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Functional role of CD11c$^+$ monocytes in atherogenesis associated with hypercholesterolemia

Huaizhu Wu, MD[1,2,3], **R. Michael Gower**[4], **Hong Wang, MD, PhD**[1,5], **Xiao-Yuan Dai Perrard, MD**[1,3], **Ruidong Ma**[6], **Daniel C. Bullard, PhD**[7], **Alan R. Burns, PhD**[2], **Antoni Paul, PhD**[8], **C. Wayne Smith, MD**[2], **Scott I. Simon, PhD**[4], and **Christie M. Ballantyne, MD**[1,2,3]

[1]Section of Atherosclerosis and Vascular Medicine, Baylor College of Medicine, Houston, Texas

[2]Section of Leukocyte Biology, Department of Pediatrics, Baylor College of Medicine, Houston, Texas

[3]Center for Cardiovascular Disease Prevention, Methodist DeBakey Heart and Vascular Center, Houston, Texas

[4]Department of Bioengineering, University of California at Davis, Davis, California

[5]Department of Pharmacology, Temple University School of Medicine, Philadelphia, Pennsylvania

[6]Section of Thrombosis Research, Baylor College of Medicine, Houston, Texas

[7]Department of Genetics Research Division, The University of Alabama at Birmingham, Birmingham, AL

[8]Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Baylor College of Medicine, Houston, Texas

Abstract

Background—Monocyte activation and migration into the arterial wall is a key event in atherogenesis associated with hypercholesterolemia. CD11c/CD18, a β_2 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 apoE$^{-/-}$ mice to generate CD11c$^{-/-}$/apoE$^{-/-}$ mice. Using flow cytometry, we examined CD11c on blood leukocytes in apoE$^{-/-}$ hypercholesterolemic mice and found that compared to wild-type and apoE$^{-/-}$ mice on normal diet, apoE$^{-/-}$ mice on western high-fat diet (HFD) had increased CD11c$^+$ monocytes. Circulating CD11c$^+$ monocytes from HFD-fed apoE$^{-/-}$ mice 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 HFD.

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.

Address correspondence and reprint requests to Dr. Christie M. Ballantyne, Department of Medicine, Baylor College of Medicine, 6565 Fannin, M.S. A-601, Houston, TX 77030. Phone: 713-798-5034; fax: 713-798-3057; Email: E-mail: cmb@bcm.tmc.edu.. A.R.B.'s current address: College of Optometry, University of Houston, Houston, TX

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Keywords

atherosclerosis; cell adhesion molecules; leukocytes

Introduction

Atherosclerosis associated with hypercholesterolemia is a complex inflammatory process, characterized pathologically by recruitment of monocytic leukocytes in the arterial wall and lipid accumulation in monocytic 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 (PSGL-1) on monocytes, followed by firm arrest facilitated through interactions between activated β_1 and β_2 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.[4-6] However, the effect of hypercholesterolemia on monocyte activation and its contribution to atherogenesis are less defined.

The β_2 integrins, which include CD11a/CD18, CD11b/CD18, CD11c/CD18, and CD11d/CD18,[7] contribute to atherogenesis as evidenced by a significant reduction in atherosclerosis development in CD18$^{-/-}$ mice, which lack all four CD11/CD18 integrins.[4] CD11b has been used as an activation marker for monocyte/macrophages and increases in hyperlipidemia[8] but was not essential for atherosclerosis development in LDL receptor$^{-/-}$ mice.[9]

CD11c/CD18 is found on monocytes/macrophages, granulocytes, and subsets of dendritic cells (DCs) in humans,[10,11] and on DCs and subsets of monocytes/macrophages in mice.[12,13] CD11c binds a variety of ligands including ICAM-1, ICAM-2, fibrinogen, collagen, iC3b, and lipopolysaccharide,[10,11,14] 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,[14] which participates in atherogenesis.[5] CD11c has been used as an activation marker for monocytes/macrophages.[3] 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 blood monocytes in atherosclerosis development using CD11c$^{-/-}$/apolipoprotein (apo) E$^{-/-}$ mice.

Materials and Methods

Detailed methods can be found in the online-only data supplement.

Targeting construct and generation of CD11c$^{-/-}$ mice

Murine CD11c genomic clone was isolated from a 129/Sv mouse lambda library (Stratagene, La Jolla, CA) (Fig. 1A). A neomycin cassette was inserted (Fig. 1B), replacing exons 1, 2, and 3 of the CD11c gene (Fig. 1C).

The AB2.1 embryonic stem cell line was electroporated with linearized targeting construct. Cells carrying the mutation were injected into C57BL/6 blastocysts and transferred into foster

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mothers. Chimeric males were mated with C57BL/6 females. Germline transmission was confirmed by Southern blotting (Fig. 1D, 1E).

Animals and diets

CD11c mutant mice were backcrossed for 12 generations onto C57BL/6. $CD11c^{-/-}$ mice were crossbred with $apoE^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) to obtain $CD11c^{-/-}$/$apoE^{-/-}$ and $CD11^{+/+}$/$apoE^{-/-}$ mice. Male mice were used, and fed normal chow diet (ND) throughout or switched to western high-fat diet (HFD; 21% fat [w/w], 0.15% cholesterol [w/w]; Dyets Inc., Bethlehem, PA) at age 8 weeks and maintained on HFD for 12-24 weeks. 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 were used: CD11c (FITC- or PE-conjugated), CD11b (FITC-conjugated), CD49d (FITC-conjugated), Gr-1 (FITC- or PE-conjugated), CD29 (unconjugated, with a FITC-conjugated secondary Ab) from BD Biosciences (San Jose, CA), CD204 (FITC- or Alexa Fluor [AF] 647-conjugated), and CD115 (PE-conjugated) from AbD Serotec Inc. (Raleigh, NC), for FACS analysis of mouse blood leukocytes.

Characterization of mouse blood monocytes

$CD11c^{+}$ cells were purified from mouse blood mononuclear cells (MNCs) by using anti-mouse CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), cytospun onto slides, and stained with Neat Stain (Midlantic Biomedical Inc., Paulsboro, NJ) or Oil Red O (Sigma). $CD11c^{+}$/$CD204^{+}$ and $CD11c^{-}$/$CD204^{+}$ cells were isolated from mouse blood by cell sorting (FACSAria II Cell Sorter, BD Biosciences), and stained with Neat Stain.

Tissue culture

Mouse MNCs were cultured in RPMI-1640 with 20 μg/ml of human native LDL or Cu^{+}-oxidized LDL (ox-LDL) (Intracel, Frederick, MD). After 24 hours, FACS analysis was performed to examine CD11c.

Intravenous injection of DiI-ox-LDL in mice

DiI-labeled ox-LDL (DiI-ox-LDL) or DiI-labeled 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-anti-mouse CD11c or CD204 mAbs, and analyzed by FACS.

In vitro flow adhesion assay

The interaction between mouse MNCs and recombinant mouse VCAM-1/E-selectin (R&D Systems, Minneapolis, MN) was examined in a microfluidic flow chamber.[14] To quantitate the number of adherent Ly-$6C^{high}$ and Ly-$6C^{low}$ monocytes, arrested MNCs were 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]

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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.

Results

Generation of mice deficient in CD11c

Embryonic stem cells with a targeted event had a 4.0-kb Hind III fragment compared with the 9.4-kb fragment in wild-type (WT) (Fig. 1D). The expected targeted allele of 4.0 kb was identified by the 3'-flanking probe on Southern blots of DNA from mutant mice (Fig. 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 (Fig. 2A), another widely used monocyte marker.[13] Based on CD11c expression, we classified mouse monocytes as $CD11c^{+}/CD204^{+}$ and $CD11c^{-}/CD204^{+}$ subsets (Fig. 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 ($P<0.001$, Fig. 2C). Compared to WT or $apoE^{-/-}$ on ND, $apoE^{-/-}$ mice on HFD had significantly increased proportion of $CD11c^{+}/CD204^{+}$ monocytes in total leukocytes (Fig. 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 to WT (Fig. 2C).

