Immune-deficient Mice Develop Typical Atherosclerotic Fatty Streaks when Fed an Atherogenic Diet

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Abstract

Inbred strain C57BL/6J mice develop typical atherosclerotic fatty streaks in the aorta after 15 wk on a high fat, high cholesterol diet. To investigate the effects of the immune system on the development of fatty streaks in this model, C57BL/6J mice with a normal immune system were compared with C57BL/6J mice carrying mutations resulting in various immune deficiencies. These included mice with severe combined immune deficiency, athymic "nude" mice, class I MHC deficient mice, and class II MHC deficient mice. Despite similar lipoprotein profiles, lesion development in the immune compromised strains was similar to or increased compared with normal C57BL/6J mice. Class I MHC deficient mice demonstrated a threefold increase in lesion area (22,961±6,653 vs 8,868±1,817 μm^2, $P = 0.01$). Immunohistochemical analysis of lesions showed characteristic features of atherosclerosis with vascular cell adhesion molecule-1 expression, immunoglobulin deposition, monocyte infiltration, and smooth muscle cell proliferation. These data indicate that the classical immune system, while not essential for atherosclerotic fatty streak development, may act to suppress the development of lesions. (*J. Clin. Invest.* 1994. 94:2516–2520.) Key words: atherosclerosis • mouse models • lymphocytes • immune system

Introduction

Atherosclerosis is a disease of the large arteries that is the major cause of heart disease and stroke. Early atherosclerotic lesions, termed fatty streaks, consist of subendothelial accumulations of plasma lipoprotein-derived cholesterol and leukocytes, primarily monocyte-macrophages, which have crossed the endothelial barrier. Advanced lesions are characterized by the presence of a fibrous "cap" consisting of connective tissue and smooth muscle cells and are frequently associated with calcification. Several lines of evidence suggest that the immune system is involved in atherogenesis. First, atherosclerotic lesions frequently contain activated T lymphocytes, in addition to monocyte-macrophages (1), which express the memory phenotype (2). Second, immunoglobulins are associated with lesions, and antibodies specific for oxidized low density lipoproteins (LDL) commonly occur in human and animal models (3). Such oxidized LDL species are present in fatty streak lesions, and it has been suggested that the uptake of LDL by macrophages and smooth muscle cells to generate foam cells may be mediated in part by interactions involving immunoglobulins (4). Third, endothelial cells and vascular smooth muscle cells express both class I and II MHC antigens. In particular, endothelial cells exhibit inducible class II MHC molecule expression (5), allowing them to act efficiently as antigen presenting cells (6), and the expression of class II MHC on endothelium has been associated with local infiltration of lymphocytes and monocyte-macrophages into atherosclerotic lesions (1). Fourth, the cellular immune system is clearly involved in the development of transplant coronary disease, a progressive, diffuse atherosclerotic process occurring in heart allografts (7).

To test the role of various immune functions in atherogenesis, we used four naturally occurring or gene-targeted mouse models exhibiting specific immune deficiencies. These included: first, mice with severe combined immunodeficiency (SCID)[1] which lack both T and B lymphocyte populations (8), although natural killer cell and monocyte-macrophage activities are normal (9); second, athymic (nude) mice which lack cellular immunity but retain normal or near normal serum immunoglobulins (10); third, class I MHC deficient mice, created by gene targeting of the β_2 microglobulin locus (11), which lack cytolytic T cells and have impaired natural killer cell activity (12); and, fourth, class II MHC deficient mice, created by gene targeting of mouse Ia genes, which lack $CD4^+$ T helper cells and are unable to respond to T cell–dependent antigenic stimuli, while maintaining normal B lymphocyte numbers and near normal serum immunoglobulin levels (13, 14). Each of these mutations was carried on the genetic background of the inbred strain C57BL/6, a strain susceptible to the development of fatty streak lesions when maintained on an atherogenic diet (15). Using these immune deficient mice, we now report studies indicating that the early stages of atherogenesis do not require either cellular or humoral immune function, which may act to suppress lesion development.

