Leukocyte CD11b expression is not essential for the development of atherosclerosis in mice

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Abstract **CD11b is an α chain of the leukocyte β_2-integrin, Mac-1, which mediates binding and extravasation of leukocytes. Because this event is critical in atherosclerosis, we examined the role of CD11b in lesion formation. Atherosclerosis-susceptible, low density lipoprotein receptor-deficient (LDL-R$^{-/-}$) mice were irradiated and repopulated with bone marrow cells from CD11b-deficient (CD11b$^{-/-}$) mice. After 4 weeks, <2% of the peripheral blood leukocytes of the CD11b$^{-/-}$ bone marrow-transplanted LDL-R$^{-/-}$ mice expressed CD11b, whereas ~25% of the CD11b$^{+/+}$ bone marrow-transplanted LDL-R$^{-/-}$ mice expressed CD11b. After consuming a high-fat diet for 16 weeks the mean lesion aortic valve area, cholesterol accumulation in the aorta, and the degree of intimal macrophage infiltration were similar in mice reconstituted with either CD11b$^{+/+}$ or CD11b$^{-/-}$ bone marrow cells. The studies confirm that CD11b expression of bone marrow-derived cells does not influence the development of atherosclerosis in hypercholesterolemic LDL-R$^{-/-}$ mice.**—Kubo, N., W. A. Boisvert, C. M. Ballantyne, and L. K. Curtiss. **Leukocyte CD11b expression is not essential for the development of atherosclerosis in mice.** ***J. Lipid Res.*** **2000.** 41: **1060–1066.**

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Multiple types of adhesion molecules are used by peripheral blood leukocytes to adhere to and migrate through the endothelium. The integrin family of adhesion molecules participates in cellular recruitment at sites of inflammation in an activation-dependent or -independent fashion (1, 2). Although similar families of integrins are expressed on identical lineages of leukocytes, they functionally identify different populations. For example, myeloid cells of both neutrophils and monocytes in the periphery express β_2-integrins, which share a common β chain (CD18) and one of many noncovalently associated α chains, i.e., CD11a (LFA-1), CD11b (Mac-1), CD11c (p150, 95), and CD11d (3, 4). CD11b is abundant on both neutrophils and monocytes (5), is known as the C3bi complement receptor (6), and is expressed on activated CD8$^+$ T cells (7) as well as natural killer (NK) cells (8). In vitro studies using antibodies specific for the α chains or the common β chain, CD18, indicate that β_2-integrins on monocytes participate in their adherence to endothelial cells (9–11).

Monocytes, T cells (12, 13), and NK cells (14) accumulate in the intima of early fatty streak lesions where monocyte-derived, lipid-rich macrophages predominate (15). The presence of smaller numbers of proliferating and apoptotic intimal macrophages (16) suggests that macrophage foam cell accumulation reflects active migration of cells into and minimal migration out of the vessel wall. A monoclonal antibody specific for CD11b inhibits myelomonocytic cells in vivo in models of inflammation (17). Other molecules participate in monocyte adherence, including very late antigen VLA-4 (a β_1-integrin that interacts with vascular cell adhesion molecule type 1 [VCAM-1]) (18), and the selectin systems (11, 19). It has been shown that P-selectin (20) alone or in combination with E-selectin (21) retards the development of atherosclerotic lesions in vivo, and endothelial cell ICAM-1 staining has been reported (22). However, direct participation of the CD11b integrin or CD11b-positive leukocytes in atherosclerosis has not been explored.

The generation of mice deficient in CD11b has provided an opportunity to study multiple functions of CD11b in vivo (23). We chose the C57BL/6, low density lipoprotein receptor-deficient (LDL-R$^{-/-}$) mouse model (24) to evaluate the role of leukocyte CD11b in atherosclerosis. We used bone marrow transplantation of atherosclerosis-susceptible, LDL-R$^{-/-}$ recipient mice to examine the role of CD11b in intimal monocyte/macrophage accumulation and atherosclerotic lesion formation. Impor-

Abbreviations: AEC, 9-amino-3-ethylene-carbazole; BMT, bone marrow transplantation; BSA, bovine serum albumin; FBS, fetal bovine serum; FITC, fluorescein 5-isothiocyanate; HFD, high-fat diet; ICAM-1, intercellular adhesion molecule type 1; LDL-R, low density lipoprotein receptor; NK, natural killer; PBS, phosphate-buffered saline; VCAM-1, vascular cell adhesion molecule type 1; SPF, specific pathogen free; TLC, thin-layer chromatography.

