

## **Role of macrophage-expressed adipocyte fatty acid binding protein in the development of accelerated atherosclerosis in hypercholesterolemic mice**

Matthew D. Layne<sup>\*,†,§</sup>, Anand Patel<sup>\*</sup>, Yen-Hsu Chen<sup>\*</sup>, Vivienne I. Rebel<sup>†,§</sup>, Irvith M. Carvajal<sup>\*</sup>, Andrea Pellacani<sup>\*,§</sup>, Bonna Ith<sup>\*</sup>, Dezheng Zhao<sup>#</sup>, Barbara M. Schreiber<sup>#</sup>, Shaw-Fang Yet<sup>\*,§</sup>, Mu-En Lee<sup>\*,§</sup>, Judith Storch<sup>\*\*</sup>, and Mark A. Perrella<sup>\*,†,§</sup>

<sup>\*</sup>Program of Developmental Cardiovascular Biology, Cardiovascular Division, and <sup>†</sup>Pulmonary and Critical Care Division, Brigham and Women's Hospital; <sup>‡</sup>Department of Cancer Biology, Dana-Farber Cancer Institute; Departments of <sup>§</sup>Medicine and <sup>||</sup>Pediatrics, Harvard Medical School, Boston, Massachusetts; <sup>#</sup>Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts; and <sup>\*\*</sup>Department of Nutritional Sciences, Rutgers University, New Brunswick, New Jersey

Corresponding author: Mark A. Perrella, Pulmonary and Critical Care Division, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. E-mail: mperrella@rics.bwh.harvard.edu

### **ABSTRACT**

Atherosclerosis is an inflammatory disease process associated with elevated levels of plasma cholesterol, especially low-density lipoproteins. The latter become trapped within the arterial wall and are oxidized and taken up by macrophages to form foam cells. This process is an initiating event for atherosclerosis. Fatty acid binding proteins (FABP) are involved in fatty acid metabolism and cellular lipid transport, and adipocyte FABP (aP2) is also expressed in macrophages. We recently generated mice lacking both apolipoprotein (Apo)E and aP2 (ApoE<sup>-/-</sup>aP2<sup>-/-</sup>) and found that these mice, compared with ApoE<sup>-/-</sup> mice, developed markedly smaller atherosclerotic lesions that contained fewer macrophages. Here we investigated the mechanism(s) responsible for this prevention of atherosclerotic lesion formation. Bone marrow transplantations were performed in ApoE<sup>-/-</sup> mice, receiving cells from either ApoE<sup>-/-</sup> or ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice. The lack of aP2 in donor marrow cells led to the development of smaller (5.5-fold) atherosclerotic lesions in the recipient mice. No differences were found in plasma cholesterol, glucose, or insulin levels between recipients of bone marrow cells from ApoE<sup>-/-</sup> or ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice. However, the expression of chemoattractant and inflammatory cytokines was decreased in macrophages from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice compared with ApoE<sup>-/-</sup> mice, which may contribute to the decrease in atherosclerotic lesion formation. Taken together, we demonstrate the importance of macrophage aP2 in the development of atherosclerotic lesions.

Key words: atheroma • inflammatory cytokines • chemokines

An important risk factor for the development of atherosclerosis is the presence of high plasma levels of cholesterol (1, 2). Of particular importance among the cholesterol lipoproteins for the development of atherosclerotic lesions is an elevation in low-density lipoproteins (LDL). Nevertheless, beyond this metabolic derangement, atherosclerosis is a disease of inflammation (2).

The earliest noted atherosclerotic lesions, fatty streaks, consist of monocyte-derived macrophages and T-lymphocytes (3). Throughout the course of atherosclerotic lesion formation, mononuclear cells (predominantly monocytes and macrophages [4]) are contributing to the development of more advanced lesions. LDL within the vessel wall become oxidized (oxLDL), and are taken up by macrophages via scavenger receptors on their cell surface (2). The uptake of oxLDL leads to the development of foam cells. A continued inflammatory response, including the activation of mononuclear cells that produce chemoattractant (chemokines), proinflammatory, and growth-inducing cytokines, leads to more advanced, complex lesions (2). The importance of macrophages in atherosclerotic lesion formation has been shown by using genetic models of hypercholesterolemia in mice. Hypercholesterolemic mice generated with a deficiency in apolipoprotein (Apo)E (5, 6) and also a deficiency in macrophage colony-stimulating factor (resulting in a decreased number of peripheral blood monocytes) (7, 8) have shown a significant reduction in lesion size compared with a deficiency in ApoE alone. Similarly, targeted disruption of the gene-encoding macrophage scavenger receptor-A in mice led to a reduction in lesion size in ApoE deficient mice (9). Other investigators have shown in hypercholesterolemic mice that a lack of monocyte chemoattractant protein (MCP)-1 (10), or its receptor CCR2 (11), caused a decrease in macrophage infiltration and lesion size; whereas overexpression of MCP-1 in bone marrow-derived cells of ApoE<sup>-/-</sup> mice accelerated the development of atherosclerotic lesions (12).

Recently, we have demonstrated that adipocyte fatty acid binding protein (AFABP or aP2) is an important factor in the development of atherosclerosis (13). Fatty acid binding proteins (FABP) belong to a family of low-molecular-weight cytoplasmic proteins that are involved in intracellular trafficking of fatty acids to their sites of use and cellular lipid transport (14). In addition, FABP have been linked with enzymes of lipid metabolism (15–17). Although aP2 is expressed highly in differentiated adipocytes (18), recently it has been shown that aP2 is also expressed in macrophages (13, 19)—a cell type critical in the evolution of atherosclerosis—and that aP2 is up-regulated during macrophage differentiation (13). By crossing aP2-deficient (aP2<sup>-/-</sup>) mice with ApoE-deficient (ApoE<sup>-/-</sup>) mice, we determined that a lack of aP2 led to smaller, less complex lesions (13) in ApoE<sup>-/-</sup> mice fed a high-fat, Western diet. Moreover, we found that atherosclerotic lesions in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice have a marked reduction in macrophage accumulation.

Another risk factor for the development of atherosclerosis is diabetes (20, 21). When aP2-deficient mice were characterized initially, it was reported that these mice that were fed a high-fat diet lacked obesity-induced hyperinsulinemia and insulin resistance that was present in wild-type (WT), control mice (22). However, a later study showed no difference in insulin resistance between aP2-deficient and wild-type mice fed the same high-fat diet (23). It was suggested that keratinocyte fatty acid binding protein (KFABP) could compensate functionally for the lack of aP2 during high-fat feedings (23).

The present study was designed to elucidate further the mechanism(s) responsible for the prevention of advanced atherosclerotic lesion formation in hypercholesterolemic mice lacking aP2. In particular, we wanted to determine whether the reduction in lesion formation in the absence of aP2 was related to an alteration in bone marrow-derived macrophages, cholesterol levels, or glucose/insulin levels.

## **MATERIALS AND METHODS**

### **Mice**

aP2<sup>-/-</sup> mice back-crossed and fixed on a C57BL/6 background (24) were mated with ApoE<sup>-/-</sup> mice (C57BL/6J-ApoE<sup>tm1Unc</sup>, Jackson Laboratories, Bar Harbor, ME) to generate mice heterozygous for ApoE and aP2 (ApoE<sup>+/-</sup> aP2<sup>+/-</sup>). These mice were then bred to generate the mice for this study, which include WT, ApoE<sup>-/-</sup>, aP2<sup>-/-</sup>, or ApoE<sup>-/-</sup> aP2<sup>-/-</sup>. The mice were genotyped by polymerase chain reaction analysis, which was confirmed by Southern blot analysis.