Characteristics of $CD11c^{+}$ monocytes in hypercholesterolemic mice

Scatter pattern (forward scatter [FSC: cell size] versus side scatter [SSC: 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 (Fig. 3A, 3B). As expected, R2/monocytes but not R1/granulocytes contained monocytes ($CD204^{+}$) in WT (Fig. 3A) and $apoE^{-/-}$ mice on ND (data not shown). In contrast, blood from $apoE^{-/-}$ mice on HFD contained monocytes in both R1 and R2 (Fig. 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 ($P<0.001$, Fig. 3C). The $CD11c^{+}$ subset accounted for 23% of monocytes in R2 of $apoE^{-/-}$ mice on HFD, which was lower than in WT (Fig. 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 (Fig. 3B, 3C). $CD11c^{+}$ monocytes in R1 and R2 of $apoE^{-/-}$ mice on HFD were $CD11b^{+}$, Gr-1^{low}, $CD49d^{+}$ (Fig. 3B), $F4/80^{+}$, Mac-3^{-}, and $CD205^{-}$ (Fig. I in the online data supplement), confirming their characteristics as monocytes, but not $CD205^{+}$ DCs or granulocytes, which are Gr-1^{high}. 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, Fig. 3D; $P=0.012$ for CD29, Fig. 3E), with higher CD11b and CD29 levels on $CD11c^{+}$ monocytes in R1 of $apoE^{-/-}$ mice on HFD (Fig. 3D, 3E). Therefore, compared to 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.

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Morphologic examination revealed that most $CD11c^+$ cells from blood of $apoE^{-/-}$ 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 (Fig. 3F). These lipid vacuoles were not found in $CD11c^+$ cells from blood of WT (Fig. 3F). Further examination showed that $CD11c^+/CD204^+$ (and $CD11c^-/CD204^+$) cells in both R1 and R2 of $apoE^{-/-}$ on HFD displayed morphology of monocytes (Fig. 3G). $CD11c^+$ monocytes in R1 appeared to have more intracellular lipid vacuoles than the monocytes in R2 (Fig. 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 to 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 (Fig. 4A).

To examine the effect of ox-LDL on CD11c expression in vivo, DiI-ox-LDL was injected intravenously into $apoE^{-/-}$ mice on ND, and changes in CD11c expression on monocytes that bound ox-LDL were traced by detection of DiI with CD11c by FACS. DiI-ox-LDL injection resulted in obvious DiI signal in leukocytes (Fig. 4B), indicating binding and/or uptake of the exogenous ox-LDL by the leukocytes. As expected, only $CD204^+$ monocytes (up to 70%) exhibited $DiI(ox\text{-}LDL)^+$ at 1 hour and 24 hours after injection (Fig. 4B, top row). CD11c staining indicated that both $CD11c^+$ and $CD11c^-$ monocytes exhibited DiI^+ at 1 hour. At 24 hours, however, the vast majority of DiI^+ cells were $CD11c^+$ (Fig. 4B, middle row), with a significant increase in the proportion of $CD11c^+$ cells in total DiI^+ cells compared to 1 hour (Fig. 4C), indicating that the interaction between ox-LDL and monocytes drove $CD11c^-$-to-$CD11c^+$ monocyte differentiation. In contrast, injection of DiI-native LDL did not change the ratio of $CD11c^+/DiI^+$ to $CD11c^-/DiI^+$ cells at 24 hours compared to 1 hour after injection (Fig. 4B, bottom row; Fig. 4C).

The 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 $CD11c^{+/+}/apoE^{-/-}$ or $CD11c^{-/-}/apoE^{-/-}$ mice were perfused through a microfluidic flow chamber coated with E-selectin/VCAM-1 at a shear stress of 2 dyne/cm^2. Immunofluorescence analysis confirmed that for both genotypes, ~90% of the arrested MNCs were $CD204^+$ monocytes (Fig. 5A, 5B).

We first examined the proportion of firmly arrested Ly-$6C^{high}$ and Ly-$6C^{low}$ monocytes by introducing AF647-CD204 and PE-Gr-1-labeled MNCs, and recording firmly arrested $CD204^+$/Ly-$6C^{high}$ and $CD204^+$/Ly-$6C^{low}$ monocytes. We found that 63% of arrested cells from $CD11c^{+/+}/apoE^{-/-}$ mice were Ly-$6C^{high}$ monocytes, compared with 80% of arrested cells from $CD11c^{-/-}/apoE^{-/-}$ mice. The most striking difference between the two genotypes was the proportion of firmly arrested Ly-$6C^{low}$ monocytes, which comprised 11% of arrested cells in $CD11c^{-/-}/apoE^{-/-}$ mice and more than 25% in $CD11c^{+/+}/apoE^{-/-}$ mice (Fig. 5A, 5B).

In this model, we expected that MNCs from both genotypes would demonstrate a similar capacity of rolling, since recruitment via E-selectin is mediated by ligands such as PSGL-1 that are expressed on most MNCs.[17] Indeed, the numbers of rolling MNCs per field were not significantly different between the two genotypes (28.2±5.9 for $CD11c^{+/+}/apoE^{-/-}$ [n=6] versus 21.3±3.8 for $CD11c^{-/-}/apoE^{-/-}$ [n=8]). However, the numbers of MNCs that transitioned from rolling to firm arrest differed significantly ($P<0.001$, Fig. 5C). More than twice as many MNCs

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from CD11c$^{+/+}$/apoE$^{-/-}$ mice transitioned from rolling to firm arrest on VCAM-1 compared to MNCs from CD11c$^{-/-}$/apoE$^{-/-}$ mice (Fig. 5C). Treatment of MNCs from CD11c$^{-/-}$/apoE$^{-/-}$ mice with anti-VLA-4 mAbs (clones 9C10 [MFR4.B] and R1-2) decreased adhesion by 67%, and anti-VLA-4 plus anti-CD18 mAbs decreased arrest to baseline levels as observed in the absence of VCAM-1, suggesting that at least a portion of MNC arrest was mediated by other CD11/CD18 integrins (Fig. 5C). These data indicate that monocytes in MNCs utilize both CD11c and VLA-4 in a cooperative manner to transition from E-selectin-mediated rolling to firm arrest on VCAM-1.

To determine whether CD11c modulates cell avidity via VLA-4 enabling MNCs to resist shear stress over time, we exposed arrested cells to a 5-fold stepped increase in shear stress from 2 to 10 dyne/cm^2 and measured the fraction remaining adherent after 1 minute at 10 dyne/cm^2. Less than 50% of cells from CD11c$^{-/-}$/apoE$^{-/-}$ mice remained arrested on VCAM-1, compared to approximately 75% of cells from CD11c$^{+/+}$/apoE$^{-/-}$ mice (Fig. 5D), indicating that CD11c binding to VCAM-1 is required for high-avidity sustained adhesion.

The role of CD11c in atherosclerosis development

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

Next, we determined whether CD11c contributes to atherosclerosis development. Plasma total cholesterol levels were not significantly different between CD11c$^{+/+}$/apoE$^{-/-}$ and CD11c$^{-/-}$/apoE$^{-/-}$ mice before or after HFD (Table I in the online data supplement). At 12 weeks after HFD, CD11c$^{+/+}$/apoE$^{-/-}$ 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 (Fig. 6E). In contrast, CD11c$^{-/-}$/apoE$^{-/-}$ mice developed smaller atherosclerotic lesions in the whole aorta and aortic arch (Fig. 6F, 6G). Compared to CD11c$^{+/+}$/apoE$^{-/-}$, CD11c$^{-/-}$/apoE$^{-/-}$ mice also exhibited less macrophage accumulation in atherosclerotic lesions of aortic sinus (Fig. 6H, 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 MCSF 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^{low}/CX3CR^{high} and Ly-6C^{high}/CX3CR^{low} monocytes,[12,13,18,19] most of the CD11c$^+$ monocytes were Ly-6C^{low}; however, a subpopulation of CD11c$^+$ monocytes were Ly-6C^{high} (Fig. I in the online data supplement). We observed that both CD11c$^+$ and CD11c$^-$ monocytes were increased in circulation of apoE$^{-/-}$ mice on HFD compared to WT or apoE$^{-/-}$ on ND. Noticeably, in apoE$^{-/-}$ mice on HFD, although most CD11c$^-$ monocytes remained in the classical monocyte regions, most CD11c$^+$ monocytes exhibited increased granularity and shifted to the granulocyte region as examined by FACS.