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tantly, we have shown previously that repopulation of LDL-R$^{-/-}$ mice with LDL-R-expressing wild-type bone marrow cells does not influence their atherogenesis (25).

METHODS

Animals and facilities

Wild-type C57BL/6 mice were obtained from the rodent breeding facility of the Scripps Research Institute (La Jolla, CA). LDL-R$^{-/-}$ mice on a C57BL/6 background were initially purchased from Jackson Laboratories (Bar Harbor, ME) and bred in-house. CD11b-deficient mice on a C57BL/6 background were generated as described (23), and were transferred from the Speros P. Martel Laboratory of Leukocyte Biology (Baylor College of Medicine, Houston, TX) to the specific pathogen-free (SPF) facility of the Scripps Research Institute. Mice were housed at four per cage, were kept on a 12-h light–dark cycle, and were fed ad libitum either a chow diet (diet 5015; Harlan Teklad, Madison, WI) or an atherogenic high-fat diet (HFD) containing 15.8% (w/w) fat, 1.25% (w/w) cholesterol, and no cholate (diet 94059; Harlan Teklad). Routine serologic tests of sentinel mice for specific pathogens remained negative throughout the experimental period. The animals were bled under Metofane-induced anesthesia after a 14-h fast by retroorbital puncture into heparin-coated capillary tubes. All procedures were performed in accordance with institutional guidelines.

Plasma cholesterol analysis

The blood was centrifuged at 3000 *g* for 5 min at 4°C, the cells were used immediately for fluorescence-activated cell sorting (FACS) as described below and the separated plasma was stored at −20°C. Enzymatic measurements of total plasma cholesterol were performed with a kit from Sigma (St. Louis, MO) according to the manufacturer directions.

Bone marrow transplantation

Bone marrow transplantation (BMT) was performed as described previously (25–27). Briefly, twenty-four 6-week-old male LDL-R$^{-/-}$ mice were subjected to a single dose of 1000 rad of total body irradiation to eliminate their bone marrow-derived cells, including stem cells. Donor marrow cells used for repopulation of the recipient irradiated mice were isolated from a male CD11b$^{-/-}$ mouse and a male wild-type CD11b$^{+/+}$ mouse. Four hours after irradiation the LDL-R$^{-/-}$ mice were injected intravenously with 2×10^6 bone marrow cells from either a CD11b$^{-/-}$ and/or a wild-type CD11b$^{+/+}$ donor. The groups were designated CD11b$^{-/-}$ BMT and CD11b$^{+/+}$ BMT mice, respectively. After bone marrow reconstitution, the mice were fed a chow diet for 4 weeks while they were repopulated with donor bone marrow. Four weeks later they were bled and fed the atherogenic HFD for an additional 16 weeks.

Flow cytometry

The cell pellet obtained from 0.1 mL of whole blood was washed in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (2% FBS) and a 0.1-mL suspension incubated at 4°C with 1 μl of Fc receptor blocking solution (Fc block: CD16/32, clone 2.4G2; PharMingen, San Diego, CA) to prevent nonspecific binding. The cells were stained with fluorescein 5-isothiocyanate (FITC)-labeled anti-mouse CD11b (clone Ml/70; Serotec, Raleigh, NC) for 30 min in 70 μl of PBS with 2% fetal bovine serum. The cells were washed and the red blood cells lysed by exposure to 0.14 M ammonium chloride. Data were collected and analyzed by FACS (Becton Dickinson, Mountain View, CA) and CellQuest software. At least 10,000 events were analyzed for forward and side scatter gating and subsequently analyzed for CD11b.

