role of CD11c in monocyte adhesion, MNCs from blood of CD11c<sup>+/+</sup>/apoE<sup>−/−</sup> or CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice were perfused through a microfluidic flow chamber coated with E-selectin/VCAM-1 at a shear stress of 2 dyne/cm<sup>2</sup>. Immunofluorescence analysis confirmed that for both genotypes, ≈90% of the arrested MNCs were CD204<sup>+</sup> monocytes (Figure 5A and 5B).

We first examined the proportion of firmly arrested Ly-6<sup>Chigh</sup> and Ly-6<sup>Clow</sup> monocytes by introducing AF647–CD204 and PE–Gr-1–labeled MNCs and recording firmly arrested CD204<sup>−</sup>/Ly-6<sup>Chigh</sup> and CD204<sup>−</sup>/Ly-6<sup>Clow</sup> monocytes. We found that 63% of arrested cells from CD11c<sup>+/+</sup>/apoE<sup>−/−</sup> mice were Ly-6<sup>Chigh</sup> monocytes compared with 80% of arrested cells from CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice. The most striking difference between the 2 genotypes was the proportion of firmly arrested Ly-6<sup>Chigh</sup> monocytes, which comprised 11% of arrested cells in CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice and >25% in CD11c<sup>+/+</sup>/apoE<sup>−/−</sup> mice (Figure 5A and 5B).

In this model, we expected that MNCs from both genotypes would demonstrate a similar capacity of rolling because cells from CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice were perfused over E-selectin/VCAM-1 in a microfluidic flow chamber at 2 dyne/cm<sup>2</sup>. A and B, Bivari-ate plots of the intensity of Gr-1 and CD204 staining, in the whole aorta; the most severe lesions developed in monocyte arrest on E-selectin/VCAM-1 compared with MNCs from CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice. The most striking differ-

Role of CD11c in Atherosclerosis Development

Immunofluorescence staining showed high expression of CD11c in atherosclerotic lesions from CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice on HFD (12 weeks) (Figure 6A). CD11c<sup>+</sup> cells in the lesions were CD205<sup>−/−</sup> (Figure 6B) but MOMA-2<sup>−/−</sup> (Figure 6C and 6D), indicating that these CD11c<sup>+</sup> cells were more likely to be macrophages than CD205<sup>−/−</sup> DCs.

Next, we determined whether CD11c contributes to atherosclerosis development. Plasma total cholesterol levels were not significantly different between CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> and CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice before or after HFD (Table I in the online-only Data Supplement). At 12 weeks after HFD, CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice developed obvious atherosclerotic lesions, which appeared as red plaques with Sudan IV staining, in the whole aorta; the most severe lesions developed in the aortic arch (Figure 6E). In contrast, CD11c<sup>−/−</sup>/apoE<sup>−/−</sup> mice developed smaller atherosclerotic lesions in the whole aorta and aortic arch (Figure 6F and 6G). Compared
with CD11c"/apoE" mice also exhibited less macrophage accumulation in atherosclerotic lesions of aortic sinus (Figure 6H and 6I). These data revealed an essential role of CD11c in monocyte/macrophage infiltration into atherosclerotic lesions and in atherosclerosis development.

Discussion
We report that hypercholesterolemia in apoE" mice on HFD was associated with an increased proportion of CD11c" monocytes in the circulation. These CD11c" monocytes contained abundant lipid in the cytoplasm and expressed high levels of CD11b and CD29, which are associated with cell activation. CD11c deficiency decreased firm adhesion of monocytes on VCAM-1/E-selectin and reduced atherosclerosis in apoE" mice. These data demonstrate an important role of CD11c in atherosclerosis development associated with hypercholesterolemia.

A variety of membrane proteins have been used to identify mouse monocytes.12,13,18–20 We used CD204, also known as scavenger receptor type A, as a primary mouse monocyte marker. The coexpression of CD204 with the macrophage colony stimulating factor receptor CD115 indicates that these blood cells correspond to the monocyte population, in agreement with Tacke et al.13 Compared with previous classification as Ly-6C"/CX3CR" and Ly-6C"/CX3CR" monocytes,12,13,18,19 most of the CD11c" monocytes were Ly-6C"; however, a subpopulation of CD11c" monocytes were Ly-6C" (Figure I in the online-only Data Supplement). We observed that both CD11c" and CD11c" monocytes were increased in circulation of apoE" mice on HFD compared with WT or apoE" on ND. Noticeably, in apoE" mice on HFD, although most CD11c" monocytes remained in the classic monocyte regions, most CD11c" monocytes exhibited increased granularity and shifted to the granulocyte region as examined by FACS.