### **Bone marrow transplantation**

Donor ApoE<sup>-/-</sup> or ApoE<sup>-/-</sup> aP2<sup>-/-</sup> mice, 8 to 10 weeks of age, were killed, and bone marrow cells were harvested from the femurs and tibias as described (25, 26). Recipient ApoE<sup>-/-</sup> mice, 4 to 5 weeks of age, were irradiated with 1100 cGy of gamma irradiation in a single dose and then injected with  $2 \times 10^6$  bone marrow cells via tail vein injection (26). Control experiments were performed to evaluate hematopoietic reconstitution by using the previously mentioned doses of irradiation and bone marrow cell replacement in C57BL/6J mice 4 to 5 weeks of age. We determined by flow cytometry techniques after transplantation that 86% of circulating bone marrow-derived cells came from the donor marrow (data not shown).

### **Atherogenic diet**

After completion of the bone marrow transplantations, the mice were placed on a Western diet (Teklad Adjusted Calories Western-type diet, 88137, Harlan-Teklad, Madison, WI) containing 21% fat by weight to accelerate the development of atherosclerosis.

### **Immunohistochemical staining and morphometry**

After 12 weeks on the high-fat, Western diet, the mice were anesthetized and their vasculature was perfused with phosphate buffered saline and histologic analysis of the vasculature was performed. The arch of the aorta and the right brachiocephalic artery were dissected out, fixed by immersion in methyl Carnoy's solution, dehydrated, and then embedded in paraffin. We analyzed sections of tissue from the same anatomical level of the right brachiocephalic artery. Vessel sections from the mice were stained with Verhoeff's stain for elastin (27) to assess lesion area and luminal occlusion. The areas of the atherosclerotic lesions and the lumens were measured by computerized planimetry. The percentage of luminal occlusion was calculated as the area of lesion divided by the entire area within the internal elastic lamina, multiplied by 100.

## **Plasma cholesterol, glucose, and insulin levels**

Mice that were fed the high-fat, Western diet for 12 weeks were fasted for 4 h in the morning. Blood was then drawn from the retro-orbital venous plexus and placed into ethylenediaminetetraacetic acid (EDTA)-coated tubes on ice. The samples were then centrifuged and the plasma was removed and stored at  $-20^{\circ}\text{C}$  until further analyses. We determined total cholesterol with a commercially available enzymatic reaction kit (Sigma Chemical Co., St. Louis, MO). Plasma glucose levels were determined by the glucose oxidase and peroxidase method as described by the manufacturer (Sigma). We measured plasma glucose levels by using a microplate analyzer at an absorbance wavelength of 505 nm. Plasma insulin levels were measured by an ultrasensitive rat insulin ELISA method (ALPCO Diagnostics, Windham, NY), according to the manufacturer recommendations for mouse plasma.

## **Cholesterol esterification and uptake in macrophages**

We harvested peritoneal macrophages from wild-type or  $\text{aP2}^{-/-}$  mice as described (28). Staining with  $\alpha$ -naphthyl butyrate esterase (Sigma) revealed the cells to be of macrophage origin (data not shown). Oxidized LDL was obtained from Biomedical Technologies, Inc. (Stoughton, MA) The extent of LDL oxidation was determined colorimetrically by using the TBARS (thiobarbituric acid reactive substances) assay with malondialdehyde (MDA) as a standard. The preparations of oxLDL used in these studies contained 17.9–19.2 nM of MDA/mg protein. Peritoneal macrophages were cultured for 8 or 24 h in the presence or absence of oxLDL (50  $\mu\text{g}$  protein/ml) and [ $^{14}\text{C}$ ]oleic acid complexed to albumin as previously described (28). At the time of harvest, the lipids were then extracted from the cells and analyzed [ $^{14}\text{C}$ ]cholesteryl oleate accumulation, as described previously (28).

## **Northern and RNase Protection assays**

Total RNA was obtained from harvested peritoneal macrophages by guanidinium isothiocyanate extraction (29). Northern blot analysis was performed as described (29) by using probes for  $\text{aP2}$ , KFABP, and normalized by hybridization to a  $^{32}\text{P}$ -labeled 28S oligonucleotide. For RNase protection assays,  $^{32}\text{P}$ -labeled anti-sense RNA probes were generated from custom RiboQuant<sup>TM</sup> Multi-Probe Template Sets (PharMingen, San Diego, CA) and hybridized with the target RNA according to the manufacturer recommendations. The RNase protected probes were then purified and resolved on denaturing polyacrylamide gels. The gels were developed subsequently on film, and the expressed mRNA species were identified by bands corresponding to the expected fragment lengths. Signal intensities were measured by densitometric analysis by using image software (National Institutes of Health, Bethesda, MD), and the signals were normalized to GAPDH. The experiments were performed in duplicate, and data are presented as a percentage change in mRNA levels  $\pm$  SD for chemoattractant and inflammatory cytokines that decreased significantly.

## Nitrite assay

To determine the amount of nitric oxide (NO) produced by peritoneal macrophages, we measured a stable product of NO oxidation,  $\text{NO}_2^-$  (nitrite), by a standard method (30) as described previously (29). Briefly, cells were cultured in medium without phenol red for the experiments assessing nitrite concentrations. An aliquot of cell supernatant was mixed with an equal volume of Griess reagent (one part 0.1% naphthylethylenediamine dihydrochloride added to one part 1% sulfanilamide in 5% phosphoric acid) and allowed to stand at room temperature for 10 min. Nitrite levels in the cell supernatants were measured subsequently with a microplate reader at an absorbance wavelength of 560 nm and were converted to micromolar units ( $\mu\text{M}$ ) per  $\mu\text{g}$  protein.

## RESULTS

### **Bone marrow cells from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice transplanted into ApoE<sup>-/-</sup> mice promotes a marked reduction in atherosclerotic lesion formation**

To determine whether bone marrow-derived aP2 was important in the development of atherosclerosis, we transplanted bone marrow cells from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice into ApoE<sup>-/-</sup> mice. As a control, bone marrow cells from ApoE<sup>-/-</sup> mice were transplanted into ApoE<sup>-/-</sup> mice. The mice were placed on a high-fat, Western diet immediately after the transplantation. After 12 weeks, the mice were killed and the right brachiocephalic arteries were analyzed for the presence of lesions. Representative vessels from ApoE<sup>-/-</sup> mice receiving marrow cells from ApoE<sup>-/-</sup> donor mice are depicted in [Figure 1 A, B](#). Large atheromas were present in the brachiocephalic arteries from these mice, producing lesions occluding the vessel by  $42 \pm 8\%$  ([Fig. 2A](#), black bar,  $n=6$ ). In comparison, vessels from ApoE<sup>-/-</sup> mice receiving cells from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> donor mice contained significantly smaller lesions ([Fig. 1C, D](#),  $P<0.0006$ ) occluding the brachiocephalic arteries by only  $9 \pm 2\%$  ([Fig. 2A](#), white bar,  $n=9$ ). Lesions in the brachiocephalic arteries of ApoE<sup>-/-</sup> mice receiving ApoE<sup>-/-</sup>aP2<sup>-/-</sup> marrow cells were 5.5-fold smaller by area ( $P<0.0012$ ) than ApoE<sup>-/-</sup> mice receiving cells from ApoE<sup>-/-</sup> mice ([Fig. 2B](#)). These results strongly suggest that bone marrow-derived cells are the primary mediators of the previously observed anti-atherosclerotic effects in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice (13).

### **No difference in plasma cholesterol levels of ApoE<sup>-/-</sup> mice receiving bone marrow cells from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> or ApoE<sup>-/-</sup> donors**

We showed previously an overall reduction in total circulating cholesterol levels in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice compared with ApoE<sup>-/-</sup> mice (13). However, because total cholesterol levels remained high in both groups compared with wild-type mice, we could not explain the reduction in lesion size by the modest (38%) decrease in plasma cholesterol levels in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice. Thus, to clarify this issue further, we measured total circulating cholesterol levels in ApoE<sup>-/-</sup> mice receiving bone marrow transplantations ([Fig. 3](#)). There was no difference ( $P=0.95$ ) in total cholesterol levels between mice receiving marrow cells from ApoE<sup>-/-</sup> donors ( $1291 \pm 157$  mg/dl,  $n=6$ ) and mice receiving marrow cells from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> donors ( $1280 \pm 85$  mg/dl,  $n=11$ ). These data confirm that an alteration in circulating cholesterol levels cannot account for the decrease in lesion size previously found in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice (13).

