Lack of macrophage fatty-acid–binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis

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The adipocyte fatty-acid–binding protein, aP2, has an important role in regulating systemic insulin resistance and lipid metabolism. Here we demonstrate that aP2 is also expressed in macrophages, has a significant role in their biological responses and contributes to the development of atherosclerosis. Apolipoprotein E (ApoE)-deficient mice also deficient for aP2 showed protection from atherosclerosis in the absence of significant differences in serum lipids or insulin sensitivity. aP2-deficient macrophages showed alterations in inflammatory cytokine production and a reduced ability to accumulate cholesterol esters when exposed to modified lipoproteins. *Apoe*$^{-/-}$ mice with *Ap2*$^{+/+}$ adipocytes and *Ap2*$^{-/-}$ macrophages generated by bone-marrow transplantation showed a comparable reduction in atherosclerotic lesions to those with total aP2 deficiency, indicating an independent role for macrophage aP2 in atherogenesis. Through its distinct actions in adipocytes and macrophages, aP2 provides a link between features of the metabolic syndrome and could be a new therapeutic target for the prevention of atherosclerosis.

Adipocyte fatty-acid–binding protein, aP2 (encoded by *Ap2*), is a member of the intracellular fatty-acid–binding protein (FABP) family. Cytoplasmic FABPs are small proteins that are expressed in a highly tissue-specific manner and bind to fatty acids[1,2]. aP2 is primarily detected in adipose tissue and its expression is highly regulated during differentiation of adipocytes[3,4]. Moreover, expression of *Ap2* mRNA is transcriptionally controlled by fatty acids[5,6]. Recent studies in aP2-deficient mice have shown that loss of this protein has a critical impact on several aspects of the metabolic syndrome. First, lack of aP2 provides significant protection from hyperinsulinemia and insulin resistance associated with dietary or genetic obesity[7,8]. Second, aP2 contributes to improved systemic glucose and lipid metabolism in the setting of dietary or genetic obesity[7,8] and alters the rate of adipocyte lipolysis[9,10]. As both insulin resistance and abnormal lipid metabolism are risk factors for cardiovascular disease, it is possible that aP2 influences the development of atherosclerosis by modulating these factors.

Several lines of evidence indicate a striking overlap between the biology of adipocytes and macrophages. Genes that are critical in adipocytes, including those encoding transcription factors, cytokines, inflammatory molecules, fatty acid transporters and scavenger receptors, are also expressed in macrophages and have an important role in their biology[11]. For example, peroxisome-proliferator activated receptor-γ (PPAR-γ) is a member of the nuclear-receptor superfamily of ligand-activated transcription factors that regulates adipocyte development and glucose homeostasis. PPAR-γ is also expressed in activated monocyte/macrophages and might have a role in generating the inflammatory response and forming the foam cells associated with atherosclerotic lesions[12–16]; however, the mechanisms for these functions are unclear. Interestingly, aP2 expression was recently revealed in human monocytes following stimulation with PPAR-γ activators[17], and oxidized low-density lipoprotein has been reported to induce expression of aP2 in human THP-1 macrophages[18]. These observations indicate that expression of aP2 by macrophages might also influence foam-cell formation and thereby atherosclerotic processes, possibly through mechanisms independent of its metabolic effects.

Here we examined whether macrophage aP2 influences foam-cell formation and the development of atherosclerosis independent of the effects of aP2 on insulin resistance and plasma lipids. Using two different models, *Apoe*$^{-/-}$ mice intercrossed with *Ap2*$^{-/-}$ mice and the bone-marrow transplantation (BMT) model in which *Ap2*$^{-/-}$*Apoe*$^{-/-}$ mice are donors to recipient *Ap2*$^{+/+}$*Apoe*$^{-/-}$ mice, we demonstrate that aP2-deficient macrophages provide significant protection against atherosclerosis in the ApoE-deficient model in the absence of differences in glucose and lipid metabolism.