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Received for publication 10 June 1994 and in revised form 18 August 1994.

J. Clin. Invest.

0021-9738/94/12/2516/05 $2.00
Volume 94, December 1994, 2516–2520

1. *Abbreviations used in this paper:* SCID, severe combined immunodeficiency; VCAM-1, vascular cell adhesion molecule-1.

Methods

Female C57BL/6 wild-type, SCID and nude mice were obtained from The Jackson Laboratories (Bar Harbor, ME). SCID mice were obtained at 6 wk of age and placed on the atherogenic diet, as older SCID mice can become "leaky" with demonstrable immunoglobulin production (8). C57BL/6 MHC class I and II deficient mice (14) were obtained from GenPharm International (Mountain View, CA). At ~ 3 mo of age, mice carrying the four immune deficiency mutations, as well as C57BL/6J control mice, were transferred from a low fat chow diet to an atherogenic diet (Food-Tek, Morris Plains, NJ) containing 15% saturated fat, 1.25% cholesterol, and 0.5% cholic acid as previously described (15, 16). Animals were kept in accordance with standard animal care requirements, housed in autoclaved filter top cages with autoclaved water. Mice were maintained on a 12-h light-dark cycle, and animals remained healthy for the duration of the study. Mice were bled at the time of initiation of the feeding of the atherogenic diet and immediately before time of death to monitor levels of plasma lipoproteins by retro-orbital puncture. Total plasma cholesterol, high density lipoprotein (HDL) cholesterol, and total triglycerides were measured as described previously (16). The lack of circulating immunoglobulins G and M was confirmed in the SCID mice by immunoblotting serum with antibodies to mouse IgG and IgM (Pierce, Rockford, IL) using methods previously described (17).

After 15 wk on the atherogenic diet, the mice were killed, and the basal half of the ventricles and ascending aorta were removed, embedded in OCT (Tissue Tek, Elkhart, IN), frozen on dry ice, and stored at −70°C until sectioned. Serial 10-μm sections were collected on poly-D-lysine–coated slides. Sections were stained with oil red O and hematoxylin, counterstained with fast green, and examined by light microscopy. Lesion area was calculated using serial sections of the first 400 μm of the ascending aorta and an eyepiece grid by an observer blinded to the animal groupings. Groups were compared using the Student's t test with significance defined at $P < 0.05$.

Immunohistochemical analysis was performed as described previously (18). Briefly, cryostat sections (10 μm) were fixed in acetone or 4% paraformaldehyde, incubated with primary antibodies to mouse macrophages (monoclonal rat anti–mouse Mac-1 from Boehringer Mannheim, Indianapolis, IN; monoclonal rat anti–mouse F4/80 from Serotec Ltd., Oxford, UK), mouse immunoglobulin M (monoclonal rat anti–mouse from Zymed Labs, Inc., South San Francisco, CA), mouse immunoglobulin G (monoclonal rat anti–mouse from Zymed Labs, Inc.), smooth muscle tropomyosin (polyclonal rabbit anti–chicken tropomyosin from Sigma Immunochemicals, St. Louis, MO), vascular cell adhesion molecule-1 (VCAM-1) (monoclonal rat anti–mouse VCAM-1 from PharMingen, San Diego, CA), and apolipoprotein B (monospecific polyclonal rabbit anti–rat apolipoprotein B [18]). After three washes in PBS, the sections were processed using an avidin-biotin peroxidase technique (ABC; Vector Labs, Inc., Burlingame, CA) and counterstained with hematoxylin. Controls included omission of the primary antibody and use of nonimmune sera.