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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 (Fig. 3B, 3G). This is also supported by the observation that granularity of adipocytes undergoing differentiation increases with increasing accumulation of intracellular lipid.[22] The 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 up-regulated 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, β_1 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 (i.e., ~40%) from CD11c binding VCAM-1 was detected by blocking ICAM-1, CD11a, CD11b, and VLA-4.[14] Using a reduced inflammatory substrate model of recombinant 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 (~15 cells/field) or CD11c (~10 cells/field) alone did not add up to the total (~40 cells/field). Hence, while 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-6C^{low} monocytes were preferentially recruited to the arterial wall in atherosclerosis.[19] More recent evidence indicated that CX3CR^{low}/Ly-6C^{high} monocytes preferentially home to regions of atherosclerosis.[12,13,17] Ly-6C^{high} monocytes typically express lower CD11c levels than Ly-6C^{low} monocytes.[12,13,17] However, little is known about the functionality of CD11c on these two monocyte subsets during recruitment. Our current study is the first to show that CD11c plays a functional role in the firm arrest of both Ly-6C^{low} and Ly-6C^{high} monocytes

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on VCAM-1 in shear flow. The greater diminution in firm arrest of Ly-6C^{low} monocytes in CD11c$^{-/-}$/apoE$^{-/-}$ mice suggests that CD11c plays an important role in the trafficking of this subset. The ~50% decrease in monocyte recruitment in CD11c$^{-/-}$/apoE$^{-/-}$ mice along with the immunophenotyping data indicates that CD11c present on a subpopulation of Ly-6C^{high} 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 there is macrophage heterogeneity in mouse atherosclerotic lesions, as CD11c^{-} macrophages are also present. Based on the expression of CD68 and lack of CD83, a marker for mature DCs, Liu et al considered the CD11c^{+} cells in mouse aortic wall as immature DCs.[28] 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 showed that Ly-6C^{low}/CX3CR1^{high} monocytes entered atherosclerotic lesions and were more prone to becoming macrophage-like cells expressing CD11c.[13] Liu et al found that deficiency of CX3CR1 impaired CD11c^{+} cell accumulation in arterial intima and reduced atherosclerosis.[28]

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Libby P. Inflammation in atherosclerosis. Nature 2002;420:868–874. [PubMed: 12490960]
2. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007;7:678–689. [PubMed: 17717539]
3. Zhao L, Moos MP, Grabner R, Pedrono F, Fan J, Kaiser B, John N, Schmidt S, Spanbroek R, Lotzer K, Huang L, Cui J, Rader DJ, Evans JF, Habenicht AJ, Funk CD. The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm. Nat Med 2004;10:966–973. [PubMed: 15322539]
4. Nageh MF, Sandberg ET, Marotti KR, Lin AH, Melchior EP, Bullard DC, Beaudet AL. Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice. Arterioscler Thromb Vasc Biol 1997;17:1517–1520. [PubMed: 9301629]
5. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 2001;107:1255–1262. [PubMed: 11375415]
6. Ting HJ, Stice JP, Schaff UY, Hui DY, Rutledge JC, Knowlton AA, Passerini AG, Simon SI. Triglyceride-rich lipoproteins prime aortic endothelium for an enhanced inflammatory response to tumor necrosis factor-α. Circ Res 2007;100:381–390. [PubMed: 17234968]
7. Wu H, Rodgers JR, Perrard X-YD, Perrard JL, Prince JE, Abe Y, Davis BK, Dietsch G, Smith CW, Ballantyne CM. Deficiency of CD11b or CD11d results in reduced staphylococcal enterotoxin-induced T cell response and T cell phenotypic changes. J Immunol 2004;173:297–306. [PubMed: 15210787]
8. van Oostrom AJ, Rabelink TJ, Verseyden C, Sijmonsma TP, Plokker HW, De Jaegere PP, Cabezas MC. Activation of leukocytes by postprandial lipemia in healthy volunteers. Atherosclerosis 2004;177:175–182. [PubMed: 15488881]
9. Kubo N, Boisvert WA, Ballantyne CM, Curtiss LK. Leukocyte CD11b expression is not essential for the development of atherosclerosis in mice. J Lipid Res 2000;41:1060–1066. [PubMed: 10884286]
10. Ingalls RR, Golenbock DT. CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. J Exp Med 1995;181:1473–1479. [PubMed: 7535339]
11. Postigo AA, Corbi AL, Sanchez-Madrid F, de Landazuri MO. Regulated expression and function of CD11c/CD18 integrin on human B lymphocytes: relation between attachment to fibrinogen and triggering of proliferation through CD11c/CD18. J Exp Med 1991;174:1313–1322. [PubMed: 1683891]
12. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. J Clin Invest 2007;117:195–205. [PubMed: 17200719]
13. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, Garin A, Liu J, Mack M, van Rooijen N, Lira SA, Habenicht AJ, Randolph GJ. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. J Clin Invest 2007;117:185–194. [PubMed: 17200718]
14. Sadhu C, Ting HJ, Lipsky B, Hensley K, Garcia-Martinez LF, Simon SI, Staunton DE. CD11c/CD18: novel ligands and a role in delayed-type hypersensitivity. J Leukoc Biol 2007;81:1395–1403. [PubMed: 17389580]
15. Wang H, Jiang X, Yang F, Gaubatz JW, Ma L, Magera MJ, Yang X, Berger PB, Durante W, Pownall HJ, Schafer AI. Hyperhomocysteinemia accelerates atherosclerosis in cystathionine beta-synthase and apolipoprotein E double knock-out mice with and without dietary perturbation. Blood 2003;101:3901–3907. [PubMed: 12506016]
16. Paul A, Ko KW, Li L, Yechoor V, McCrory MA, Szalai AJ, Chan L. C-reactive protein accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. Circulation 2004;109:647–655. [PubMed: 14744975]

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17. An G, Wang H, Tang R, Yago T, McDaniel JM, McGee S, Huo Y, Xia L. P-selectin glycoprotein ligand-1 is highly expressed on Ly-6Chi monocytes and a major determinant for Ly-6Chi monocyte recruitment to sites of atherosclerosis in mice. Circulation 2008;117:3227–3237. [PubMed: 18519846]

18. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 2003;19:71–82. [PubMed: 12871640]

19. Deem TL, Ley K. Dendritic cells preferentially accumulate in atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2006;26:e–51.