Even though there was no difference in circulating cholesterol levels, we performed studies to determine whether macrophage uptake of cholesterol might be decreased in the absence of aP2. Peritoneal macrophages were harvested from wild-type (aP2<sup>+/+</sup> = WT) and aP2<sup>-/-</sup> mice and then were stimulated with oxLDL. OxLDL caused a significant increase in cholesteryl ester accumulation at 8 and 24 h ( $P < 0.05$ , 0  $\mu\text{g/ml}$  vs. 50  $\mu\text{g/ml}$  oxLDL) in peritoneal macrophages from both aP2<sup>+/+</sup> and aP2<sup>-/-</sup> mice (Fig. 4), and, although there were slight differences in [<sup>14</sup>C] cholesteryl oleate accumulation in aP2<sup>+/+</sup> vs. aP2<sup>-/-</sup> macrophages, none were likely to account for the large decrease in lesion forming capacity of aP2<sup>-/-</sup> macrophages. The uptake of fluorescently labeled acetylated LDL (DiI-Ac-LDL, Biomedical Technologies, Inc.) was also similar among wild-type, ApoE<sup>-/-</sup>, aP2<sup>-/-</sup>, and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> macrophages (data not shown). In addition, macrophages from ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice that were exposed to high levels of cholesterol *in vivo* showed no marked difference in cholesteryl ester accumulation (data not shown). These data show no difference in cholesterol uptake by aP2-deficient macrophages that could account for the dramatic decrease in lesion formation.

### **No difference in plasma glucose or insulin levels of ApoE<sup>-/-</sup> mice in the presence or absence of aP2**

To determine whether an alteration in glucose metabolism may contribute to the reduced atherosclerotic lesion size in ApoE<sup>-/-</sup> mice lacking aP2, we measured fasting levels of plasma glucose and insulin in ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice. There was no difference in fasting glucose (Fig. 5A) or insulin (Fig. 5B) levels between ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice that had been on a high-fat, Western diet for 12 weeks. Moreover, ApoE<sup>-/-</sup> mice receiving bone marrow cells from ApoE<sup>-/-</sup> or ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice did not show a difference in fasting glucose (Fig. 5C) or insulin (Fig. 5D) levels on the same diet. Taken together, these data indicate no obvious differences in systemic glucose metabolism that could explain the decrease in atherosclerotic lesion formation in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice.

### **KFABP mRNA levels are not altered in macrophages derived from aP2<sup>-/-</sup> mice**

We wanted to examine whether KFABP expression was regulated in peritoneal macrophages derived from ApoE<sup>-/-</sup>, aP2<sup>-/-</sup>, and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice. RNA was harvested from wild-type, ApoE<sup>-/-</sup>, aP2<sup>-/-</sup>, and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> macrophages and analyzed by Northern blot. Peritoneal macrophages from wild-type and ApoE<sup>-/-</sup> mice express aP2 mRNA (Fig. 6). As expected, macrophages from aP2<sup>-/-</sup> and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> did not express aP2 mRNA. We did not observe any differences in KFABP mRNA expression normalized to 28S in the macrophages derived from mice of the various genotypes (Fig. 6), indicating that KFABP was not up-regulated in the absence of aP2.

### **Decrease in expression of inflammatory cytokines and chemokines, but no alteration in NO production, in aP2 deficient macrophages**

Peritoneal macrophages were harvested from ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice and were exposed to vehicle or oxLDL for 48 h. After this, total RNA was extracted to perform RNase protection

assays. Levels of mRNA for the proinflammatory cytokine tumor necrosis factor (TNF)- $\alpha$  and macrophage inflammatory protein (MIP)-1 ( $\alpha$  and  $\beta$ ) were decreased ( $58 \pm 5$  %,  $45 \pm 9$  %, and  $46 \pm 11$  %, respectively) in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> cells compared with ApoE<sup>-/-</sup> cells (Fig. 7A). In addition, a dramatic decrease in chemokine expression for MCP-1 occurred in the absence of aP2. Levels of MCP-1, particularly in the presence of oxLDL, were decreased considerably ( $62 \pm 7$  %) in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> cells (Fig. 7A).

Finally, we assessed NO production by measuring nitrite levels in wild-type, aP2<sup>-/-</sup>, ApoE<sup>-/-</sup>, and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> macrophages stimulated with lipopolysaccharide (LPS, 1  $\mu$ g/ml) from *Escherichia coli* (serotype 026:B6) for 18 h (Fig. 7B). Control (nonstimulated) macrophages of all genotypes did not exhibit detectable levels of nitrite production (Fig. 7B). Overall, macrophages isolated from all genotypes treated with LPS produced high levels of nitrite (Fig. 7B), although this induction was slightly lower in macrophages from ApoE<sup>-/-</sup> mice. Macrophages from ApoE<sup>-/-</sup> mice that were also deficient in aP2 (ApoE<sup>-/-</sup>aP2<sup>-/-</sup>) showed nitrite inducibility that did not differ from wild-type or aP2<sup>-/-</sup> macrophages (Fig. 7B). These data suggest that macrophages deficient in aP2 are fully activated by inflammatory stimuli, yet they show a decreased message for inflammatory cytokines and chemokines.

## DISCUSSION

The present study extends our previous observations to determine the mechanism(s) responsible for the decrease in atherosclerotic lesion formation in hypercholesterolemic mice deficient in aP2 (13). Of particular interest, bone marrow cells transplanted from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice into ApoE<sup>-/-</sup> mice could reduce brachiocephalic lesion size in mice fed a high-fat, Western diet (Fig. 1C, D compared with Fig. 1A, B) to a similar degree as found previously for ApoE<sup>-/-</sup> mice entirely deficient in aP2 (13). The absence of aP2 in bone marrow-derived cells of ApoE<sup>-/-</sup> mice reduced luminal occlusion by 82% (Fig. 2A) and lesion area by 77% (Fig. 2B) compared with ApoE<sup>-/-</sup> mice that have wild-type concentrations of aP2 after bone marrow transplantation. Thus, even in the presence of adipocyte aP2 expression in other cell types, the absence of macrophage expression reproduced the phenotype observed in the ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice (13). These data establish the importance of aP2 from bone marrow-derived cells in mediating atherosclerotic lesion formation. Moreover, because monocytes and macrophages constitute ~80% of inflammatory cells in atherosclerotic lesions (4) and aP2 is expressed strongly in isolated macrophages (13, 19) and atherosclerotic lesions (13), these data strongly support our hypothesis regarding the importance of macrophage aP2 in the development of atherosclerosis.

Previously, we noted that plasma cholesterol levels were ~38% reduced in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice compared with ApoE<sup>-/-</sup> mice (13). Although these lower levels still remained fivefold higher than values reported for wild-type mice (5) and no changes in lipoprotein profile were observed (13), we could not exclude completely this modest decrease in plasma cholesterol levels as a contributing factor in the reduced lesion size of ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice. However, in the present study, ApoE<sup>-/-</sup> mice hematologically reconstituted with bone marrow cells from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice had small, trivial lesions even in the setting of very high plasma cholesterol levels (Fig. 3). In fact, plasma cholesterol levels for these mice were almost identical to ApoE<sup>-/-</sup> mice transplanted with syngeneic ApoE<sup>-/-</sup> cells (Fig. 3), which had dramatically larger lesions (Fig. 1 and Fig. 2). These data suggest that the beneficial effects of aP2 deficiency on preventing

atherosclerotic lesion formation are not directly a result of lowering the plasma cholesterol levels, but are mediated by bone marrow-derived cells.