Assessment of atherosclerosis in aortas and aortic valves

Methods used to assess atherosclerosis in aortic valves are detailed elsewhere (28, 29). In brief, the heart was dissected out, cleaned of fascia, and stored at −80°C. O.C.T.-embedded hearts were sectioned in a cryostat until all three leaflets were visible within the aortic valve. From this point, 10-μm sections were collected for the next 300 μm of the valve region, and each section was collected on a Superfrost slide (Fisher Scientific, Tustin, CA). The lipid-rich lesions were visualized by staining the sections with oil red O followed by counterstaining with hematoxylin. A total of five sections taken every 40 μm was used to quantitate individual mouse lesion areas, using a computer-assisted video imaging system. The lesion areas of the five sections were used to calculate the mean lesion area per mouse.

After excising the heart, the aorta was stripped of its adventitial fat and dissected out from the right common carotid artery to the superior mesenteric artery. The aortas were weighed and subjected to lipid extraction as described previously (30). The extracts were used to measure free cholesterol and cholesteryl ester by thin-layer chromatography (TLC) as described (30). Cholesterol and cholesteryl ester standards were applied at 20, 10, 5, 2.5, and 1.25 μg per lane to enable quantitation of the bands. The free cholesterol and cholesteryl ester bands were scanned with a laser scanning densitometer and quantitated with ImageQuant software (30).

Immunohistochemistry

Heart tissue cryosections were fixed in acetone at −20°C for 2 min and immersed in PBS for 5 min to rehydrate the tissues. All further incubations were performed at room temperature in a humid chamber, except for the incubation with primary antibodies. After blocking endogenous biotin in the sections by use of a blocking kit (Vector Laboratories, Burlingame, CA), the sections were incubated for 30 min with normal rabbit serum (Dako, Carpinteria, CA) diluted in PBS containing 0.5% bovine serum albumin (BSA). After blot drying, the sections were incubated at 4°C with a 100-ng/mL dilution of the mouse macrophage-specific monoclonal antibody MOMA-2 (Serotec), or with a 1:100 dilution of anti-CD11b antibody (clone M-70.15.11.H2.5; a generous gift from R. Hyman, Salk Institute, La Jolla, CA). After a thorough wash, endogenous peroxidase was blocked for 2 min with a blocking agent (Zymed, South San Francisco, CA). The slides were incubated with biotinylated rabbit anti-rat IgG at 10 μg/mL, and exposed to Vectastain ABC Elite solution (Vector Laboratories) for 30 min and developed with 9-amino-3-ethylenecarbazole (AEC; Vector Laboratories). Sections were counterstained with hematoxylin and mounted with an aqueous mounting medium (Shandon Lipshaw, Pittsburgh, PA).

Statistical analysis

Lesion areas in the aortic valve were compared between the two groups of mice with the Mann–Whitney U test.

RESULTS

To examine the in vivo role of CD11b expression in atherosclerosis, male LDL-R$^{-/-}$ mice were subjected to lethal amounts of total body irradiation and repopulated with