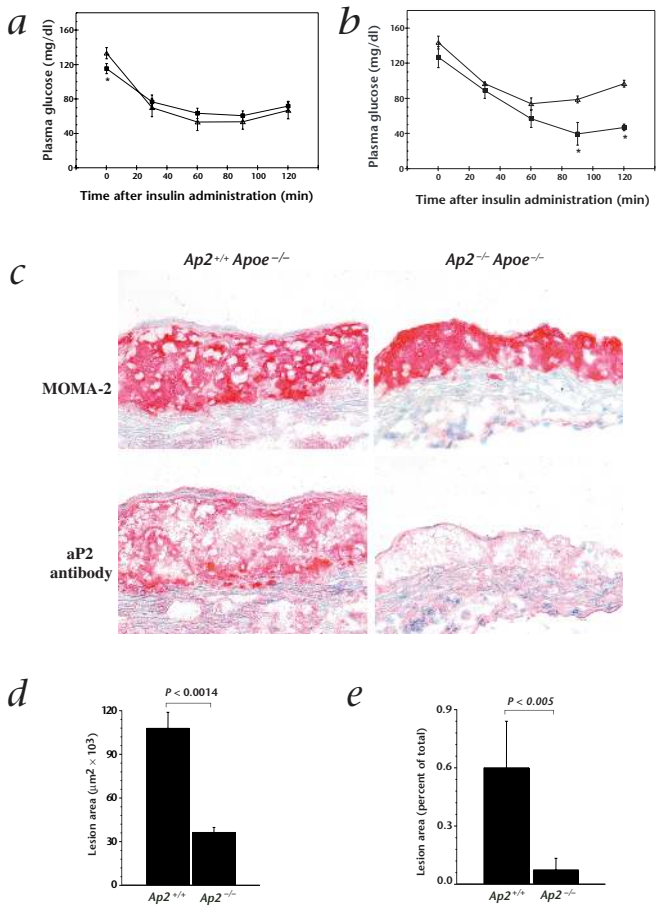

Fig. 1 Insulin sensitivity and atherosclerosis in $Ap2^{+/+}Apoe^{-/-}$ and $Ap2^{-/-}Apoe^{-/-}$ mice on chow diet. ***a*** and ***b***, Insulin tolerance tests were performed in 15 male (*a*) and 7 female (*b*) 14-week-old $Apoe^{-/-}$ (△) and $Ap2^{-/-}Apoe^{-/-}$ (■), mice. Data are mean ± s.e.m. *, $P < 0.05$. ***c***, Immunocytochemical detection of macrophages and aP2 expression in the proximal aorta of $Ap2^{+/+}Apoe^{-/-}$ (left) and $Ap2^{-/-}Apoe^{-/-}$ (right) mice. Macrophages are stained with MOMA-2 (upper), and aP2 is detected with polyclonal rabbit antiserum against mouse aP2 (lower). ***d*** and ***e***, Quantification of atherosclerotic lesion area in the proximal (*d*) and *en face* aorta (*e*) in $Ap2^{+/+}Apoe^{-/-}$ and $Ap2^{-/-}Apoe^{-/-}$ mice on chow diet. Data are represented as the average mean lesion area for each group.

Metabolism and atherosclerosis in $Ap2^{-/-}Apoe^{-/-}$ mice

Feeding C57BL/6 mice a high-fat diet promotes the development of obesity and insulin resistance[19], which could impact the development of atherosclerosis[20]. We therefore initially used ApoE-deficient mice, which on a normal chow diet develop spontaneous atherosclerotic lesions that are largely independent of obesity, hyperglycemia and hyperinsulinemia. To analyze the role of aP2 in atherosclerotic lesion formation, we intercrossed $Ap2^{-/-}$ mice with ApoE-deficient mice. At 4 weeks, we divided littermates into an experimental group of $Ap2^{-/-}Apoe^{-/-}$ ($n = 7$) and a control group of $Ap2^{+/+}Apoe^{-/-}$ ($n = 7$) mice and fed them a regular chow diet (< 4.5% fat) for 14 weeks. After insulin tolerance tests and steady-state biochemical measurements, we analyzed the extent of aortic atherosclerosis at 18 weeks. After a four-hour fast, total serum cholesterol (445 ± 36 versus 478 ± 12; mg/dl ± s.e.m.) and triglyceride (142 ± 27 versus 193 ± 13; mg/dl ± s.e.m.) levels were elevated as a result of ApoE deficiency in both $Ap2^{+/+}$ and $Ap2^{-/-}$ groups, respectively, without significant differences between genotypes. In addition, serum-glucose levels did not differ significantly between the experimental and control mice (117 ± 5.9 and 126.3 ± 13; mg/dl ± s.e.m.; $P = 0.53$). There was a reduction in total cholesterol, triglyceride and glucose, but not insulin levels in the $Ap2^{-/-}Apoe^{-/-}$ group compared with $Ap2^{+/+}Apoe^{-/-}$ controls after 24 hours fasting (data not shown). Insulin tolerance studies showed that insulin sensitivity did not differ between the male $Ap2^{-/-}Apoe^{-/-}$ and $Ap2^{+/+}Apoe^{-/-}$ mice (Fig. 1*a*). We saw similar results in the females, except that insulin tolerance tests indicated a small improvement in the $Ap2^{-/-}Apoe^{-/-}$ mice compared with $Apoe^{-/-}$ controls (Fig. 1*b*). These data showed that except for a small change in insulin sensitivity in the females, the steady state metabolic status of these mice was similar.

All of the mice developed moderate, fatty streak lesions, consisting predominantly of macrophage-derived foam cells, as determined by immunocytochemistry using the monoclonal antibody against mouse macrophages[21], MOMA-2 (Fig. 1*c*, upper panels). The macrophage-derived foam cells stained strongly positive for aP2 in the lesions of the $Ap2^{+/+}Apoe^{-/-}$ but not in the $Ap2^{-/-}Apoe^{-/-}$ mice (Fig. 1*c*, lower panels). Quantitative analysis of the proximal aorta revealed that the mean atherosclerotic lesion area in $Ap2^{-/-}Apoe^{-/-}$ males was reduced by 66% compared with $Ap2^{+/+}Apoe^{-/-}$ males (36,334 ± 3,492 versus 107,815 ± 11,144; μm^2/section ± s.e.m.; $P < 0.0014$; Fig. 1*d*). A similar reduction (87.6%) was found by *en face* analysis of the extent of atherosclerosis in the entire aorta in $Ap2^{-/-}Apoe^{-/-}$ males compared with $Ap2^{+/+}Apoe^{-/-}$ controls (0.60 ± 0.15 versus 0.074 ± 0.42; percent ± s.e.m.; $P < 0.005$; Fig. 1*e*). Thus, in the setting of a normal chow diet, male ApoE-deficient mice lacking aP2 were protected from the development of macrophage-derived foam cells and atherosclerosis. In a separate experiment, the extent of atherosclerosis in the proximal aorta was reduced by 35% in male and by 26% in female $Ap2^{-/-}Apoe^{-/-}$ mice fed the Western diet for 14 weeks compared with $Ap2^{+/+}Apoe^{-/-}$ controls ($P < 0.0024$ and $P < 0.006$, respectively; data not shown).