In the development of atherosclerosis, circulating monocytes enter the arterial wall, differentiate into macrophages, and then scavenge cholesteryl esters that originate from plasma lipoproteins to become lipid-filled foam cells (1, 2). When these foam cells accumulate within the intima, the first identifiable lesion of atherosclerosis develops. One consideration that we explored was that, in the absence of aP2, macrophages would not be able to scavenge cholesterol appropriately. In this scenario, the development of foam cells would be altered and the initiation of the atherosclerotic process, prevented (2). Our experiments revealed only slight differences in cholesterol esterification in aP2<sup>-/-</sup> macrophages exposed to oxLDL *in vitro*, and only at an early (8 h) time point (Fig. 4). After exposure to oxLDL for longer periods (24 h), no difference in cholesterol ester accumulation was found between aP2<sup>-/-</sup> and aP2<sup>+/+</sup> macrophages. While this article was in review, Makowski and colleagues reported that, on a chow diet, ApoE<sup>-/-</sup> mice receiving bone marrow cells from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice had smaller lesions (31). When studying aP2-deficient macrophages in the presence or absence of acetylated LDL *in vitro*, they found a small reduction in cholesterol ester mass. Taken together, we believe in the setting of chronic hypercholesterolemia in the ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup> aP2<sup>-/-</sup> mice *in vivo*, it is unlikely that a differential ability of aP2<sup>-/-</sup> macrophages to accumulate cholesterol esters could account for the dramatic differences in atherosclerotic lesion formation.

As mentioned previously, diabetes is an important risk factor in the development of atherosclerosis (20, 21). Hotamisligil and colleagues reported previously that a lack of aP2 in mice prevented the development of obesity-induced insulin resistance and diabetes (22). We, therefore, investigated whether an alteration in fasting plasma glucose and insulin levels may contribute to the smaller lesion size in ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice that were fed a high-fat, Western diet. Figure 5 shows that plasma glucose and insulin levels were not different between ApoE<sup>-/-</sup>aP2<sup>-/-</sup> and ApoE<sup>-/-</sup> mice (Fig. 5A, B) of comparable weight. There was also no difference in plasma glucose and insulin levels in ApoE<sup>-/-</sup> mice receiving bone marrow cells from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> donors and those receiving cells from ApoE<sup>-/-</sup> donors (Fig. 5C, D). Furthermore, similar to the findings of Shaughnessy and colleagues (23), we did not find a difference in fasting plasma glucose and insulin levels in wild-type and aP2<sup>-/-</sup> mice (data not shown). Taken together, these data suggest that the beneficial effect of aP2 deficiency in ApoE<sup>-/-</sup> mice fed a high-fat, Western diet cannot be explained by a difference in fasting plasma glucose levels or a lack of insulin resistance.

Finally, we wanted to determine whether the expression of inflammatory factors that are known to modulate atherosclerotic lesion formation—proinflammatory and chemoattractant cytokines—are altered in the absence of aP2 (4, 32). Indeed, the mRNA levels for TNF- $\alpha$  (inflammatory cytokine) and MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCP-1 (chemokines) were reduced in macrophages harvested from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice compared with macrophages from ApoE<sup>-/-</sup> mice (Fig. 7A). Moreover, the reduction in inflammatory and chemoattractant cytokines was present when macrophages were stimulated with oxLDL. We demonstrated previously that atherosclerotic lesions from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice fed a high-fat, Western diet were small and contained few macrophages compared with lesions from ApoE<sup>-/-</sup> mice. These data are consistent with studies showing that hypercholesterolemic mice deficient in MCP-1 (10), or its receptor CCR2 (11),

developed smaller and less macrophage-rich lesions. To make sure that the decreased levels of inflammatory mediators were not related to a general suppression of macrophage activation in cells deficient in aP2, we assessed NO production in wild-type, aP2<sup>-/-</sup>, ApoE<sup>-/-</sup>, and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> macrophages stimulated with LPS (Fig. 7B). Our data show that macrophages from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice activate and produce NO at a level comparable with wild-type cells (Fig. 7B). Taken together, we demonstrate the importance of macrophage aP2 in the development of atherosclerotic lesions. Moreover, our data suggest that decreased expression of inflammatory and chemoattractant cytokines in macrophages from ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice may contribute to the decrease in atherosclerotic lesion formation.

## ACKNOWLEDGMENTS

The aP2-deficient mice were generated in the laboratory of Bruce Spiegelman, and these mice were provided to our laboratory by Gökhan Hotamisligil for breeding with ApoE-deficient mice in order to generate the ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice.

## REFERENCES

1. Ross, R. (1995) Arteriosclerosis: an overview. In *Molecular Cardiovascular Medicine* (Haber, E., ed.) Vol. 2. Scientific American, Inc.: New York, pp. 11–30
2. Ross, R. (1999) Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340**, 115–126
3. Stary, H. C., Chandler, A. B., Glagov, S., Guyton, J. R., Insull, W. J., Rosenfeld, M. E., Schaffer, S. A., Schwartz, C. J., Wagner, W. D., and Wissler, R. W. (1994) A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* **89**, 2462–2478
4. Gerszten, R. E., Mack, F., Sauty, A., Rosenzweig, A., and Luster, A. D. (2000) Chemokines, leukocytes, and atherosclerosis. *J. Lab. Clin. Med.* **136**, 87–92
5. Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J. G., Rubin, E. M., and Breslow, J. L. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* **71**, 343–353
6. Zhang, S. H., Reddick, R. L., Piedrahita, J. A., and Maeda, N. (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* **258**, 468–471

7. Smith, J. D., Trogan, E., Ginsberg, M., Grigaux, C., Tian, J., and Miyata, M. (1995) Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc. Natl. Acad. Sci. USA* **92**, 8264–8268
8. Qiao, J. H., Tripathi, J., Mishra, N. K., Cai, Y., Tripathi, S., Wang, X. P., Imes, S., Fishbein, M. C., Clinton, S. K., Libby, P., Lusis, A. J., and Rajavashisth, T. B. (1997) Role of macrophage colony-stimulating factor in atherosclerosis: studies of osteopetrotic mice. *Am. J. Path.* **150**, 1687–1699
9. Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cynshi, O., Wada, Y., Honda, M., Kurihara, H., Aburatani, H., Doi, T., Matsumoto, A., Azuma, S., Noda, T., Toyoda, Y., Itakura, H., Yazaki, Y., Horiuchi, S., Takahashi, K., Kruijt, J. K., van Berkel, T. J. C., Steinbrecher, U. P., Ishibashi, S., Naeda, N., Gordon, S., and Kodama, T. (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature (London)* **386**, 292–296
10. Gu, L., Okada, Y., Clinton, S. K., Gerard, C., Sukhova, G. K., Libby, P., and Rollins, B. J. (1998) Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol. Cell* **2**, 275–281
11. Boring, L., Gosling, J., Cleary, M., and Charo, I. F. (1998) Decreased lesion formation in CCR2<sup>-/-</sup> mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature (London)* **394**, 894–897
12. Aiello, R. J., Bourassa, P.-A.K., Lindsey, S., Weng, W., Natoli, E., Rollins, R. J., and Milos, P. M. (1999) Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **19**, 1518–1525
13. Perrella, M. A., Pellacani, A., Layne, M. D., Patel, A., Zhao, D., Schreiber, B. M., Storch, J., Feinberg, M. W., Hsieh, C.-M., Haber, E., and Lee, M.-E. (2001) Absence of adipocyte fatty acid binding protein prevents the development of accelerated atherosclerosis in hypercholesterolemic mice. *FASEB J.* (June 8, 2001) 10.1096/fj.01-0017fje.
14. Storch, J., and Thumser, A. E. (2000) The fatty acid transport function of fatty acid-binding proteins. *Biochim. Biophys. Acta* **1486**, 28–44
15. Ockner, R. K., and Manning, J. A. (1976) Fatty acid binding protein. Role in esterification of absorbed long chain fatty acid in rat intestine. *J. Clin. Invest.* **58**, 632–641
16. Lunzer, M. A., Manning, J. A., and Ockner, R. K. (1977) Inhibition of rat liver acetyl coenzyme A carboxylase by long chain acyl coenzyme A and fatty acid. Modulation by fatty acid-binding protein. *J. Biol. Chem.* **252**, 5483–5487
17. Catala, A. (1986) Stearic acid desaturation in rat liver microsomes: stimulation by fatty acid binding protein. *Acta. Physiol. Pharmac.* **36**, 19–27