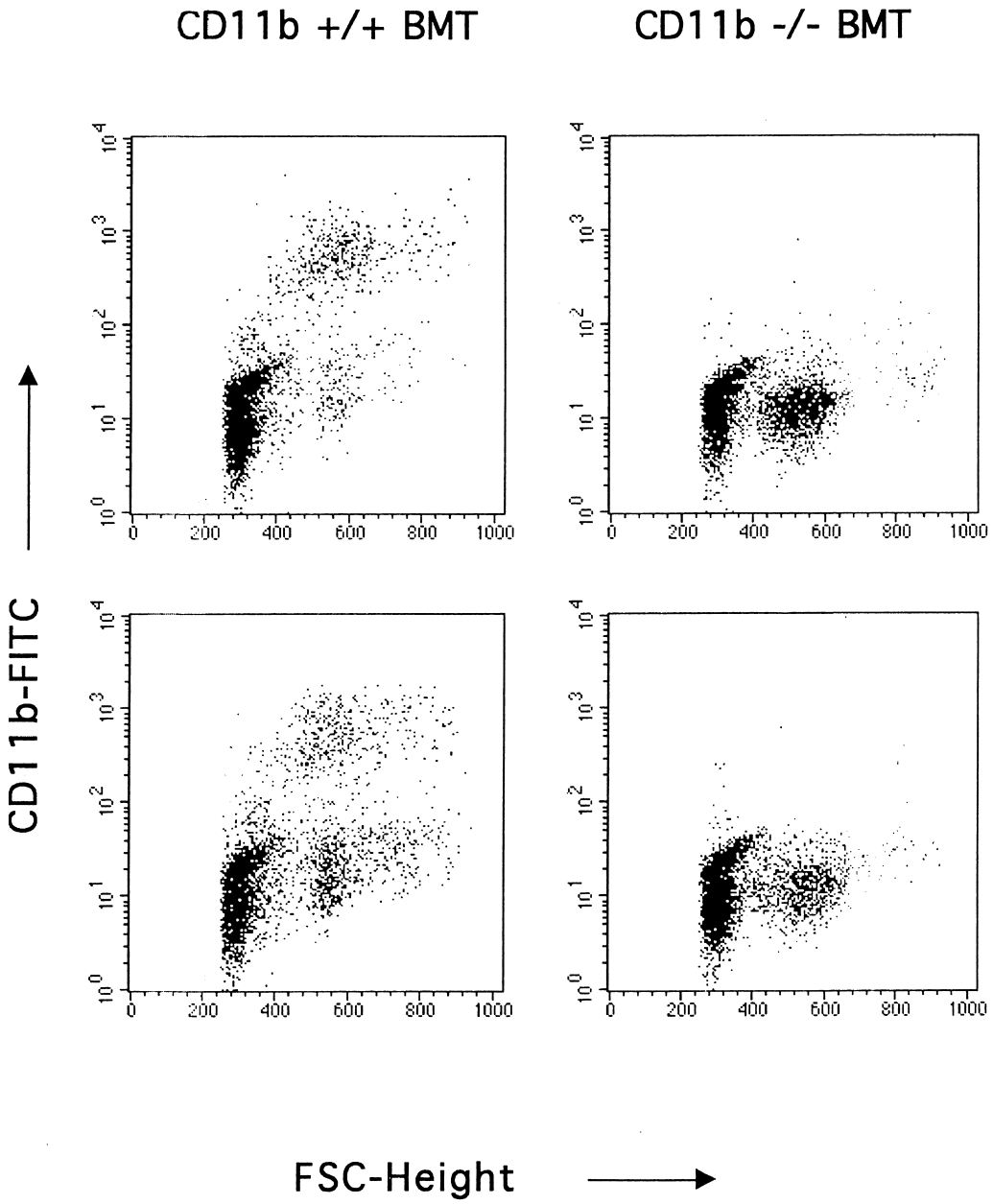

Fig. 1. Flow cytometric (FACS) analysis of CD11b^{+} peripheral blood leukocytes. Peripheral blood was obtained 4 weeks after bone marrow transplantation from all mice. Shown are the analyses from two representative male LDL-R$^{-/-}$CD11b$^{+/+}$ BMT mice (left) and two representative male LDL-R$^{-/-}$CD11b$^{-/-}$ BMT mice (right). The leukocytes were stained with antibody to CD11b and analyzed by FACS. Note that the proportion of cells that stained positive for CD11b (FITC intensity $>10^2$) in the CD11b$^{+/+}$ BMT mice were absent in the CD11b$^{-/-}$ BMT mice.

donor bone marrow cells isolated from either male CD11b-deficient (CD11b$^{-/-}$) or male wild-type (CD11b$^{+/+}$) mice. After consuming a chow diet for 4 weeks, FACS analysis of the peripheral blood leukocytes confirmed that <2% of the leukocytes from CD11b$^{-/-}$ BMT mice expressed CD11b. In contrast, normal numbers (~25%) of leukocytes expressed CD11b in the CD11b$^{+/+}$ BMT recipients (**Fig. 1**). This analysis, which was performed on all animals, verified successful donor-specific repopulation of all transplanted animals. Moreover, CD11b status did not affect the number of total circulating leukocytes. Total leukocyte counts were not different between the two groups 4 weeks after the BMT (2,759 ± 1,212 for CD11b$^{+/+}$ BMT and 3,250 ± 289 for CD11b$^{-/-}$ BMT mice). All mice survived the study and appeared healthy at time of death.