Results

Strain C57BL/6 mice fed a high cholesterol, high saturated fat diet containing cholic acid develop hypercholesterolemia associated with the development of aortic and coronary atherosclerotic plaques (15, 16, 18). To investigate the role of the cellular and humoral immune system on the development of atherosclerosis, we used four spontaneous or gene-targeted mice strains with immune deficiencies. SCID C57BL/6 mice lack both cellular- and antibody-mediated immunity at a young age; nude or athymic mice lack a cellular immune system; class I MHC deficient mice also lack T cytotoxic/suppressor lymphocytes; and class II MHC deficient mice lack T helper lymphocytes, but may also be expected to have defects in antigen presentation by macrophages and endothelial cells. The SCID mice were demonstrated to lack significant circulating immunoglobulins G and M at the time of death as determined by immunoblotting (data not shown).

The levels of plasma lipoproteins in mice maintained on the chow diet were not significantly different among the five groups of animals, exhibiting the following values (mean±SE): total cholesterol, 73±3 mg/dl; HDL cholesterol, 49±2 mg/dl; and total triglycerides, 35.3±2.1 mg/dl. After 15 wk on the atherogenic diet, the levels of total cholesterol and non-HDL cholesterol increased greatly, whereas the levels of HDL cholesterol decreased slightly. With the exception of the nude mice, the levels of lipoproteins did not differ significantly among the groups. Thus, among the four groups, excluding the nude mice, the animals exhibited the following values: total cholesterol, 250±9 mg/dl; HDL-cholesterol, 47±2 mg/dl; and total triglycerides, 6.2±0.5 mg/dl. The nude C57BL/6J mice had significantly lower total cholesterol levels (200±5 mg/dl, $P < 0.005$ vs control mice), whereas HDL-cholesterol and triglyceride levels were similar to the other groups. The explanation for the altered levels of lipoproteins in nude mice is unknown, although immune function has been associated previously with alterations in lipoprotein metabolism (19, 20).

Atherosclerotic lesion development was quantitatively evaluated in serial 10-μm-thick cryosections of the first 400 μm of the ascending aorta as previously described (15, 16, 18). Sections were stained for lipid with oil red O, and the mean lesion area per section was determined. Table I compares the lesion area in the four immunologically deficient strains, the C57BL/6J background strain, and the relatively atherosclerosis-resistant C3H/HeJ strain, the latter data added as a negative control. The lesion areas in the four SCID mice and the five nude mice were not significantly different from those of the C57BL/6J background strain. In class I MHC deficient mice, there was a threefold increase in aortic lesion area compared with C57BL/6J control mice (22,960±6,650 vs 8,870±1,760 μm^2/section, $P = 0.01$). Class II MHC deficient animals demonstrated similar lesion area to the C57BL/6J background strain. All five C57BL/6J groups exhibited significantly greater lesion development than the resistant C3H/HeJ strain or strain C57BL/6J mice fed a chow diet (Table I).

The lesions in each of the C57BL/6J immune deficient mice resembled control C57BL/6J mice with respect to lesion composition. Using previously described immunohistochemical procedures (18), macrophage antigens (Mac-1 and F4/80) (Fig. 1 *H*) and apolipoprotein B, the major protein component of LDL (data not shown), were observed to colocalize with lipid staining in lesions of each group of C57BL/6J mice maintained on the atherogenic diet. The lesions were also positive for the adhesion molecule VCAM-1 (Fig. 1 *B*) which has been observed previously in human coronary atherosclerotic plaques (21) and rabbit atheroma (22). The expression of VCAM-1 was seen in both endothelium and vascular smooth muscle cells as is prominent in mouse atherosclerotic plaques (18) and in rabbit advanced lesions (23). The lesions of all the groups except the SCID mice exhibited prominent accumulation of IgM (Fig. 1, *F* and *G*). The absence of such staining in SCID mice was expected since they also lacked circulating immunoglobulins G and M. Some of the lesions in strain C57BL/6J mice became clearly "raised" as compared with the flanking regions

Table I. Extent of Aortic Atherosclerotic Lesions in Control and Immune Deficient Mice