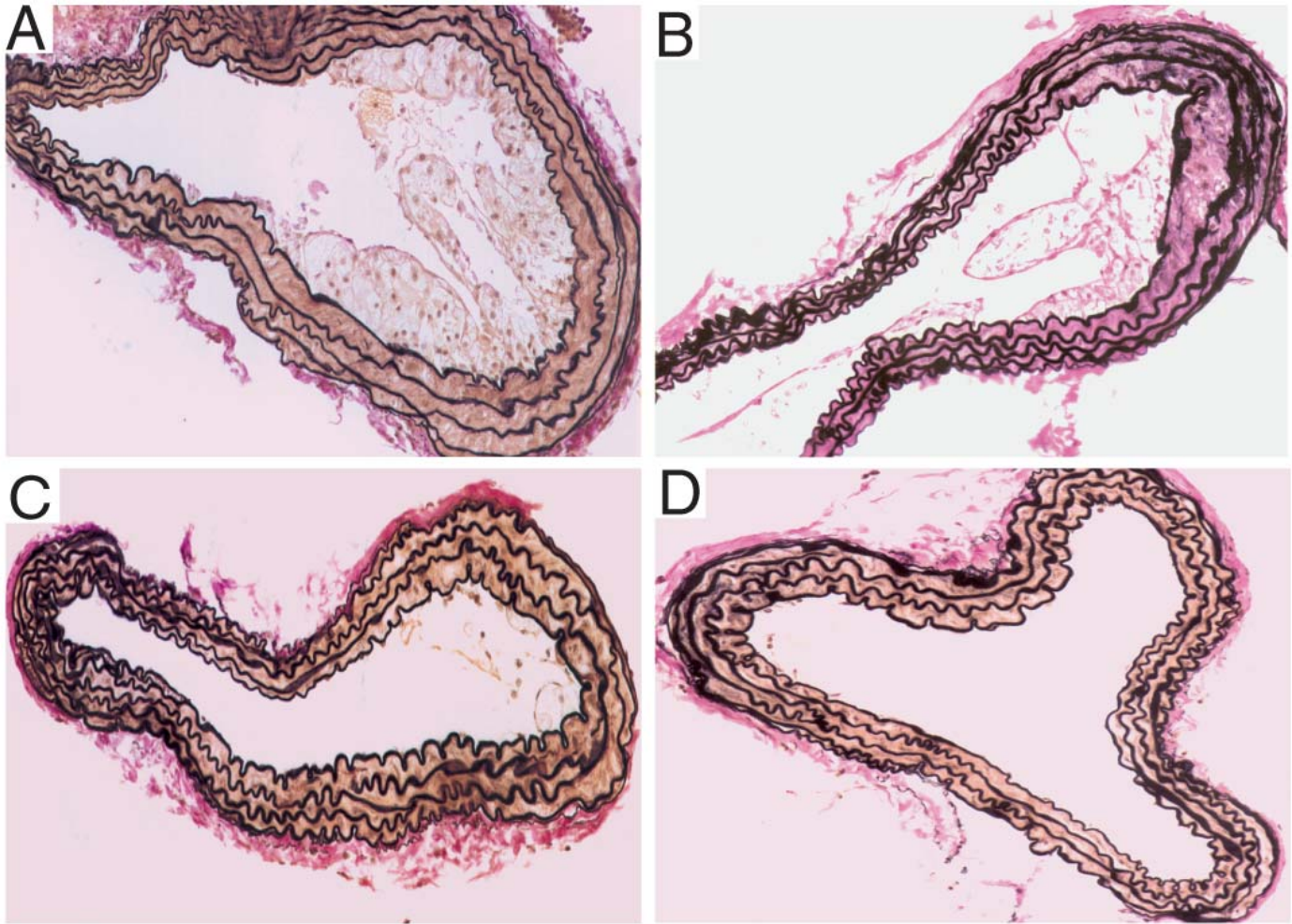
18. Spiegelman, B. M., and Green, H. (1980) Control of specific protein biosynthesis during the adipose conversion of 3T3 cells. *J. Biol. Chem.* **255**, 8811–8818
19. Pelton, P. D., Zhou, L., Demarest, K. T., and Burris, T. P. (1999) PPAR $\gamma$  activation induces the expression of the adipocyte fatty acid binding protein gene in human macrophages. *Biochem. Biophys. Res. Commun.* **261**, 456–458
20. Glasser, S. P., Selwyn, A. P., and Ganz, P. (1996) Atherosclerosis, risk factors and vascular endothelium. *Am. Heart J.* **131**, 379–384
21. Orford, J. L., Kinlay, S., Fernandes, J., Behrendt, D., Ganz, P., and Selwyn, A. P. (2001) Manipulating the vascular biology of coronary atherosclerosis in diabetes: new opportunities. *J. Lab. Clin. Med.* **137**, 82–92
22. Hotamisligil, G. S., Johnson, R. S., Distel, R. J., Ellis, R., Papaioannou, V. E., and Spiegelman, B. M. (1996) Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* **274**, 1377–1379
23. Shaughnessy, S., Smith, E. R., Kodukula, S., Storch, J., and Fried, S. K. (2000) Adipocyte metabolism in adipocyte fatty acid binding protein knockout (aP2 $^{-/-}$ ) mice after short-term high-fat feeding. Functional compensation by the keratinocyte fatty acid binding protein. *Diabetes* **49**, 904–911
24. Scheja, L., Makowski, L., Uysal, T., Wiesbrock, S. M., Shimshek, D. R., Meyers, D. S., Morgan, M., Parker, R. A., and Hotamisligil, G. S. (1999) Altered insulin secretion associated with reduced lipolytic efficiency in aP2 $^{-/-}$  mice. *Diabetes* **48**, 1987–1994
25. Thiele, D. L., Eigenbrodt, M. L., Bryde, S. E., Eigenbrodt, E. H., and Lipsky, P. E. (1989) Intestinal graft-versus-host disease is initiated by donor T cells distinct from classic cytotoxic T lymphocytes. *J. Clin. Med.* **84**, 1947–1956
26. Rebel, V. E., Hartnett, S., Hill, G. R., Laxo-Kallanian, S. B., Ferrara, J. L., and Sieff, C. A. (1999) Essential role for the p55 tumor necrosis factor receptor in regulating hematopoiesis at a stem cell level. *J. Exp. Med.* **190**, 1493–1504
27. Shi, C., Russell, M. E., Bianchi, C., Newell, J. B., and Haber, E. (1994) Murine model of accelerated transplant arteriosclerosis. *Circ. Res.* **75**, 199–207
28. Schreiber, B. M., Martin, B. M., Hollander, W., and Franzblau, C. (1988) b-VLDL-induced alterations in growth potentiating activity produced by mononuclear phagocytes. *Atherosclerosis* **69**, 69–79
29. Perrella, M. A., Yoshizumi, M., Fen, Z., Tsai, J. C., Hsieh, C. M., Kourembanas, S., and Lee, M. E. (1994) Transforming growth factor- $\beta$ 1, but not dexamethasone, down-regulates nitric-

oxide synthase mRNA after its induction by interleukin-1b in rat smooth muscle cells. *J. Biol. Chem.* **269**, 14595–14600

30. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannebaum, S. R. (1982) Analysis of nitrate, nitrite and [<sup>15</sup>N] nitrate in biological fluids. *Anal. Biochem.* **126**, 131–138
31. Makowski, L., Boord, J. B., Maeda, K., Babaev, V. R., Uysal, K. T., Morgan, M. A., Parker, R. A., Suttles, J., Fazio, S., Hotamisligil, G. S., and Linton, M. F. (2001) Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nat. Med.* **7**, 699–705
32. Wilcox, J. N., Nelken, N. A., Coughlin, S. R., Gordon, D., and Schall, T. J. (1994) Local expression of inflammatory cytokines in human atherosclerotic plaques. *J. Atheroscler. Thromb.* **1**, S10–S13

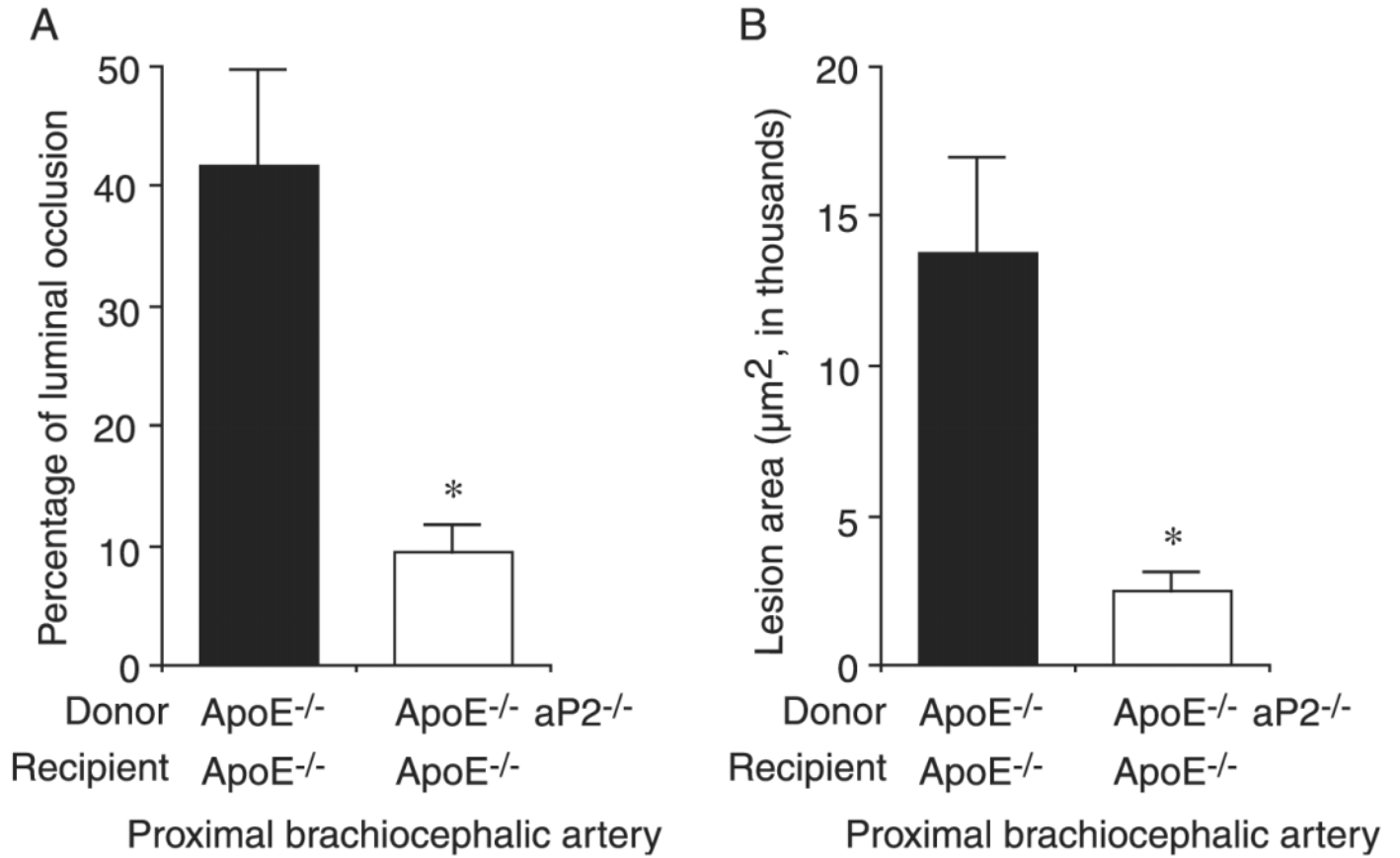
*Received June 12, 2001; revised August 7, 2001.*

Fig. 1



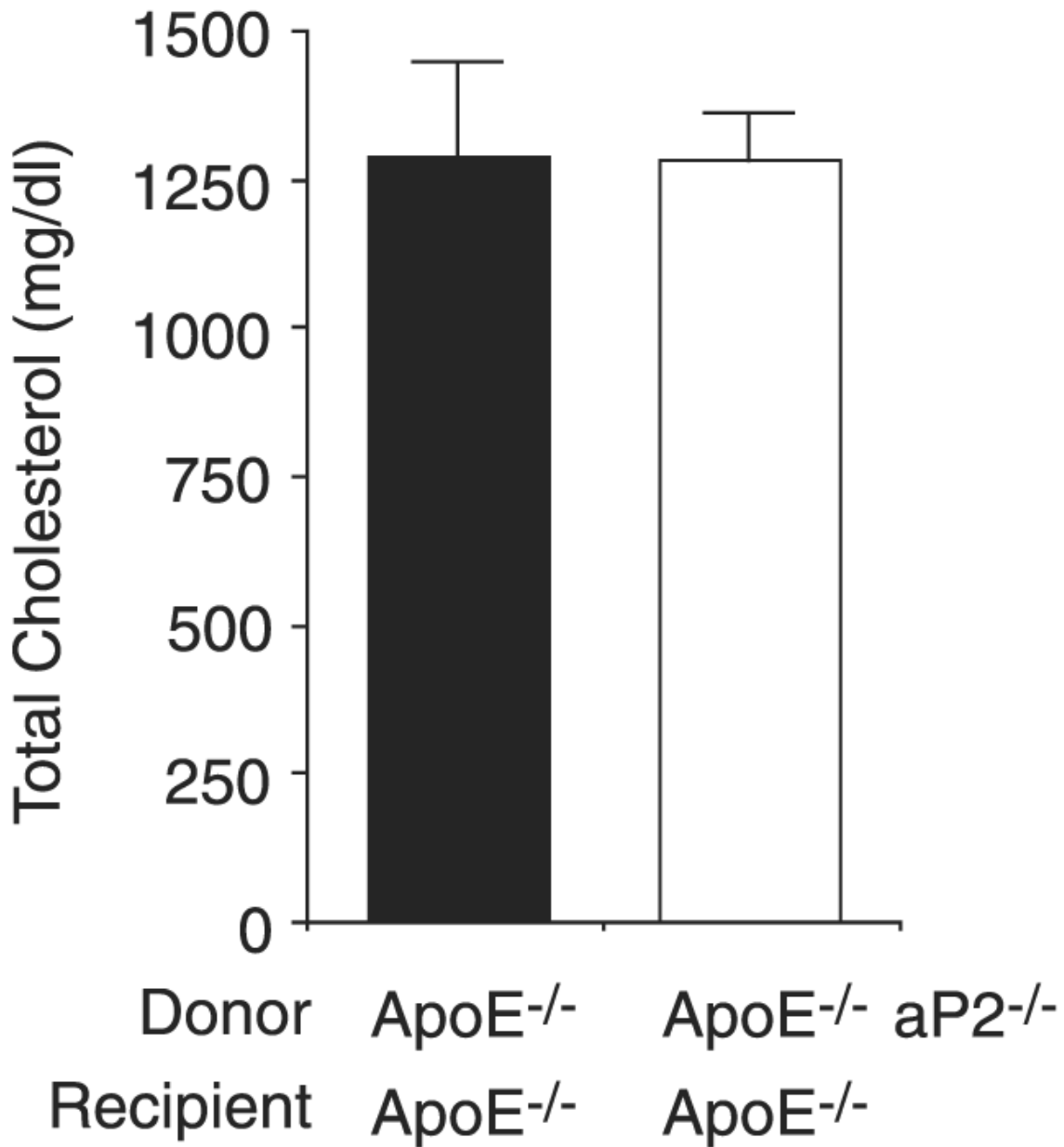
**Figure 1. Absence of aP2 in bone marrow-derived cells of ApoE<sup>-/-</sup> mice leads to decreased atherosclerotic lesion formation.** Staining for elastin (black) was performed in proximal brachiocephalic arteries ( $\times 100$ ) from representative ApoE<sup>-/-</sup> mice receiving bone marrow cells from ApoE<sup>-/-</sup> (A and B) or ApoE<sup>-/-</sup> aP2<sup>-/-</sup> mice (C and D).