When successful bone marrow reconstitution was confirmed, the plasma cholesterol levels were examined. The plasma cholesterol levels were between 200 and 400 mg/dL (**Fig. 2**). After the mice were fed a HFD for an additional 16 weeks, all animals responded to the HFD with elevated total cholesterol levels of greater than 1,000 mg/

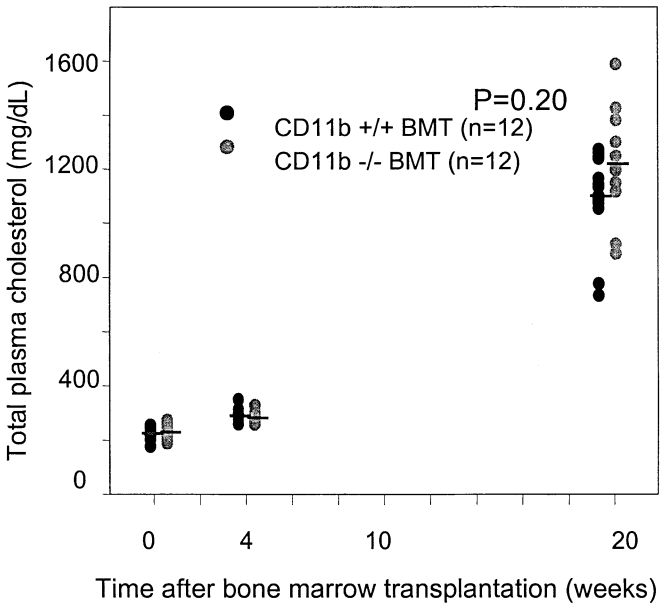

Fig. 2. Plasma total cholesterol levels in irradiated male LDL-R$^{-/-}$ recipient mice transplanted with CD11b$^{-/-}$ (n = 12) versus CD11b$^{+/+}$ (n = 12) donor bone marrow cells. The animals received the irradiation and bone marrow reconstitution at time 0 and were fed a chow diet. Four weeks later, the animals were bled and fed a high-fat diet for an additional 16 weeks. All animals were bled and killed at 20 weeks. $P \geq 0.200$ for total cholesterol levels of CD11b$^{+/+}$ BMT versus CD11b$^{-/-}$ BMT animals at 0, 4, and 20 weeks.

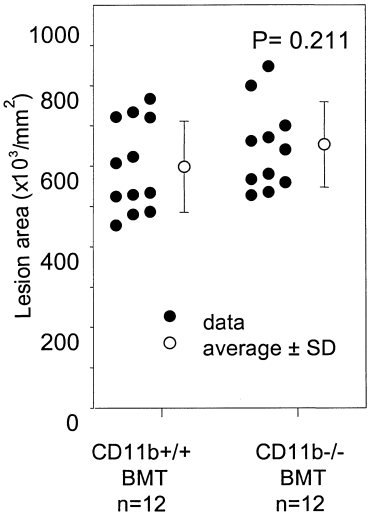

Fig. 3. Quantification of the size of aortic valve lesion areas in individual mice. Five oil red O-stained valve lesion areas were quantitated from each animal, using a computer-assisted video imaging system as described in Materials and Methods. $P = 0.211$ for mean lesion areas of CD11b$^{+/+}$ BMT versus CD11b$^{-/-}$ BMT animals at 20 weeks.

dL. Moreover, these levels remained similar between the two groups throughout the study (Fig. 2).

The mean aortic valve lesion area of each animal was measured after the animals had consumed the HFD for 16 weeks. Oil red O-stained serial sections of each frozen heart were examined by computer-assisted video imaging. As shown in **Fig. 3**, where each point represents the mean lesion area of five valve sections from each mouse, the average lesional area of the CD11b$^{+/+}$ BMT mice was 583,220 ± 112,760 μm^2 (n = 12) compared with 642,150 ± 105,990 μm^2 for the CD11b$^{-/-}$ BMT mice (n = 12), $P = 0.211$. Thus, no differences in aortic valve lesion areas were observed between the two groups of bone marrow-transplanted mice.

The entire aorta from the aortic arch to the iliac bifurcation was isolated from each mouse and subjected to lipid extraction. The extracts were used to quantitate the amount of free cholesterol and cholesteryl ester accumulation in the aorta via TLC analysis. The mean free cholesterol in the aorta was 2.8 ± 0.6 and 3.1 ± 0.6 μg/mg of tissue and the mean cholesteryl ester was 4.7 ± 1.8 and 5.1 ± 1.6 μg/mg of tissue in the CD11b$^{+/+}$ BMT mice and CD11b$^{-/-}$ BMT mice, respectively. Neither the free cholesterol nor the cholesteryl ester accumulation in the aorta was statistically different between the two groups.