Atherogenesis is thought to develop from lipid uptake when macrophages convert to foam cells in the arterial wall. However, our study revealed extensive lipid accumulation inside blood monocytes of apoE" mice on HFD. Similarly, patients with familial hypercholesterolemia have been noted to have cytoplasmic lipid vacuoles in blood monocytes.21 More lipid vacuoles inside the monocytes in the granulocyte/R1 region suggest that lipid accumulation within these monocytes may cause the increased granularity (Figure 3B and 3G). This is also supported by the observation that granularity of adipocytes undergoing differentiation increases with increasing accumulation of intracellular lipid.22
predominance of CD11c\(^+\) (over CD11c\(^-\)) monocytes in R1 indicates that most of the monocytes with lipid accumulation were CD11c\(^-\). From the data that ox-LDL upregulated CD11c on mouse monocytes, which is consistent with another study with human monocytes/macrophages,\(^{23}\) we infer that the interaction of ox-LDL with monocytes, which results in cellular lipid accumulation, drives CD11c\(^-\)-to-CD11c\(^+\) monocyte differentiation. The consequences of cellular lipid accumulation, which have been well characterized in macrophages,\(^{24}\) are not clear and merit further studies in blood monocytes. One consequence of this uptake is upregulation of membrane-expressed CD11b, \(\beta\) integrin (CD29), and CD11c that is presumably released from cytoplasmic pools as these monocytes become activated. Both humans and mice with severe hypercholesterolemia exhibit increased foam cells in arteries (atherosclerosis) and in skin (cutaneous xanthomas).\(^{25}\) Extensive cutaneous xanthomas have also been described in a human disorder, necrobiotic xanthogranuloma, which is characterized by intracellular lipid accumulation in peripheral monocytes.\(^{26}\) Therefore, lipid accumulation in circulating monocytes, leading to monocyte activation, may play an important role in development of atherosclerosis and cutaneous xanthomas.

An increased propensity for CD11c in monocytes to convert from rolling to stable adhesion and exhibit shear-strengthened adhesion is demonstrated in our shear flow assay. An earlier study showed that CD11c mediates human monocyte arrest on inflamed aortic ECs, and a distinct contribution (ie, \(\approx40\%\)) from CD11c binding VCAM-1 was detected by blocking ICAM-1, CD11a, CD11b, and VLA-4.\(^{14}\) Using a reduced inflammatory substrate model of recombiant mouse E-selectin/VCAM-1, we observed that mAb blocking of VLA-4 on MNCs of CD11c\(^-\)/apoE\(^-\) mice decreased firm arrest by 86% and together with anti-CD18 diminished adhesion to an integrin-independent baseline level.

Remarkably, monocyte adhesion attributable to VLA-4 (\(\approx15\) cells per field) or CD11c (\(\approx10\) cells per field) alone did not add up to the total (\(\approx40\) cells per field). Hence, although VLA-4 is the principal ligand for VCAM-1, its efficacy for mediating monocyte firm arrest is significantly diminished in the absence of CD11c. This was underscored in the assay of adhesive strength. Thus, monocyte arrest in regions of atherosclerosis is orchestrated through cooperation between CD11c and VLA-4, which may involve both adhesion and signaling functions of these receptors. Regulation of one adhesion receptor by another is an efficient means by which a leukocyte can modulate bond lifetime and strength, thereby increasing the efficiency of adhesion.\(^{27}\) A mechanistic interpretation of our finding is that CD11c ligation of VCAM-1 produces an outside-in signal that increases VLA-4 adhesive function.