Mouse strain, Diet	Aortic lesion area*	Raised lesions‡	Advanced lesions§
	μm^2/section		
C57BL/6J, Chow (n = 7)	18±18	0/7 (0%)	0/7 (0%)
C3H/HeJ, Atherogenic (n = 30)	619±151	0/30 (0%)	0/30 (0%)
C57BL/6J, Atherogenic (n = 15)	8868±1817	11/15 (73%)	1/15 (7%)
SCID C57BL/6J, Atherogenic (n = 4)	16180±7300	3/4 (75%)	1/4 (25%)
Nude C57BL/6J, Atherogenic (n = 5)	7436±1309	4/5 (80%)	0/5 (0%)
Class I MHC deficient, Atherogenic (n = 9)	22961±6653‖	9/9 (100%)	2/9 (22%)
Class II MHC deficient, Atherogenic (n = 17)	6581±1287	16/17 (94%)	3/17 (18%)

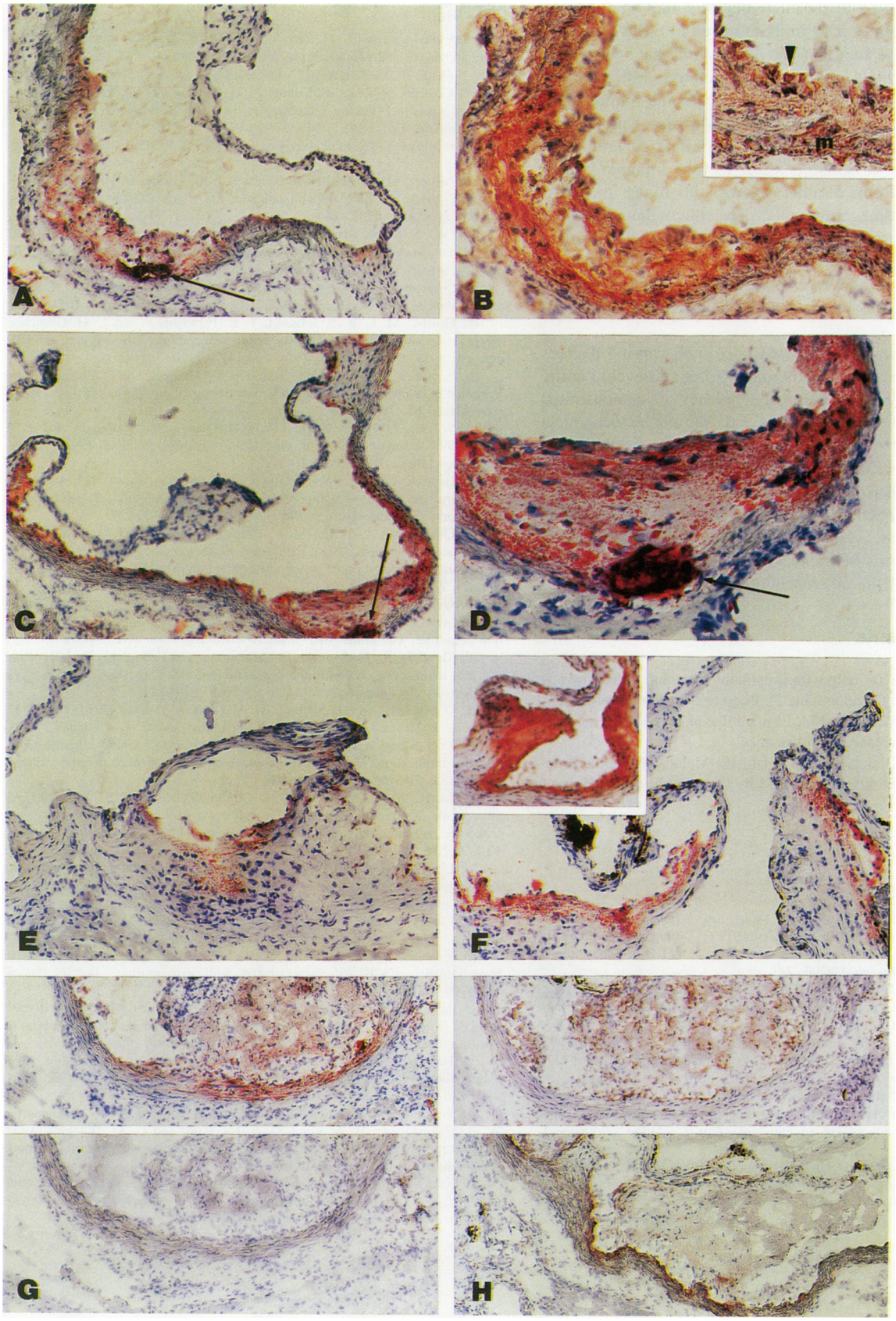
* Aortic lesion area is the mean area per section exhibiting lipid staining with oil red O. Mean±SE. ‡ Raised lesions were identified histologically as fatty lesions which were raised when compared with the flanking regions of the aortic wall. Mice exhibiting one or more raised lesions were considered positive. Values are expressed as the number of positive animals/total (percent positive). § Advanced lesions were identified as lesions exhibiting a fibrous cap staining positively with the smooth muscle cell antigen tropomyosin. Mice exhibiting one or more advanced lesions were considered positive. Values are expressed as the number of positive animals/total (percent positive). ‖ P = 0.01 vs controls.