Our qualitative estimate of the degree of lipid accumulation, as assessed by oil red O staining of the aortic valve lesions, was indistinguishable as well (**Fig. 4**). The lesions were morphologically fibrofatty streaks with proliferating smooth muscle caps and necrotic cores. Macrophage infiltration, identified by the macrophage-specific MOMA-2 antibody staining, was similar in both groups (**Fig. 5A** and **C**). Yet, as expected, staining with anti-CD11b showed a pattern of macrophage distribution that was similar to the MOMA-2 staining in the CD11b$^{+/+}$ BMT group (Fig. 5B) but showed only background staining with the CD11b$^{-/-}$ BMT group (Fig. 5D). This confirmed that leukocyte-specific expression of CD11b had no influence on either macrophage trafficking or the extent of atherosclerosis in the male LDL-R$^{-/-}$mice fed a HFD.

DISCUSSION

Monocyte adherence is mediated by the binding of highly regulated cell surface adhesion molecules. The β_2-integrins and their ligand interactions participate in the attachment of monocytes to endothelial cells (1, 2). Experiments using antibodies specific for CD11b established that CD11b as well as LFA-1 are members of the CD18 integrins on monocytes that participate in monocyte adherence to endothelial cells (9–11). The role of β_2-integrins as well as VLA-4 (the integrin that interacts with VCAM-1) in endothelial cell adherence has been demonstrated in vitro with the use of blocking antibodies specific for common β or individual α subunits (18). In addition, passive transfer of a monoclonal antibody specific for CD11b (CR3) inhibits myelomonocytic cells in an animal model of inflammation (17). These studies support a role for cell adhesion molecules in the firm attachment of monocyte/macrophages to endothelial cells at inflammatory sites.