Monocyte adhesion to ECs and migration into the arterial wall are key events in atherogenesis.\(^{1}\) It is currently debated which monocyte subsets in the apoE\(^-\) mouse model contribute more to atherosclerosis formation. An earlier report showed that CX3CR1\(^{high}\)/Ly-6\(^{chigh}\) monocytes were preferentially recruited to the arterial wall in atherosclerosis.\(^{19}\) More recent evidence indicated that CX3CR1\(^{low}\)/Ly-6\(^{clow}\) monocytes preferentially home to regions of atherosclerosis.\(^{12,13,17}\) Ly-6\(^{chigh}\) monocytes typically express lower CD11c levels than Ly-6\(^{clow}\) monocytes.\(^{12,13,17}\) However, little is known about the functionality of CD11c on these 2 monocyte subsets during recruitment. Our present study is the first to show that CD11c plays a functional role in the firm arrest of both Ly-6\(^{clow}\) and Ly-6\(^{chigh}\) monocytes on VCAM-1 in shear flow. The greater diminution in firm arrest of Ly-6\(^{chigh}\) monocytes in CD11c\(^-\)/apoE\(^-\) mice suggests that CD11c plays an important role in the trafficking of this subset. The \(\approx50\%\) decrease in monocyte recruitment in CD11c\(^-\)/apoE\(^-\) mice along with the immunophenotyping data indicates that CD11c present on a subpopulation of Ly-6\(^{chigh}\) monocytes aids in the firm arrest of these cells on VCAM-1. In addition, CD11c\(^+\) monocytes express high levels of VLA-4. As we show, CD11c and VLA-4 act synergistically to increase monocyte recruitment; we hypothesize that uptake of lipid and concomitant increase in expression of CD11c, CD11b, and CD29 correlate with efficient recruitment to incipient sites of plaque formation in arteries. In support of this hypothesis, we found abundant CD11c\(^+\) cells in mouse atherosclerotic lesions. Consistently, others have also shown that CD11c is widely expressed in mouse atherosclerotic lesions and colocalized with macrophage markers such as CD68.\(^{13,28}\) Clearly macrophage heterogeneity exists in mouse atherosclerotic lesions, as CD11c\(^-\)
macrophages are also present. On the basis of the expression of CD68 and lack of CD83, a marker for mature DCs, Liu et al.\(^{28}\) considered the CD11c\(^{+}\) cells in mouse aortic wall as immature DCs. Although CD11c has been considered a DC marker, its expression is not always restricted to DCs, as observed in CD11c expression by lung macrophages.\(^{13}\) The coexpression of CD11c with MOMA-2, another macrophage marker, in atherosclerotic lesions suggests that these CD11c\(^{+}\) cells are more likely to be macrophages. A crucial role of CD11c in atherogenesis is indicated by our observations that CD11c\(^{-/}\)/apoE\(^{-/}\) mice exhibited diminished adhesion of monocytes (particularly Ly-6C\(^{low}\) monocytes) to VCAM-1/E-selectin, reduced macrophage content in atherosclerotic lesions, and decreased atherosclerosis. Tacke et al.\(^{13}\) showed that Ly-6C\(^{low}/CX3CR1\(^{high}\) monocytes entered atherosclerotic lesions and were more prone to becoming macrophage-like cells expressing CD11c. Liu et al.\(^{28}\) found that deficiency of CX3CR1 impaired CD11c\(^{+}\) cell accumulation in arterial intima and reduced atherosclerosis.

We realize that multiple hypotheses have been tested in our study, and some of them have been considered without making a formal adjustment of the chosen level of significance. However, the consistent and biologically plausible pattern of significant results suggests that our conclusions are not based on false-positives. We have provided exact \(P\) values for \(t\) tests and the whole ANOVAs so that readers may make their own adjustments and draw their own conclusions.

In summary, we propose that CD11c promotes atherogenesis associated with hypercholesterolemia in significant ways involving monocyte activation and adhesion, as depicted in Figure 7. In severe hypercholesterolemia, uptake of modified LDL by blood monocytes through scavenger receptors and other undefined pathways causes intracellular lipid accumulation and drives CD11c\(^{-/-}\)-to-CD11c\(^{+}\) monocyte differentiation. This in turn leads to an increase in circulating CD11c\(^{+}\) “foamy monocytes,” which are partially activated to further upregulate CD11b and CD29. These monocytes are proadhesive to ECs and prone to transmigrate into the arterial wall through cooperative function of CD11c and VLA-4 with VCAM-1, thereby playing a critical role in the development of atherosclerosis and cutaneous xanthomas associated with hypercholesterolemia.