FABP expression and function in macrophages

Given that atherosclerotic lesions showed strong presence of aP2 and the differences in atherosclerosis occurred in the absence of significant metabolic alterations, macrophage aP2 expression might be responsible for the observed differences in atherosclerosis. We therefore investigated aP2 expression and regulation in primary isolated macrophages and macrophage cell lines. In both THP-1 and U-937 human monocyte/macrophage cell lines, we did not detect aP2 expression in resting cells but did so after stimulation by PMA (phorbol 13-myristate 12-acetate) to induce differentiation (Fig. 2*a* and *b*). Similarly, primary human monocytes did not express aP2 protein, but we observed high levels upon stimulation with PMA (Fig. 2*d*). In contrast, we did not detect aP2 expression by northern-blot analysis of resting or stimulated EL4 T-cell or M12 B-cell lines (data not shown). The keratinocyte FABP, mal1, which is the second isoform expressed in adipocytes, was also present in these cells and was regulated in an essentially identical manner upon PMA stimulation, although low levels were detectable in the resting state in THP-1 and U-937 cell lines (Fig. 2*a* and *b*). Expression of both aP2 and mal1 was also observed in isolated peritoneal mouse macrophages. Unlike the compensatory regulation in adipocytes, mRNA encoding mal1 did not appear to be significantly upregulated in $Ap2^{-/-}$ primary macrophages (Fig. 2*c*). We then investigated whether the regulatory elements that direct expression of aP2 in adipocytes are sufficient to confer expression in macrophages of transgenic mice. For this we used three independent lines expressing genes encoding uncoupling protein (UCP)-1,