of the aortic wall (for example, Fig. 1, *A* and *C*), and a fraction of these appeared relatively "advanced" as judged by the presence of a fibrous cap that stained positively for the smooth muscle antigen tropomyosin (Fig. 1 *B*). Neither the C57BL/6J mice maintained on a chow diet nor the atherosclerosis resistant C3H/HeJ mice maintained on the atherogenic diet exhibited raised or advanced lesions (Table I). All four groups of immune deficient mice exhibited a similar frequency of raised lesions as the C57BL/6J background strain (Table I), and, with the exception of nude mice, all exhibited relatively advanced lesions (Table I). The failure to observe advanced lesions in the nude mice could well be due to the small number of animals studied (Table I) or the lower total plasma cholesterol noted in the nude mice. Staining of normal medial smooth muscle cells was observed with the anti-tropomyosin antibody, and serial sections did not stain with isotypic secondary antibodies alone (data not shown). A number of the lesions exhibited calcification (Fig. 1, *A, C,* and *D*). The identity of the calcium deposits was confirmed by alizarin red S and von Kossa techniques as described previously (18) (data not shown).

Discussion

The development of atherosclerotic lesions in immune deficient mice with typical lipid deposition, adhesion molecule expression, cellular infiltration, calcification, and smooth muscle cell proliferation suggests that a complete immune system is not required for at least the early stages of atherogenesis. Thus, the presence of immunoglobulins and T lymphocytes in atherosclerotic lesions is unlikely to be causal in the disease process. In our study, high levels of IgM were present in the lesions of all strains examined with the expected exception of SCID mice; nevertheless, SCID mice exhibited about the same spectrum of lesion develoment as the C57BL/6J background strain (Fig. 1). Immunohistochemical studies indicated that lymphocytes are minor cellular constituents of aortic lesions in mice, the major infiltrating cells being monocyte-macrophages (18). The recruitment of monocyte-macrophages and their transformation to foam cells, as well as the proliferation of smooth muscle cells in a subset of lesions, was not impaired in the immune deficient mice. Therefore, these processes are likely to be mediated by inflammatory mechanisms involving the vascular endothelium rather than the cellular and humoral aspects of the classical immune system. It is noteworthy that athymic mice and irradiated mice, the latter resembling SCID immune deficient mice, demonstrate normal macrophage function in response to infection (24). Our results suggest that the immune system plays a modulating role in atherogenesis and that it may exert a suppressive effect on atherosclerosis development. The small number of SCID mice examined showed a nonsignificant trend toward an increase in lesion area. Likewise, the nude mice, while demonstrating similar lesion area, did so with a significantly lower total plasma cholesterol, suggesting that the lack of cellular immunity may be associated with an increase in lesion development. The small number of SCID and nude mice examined significantly limits our ability to precisely define the relative contributions of humoral and cellular immunity to lesion formation and deserves further study. Although the increase in lesions in class I MHC deficient mice requires confir-