20. Combadière C, Potteaux S, Rodero M, Simon T, Pezard A, Esposito B, Merval R, Proudfoot A, Tedgui A, Mallat Z. Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C^{hi} and Ly6C^{lo} monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. Circulation 2008;117:1649–1657. [PubMed: 18347211]

21. Dresel HA, Via DP, Stohr M, Elchner U, Gnasso A, Postiglione A, Blin N, Augustin J, Schettler G. Observations on leukocytes from patients with severe familial hypercholesterolemia. Arteriosclerosis 1986;6:259–264. [PubMed: 3707427]

22. Lee YH, Chen SY, Wiesner RJ, Huang YF. Simple flow cytometric method used to assess lipid accumulation in fat cells. J Lipid Res 2004;45:1162–1167. [PubMed: 14993237]

23. Cho HJ, Shashkin P, Gleissner CA, Dunson D, Jain N, Lee JK, Miller Y, Ley K. Induction of dendritic cell-like phenotype in macrophages during foam cell formation. Physiol Genomics 2007;29:149–160. [PubMed: 17244792]

24. Tabas I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. J Clin Invest 2002;110:905–911. [PubMed: 12370266]

25. Feingold KR, Elias PM, Mao-Qiang M, Fartasch M, Zhang SH, Maeda N. Apolipoprotein E deficiency leads to cutaneous foam cell formation in mice. J Invest Dermatol 1995;104:246–250. [PubMed: 7829881]

26. Matsuura F, Yamashita S, Hirano K, Ishigami M, Hiraoka H, Tamura R, Nakagawa T, Nishida M, Sakai N, Nakamura T, Nozaki S, Funahashi T, Matsumoto C, Higashiyama M, Yoshikawa K, Matsuzawa Y. Activation of monocytes in vivo causes intracellular accumulation of lipoprotein-derived lipids and marked hypocholesterolemia--a possible pathogenesis of necrobiotic xanthogranuloma. Atherosclerosis 1999;142:355–365. [PubMed: 10030387]

27. Simon SI, Hu Y, Vestweber D, Smith CW. Neutrophil tethering on E-selectin activates β_2 integrin binding to ICAM-1 through a mitogen-activated protein kinase signal transduction pathway. J Immunol 2000;164:4348–4358. [PubMed: 10754335]

28. Liu P, Yu YR, Spencer JA, Johnson AE, Vallanat CT, Fong AM, Patterson C, Patel DD. CX3CR1 deficiency impairs dendritic cell accumulation in arterial intima and reduces atherosclerotic burden. Arterioscler Thromb Vasc Biol 2008;28:243–250. [PubMed: 18079406]

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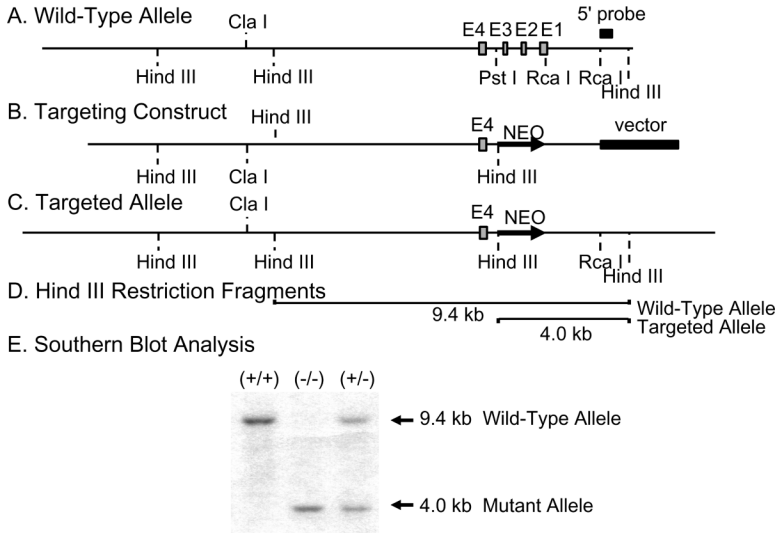

Figure 1. CD11c targeting construct and homologous recombination

A: Partial restriction map of murine CD11c gene. Boxes represent exons 1-4. The 5′ probe was generated from flanking DNA not used in the targeting construct. B: The CD11c replacement construct 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 a neomycin resistance cassette (NEO). C: Map of the predicted homologous recombination event. D: HindIII digestion results in a 4.0-kb fragment in mutant and a 9.4-kb fragment in wild-type (WT). E: Genotyping of WT (+/+), mutant (-/-), and heterozygous (+/-) mice by Southern blot analysis using the probe indicated in (A).

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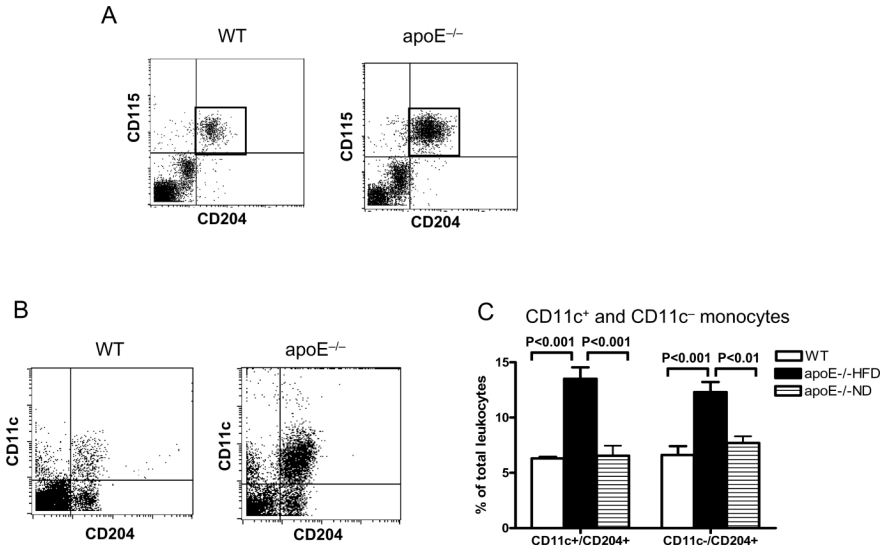

Figure 2. Increased CD11c^{+} monocytes in blood of hypercholesterolemic mice

A: representative FACS of CD204 and CD115 expression on blood leukocytes of WT and apoE$^{-/-}$ HFD. B: Representative FACS of CD11c and CD204 expression on blood leukocytes of WT and apoE$^{-/-}$ on HFD, indicating CD11c^{+}/CD204^{+} and CD11c^{-}/CD204^{+} monocyte subsets. C: Compared to WT or apoE$^{-/-}$ on ND, apoE$^{-/-}$ mice on HFD (12 weeks) showed significantly increased CD11c^{+}/CD204^{+} and CD11c^{-}/CD204^{+} monocytes. n=9-12 mice/group.

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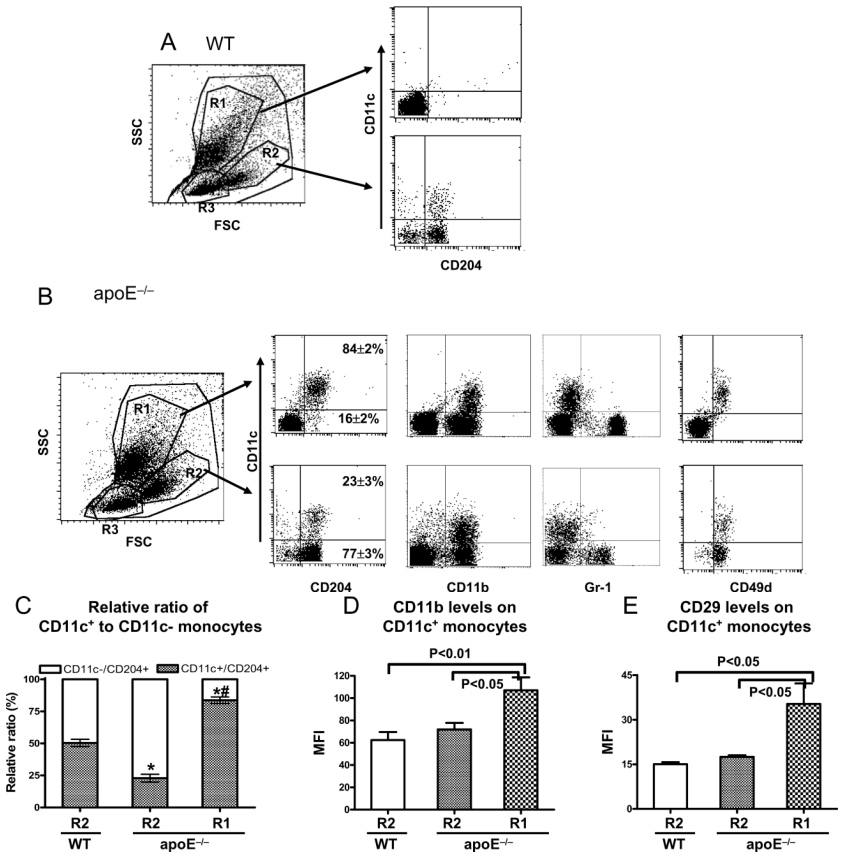