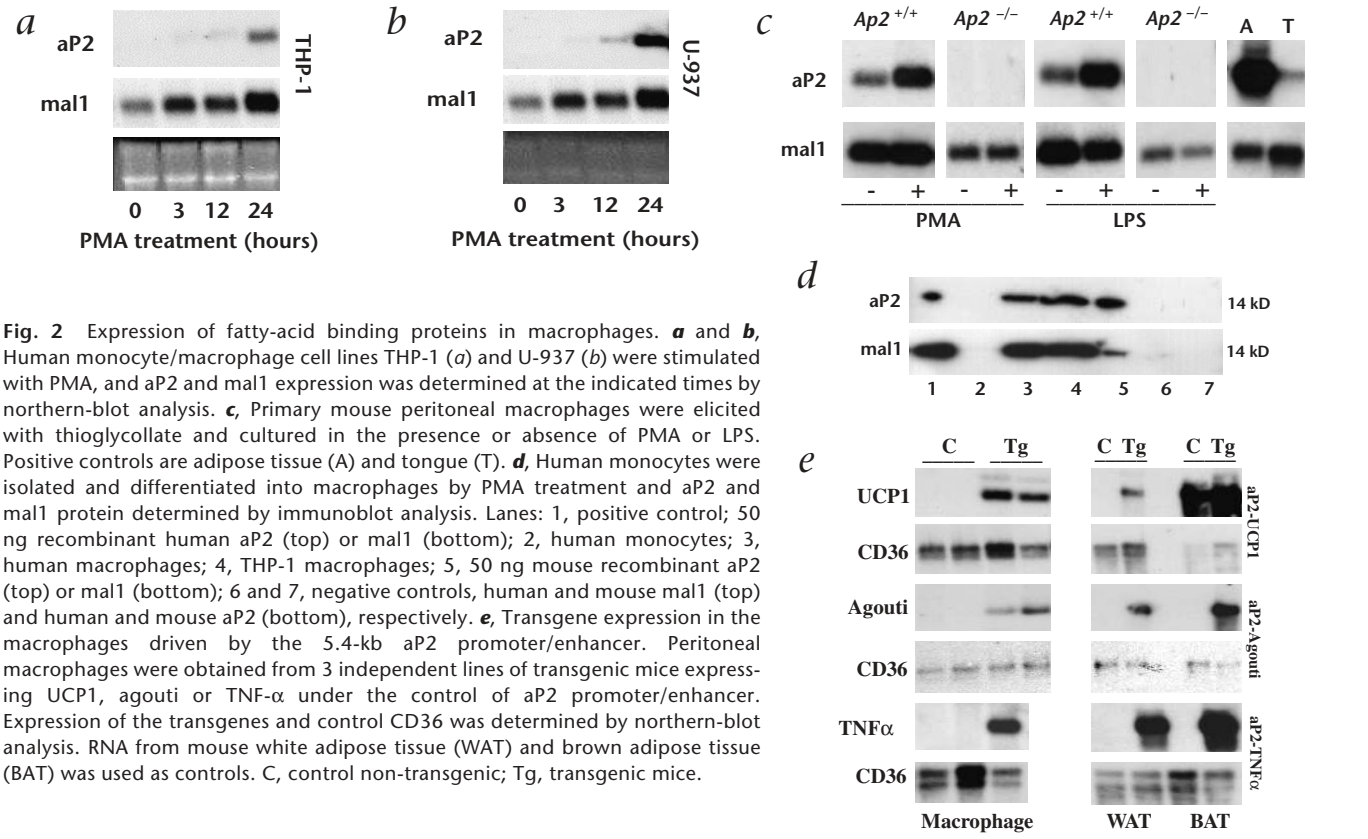

Fig. 2 Expression of fatty-acid binding proteins in macrophages. ***a*** and ***b***, Human monocyte/macrophage cell lines THP-1 (*a*) and U-937 (*b*) were stimulated with PMA, and aP2 and mal1 expression was determined at the indicated times by northern-blot analysis. ***c***, Primary mouse peritoneal macrophages were elicited with thioglycollate and cultured in the presence or absence of PMA or LPS. Positive controls are adipose tissue (A) and tongue (T). ***d***, Human monocytes were isolated and differentiated into macrophages by PMA treatment and aP2 and mal1 protein determined by immunoblot analysis. Lanes: 1, positive control; 50 ng recombinant human aP2 (top) or mal1 (bottom); 2, human monocytes; 3, human macrophages; 4, THP-1 macrophages; 5, 50 ng mouse recombinant aP2 (top) or mal1 (bottom); 6 and 7, negative controls, human and mouse mal1 (top) and human and mouse aP2 (bottom), respectively. ***e***, Transgene expression in the macrophages driven by the 5.4-kb aP2 promoter/enhancer. Peritoneal macrophages were obtained from 3 independent lines of transgenic mice expressing UCP1, agouti or TNF-α under the control of aP2 promoter/enhancer. Expression of the transgenes and control CD36 was determined by northern-blot analysis. RNA from mouse white adipose tissue (WAT) and brown adipose tissue (BAT) was used as controls. C, control non-transgenic; Tg, transgenic mice.

agouti and tumor necrosis factor (TNF)-α under the control of the 5.4-kb aP2 promoter/enhancer. Primary macrophages from all three transgenic lines showed high levels of expression of these transgenes (Fig. 2*e*). The aP2 promoter/enhancer thus directs expression in macrophages as well as adipocytes.

We next investigated whether aP2 deficiency has functional consequences in macrophage biology that might be relevant to the development of atherosclerosis. We first determined the expression of several inflammatory cytokines, including TNF-α, interleukin (IL)-1β and IL-6 in $Ap2^{-/-}$ macrophages and wild-type controls. In the resting state, expression of these cytokines was significantly reduced in the aP2-deficient macrophage cell lines compared with $Ap2^{+/+}$ controls (Fig. 3*a*). Moreover, whereas an increase in TNF-α, IL-1β, and IL-6 expression was evident in the wild-type cells upon PMA stimulation, we observed no changes in the $Ap2^{-/-}$ cells, demonstrating a reduced inflammatory capacity of $Ap2^{-/-}$ macrophages (Fig. 3*a*).

To examine the potential function of aP2 in macrophage lipid deposition, cholesterol ester accumulation was assayed in acetylated low-density lipoprotein (Ac-LDL)-loaded macrophages derived from wild-type and $Ap2^{-/-}$ mice. Lipids extracted from cells were assayed using a Nile Red fluorescent-lipid thin-layer chromatography (TLC). $Ap2^{-/-}$ macrophages responded to Ac-LDL loading with 37% less accumulation of cholesterol ester compared with the $Ap2^{+/+}$ controls ($P < 0.001$; Fig. 3*b*). In the absence of lipid loading, the cholesterol ester levels in the $Ap2^{-/-}$ cells were 29% lower than in $Ap2^{+/+}$ cells ($P < 0.01$). We also evaluated the levels of aP2 and mal1 protein expression in the $Ap2^{-/-}$ and wild-type macrophages. Under the conditions of the lipid accumulation studies, the aP2 protein level in the wild-type line (2 ng/μg cell protein) was approximately 22% that of mal1 (9 ng/μg). In the $Ap2^{-/-}$ line, aP2 was not detectable and mal1 was 20.9 ng/μg. Exposure to Ac-LDL did not affect the expression levels of either

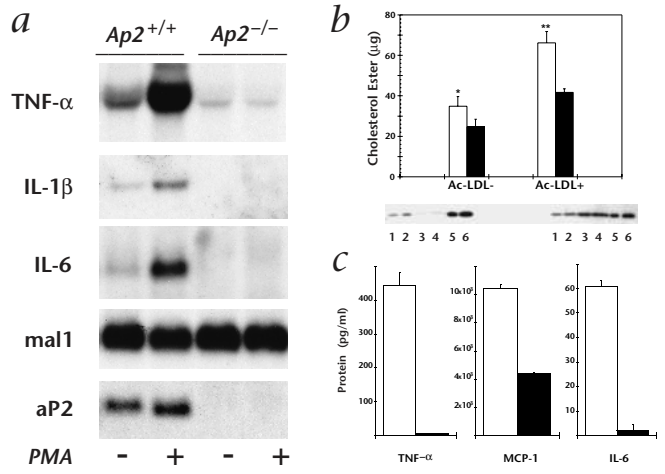

Fig. 3 Expression of inflammatory cytokines and cholesterol ester levels in $Ap2^{-/-}$ macrophages. ***a***, TNF-α, IL-1β and IL-6 mRNA levels were determined following PMA treatment. The bottom two blots show mal1 and aP2 mRNA expression. ***b*** and ***c***, Accumulation of cholesterol esters (*b*) and cytokine secretion (*c*) in control (□) and $Ap2^{-/-}$ (■) macrophages. Cholesterol ester levels were determined before and after treatment with Ac-LDL. Cytokine levels were determined by ELISA in the conditioned medium following treatment with Ac-LDL. The graph shows mean ± s.e. from 10 independent experiments. Immunoblot analysis of aP2 (L) and mal1 (R) protein expression is shown in *b*, lower. Lanes: 1, $Ap2^{+/+}$ without Ac-LDL; 2, $Ap2^{+/+}$ with Ac-LDL; 3, $Ap2^{-/-}$ without Ac-LDL; 4, $Ap2^{-/-}$ with Ac-LDL; 5&6, 20 and 40 ng of recombinant murine aP2 (left) or Mal1 (right) standards.