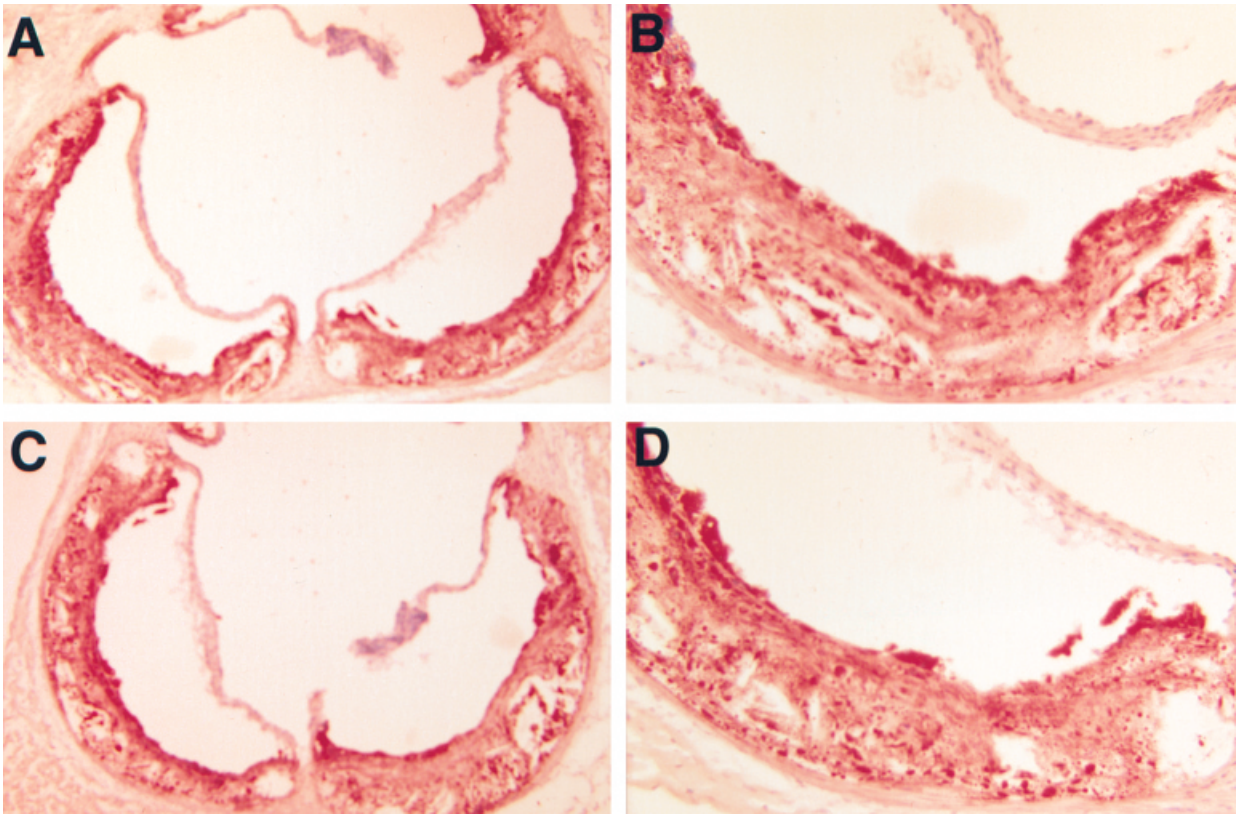

Fig. 4. Lipid accumulation and macrophage infiltration of the aortic valve lesion of LDL-R$^{-/-}$ recipient mice transplanted with CD11b$^{+/+}$ (A and B) versus CD11b$^{-/-}$ (C and D) donor bone marrow. Representative aortic valve sections (10 μm) were stained for lipid with oil red O. The red staining shows neutral lipid accumulation in the lesions. Original magnification: (A and C) ×100; (B and D) ×250.

However, previous in vitro studies are complicated by the possibility that tissue culture exposure conditions can alter the expression and activation of adhesion molecules. The in vivo studies are limited by clearance of the passive antibodies. Thus, although valuable, these observations are less reliable in identifying the key molecules that mediate leukocyte adhesion in vivo in atherosclerosis.

We utilized a bone marrow chimera model of atherosclerosis-susceptible LDL-R$^{-/-}$ mice to examine the role of leukocyte-specific CD11b in lesion development. Examination of aortic valves for atherosclerotic lesions revealed that the mean lesion areas as well as the degrees of macrophage infiltration were similar in mice that did or did not express leukocyte CD11b. Free and esterified cholesterol accumulation in the aortas also were similar between the two groups. Thus, leukocyte CD11b expression did not affect monocyte/macrophage accumulation and lesion development in the bone marrow-transplanted hypercholesterolemic LDL-R$^{-/-}$ mice. This strongly suggested that leukocyte CD11b plays a minor or a redundant role in atherosclerosis.

The accumulation of cells into inflammatory tissues involves multiple steps that require a variety of adhesion and adhesion-related molecules including selectins, integrins, and chemokines (30). In atherosclerotic lesions the balance between migration (entry) and emigration (exit) of cells can influence the extent of retention of each particular cell type. Thus, if cell entry were suppressed in the CD11b$^{-/-}$ BMT mice, a reduction in monocyte/macrophage accumulation would be expected, particularly in light of evidence that cell proliferation and cell emigration or death are uncommon (16). However, because cellular accumulation was not suppressed in CD11b$^{-/-}$ BMT mice, other integrin pathways must be operative. CD11b has overlapping functions with other CD18 integrins and other pathways are clearly sufficient for the accumulation of monocyte/macrophages in lesions. Interestingly, it has been reported that mice deficient in CD18, i.e., that lack all the CD11/CD18 integrins, are resistant to diet-induced atherosclerosis (31). These results, in conjunction with our findings, suggest that other CD11/CD18 molecules must participate in lesion progression (presumably via cell recruitment to the lesional area). Because ICAM-1 deficiency also resulted in reduced atherosclerosis (31) and ICAM-1 interacts with LFA-1, perhaps LFA-1 is intimately involved in atherosclerosis. Further studies are needed to confirm this observation. The cell recruitment picture is further complicated by the findings that selectins (20, 21) as well as chemoattractant receptors such as CXCR-2 (27) and CCR2 (32) have a significant impact on the disease process.