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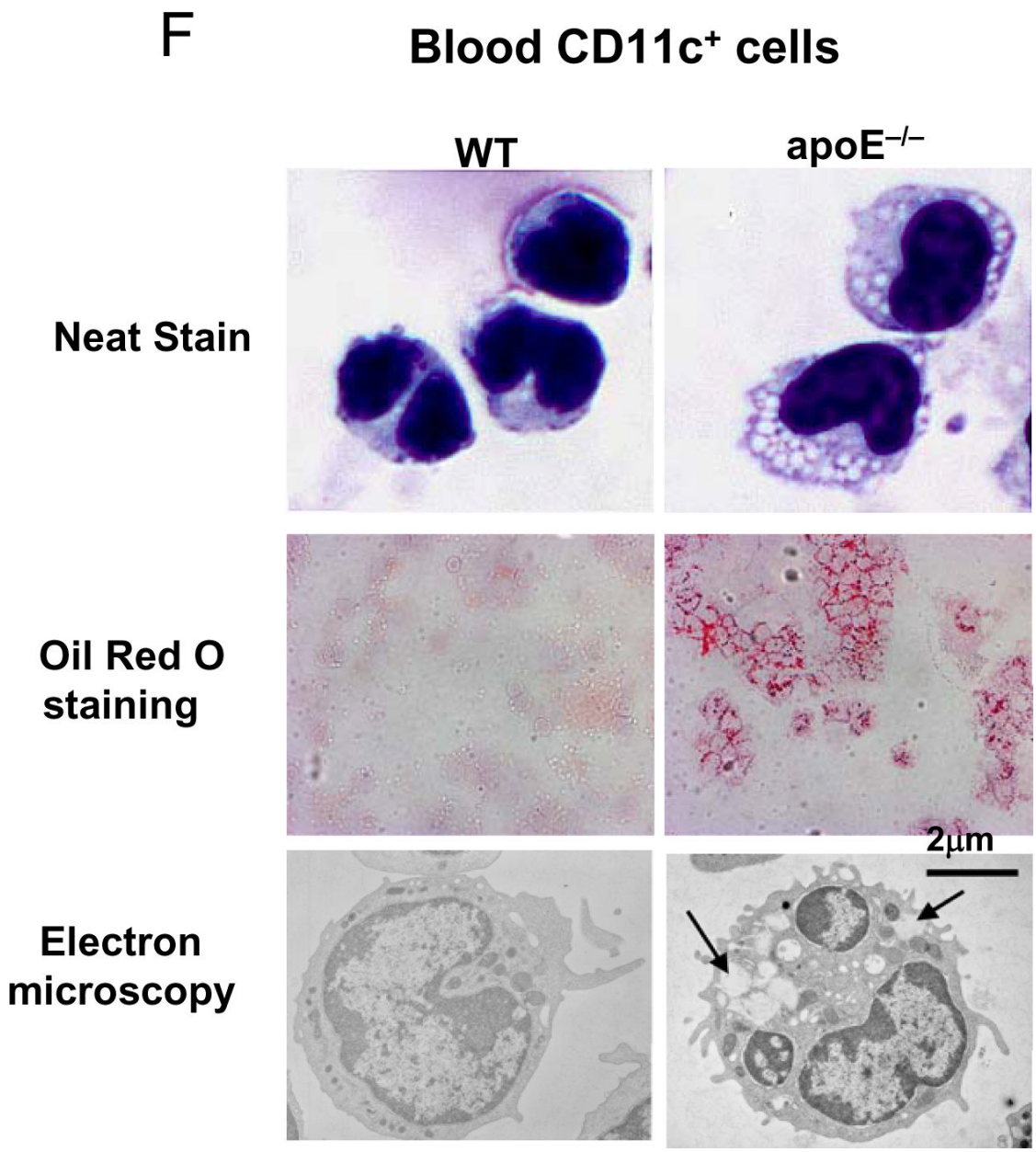

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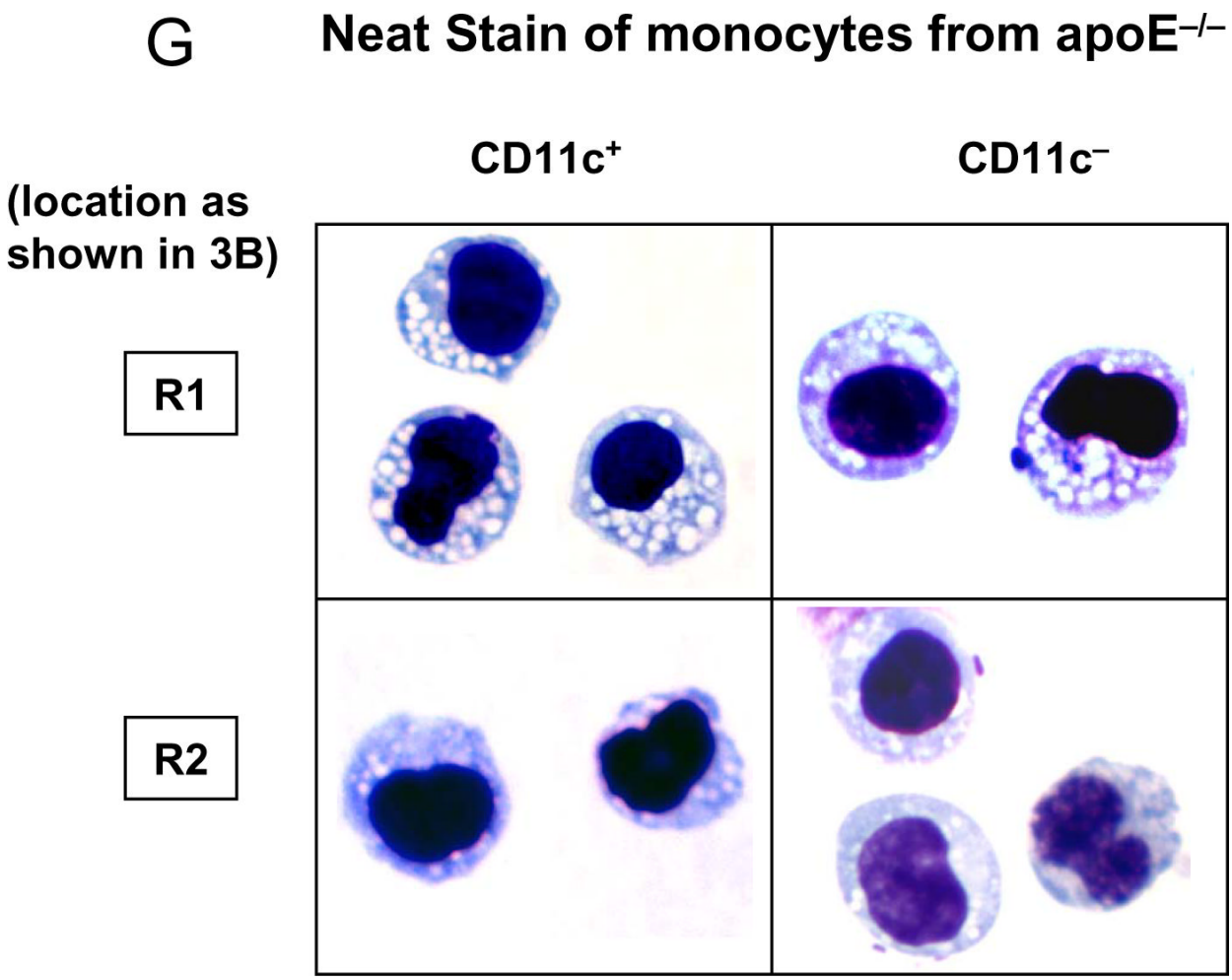

Figure 3. Characteristics of monocytes from blood of apoE$^{-/-}$ mice on HFD

A and B: Representative scatter pattern (cell size [FSC] versus granularity [SSC]) of total blood leukocytes and some phenotypic characteristics of blood CD11c^{+} cells from WT and apoE$^{-/-}$ mice on HFD analyzed by FACS. C: Relative ratio of CD11c^{+} to CD11c^{-} monocytes in apoE$^{-/-}$ mice on HFD (R1/granulocyte and R2/monocyte regions) or WT, n=9-12 mice/group; *P<0.001 vs. WT, #P<0.001 vs. apoE$^{-/-}$ R2. D and E: CD11c^{+} monocytes in R1 from apoE$^{-/-}$ mice on HFD had higher levels of CD11b and CD29 than CD11c^{+} monocytes in R2; n=5 for each of WT and apoE$^{-/-}$ on HFD. F and G: Representative images of CD11c^{+} cells isolated from blood of WT or apoE$^{-/-}$ on HFD by magnetic separation (F), and CD11c^{+} and CD11c^{-} monocytes isolated from R1 and R2 regions of apoE$^{-/-}$ on HFD by cell sorting (G). Original magnification: ×600 (Neat Stain), ×100 (Oil Red O).