Table 1 Serum lipids and glucose in *AP2*$^{-/-}$ BMT mice

Group	Serum study	Baseline	4 wk post-BMT	8 wk post-BMT	13 wk post-BMT
Ap2$^{+/+}$Apoe$^{-/-}$→Apoe$^{-/-}$ (male), *n* = 8	Cholesterol	350 ± 46	416 ± 80	423 ± 52	448 ± 109
	Triglycerides	147 ± 36	169 ± 54	120 ± 39	137 ± 49
	Glucose			110 ± 18	127 ± 39
Ap2$^{-/-}$Apoe$^{-/-}$→Apoe$^{-/-}$ (male), *n* = 9	Cholesterol	331 ± 43	450 ± 37	371 ± 66	395 ± 88
	Triglycerides	135 ± 40	144 ± 50	129 ± 32	135 ± 31
	Glucose			110 ± 9	135 ± 27
Ap2$^{+/+}$Apoe$^{-/-}$→Apoe$^{-/-}$ (female), *n* = 11	Cholesterol	282 ± 64	406 ± 67	454 ± 56	300 ± 45
	Triglycerides	63 ± 15	69 ± 19	81 ± 27	98 ± 46
	Glucose			101 ± 31	94 ± 18
Ap2$^{-/-}$Apoe$^{-/-}$→Apoe$^{-/-}$ (female), *n* = 8	Cholesterol	265 ± 59	443 ± 85	432 ± 110	283 ± 92
	Triglycerides	53 ± 12	80 ± 16	85 ± 22	118 ± 48
	Glucose			90 ± 42	107 ± 14

Fasting (4 h) total serum-cholesterol, -triglyceride and -glucose levels in male and female *Apoe*$^{-/-}$ mice before and after BMT with *Ap2*$^{+/+}$*Apoe*$^{-/-}$ or *Ap2*$^{-/-}$*Apoe*$^{-/-}$ marrow. Values (mg/dL) are mean ± s.d.

FABP in this model (Fig. 3*b*, lower panel). These results indicate that genetic elimination of aP2 significantly decreased intracellular cholesterol ester accumulation in macrophages despite the presence of substantial amounts of mal1 protein. We also determined cytokine production from these cells under the same experimental conditions. These experiments demonstrated a strong reduction in the capacity of *Ap2*$^{-/-}$ cells to secrete TNF-α, monocyte chemotactic protein (MCP)-1, and IL-6 proteins compared with wild-type controls (Fig. 3*c*).

Bone-marrow transplantation studies

BMT studies in gene-targeted mice have been a powerful tool to examine the contribution of various genes expressed by macrophages to foam-cell formation and atherogenesis[22–25]. We therefore created *Apoe*$^{-/-}$ mice chimeric for macrophage aP2 expression using BMT to determine the contribution of macrophage aP2-deficiency in atherogenesis. Ten-week-old male *Apoe*$^{-/-}$ mice were lethally radiated (9 Gy) and transplanted with either *Ap2*$^{-/-}$*Apoe*$^{-/-}$ (experimental group, *n* = 10) or *Ap2*$^{+/+}$*Apoe*$^{-/-}$ (control group, *n* = 8) bone marrow. Similarly, 10-week-old female *Apoe*$^{-/-}$ mice were lethally radiated and transplanted with either *Ap2*$^{-/-}$*Apoe*$^{-/-}$ (experimental group, *n* = 8) or *Ap2*$^{+/+}$*Apoe*$^{-/-}$ (control group, *n* = 11) bone marrow. The transplanted mice were fed a standard chow diet (< 4.5% fat) for 13 weeks. We observed no significant differences in serum levels of glucose, cholesterol or triglycerides between the experimental and control groups in either experiment at 8 or 13 weeks after transplantation (Table 1). Moreover, the distribution of cholesterol among the serum lipoproteins as determined by size exclusion chromatography did not differ significantly between the experimental and control groups in either experiment (Fig. 4*a*). Finally, insulin tolerance tests showed that insulin sensitivity did not differ between the two groups (data not shown). Therefore, the lack of macrophage aP2 expression had no impact on fasting serum-glucose and -lipid levels or lipoprotein profiles under these dietary conditions.

Thirteen weeks after transplantation, the extent of atherosclerosis in the proximal aortas of the transplanted *Apoe*$^{-/-}$ mice was determined. Staining of cross-sections of the proximal aorta with Oil Red O revealed fatty streak lesions, which in the males consisted almost exclusively of macrophage-derived foam cells, as determined by immunocytochemical staining with MOMA-2 (Fig. 4*b*). Immunocytochemical analysis of serial sections of the proximal aorta by staining with MOMA-2 for mouse macrophages (Fig. 4*b*, upper panels), or the aP2 protein (Fig. 4*b*, lower panels) revealed