Fig. 2



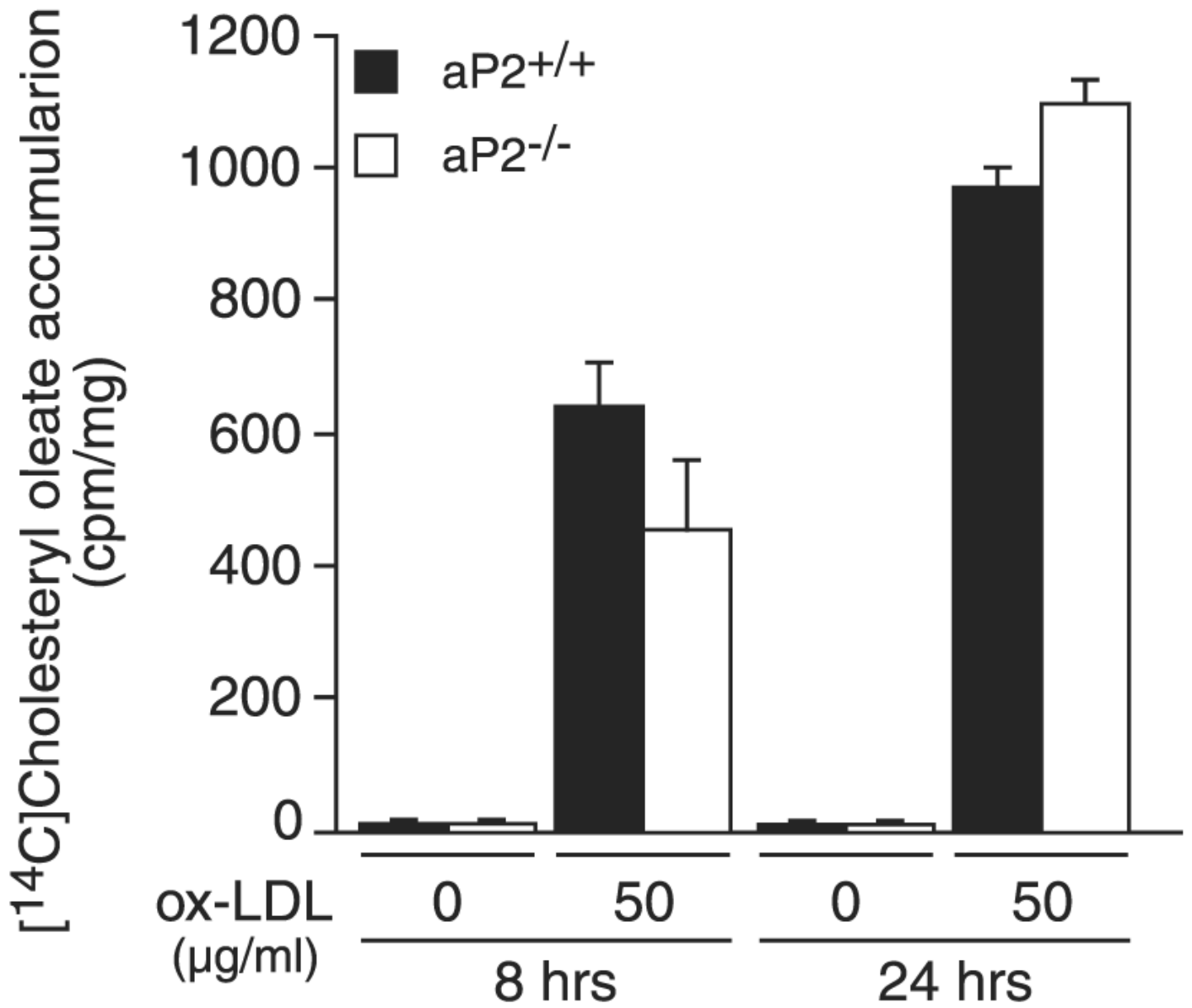
**Figure 2. Absence of aP2 in bone marrow-derived cells of ApoE<sup>-/-</sup> mice leads to decreased luminal occlusion and lesion area in brachiocephalic arteries.** Luminal occlusion (A) and cross-sectional areas (B) of atherosclerotic lesions in ApoE<sup>-/-</sup> mice receiving bone marrow cells from ApoE<sup>-/-</sup> (black bars, mean ± SE, n=6) or ApoE<sup>-/-</sup> aP2<sup>-/-</sup> (white bars, mean ± SE, n=9) donors. \*, P < 0.05 vs. ApoE<sup>-/-</sup> donors (black bars).