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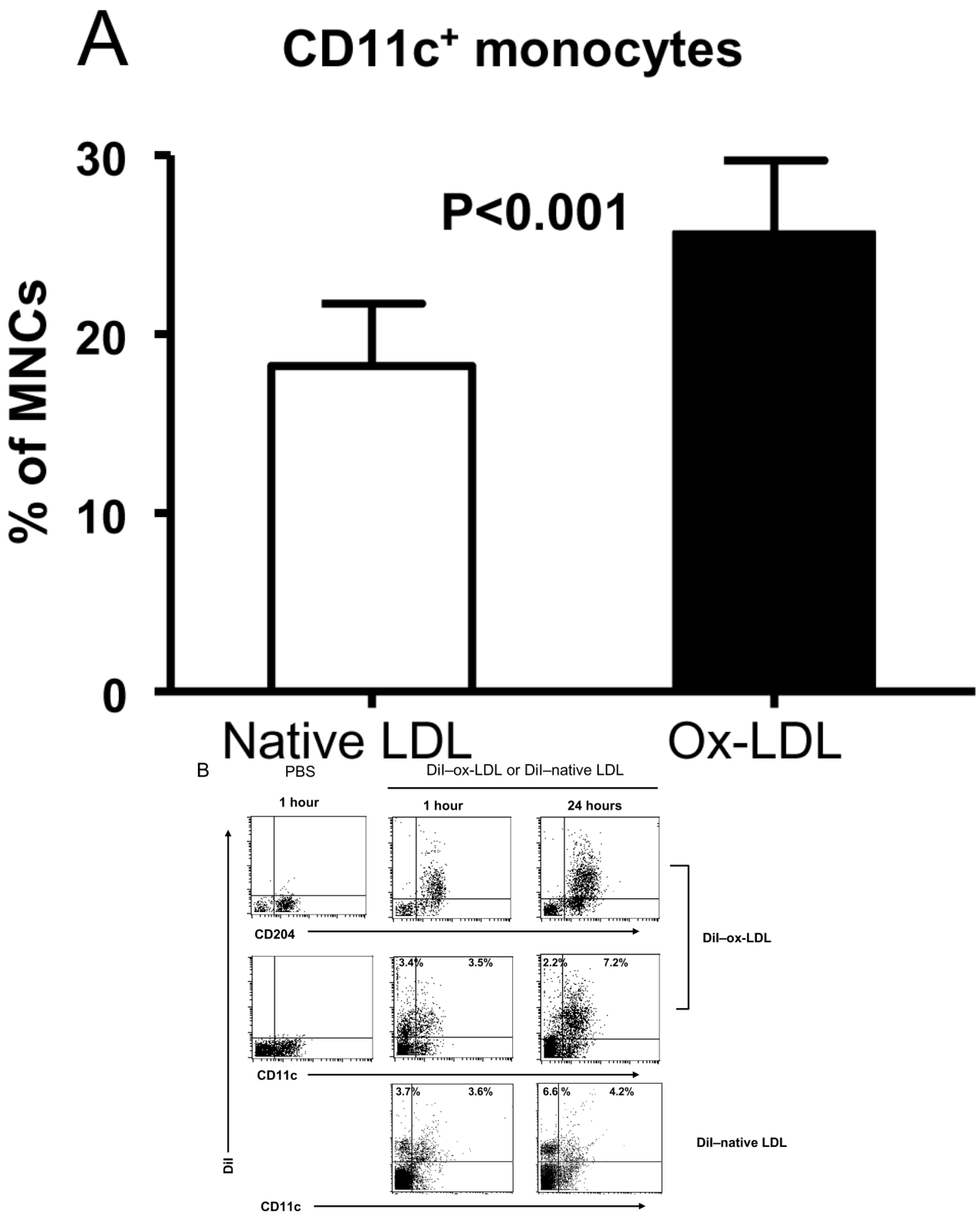

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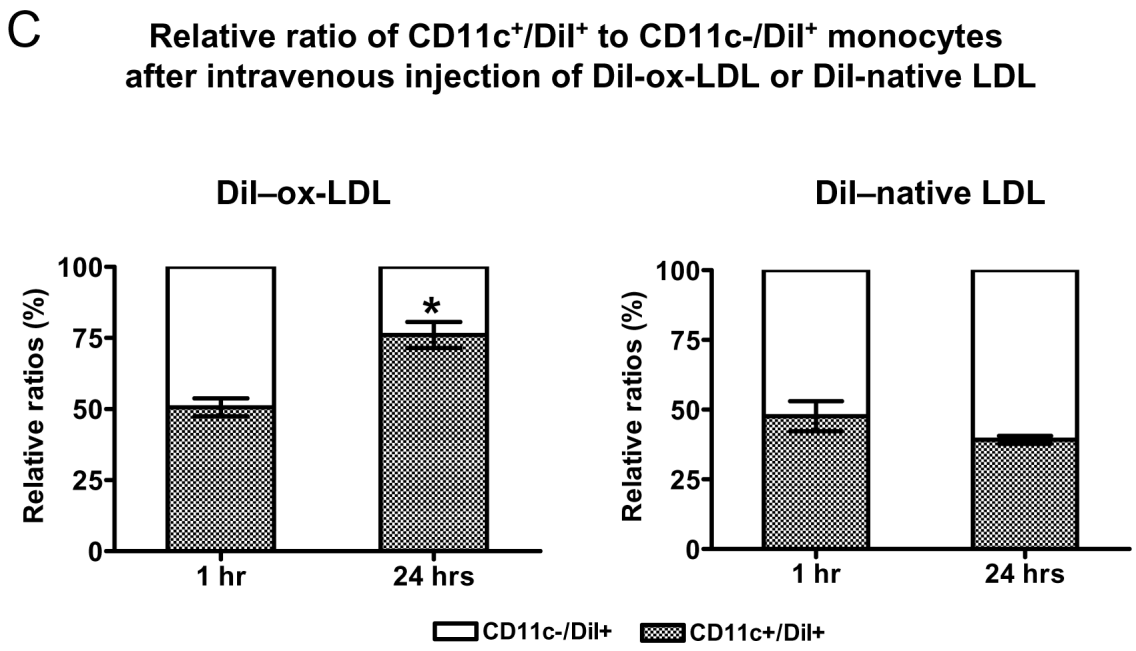

Figure 4. ox-LDL increases CD11c expression on mouse MNCs

A: Proportion of $CD11c^+$ monocytes in mouse MNCs after incubation with native LDL or ox-LDL (20 □g/ml) for 24 hours in vitro (n=8/group). B: Representative FACS of monocytes in $apoE^{-/-}$ mice on ND at different timepoints after intravenous injection of DiI-ox-LDL or DiI-native LDL with PBS as negative control. C: Relative ratio of $CD11c^+/DiI^+$ to $CD11c^-/DiI^+$ cells in blood (total DiI^+ cells assumed to be 100%) of $apoE^{-/-}$ mice at 1 hour and 24 hours after DiI-ox-LDL or DiI-native LDL injection. n=5 for DiI-ox-LDL; n=3 for DiI-native LDL; *P=0.0018 vs. 1 hr DiI-ox-LDL.