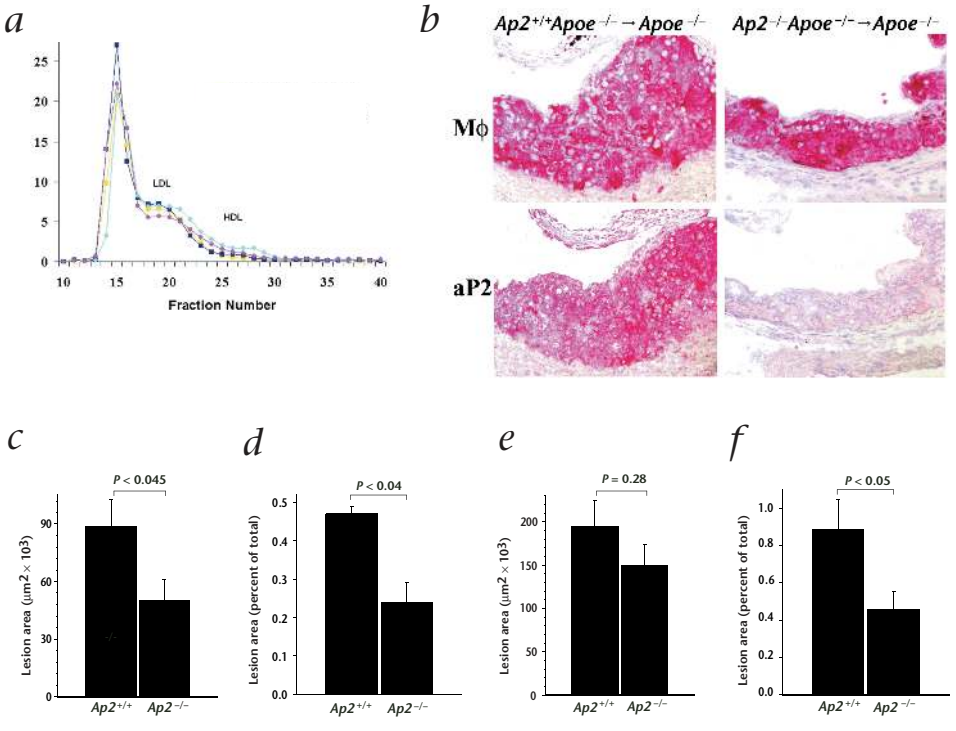

Fig. 4 Lipoprotein distribution, immunochemistry and atherosclerosis in *Ap2*$^{-/-}$ BMT mice. ***a***, Lipoprotein distribution in *Apoe*$^{-/-}$ mice with transplanted marrow after 13 wk on standard chow diet. ■, female *Ap2*$^{-/-}$*Apoe*$^{-/-}$; ■, female *Ap2*$^{+/+}$*Apoe*$^{-/-}$; ■, male *Ap2*$^{-/-}$*Apoe*$^{-/-}$; ■, male *Ap2*$^{+/+}$*Apoe*$^{-/-}$. Data are represented as an average (n = 3) percent distribution of total cholesterol for each group. Fractions 14–17 contain VLDL; fractions 18–24 are IDL/LDL; and fractions 25–29 contain HDL. Fractions 30–40 are the non–lipoprotein-associated proteins. ***b***, Immunocytochemical detection of macrophages and aP2 expression in the proximal aorta of *Apoe*$^{-/-}$ mice transplanted with *Ap2*$^{+/+}$*Apoe*$^{-/-}$ (left) or *Ap2*$^{-/-}$*Apoe*$^{-/-}$ (right) marrow. Macrophages are stained as in Fig. 1*c*. ***c–f***, Quantification of atherosclerotic lesion area in the proximal and the en face aorta, respectively for male (*c* and *d*) and female (*e* and *f*) *Apoe*$^{-/-}$ mice 13 weeks after receiving *Ap2*$^{+/+}$*Apoe*$^{-/-}$ or *Ap2*$^{-/-}$*Apoe*$^{-/-}$ marrow. The atherosclerotic lesions were stained and quantified as noted in Fig. 1. Data are represented as the average mean lesion area for each group.

that macrophage-derived foam cells in $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice colocalized with aP2 protein. Macrophages from $Ap2^{-/-} \rightarrow Apoe^{-/-}$ mice did not react with the aP2 antibody. Quantitative analysis of the extent of atherosclerotic lesions in the proximal aorta revealed that mean lesion area in male $Ap2^{-/-} \rightarrow Apoe^{-/-}$ mice was reduced by 43% compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice (50,311 ± 10,600 and 88,688 ± 13,950; μm^2/section ± s.e.m., respectively; $P < 0.045$; Fig. 4*c*). *En face* analysis of the extent of atherosclerosis in the entire aorta in $Ap2^{-/-} \rightarrow Apoe^{-/-}$ males revealed a 48% reduction compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ males (0.24 ± 0.05 versus 0.47 ± 0.095; precent ± s.e.m.; $P < 0.04$; Fig. 4*d*). Thus, male $Apoe^{-/-}$ mice reconstituted with $Ap2^{-/-}$ macrophages are protected from atherosclerosis compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice. As expected, the extent of atherosclerosis was greater in the female ApoE transplant recipients, and lesions in their proximal aorta were significantly more advanced, showing the presence of smooth muscle cells in the intima. Quantitative analysis of the extent of atherosclerotic lesions in the proximal aorta revealed a trend for a reduction in mean lesion area in female $Ap2^{-/-} \rightarrow Apoe^{-/-}$ mice compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice (150,074 ± 23,347 and 194,358 ± 29,798; μm^2/section ± s.e.m., respectively; $P = 0.288$; Fig. 4*e*). However, *en face* analysis of the extent of atherosclerosis in the entire aorta in $Ap2^{-/-} \rightarrow Apoe^{-/-}$ females revealed a significant 48% reduction in mean lesion area compared with $Ap2^{+/+} \rightarrow Apoe^{-/-}$ mice (0.46 ± 0.05 versus 0.89 ± 0.16, percent ± s.e.m.; $P < 0.04$; Fig. 4*f*). These transplantation studies demonstrate a role for macrophage aP2 expression in promoting foam-cell formation and atherogenesis *in vivo*.