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Disclosures

None.

References


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Monocyte activation and migration into the arterial wall, with subsequent differentiation into macrophages, uptake of lipid, and formation of foam cells, are key events in atherogenesis associated with hypercholesterolemia. However, the mechanisms for these processes, including monocyte migration into the arterial wall, are not fully understood. CD11c/CD18, a β2 integrin expressed on human monocytes and a subset of mouse monocytes, has been shown to play an important role in human monocyte adhesion on endothelial cells, yet its role in atherogenesis is not clear. In the present study, we first report that the proportion of CD11c+ monocytes in total leukocytes was increased in blood of apolipoprotein E−/− hypercholesterolemic mice on a Western high-fat diet. Furthermore, these circulating CD11c+ monocytes from apolipoprotein E−/− mice fed a high-fat diet exhibited abundant cytoplasmic lipid vacuoles, a morphological characteristic of “foam” cells. Compared with those with no intracellular lipid vacuoles, the CD11c+ foamy monocytes expressed higher levels of CD11b and CD29, indicating that they may be activated or partially activated. Importantly, we observed that deficiency of CD11c decreased firm adhesion of mouse monocytes on vascular cell adhesion molecule-1 and E-selectin, reduced monocyte/macrophage accumulation in atherosclerotic lesions, and decreased atherosclerosis development in apolipoprotein E−/− mice on a high-fat diet. CD11c expression increases on blood monocytes during hypercholesterolemia, plays an important role in monocyte adhesion and atherosclerosis development associated with hypercholesterolemia, and therefore could be a potential novel therapeutic target for atherosclerosis.
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**Functional role of CD11c\(^+\) monocytes in atherogenesis associated with hypercholesterolemia**

**Supplemental Methods**

*Targeting construct and generation of CD11c\(^{-/-}\) mice*

Murine CD11c cDNA was used as a probe to isolate a genomic phage clone from a 129/Sv mouse lambda library (Stratagene, La Jolla, CA). The genomic clone was characterized (Fig. 1A). A 1.6-kb genomic restriction fragment upstream of exon 1 and a 10.7-kb genomic restriction fragment downstream of exon 3 were ligated into the polylinker of pBluescript SK+ (Stratagene). A neomycin cassette driven by the mouse RNA polymerase II promoter was inserted between the two fragments (Fig. 1B). Targeted homologous recombination results in the replacement of a 0.8-kb fragment of the CD11c gene that contains exons 2 and 3, and the coding sequence of exon 1 with the 1.8-kb neomycin cassette (Fig. 1C).

The AB2.1 embryonic stem cell line was electroporated with linearized targeting construct (25 \(\mu\)g/ml).\(^1\) Following selection with G418, cells to carry the mutation were injected into day-3.5 C57BL/6 blastocysts and transferred into foster mothers. Chimeric males were mated with C57BL/6 females, and germline transmission was confirmed by Southern blotting of tail DNA after HindIII digestion (Fig. 1D, 1E).
**Animals and diets**

CD11c-mutant mice were backcrossed for 12 generations onto C57BL/6 mice. After being generated, CD11c$^{+/+}$ mice were crossbred with apoE$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) to obtain CD11c$^{-/-}$/apoE$^{-/-}$ and CD11$^{+/+}$/apoE$^{-/-}$ mice. Male mice, unless stated otherwise, were used. The mice were kept on normal chow diet (ND, PicoLab Rodent Chow 5010) until experiment, or switched to western high-fat diet (HFD; 21% fat [w/w], 0.15% cholesterol [w/w]; Dyets Inc., Bethlehem, PA) at age 8 wk, and maintained on HFD for 12–24 wks. Plasma total cholesterol levels were measured using enzymatic procedures. All animal studies were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