Figure 1. Atheromatous lesions in susceptible C57BL/6J control mice on an atherogenic diet for 15 wk (*A* and *B*) and C57BL/6J immune deficient mice (*C–H*). *A, C, D, E,* and *F* and the upper part of *G* were stained with oil red O, hematoxylin, and fast green. *B,* the inset of *F,* the lower part of *G,* and *H* were stained using an immunoperoxidase technique and counterstained with hematoxylin (18). *A* shows a large atheromatous lesion that occupies all layers of the aortic wall with calcification (*arrow*) at the base (C57BL/6J, ×33). *B* shows a sequential section of the same vessel as in *A* showing intense staining for VCAM-1 in the intima and media. The inset shows staining for smooth muscle cell tropomyosin in the intima (*arrowhead*) and media (*m*) (C57BL/6J, ×50). *C* shows extensive lipid accumulation in the aortic valve attachments and aortic free wall with calcification (*arrow*) (class I MHC deficient, ×25). *D* shows a higher magnification of the same lesion as *C* showing calcification (*arrow*) (class I MHC deficient, ×66). *E* shows lipid deposition in the base of the aortic sinus in C57BL/6J nude mice (×33). *F* shows atheromatous lesions in valve attachments in class II MHC deficient mice (×33). The inset shows positive staining of the adjacent section for IgM (×25). *G* shows a large lipid-rich lesion showing cellular proliferation in C57BL/6J SCID mice (*top,* ×25) with negative staining for IgM μ chain (*bottom,* ×25). *H* shows numerous Mac-1–positive cells within SCID lesions (*top,* ×25) associated with intense staining of the intima for VCAM-1 (*bottom,* ×25).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mation, it is interesting to speculate that this may be due to an absence of T suppressor cells in these mice (11). Such a possibility would be consistent with the observation that cyclosporin A–treated mice exhibit increased atherosclerotic lesion formation (25), since cyclosporine exerts a preferential immunosuppressive effect on T suppressor cells (26). Also, vascular responses to injury are enhanced in rats exhibiting generalized T cell depletion (27). It is also possible that the immune system may contribute to forms of atherosclerosis involving infectious agents such as cytomegalovirus (28) or chlamydia (29), and, clearly, the classic immune system is important in transplant atherosclerosis (7). It should be emphasized that there may be species differences in processes contributing to atherosclerosis, and, thus, these studies in mice should be interpreted with caution.

In summary, female C57BL/6J mice with varying degrees of immunodeficiency fed an atherogenic diet all develop aortic atherosclerotic lesions. All immune deficient mice demonstrated typical lesions on histopathology and immunohistochemical analysis. A lack of suppressor T cells in the class I MHC deficient strain was associated with an increase in lesion formation, consistent with other data suggesting that the immune system may suppress atherosclerosis. These data support a modulating role for the immune system in atherogenesis, but emphasize nonimmune functions of monocyte-macrophages as principals in the formation of the atherosclerotic lesion.

Acknowledgments

We thank C. Hedrick for help with immunoblotting and critical review of the manuscript; L. Castellani and D. Puppione for lipoprotein analysis; P.-Z. Xie, X.-P. Wang, and M. Fishbein for histologic analysis; and S. Fitzgibbon for assistance with the mice.

A. I. Fyfe was a Clinician-Scientist with the Medical Research Council of Canada. This work was supported by U. S. Public Health Service grant HL30568.

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