Discussion

Previous studies have demonstrated that deficiency of aP2, a downstream target of PPAR-γ, predominantly affects adipocytes and contributes to improved systemic glucose and lipid metabolism in the setting of dietary or genetic obesity[7,8]. Here, we demonstrate that aP2 is also strongly expressed in macrophages and modulates their biological responses. Total and macrophage-specific aP2 deficiencies led to a striking protection from the development of atherosclerosis in the $Apoe^{-/-}$ model. Based on these data, a potential mechanism underlying the metabolic syndrome emerges from coordinated modulation of the metabolic status of adipocytes and the metabolic or inflammatory status of macrophages through pathways common to both cell types.

The gene encoding aP2 is expressed at high levels in mouse and human macrophages upon activation *in vitro* and in macrophages associated with atherosclerotic lesions *in vivo*, raising the possibility that it might impact the macrophage inflammatory response and foam-cell formation. As in adipocytes, production of TNF-α is greatly reduced in $Ap2^{-/-}$ macrophages compared with wild-type controls[7]. Other inflammatory cytokines such as IL-1β and IL-6 are also suppressed in $Ap2^{-/-}$ macrophages. Moreover, $Ap2^{-/-}$ macrophages display significantly decreased intracellular cholesterol ester accumulation *in vitro* and secrete highly reduced quantities of TNF-α, MCP-1 and IL-6 upon exposure to modified lipoprotein. These results indicate that aP2 has a significant role in two important aspects of macrophage biology that are highly relevant to the pathogenesis of atherosclerosis. mal1, a close relative of aP2, presents an expression pattern similar to aP2. However, whereas the expression of mal1 mRNA in $Ap2^{-/-}$ adipocytes is greatly enhanced, compensatory regulation in $Ap2^{-/-}$ macrophages is mild. Hence, it will be important to determine the impact of mal1 and combined aP2/mal1 deficiency in metabolic regulation and atherosclerosis. Finally, macrophage aP2 expression is controlled with the same promoter/enhancer elements that confer expression in adipocytes; this might have important implications in the assessment of many transgenic lines generated with the 5.4-kb aP2 promoter/enhancer.

Both macrophages and adipocytes are sites for active lipid metabolism and signaling. Therefore, it is not surprising that FABPs have a strong impact on the biology of these cells. This is likely to involve many aspects of lipid metabolism in both cell types including transport of lipids and shuttling them to specific metabolic or signaling pathways. Recent studies with other isoforms have indicated potential regulation of nuclear hormone receptor activity in the liver through direct transportation of lipid and xenobiotic ligands[26]. Regulation of macrophage ACAT activity[27–29] or the availability of FA for esterification with cholesterol might also be altered by FABPs. Alternatively, FABPs could impact the rates of transport through quantitative or functional alterations in fatty-acid transporters or scavenger receptors. Further studies will be required to address these possibilities.

A critical question is whether the function of aP2 in the macrophage is sufficient to modulate development of atherosclerosis in a way that is distinguishable from the metabolic consequences of aP2 deficiency. We have taken several steps to address this question. First, we studied the effect of aP2 deficiency on the development of atherosclerosis in $Apoe^{-/-}$ mice on normal chow diet. On this diet, ApoE-deficient mice exhibit severe hypercholesterolemia and spontaneous atherosclerotic lesions in the absence of significant changes in body weight or serum levels of glucose or insulin. Under these conditions, male $Ap2^{-/-}Apoe^{-/-}$ mice, despite severe hypercholesterolemia, developed 66% less atherosclerosis in cross-sections of the proximal aorta and 86% smaller lesions by *en face* analysis of the extent of lesions in the entire aorta compared with control $Ap2^{+/+}Apoe^{-/-}$ mice. These reductions in the extent of atherosclerosis occurred in the absence of significant changes in serum-lipid, -glucose or -insulin levels, supporting the hypothesis that aP2 expression contributes to atherosclerosis independent of its established role in glucose and lipid metabolism.

Second, we generated macrophage-restricted aP2 deficiency through BMT experiments to examine the hypothesis that macrophage aP2 expression promotes atherogenesis independent of its expression by adipocytes. Male $Apoe^{-/-}$ mice reconstituted with $Ap2^{-/-}$ macrophages had a 43% and 49% reduction in lesion area compared with those reconstituted with $Ap2^{+/+}$ cells, as assessed by both lesion size in cross sections of the proximal aorta and in the entire aorta *en face*, respectively. As expected, the extent of atherosclerosis was greater in the proximal aortas of female ApoE-deficient transplanted mice than in the males, and the reduction in vascular lesions in female $Ap2^{-/-}Apoe^{-/-} \rightarrow Apoe^{-/-}$ mice at this site was not statistically significant. Although this observation indicates a potential gender difference regarding the effect of aP2 expression in atherosclerosis, we think that it most likely reflects the fact that the lesions in the proximal aorta of the females were more advanced. These include intermediate lesions with smooth muscle cell involvement, indicating that the contribution of macrophage aP2 expression might be lessened in the setting of more advanced lesions. In the whole aorta, a significant 48% reduction in mean lesion area was evident in female $Ap2^{-/-}Apoe^{-/-} \rightarrow Apoe^{-/-}$ mice compared with female $Ap2^{+/+}Apoe^{-/-} \rightarrow Apoe^{-/-}$ mice. This may be explained by the fact that mice develop atherosclerotic lesions first in the proximal aorta and the lesions progress distally, resulting in more advanced lesions in the proximal than the distal aorta[30]. Moreover, in response to 14 weeks on the Western-type diet, female $Apoe^{-/-}Ap2^{-/-}$ mice show reduced atherosclerosis in the proximal aorta compared with $Apoe^{-/-}Ap2^{-/-}$ con-

trols, indicating that the effects of aP2 on atherosclerosis are not gender specific. These results demonstrate a significant role for macrophage aP2 in atherogenesis independent of its expression in adipocytes and its role in glucose and lipid metabolism.