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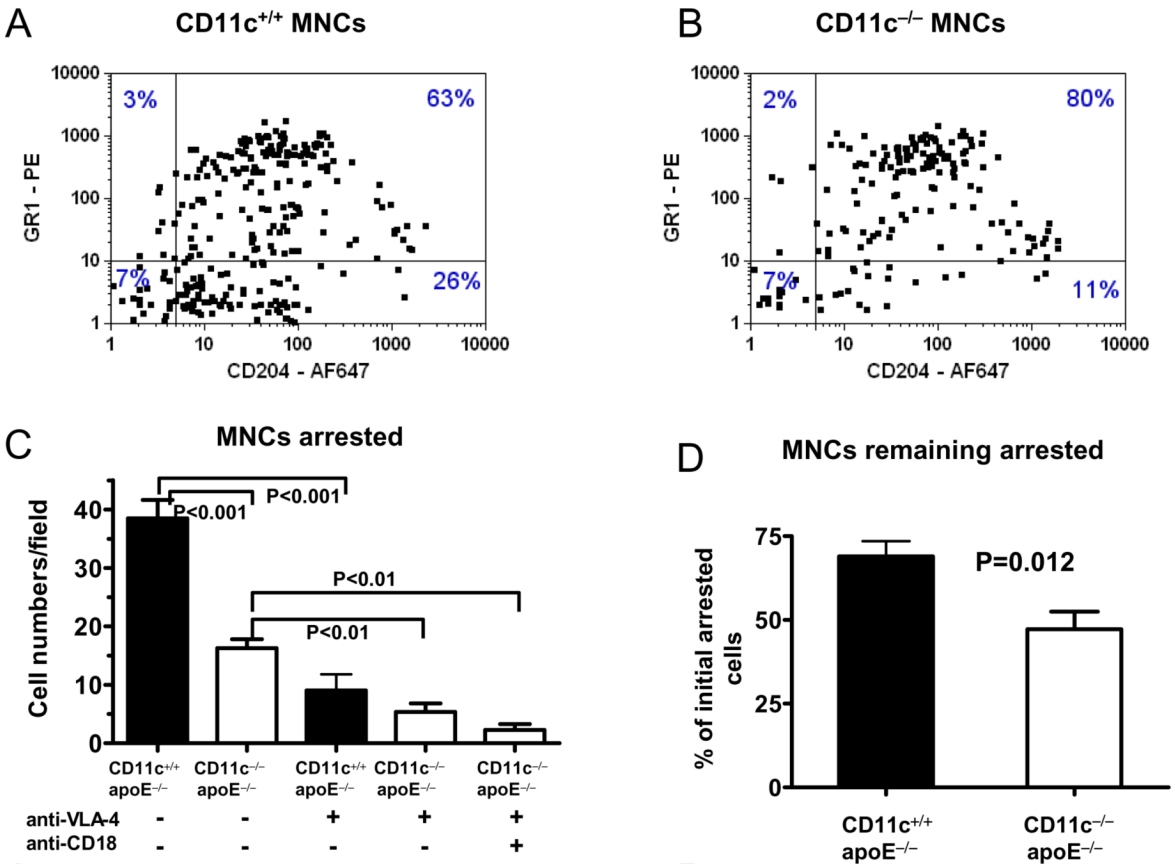

Figure 5. β_1 and $\beta_2$integrin cooperativity in monocyte arrest on E-selectin/VCAM-1 in shear flow

MNCs from blood of CD11c$^{+/+}$/apoE$^{-/-}$ and CD11c$^{-/-}$/apoE$^{-/-}$ mice were perfused over E-selectin/VCAM-1 in a microfluidic flow chamber at 2 dyne/cm^2. A and B: Bivariate plots of the intensity of Gr-1 and CD204 staining on firmly arrested MNCs from CD11c$^{+/+}$/apoE$^{-/-}$ (A) and CD11c$^{-/-}$/apoE$^{-/-}$ (B) mice. C: Number of firmly arrested MNCs per field as a function of the presence or absence of blocking antibodies to VLA-4 and CD18. D: MNCs firmly arrested at 2 dynes/cm^2 were subjected to a stepped increase in shear stress to 10 dynes/cm^2. Percentage of the arrested cells remaining 1 minute after this stepped increase is presented. Data represent mean ± standard error from 3-8 independent experiments.

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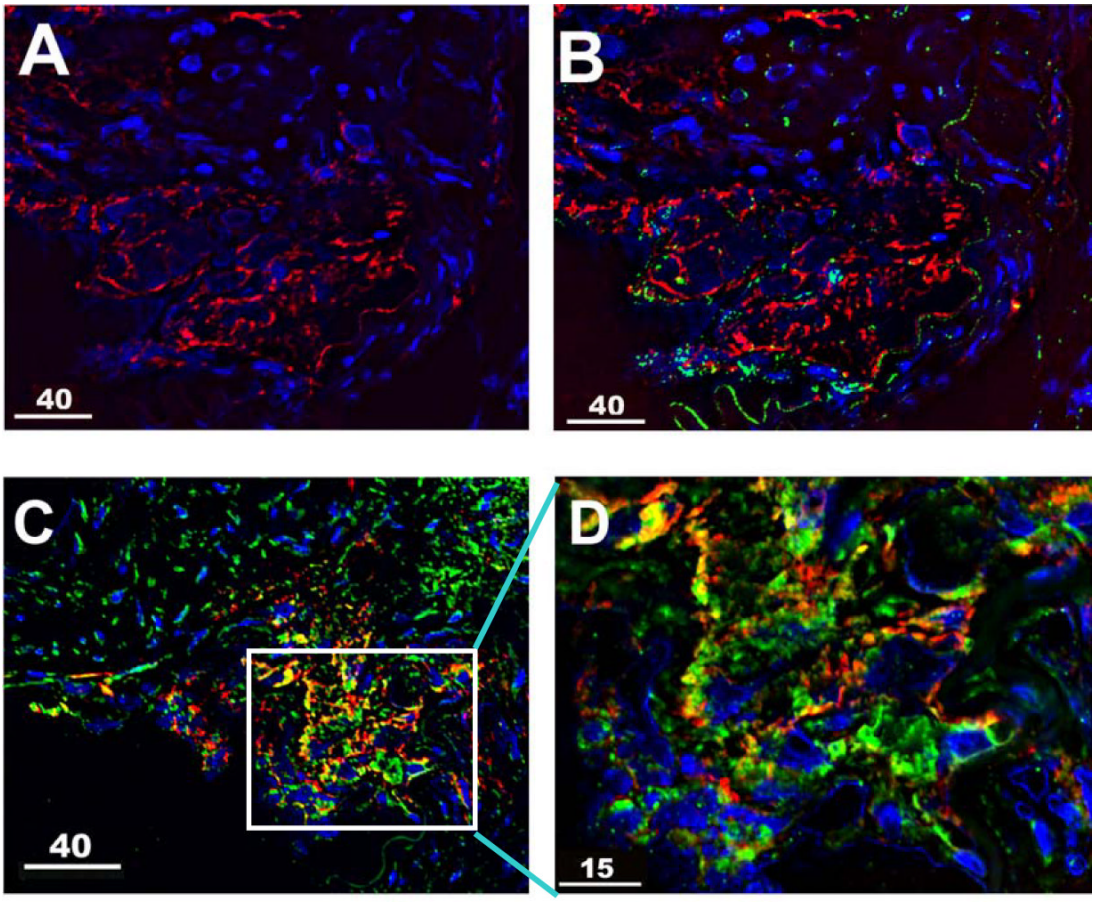