**Antibodies and flow cytometric (FACS) analysis**

The following monoclonal antibodies (mAbs) to mouse antigens, with appropriate negative controls, were used: CD11c (FITC or PE-conjugated), CD11b (FITC-conjugated), CD49d (FITC-conjugated), Gr-1 (FITC-conjugated), Ly-6C (FITC-conjugated), Mac-3 (FITC-conjugated), and CD29 (unconjugated, with a FITC-conjugated secondary Ab) from BD Biosciences (San Jose, CA); CD204 (FITC-conjugated), CD115 (PE-conjugated), CD205 (FITC-conjugated) and F4/80 (FITC-conjugated) from AbD Serotec Inc. (Raleigh, NC). Mouse whole blood, which was drawn through retro-orbital puncture, was used for FACS analysis. Whole blood, after dilution with equal volume of PBS, was incubated with various combinations of fluorescence-conjugated mAbs as described in Results or with appropriate isotype negative controls for 20 min. After staining, the samples were incubated with BD FACS Lysing Solution (BD Biosciences) for 10 min to lyse red blood cells, then were washed 3 times with PBS. Finally, the stained cells were resuspended in 1% paraformaldehyde in PBS. Data were collected with a FACScan and analyzed with CellQuest software (BD Biosciences).\(^1\) Based on scatter
pattern (forward scatter [FSC] vs. side scatter [SSC]), total leukocytes were gated and data were expressed as the percentage of positive cells in total leukocytes. In some cases, data were analyzed in the classical monocyte (high FSC/low SSC), granulocyte (medium FSC/high SSC), or lymphocyte (low FSC/low SSC) gates individually as described in Results.

*Isolation of mouse CD11c*+/cell/*, Oil Red O staining, and electron microscopy*

Mononuclear cells (MNCs) were isolated from mouse blood by Histopaque solution (Histopaque 1083, Sigma-Aldrich) and were incubated with anti-mouse CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C. CD11c*+/cell/* cells were purified by magnetic separation.

CD11c*+/cell/* cells in PBS were cytospun onto a slide and stained with Neat Stain (Midlantic Biomedical, Inc., Paulsboro, NJ). For Oil Red O staining, the CD11c*+/cell/* cells were fixed in 10% Formalin for 10 min. After being dehydrated with 100% propylene glycol, the cells were stained with Oil Red O (Sigma) for 10 min at 37°C, then washed with 85% propylene glycol and water and finally counterstained in hematoxylin (Shandon Lipshaw, Pittsburgh, PA).

Electron microscopy was performed on mouse CD11c*+/cell/* cells with a JEOL 200CX electron microscope as described previously.2

*Isolation of CD11c*+/cell/* and CD11c*−/cell/* monocytes by cell sorting*

Mouse whole blood was labeled with FITC-conjugated anti-mouse CD204 and PE-conjugated anti-mouse CD11c as described above, followed by lysis of red blood cells with BD Lysing buffer (BD Biosciences) and washing 3 times with PBS. The stained cells were resuspended in PBS supplemented with 0.5% BSA. CD11c+/CD204* and CD11c−/CD204* cells were sorted
from classical monocyte and granulocyte gates (based on SSC vs. FSC) separately using a FACSArray II Cell Sorter (BD Biosciences). The sorted cells were spun, resuspended in PBS, cytospun onto slides, and stained with Neat Stain.

**In vitro effect of oxidized LDL on CD11c expression on mouse leukocytes**

Mouse MNCs were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, in the presence of 20 μg/ml of human native LDL or oxidized LDL (ox-LDL) (Intracel, Frederick, MD). At 24 hours after the culture, FACS analysis was performed on the MNCs to examine CD11c.

**Intravenous injection of DiI–oxidized LDL in mice**

DiI-labeled oxidized human LDL (DiI–ox-LDL) or DiI–native LDL (Intracel) (3 μg/g body weight) was injected into mice intravenously. At 1 and 24 hours after injection, blood was collected, stained with FITC-conjugated anti-mouse CD11c or CD204 mAbs, and analyzed by FACS.

**In vitro flow adhesion assay**

**Crosslinking recombinant proteins to glass**

In preparation for protein crosslinking, 35 mm round coverslips (Bellco, Vineland, NJ) were cleaned in a 2:1 solution of 98% sulfuric acid and 30% hydrogen peroxide for 10 minutes and rinsed in water. Clean coverslips were dipped in a 2% solution of 3-aminopropyltriethoxysilane (Pierce, Rockford IL) for 30 seconds and then rinsed with acetone. Protein A/G (Pierce, Rockford, IL) was then crosslinked to the glass surface in the presence of excess BS3 crosslinker
(Pierce, Rockford, IL) overnight at 4°C. The following day the reaction was quenched with Tris buffer. Recombinant mouse VCAM-1/Fc chimera and E-selectin/Fc chimera (R&D Systems, Minneapolis, MN) were incubated on the coverslip at equal concentrations of 10 μg/ml for 1 hour. In order to block nonspecific binding of leukocytes, coverslips were incubated with Sea Block (Pierce, Rockford IL) for 30 minutes.