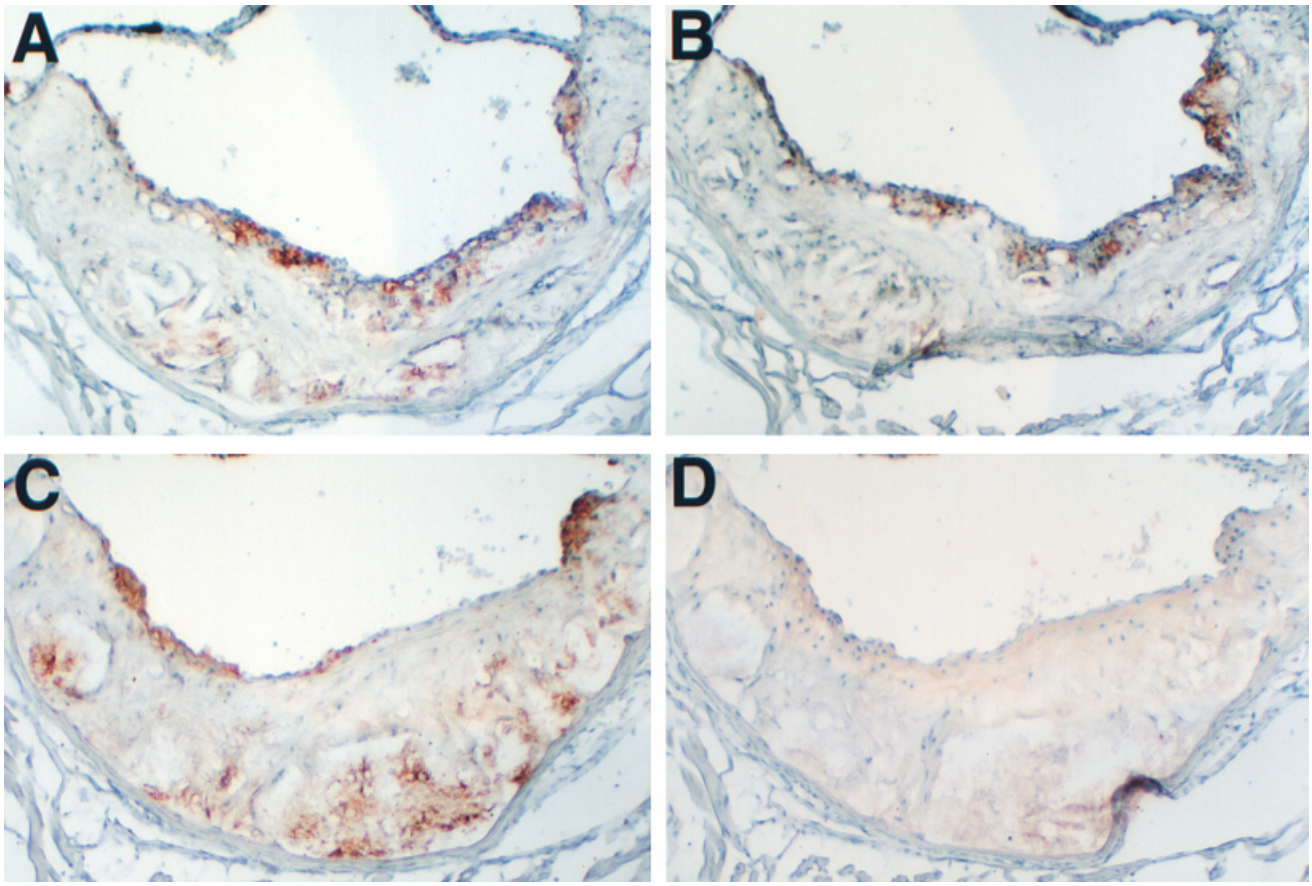

Fig. 5. Immunohistochemical detection of MOMA-2- and CD11b-positive cells in the aortic valve. Representative serial sections from a $CD11b^{+/+}$ BMT mouse (A and B) or $CD11b^{-/-}$BMT mouse (C and D) were stained with either MOMA-2 antibody (A and C) or anti-CD11b antibody (B and D). Whereas MOMA-2-positive cells were distributed similarly between the two groups, CD11b-positive cells were visible only in the sections of $CD11b^{+/+}$ BMT mice.

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