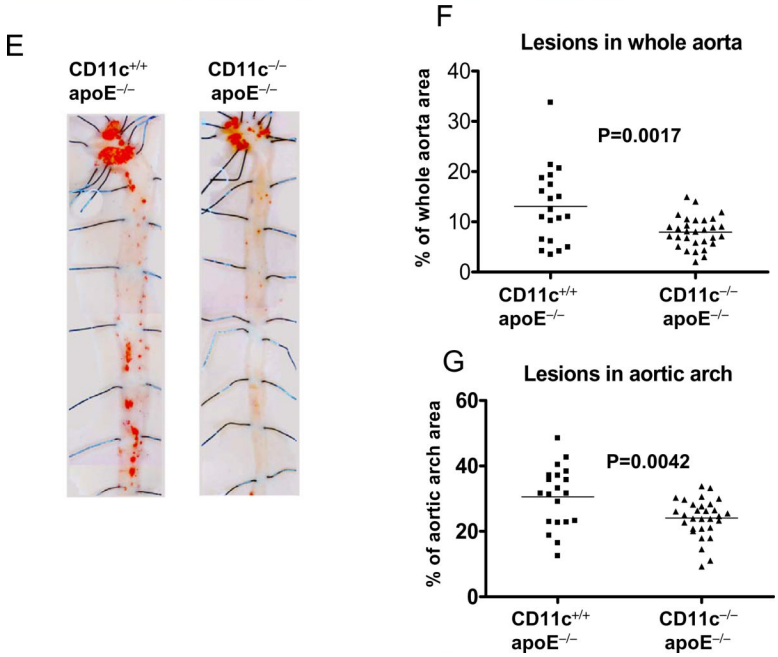

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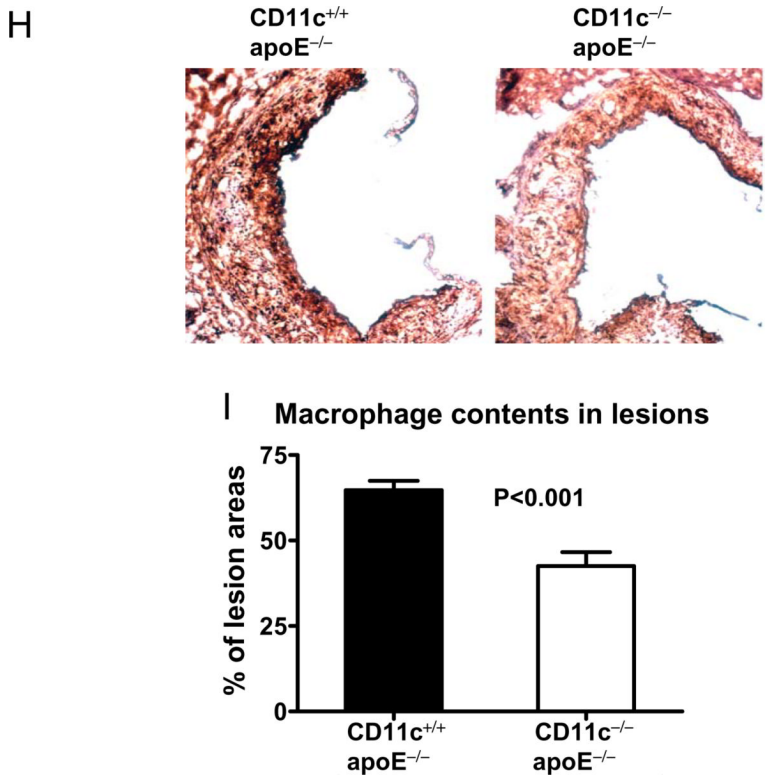

Figure 6. CD11c and atherosclerosis in apoE$^{-/-}$ mice

All samples were collected from mice on HFD for 12 weeks. A-D: Representative immunofluorescence staining on atherosclerotic lesions of CD11c$^{+/+}$/apoE$^{-/-}$ aortas, with (A) PE-anti-mouse CD11c (red), (B) PE-anti-mouse CD11c and FITC-anti-mouse CD205 (green), or (C, D) PE-anti-mouse CD11c and FITC-anti-mouse MOMA-2 (green) mAbs (yellow indicates the overlapping of the red and green), with counterstaining of DAPI (blue) for nuclei. E: Representative Sudan IV staining of mouse aortas (original magnification, × 6.5). F and G: quantification of atherosclerotic lesions in whole aortas (F) and aortic arch (G) (n=20 for CD11c$^{+/+}$/apoE$^{-/-}$, n=30 for CD11c$^{-/-}$/apoE$^{-/-}$). H: Representative macrophage staining (original magnification, × 100), and I: quantification of macrophage contents in atherosclerotic lesions of aortic sinus (n=13 for CD11c$^{+/+}$/apoE$^{-/-}$, n=11 for CD11c$^{-/-}$/apoE$^{-/-}$).

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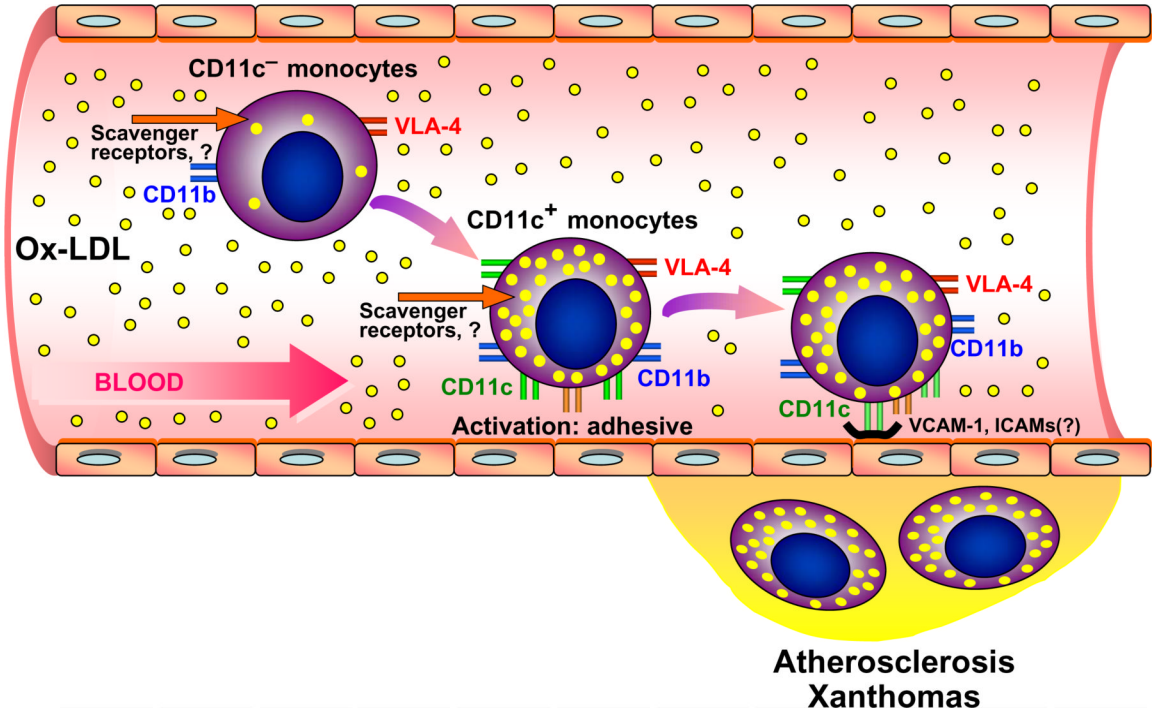

Figure 7. A model for the involvement of CD11c in atherogenesis in hypercholesterolemia

In severe hypercholesterolemia, uptake of ox-LDL by circulating monocytes (through scavenger receptors and undefined pathways) activates monocytes, and drives $CD11c^-$-to-$CD11c^+$ monocyte differentiation, forming $CD11c^+$ "foamy monocytes." These $CD11c^+$ "foamy monocytes" express high levels of VLA-4 and CD11b, and adhere efficiently to ECs through interactions of CD11c and VLA-4 with VCAM-1 on activated ECs, thereby playing an active role in development of atherosclerosis and xanthomas associated with severe hypercholesterolemia.