**MNC isolation**

Peripheral blood (0.8–1.0 ml per mouse) was drawn into heparin-coated syringe via cardiac puncture from male mice after being anesthetized with intraperitoneal injection of Ketamin (80 mg/kg)–Xylazine (12 mg/kg). The collected blood was diluted 1:1 with Hank’s balanced salt solution (HBSS) mixed with 8% sucrose. The blood was gently laid over 3 ml of Histopaque solution and then centrifuged at 500 g for 45 minutes at room temperature. After centrifugation, MNC band was collected into 15 ml conical tube and purified further from platelets by resuspending in HEPES buffer + 2% human serum albumin (HSA) and then centrifuging at 250 g for 10 minutes. After repeating this process two times, mononuclear cells were resuspended in HEPES buffer + 2% HSA + 1.5 mM CaCl₂ and kept at room temperature until use. The mononuclear cells were counted by hemocytometer.

**Microfluidic flow chamber assembly**

The vascular mimetic has been described in detail previously.³ Briefly, the device was designed such that six independent flow channels were surrounded by a series of interconnected vacuum channels allowing the device to be reversibly vacuum sealed to a glass coverslip. Master molds for the devices were fabricated by patterning SU-8 50 photoresist with a thickness of 80 μm on a
By casting PDMS (Sylgard 184) prepolymer over these masters, PDMS replicas were produced. Flow and vacuum access holes were punched directly into PDMS replicas. The flow chamber was assembled by vacuum bonding the PDMS replica over the functionalized glass coverslip resulting in 6 independent microfluidic flow chambers with dimensions of 80 μm × 600 μm × 0.75 cm (h × w × l).

**Mouse MNC adhesion assay**

Mouse MNCs were introduced into the channel at a flow rate resulting in a shear stress of 2 dynes/cm² at fluid–glass interface. To allow for enough adhesive events, cells were perfused through the chamber for 3 minutes; during this time, 5 random fields were observed (2.1 × 10⁻³ cm²). Data were acquired using video microscopy at a rate of 4 frames/second and saved to a PC using a video capture card and Image Pro Plus 4.1.

The numbers of cells rolling and firmly arrested along the length of the channel were counted using the ImageJ cell counter plug-in. Firm arrest was defined as a stationary cell whose centroid did not move greater than one cell diameter during 30 seconds. To assess the extent of shear strengthening of monocytes arrested on VCAM-1/E-selectin, shear was increased in steps of 2 dynes/cm² each minute up to 10 dynes/cm². The fraction of cells remaining firmly arrested after 1 minute at 10 dyne/cm² is reported. In certain experiments, blocking antibodies to CD49d (clones 9C10[MFR4.B] and R1-2, Biolegend, San Diego, CA) and CD18 (BD Biosciences) were used. MNCs were incubated with antibodies for 10 minutes at room temperature at a concentration of 10 μg/ml. Following incubation, MNCs and excess antibody were introduced into the flow chamber. To quantitate the number of adherent CD204⁺ MNCs (monocytes), MNCs were incubated with 10 μg/ml AlexaFluor488 conjugated CD204 antibody (AbD Serotec...
Inc.) at room temperature for 10 minutes. Cells were washed to remove excess antibody and then perfused into the flow chamber. CD204+ cells were defined as those having a mean fluorescence 3 standard deviations above the mean background fluorescence of the image.

Another experiment was performed to quantitate the number of adherent Ly-6C\textsuperscript{high} and Ly-6C\textsuperscript{low} monocytes. MNCs were sheared in the microfluidic channel over VCAM-1/E-selectin–coated glass coverslips for 3 minutes as described above. Those that achieved arrest were immunophenotyped by staining with PE-anti-mouse Gr-1 and Alexa Fluor 647–anti-mouse CD204. Firmly adherent cells were fixed to the glass coverlip with ice cold 4% paraformaldehyde. The flow chamber was then removed and the glass coverslip was mounted to a glass slide using Airvol (Air Products and Chemicals) containing DAPI (Invitrogen). Images were taken and analyzed by a deconvolution microscopy (Deltavision) and ImageJ version 1.41 to quantitate firmly arrested Ly-6C\textsuperscript{high} and Ly-6C\textsuperscript{low} monocytes.