Taken together with the previous observations regarding the impact of aP2 on glucose and lipid metabolism, our data support an important role for aP2 in understanding the molecular basis of metabolic syndrome via its coordinated action on both metabolic and inflammatory responses. These findings should provide further insights into the molecular mechanisms leading to these diseases and generate opportunities for the development of a novel class of therapeutic modalities to treat them.

Methods

Animal procedures. Mice homozygous for inactivation of *Ap2* were backcrossed 12 generations into the C57BL/6 background[8,10]. These $Ap2^{-/-}$ mice were intercrossed with the ApoE-deficient mice also in the C57BL/6 background to generate mice heterozygous at both loci. These $Ap2^{-/+}Apoe^{-/+}$ mice were then intercrossed to produce $Ap2^{-/-}Apoe^{-/-}$ mice along with $Ap2^{+/+}Apoe^{-/-}$ littermate controls. At 4 weeks, littermates were divided into an experimental group of $Ap2^{-/-}Apoe^{-/-}$ and a control group of $Ap2^{+/+}Apoe^{-/-}$ mice. For the BMT study, donor mice were derived from the same colony of $Ap2^{-/-}Apoe^{-/-}$ and $Ap2^{+/+}Apoe^{-/-}$ mice described above. Recipient mice were derived from an established colony at our mouse facility of ApoE-deficient mice on the C57BL/6 background[22,31]. Mice were fed standard chow with 4.5% fat (PMI Feeds, St. Louis, Missouri) or Western diet (diet #TD88137, Harland Teklad, Madison, Wisconson). UCP1 and agouti transgenic mice were provided by L. Kozak. Transgenic mice expressing a membrane-bound form of TNF-α driven by the aP2 promoter/enhancer in the TNF-α–deficient background were generated in one of our laboratories (Harvard). Animal care and experimental procedures were performed under approval from the Animal Care Committees of Vanderbilt and Harvard Universities.

Bone-marrow transplantation. 1 week before and 2 weeks following BMT, 100 mg/L neomycin and 10 mg/L polymyxin B sulfate (Sigma) were added to the acidified water. Bone marrow was collected from donor mice by flushing femurs with RPMI 1640 media (GIBCO, Grand Island, New York) containing 2% FBS and 5 U/ml heparin (Sigma). Recipient mice were lethally irradiated (9 Gy) by a cesium gamma source. 4 hours later, $5–10 \times 10^6$ bone-marrow cells in 0.3 ml were transplanted by tail-vein injection as described[22,32].

Serum measurements and insulin tolerance tests. Mice were fasted for either 4 h during day or 24 h overnight and blood samples were collected by retro-orbital venous plexus puncture under isoflurane anesthesia (IsoVet, Schering-Plough, Union, New Jersey). Serum was separated by centrifugation and 1 mM phenylmethylsulfonyl fluoride was added (Sigma). The serum total cholesterol and triglycerides were determined using Sigma kit #352 and kit #339 adapted for microtiter plate assay as described[33]. Blood glucose concentrations were determined on 5 μl whole blood by using gluco-analyser blood glucose strips (Medisense, Bedford, Massachussetts). Steady-state insulin concentrations were determined with a commercially available radioimmunoassay (Linco, St. Charles, Missouri). Lipoprotein assays were performed as described[30]. Insulin tolerance tests (0.5 IU/kg) were performed on conscious mice following a 6-h fast as described[7,34,35].

Immunocytochemistry and quantification of arterial lesions. To detect macrophages and the aP2 protein in arterial lesions, 5-μm serial cryosections of the proximal aorta were incubated with either a polyclonal rabbit antiserum against mouse aP2 (gift of D. Bernlohr) or rat antibody against mouse macrophages[21], MOMA-2 (Accurate, Westbury, New York) as described[25]. For quantification of arterial lesions, aortas were pinned out in an *en face* preparation as described[30,36]. 10-μm thick cryosections of the proximal aorta were stained with Oil Red O and counterstained with hematoxylin, as described[23,37]. Quantitative analysis of lipid-stained lesions was performed using an Imaging System KS 300 (Release 2.0; Kontron Electronik GmbH, Eden Prairie, Minnesota). Color threshold was used to delimit the Oil-Red-O–stained lesion area that was measured as squared microns per mouse.

Macrophage studies. PBMCs were prepared from healthy donors as described[38] and were incubated overnight in RPMI medium + 5% FBS to spontaneously differentiate into macrophages. THP-1 (ATCC #TIB 202) and U-937 (ATCC #CRL-1593.2) human monocytic leukemia cell lines were grown in RPMI with 5% FBS and differentiated by 25 or 100 nM PMA into macrophages. Immortalized $Ap2^{+/+}$ and $Ap2^{-/-}$ murine macrophage cell lines were generated in our laboratory by a modification of a described procedure[39]. Primary macrophages are isolated from the peritoneal cavity and were incubated with the indicated reagents for 24 hours. $Ap2^{+/+}$ and $Ap2^{-/-}$ mouse macrophage cell lines were grown to near confluence in 12-well plates in RPMI-1640 with 5% FBS. Supernatants were collected after 72 h of treatment with 50 μg/ml Ac-LDL (Biomedical Technologies, Stoughton, Massachusetts) and cytokine levels were determined by commercially available ELISA systems (TNF-α from R&D, Minneapolis, Minnesota; IL-6 and MCP-1 from PharMingen, San Diego, California). Macrophage cell lines were cultured and lipids were extracted from the cells for TLC analysis as described[40]. Immunoblots were conducted with rabbit antibodies against human recombinant aP2 and mal1 as described[10].

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