Fig. 3



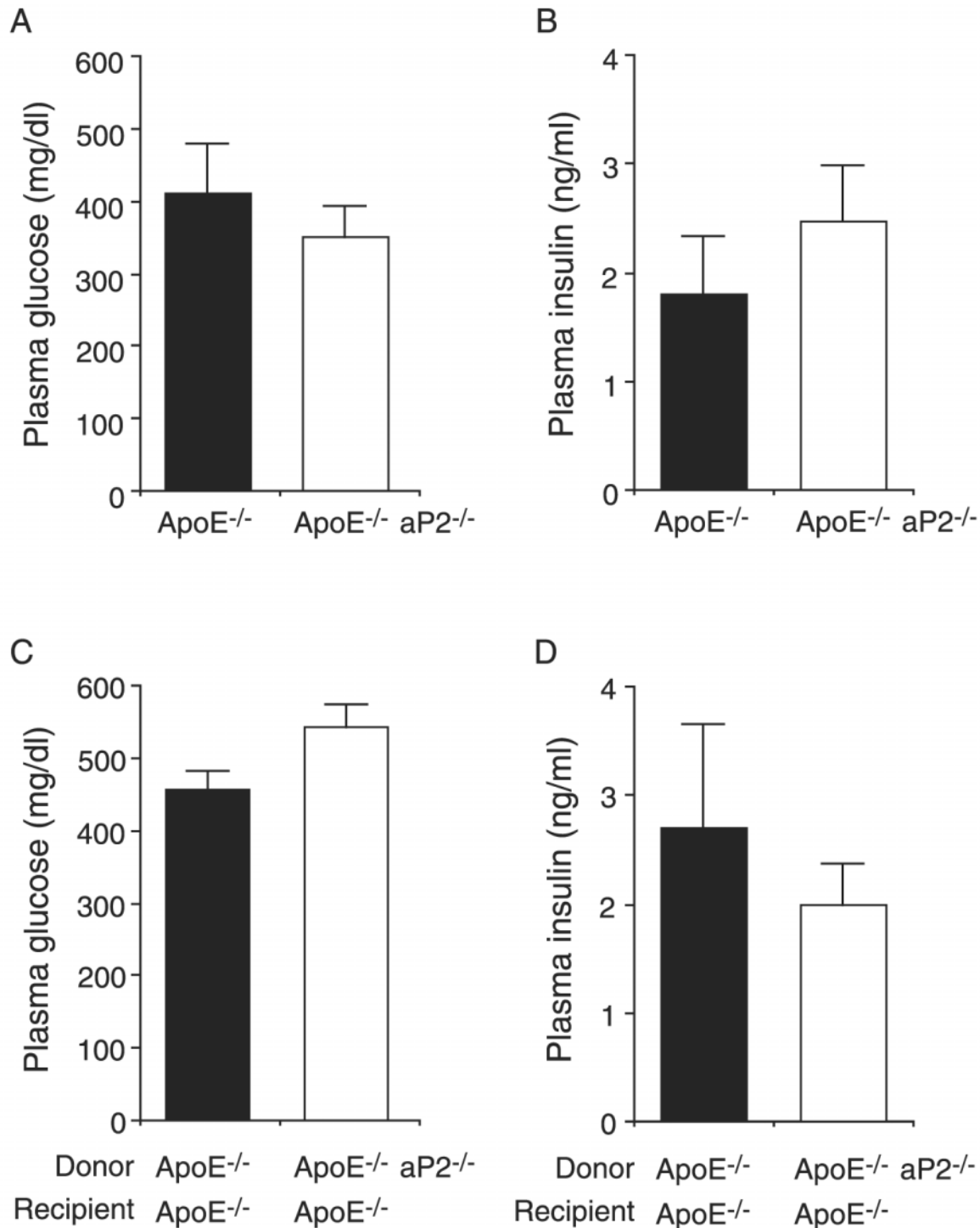
**Figure 3.** No difference in total cholesterol levels of ApoE<sup>-/-</sup> mice receiving bone marrow cells from ApoE<sup>-/-</sup> or ApoE<sup>-/-</sup> aP2<sup>-/-</sup> mice. Plasma levels of total cholesterol were analyzed in ApoE<sup>-/-</sup> mice receiving donor cells from ApoE<sup>-/-</sup> (black bars, mean  $\pm$  SE,  $n=6$ ) or ApoE<sup>-/-</sup> aP2<sup>-/-</sup> (white bars, mean  $\pm$  SE,  $n=11$ ) mice.

Fig. 4



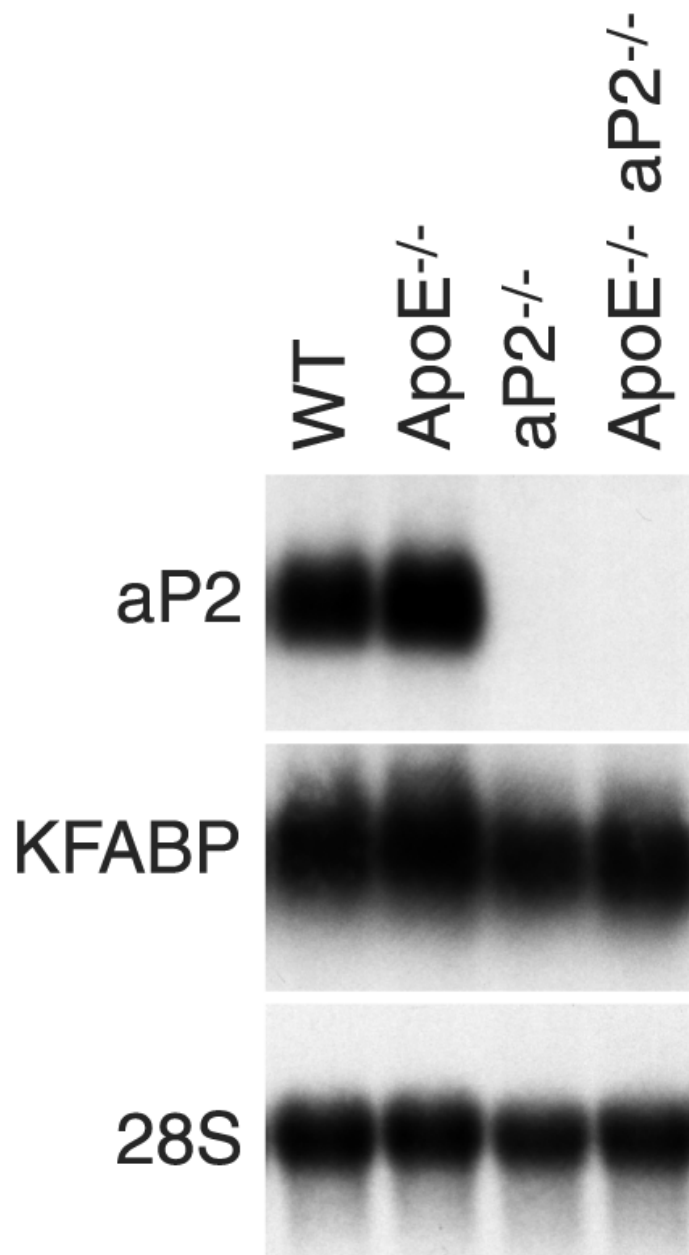
**Figure 4. Effect of aP2 on cholesterol esterification by macrophages.** Peritoneal macrophages were harvested from wild-type (aP2<sup>+/+</sup>, black bars) and aP2<sup>-/-</sup> (white bars) mice. Macrophages (*n*=3 in each group) were then stimulated with oxLDL for 8 or 24 h and were treated with [<sup>14</sup>C]oleic acid. [<sup>14</sup>C]cholesteryl oleate accumulation was assessed in the cells.

Fig. 5



**Figure 5.** No difference in plasma glucose or insulin levels in ApoE<sup>-/-</sup> mice in the presence or absence of aP2. **A** and **B**) Fasting plasma glucose and insulin levels were assessed in ApoE<sup>-/-</sup> (black bars, mean  $\pm$  SE,  $n=8$ ) and ApoE<sup>-/-</sup> aP2<sup>-/-</sup> (white bars, mean  $\pm$  SE,  $n=12$ ) mice. **C** and **D**) Fasting plasma glucose and insulin levels were also assessed in ApoE<sup>-/-</sup> mice receiving bone marrow cells from ApoE<sup>-/-</sup> (black bars, mean  $\pm$  SE,  $n=4$ ) and ApoE<sup>-/-</sup> aP2<sup>-/-</sup> (white bars, mean  $\pm$  SE,  $n=11$ ) mice.

Fig. 6



**Figure 6. KFABP mRNA levels are not altered in macrophages derived from aP2<sup>-/-</sup> or ApoE<sup>-/-</sup>aP2<sup>-/-</sup> mice.** RNA was harvested from wild-type (WT), ApoE<sup>-/-</sup>, aP2<sup>-/-</sup>, and ApoE<sup>-/-</sup>aP2<sup>-/-</sup> peritoneal macrophages and analyzed by Northern blot for aP2 and KFABP mRNA expression and normalized by hybridization to a <sup>32</sup>P-labeled 28S oligonucleotide.

**Fig. 7**

**Figure 7. Chemoattractant and inflammatory cytokine expression and nitrite production in the presence (+) or absence (-) of aP2.** **A)** Peritoneal macrophages were harvested from ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup> aP2<sup>-/-</sup> mice, and then exposed to vehicle (V) or oxLDL. Total RNA was extracted from the cells and RNase protection assays were performed for TNF- $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1, and GAPDH. **B)** Peritoneal macrophages harvested from wild-type (WT, black bar), aP2<sup>-/-</sup> (striped bar), ApoE<sup>-/-</sup> (gray bar), and ApoE<sup>-/-</sup> aP2<sup>-/-</sup> (white bar) mice were exposed to LPS (1  $\mu$ g/ml) and nitrite accumulation (mean  $\pm$  SE,  $n=3$ ) was assessed after 18 h.