\textit{Analysis of atherosclerotic lesions}

To examine CD11c expression in atherosclerotic lesions, mouse aortas were harvested and embedded in OCT (Triangle Biomedical Sciences, Durham, NC). Serial transverse cryosections were made and fixed in acetone for 5 minutes at room temperature. After blocking with 1\% BSA for 30 minutes, aortic sections were stained with PE-conjugated anti-mouse CD11c and FITC-conjugated anti-mouse CD205 or MOMA-2 mAbs (AbD Serotec Inc.) by co-incubation overnight at 4\(^\circ\)C. After washing, the sections were counterstained for nuclei with DAPI (Molecular Probes Inc., Eugene, OR) and detected with Deltavision microscope.

Atherosclerotic lesions in thoracoabdominal aortas were assessed by Sudan IV staining as previously described.\textsuperscript{5} Briefly, after 12 weeks on Western HFD, mouse aortas were removed
2 mm from the heart, excised from the aortic arch to just beyond the renal artery, and stained with saturated Sudan IV in propylene glycol. The aortic and atherosclerotic lesion area was measured using the Image Plus program (Media Cybernetics, Silver Spring, MD). The extent of atherosclerosis is expressed as the percentage of total aortic area, or percentage of the aortic arch (2 mm from the heart extending to the left subclavian artery) area, covered by atherosclerotic lesions.

Macrophages in atherosclerotic lesions were quantitated by immunohistochemistry on aortic sinus sections using a primary mAb for Mac-3 (BD Biosciences), with an appropriate isotype negative control, as described previously. Briefly, after fixation in acetone, rehydration in PBS and blockage with 3% H₂O₂ and 3% normal rabbit serum, aortic sinus sections were incubated sequentially with Mac-3 mAb and biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA). Avidin-biotin horseradish peroxidase (Vectastain ABC reagent) and ImmPACT DAB substrate kits (Vector Laboratories) were used to visualize positive cells, which were quantified using the Image Plus program, and expressed as the ratio of positively stained area to the total lesion area.

Statistics

GraphPad Prism 4 was used for statistical analyses. Values are presented as mean±SEM. Student's t-tests (for comparison between 2 groups) or one-way ANOVA (for comparisons of 3 or more groups) followed by Tukey post hoc pairwise tests were used for statistical analyses.
Supplemental Table I. Plasma lipid levels

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<th>Before high-fat diet</th>
<th>After high-fat diet</th>
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<tr>
<td></td>
<td>Triglycerides (mg/dl)</td>
<td>Cholesterol (mg/dl)</td>
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<tr>
<td>CD11c^{+/+}/apoE^{−/−}</td>
<td>125.9±6.3</td>
<td>251.9±42.7</td>
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<td>(n=6)</td>
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<tr>
<td>CD11c^{−/−}/apoE^{−/−}</td>
<td>204.7±22.7^{a}</td>
<td>375.9±55.0</td>
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<td>(n=6)</td>
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<td>95.8±8.8^{b}</td>
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<td>1130±116.7</td>
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^{a}P<0.01, ^{b}P<0.05 compared with CD11c^{+/+}/apoE^{−/−}

Enzymatic procedures were used to measure plasma levels of total cholesterol and triglyceride from CD11c^{+/+}/apoE^{−/−} or CD11c^{−/−}/apoE^{−/−} mice before or 12 weeks after western high-fat diet.
Supplemental Figure I

apoE<sup>−/−</sup>

FSC

SSC

CD11c

F4/80

Mac-3

Ly-6C

CD205
Supplemental Figure Legend:

**Fig. I. Some characteristics of CD11c⁺ cells by FACS.** CD11c⁺ cells from both granulocyte/R1 and monocyte/R2 regions of apoE⁻/⁻ mice on HFD were F4/80⁺, Mac-3⁻, and CD205⁻. Most of the CD11c⁺ cells were Ly-6C⁻⁰⁰⁷, and a small portion of CD11c⁺ cells were Ly-6C⁺.⁰⁰⁷
Supplemental References:


