

# Expression of the *Lyst*<sup>beige</sup> mutation is atheroprotective in chow-fed apolipoprotein E-deficient mice<sup>S</sup>

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**Abstract** *Lyst*<sup>beige</sup> mice crossed with hyperlipidemic low density lipoprotein receptor-deficient mice (*BgLDLr*<sup>-/-</sup>) display increased lesion area and a more stable lesion morphology. To verify that the beige phenotype is not unique to *LDLr*<sup>-/-</sup> mice, we examined atherosclerosis in beige, apolipoprotein E-deficient mutant mice (*BgApoE*<sup>-/-</sup>). Severe diet-induced hyperlipidemia in *BgApoE*<sup>-/-</sup> mice resulted in increased aortic sinus lesion areas compared with controls. Minimal aortic lesions were observed in both genotypes on a chow diet. Nevertheless, *BgApoE*<sup>-/-</sup> mice displayed drastically reduced aortic sinus lesion growth. Reconstitution with bone marrow (BM) from green fluorescent protein mice created chimeric animals that allowed for the identification of donor-derived cells within lesions. Expressing the beige mutation exclusively in BM-derived cells had no impact on plaque development, yet the beige mutation in all cells except the BM-derived cells led to significantly larger aortic sinus lesion areas. Both mRNA and secreted protein levels of monocyte chemoattractant protein 1 were altered in quiescent and phorbol ester-stimulated cultured macrophages, vascular smooth muscle cells, and aortic endothelial cells isolated from *BgApoE*<sup>-/-</sup> mice. Thus, expression of the beige mutation in all cell types involved in lesion development contributed to atheroprotection in chow-fed *ApoE*<sup>-/-</sup> mice.—Petrovan, R. J., Y. Yuan, and L. K. Curtiss. Expression of the *Lyst*<sup>beige</sup> mutation is atheroprotective in chow-fed apolipoprotein E-deficient mice. *J. Lipid Res.* 2008. 49: 429–437.

**Supplementary key words** low density lipoprotein receptor-deficient mice • bone marrow transplantation • plaque • macrophage

Studies of the pathogenesis of atherosclerosis use animal models of hyperlipidemia to identify the cellular participants of this complex and progressive disease. Although the lesions formed in mouse models do not progress to the late stages of clinical manifestations observed in humans, study of low density lipoprotein receptor-deficient (*LDLr*<sup>-/-</sup>) and apolipoprotein E-deficient (*ApoE*<sup>-/-</sup>) mice can elucidate mechanisms in the atherogenic process

(1, 2). In both of these models, atherosclerosis is defined by macrophage accumulation and foam cell formation. Whereas in *LDLr*<sup>-/-</sup> mice fed a high-fat diet (HFD) extensive lesions are observed throughout the aortic root, the entire aorta, and in the coronary arteries, *ApoE*<sup>-/-</sup> mice spontaneously develop atherosclerotic lesions even when fed a normal diet (3–6). Crosses of these atherosclerosis-prone mice with other mutant mice harboring specific molecular defects provide valuable models to study the molecular events involved in the attenuation or enhancement of atherosclerosis.

Beige mice are the animal homolog of a rare natural mutation called Chediak-Higashi syndrome (7). The mouse gene named *Lyst* (for lysosome trafficking regulator) encodes a protein that appears to be involved in the exchange of membrane material between the *trans*-Golgi network and the late endosomes (8). Mutant cells are characterized by the presence of enlarged perinuclear granules suggested to be responsible for defective cytotoxic T-lymphocyte, NK cell, and neutrophil activities, as well as by somewhat impaired macrophage function, leading to a marked immune deficiency. Earlier studies from our laboratory reported that *LDLr*<sup>-/-</sup> mice crossed with *Lyst*<sup>beige</sup> mice (*BgLDLr*<sup>-/-</sup>) display exacerbated atherosclerosis compared with *LDLr*<sup>-/-</sup> mice after consumption of a HFD for 16 weeks (9). The atherosclerosis-accentuating effects of the beige mutation occur despite a reduction of the hyperlipidemia of the *LDLr*<sup>-/-</sup> mice and independently of the impaired cytolytic activity of beige NK cells and of both T- and B-lymphocyte-mediated acquired immune responses (9). Moreover, the impaired macrophage function by itself does not account for the distinct lesion

Abbreviations: *ApoE*<sup>-/-</sup>, apolipoprotein E-deficient; *BgApoE*<sup>-/-</sup>, *Lyst*<sup>beige</sup> mice crossed with apolipoprotein E-deficient mice; BM, bone marrow; BMT, bone marrow transplantation; DAPI, 4',6-diaminophenylindole; EC, endothelial cell; GFP, green fluorescent protein; HFD, high-fat diet; *LDLr*, low density lipoprotein receptor; MCP-1, monocyte chemoattractant protein 1; PMA, phorbol ester; SMC, smooth muscle cell.

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morphology displayed by the double mutant BgLDLr<sup>-/-</sup> mice (10).

To test the hypothesis that the more severe atherosclerosis phenotype is not unique to BgLDLr<sup>-/-</sup> mice, we examined disease progression and changes in lesion morphology in beige, ApoE-deficient (BgApoE<sup>-/-</sup>) double mutant mice. ApoE<sup>-/-</sup> mice lack plasma apolipoprotein E and have high levels of plasma VLDL compared with wild-type mice. Although ApoE<sup>-/-</sup> mice develop atherosclerotic lesions when fed a chow diet, feeding ApoE<sup>-/-</sup> mice a diet enriched in fat greatly accelerates the progression of atherosclerosis and results in extensive complex lesions attributable to plasma accumulation of VLDL and intermediate density lipoproteins (6, 11, 12). Here, we sought to elucidate the impact of the beige mutation in ApoE<sup>-/-</sup> mice under conditions of both moderate and severe hypercholesterolemia and to determine whether cells of bone marrow (BM) origin play a role in modulating this impact. Disease burden was increased in BgApoE<sup>-/-</sup> mice fed a proatherogenic diet. In contrast, expression of the beige mutation in chow-fed ApoE<sup>-/-</sup> mice resulted in reduced lesions. Bone marrow transplantation (BMT) studies and primary cell culture experiments allowed us to evaluate the contribution of both BM-derived and non-BM beige cells to plaque development. Whereas BgApoE<sup>-/-</sup> mice fed a HFD had increased disease, atherosclerotic lesion development was attenuated in BgApoE<sup>-/-</sup> mice fed a chow diet, and the expression of the beige mutation in BM-derived cells as well as in aortic endothelial cells (ECs) and smooth muscle cells (SMCs) was necessary to confer this atheroprotection.

## METHODS

### Animals

Double mutant BgApoE<sup>-/-</sup> mice were generated by crossing C57Bl/6 ApoE<sup>-/-</sup> and Lys<sup>t</sup>beige mice purchased from Jackson Laboratories (Bar Harbor, ME) and bred in-house. The mice were housed under identical conditions in a sterile mouse facility, all procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. Cohorts of male ApoE<sup>-/-</sup> and BgApoE<sup>-/-</sup> animals (16 mice per group) between 8 and 10 weeks of age were used for all studies and were fed ad libitum a standard mouse chow diet (No. 7019; Harlan Teklad) or a proatherogenic HFD containing 1.25% cholesterol, 15.8% fat, and no cholate (No. 94059; Harlan Teklad) as indicated. Mice were fasted periodically, and venous blood was drawn from the retro-orbital sinus into a heparinized capillary tube. Plasma was isolated and total cholesterol levels were measured in individual samples by a colorimetric method (Sigma). For fast-performance liquid chromatography fractionation, equal volumes of samples were pooled from all mice of each experimental group.

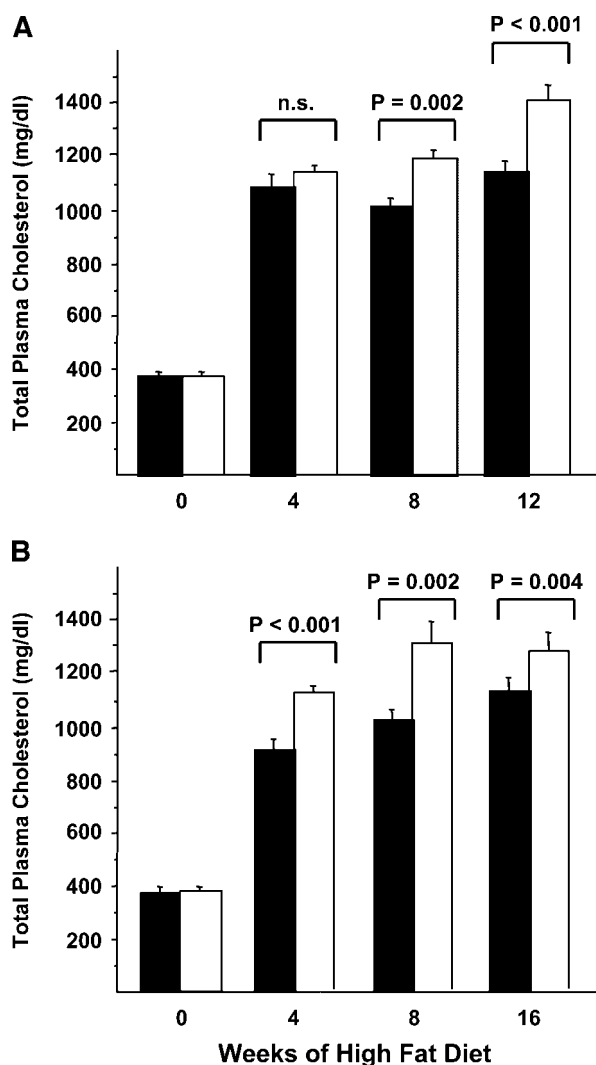
### BMT

BgApoE<sup>-/-</sup> mice were subjected to lethal total body  $\gamma$ -irradiation and reconstituted with  $3 \times 10^6$  BM cells per mouse

from either green fluorescent protein (GFP), ApoE<sup>-/-</sup> or GFP, BgApoE<sup>-/-</sup> mice, as described previously (13). In the first BMT study, the recipients were BgApoE<sup>-/-</sup> mice, so that all cells expressed the beige mutation except the BM-derived cells. In the second BMT study, irradiated ApoE<sup>-/-</sup> mice were used, so that only BM-derived cells expressed the beige mutation. All BMT mice were allowed to recover for 4 weeks, then mice were fed a chow diet for an additional 20 weeks before atherosclerosis was quantitated.

### Analysis of atherosclerosis

Atherosclerosis was assessed by multiple methodologies (13) after the mice consumed the HFD or chow diet for the times indicated for each study. Briefly, aortas were cleaned by microscopic dissection, removed, opened longitudinally, and pinned flat, followed by staining with Sudan IV and digital quantification of the lesions. Serial sections (10  $\mu$ m in thickness) were cut

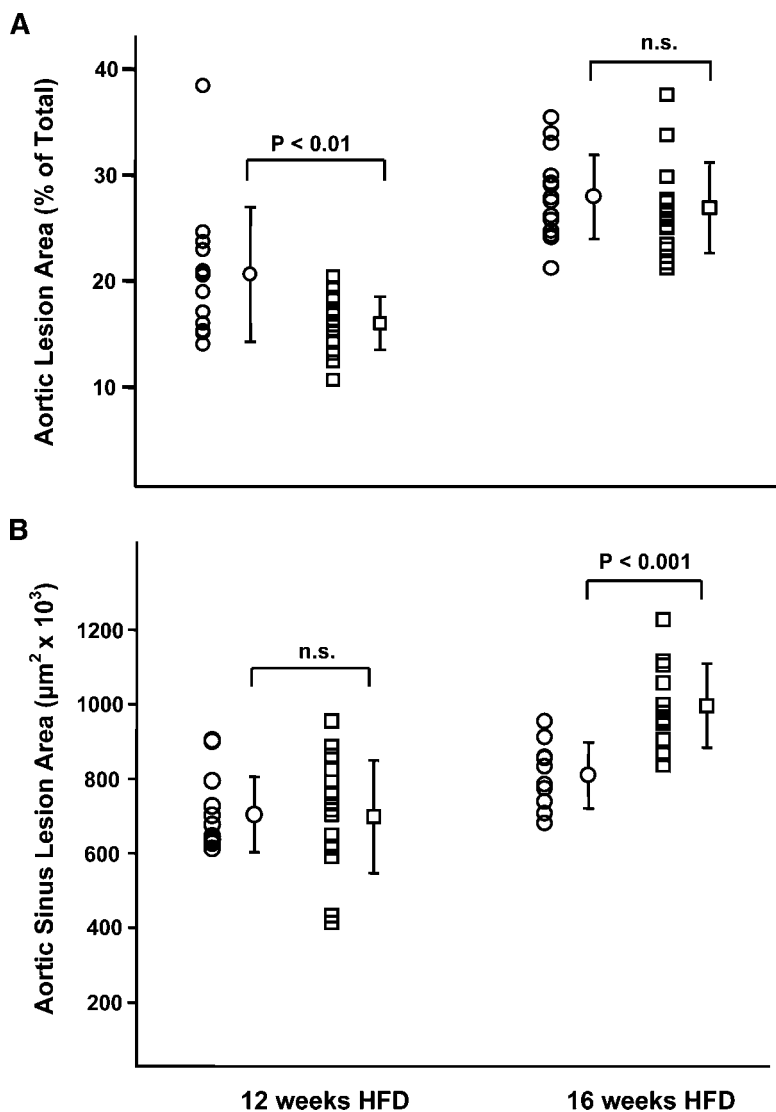


**Fig. 1.** Increased plasma cholesterol levels in Lys<sup>t</sup>beige mice crossed with apolipoprotein E-deficient mice (BgApoE<sup>-/-</sup>) fed a high-fat diet (HFD). Total cholesterol changes during 12 weeks (A) or 16 weeks (B) of proatherogenic diet feeding were measured in ApoE<sup>-/-</sup> (closed bars) and BgApoE<sup>-/-</sup> (open bars) mice. Values shown are means  $\pm$  SD (n = 14–16 mice per group).

through a 250  $\mu\text{m}$  segment of the aortic root, where all three valve leaflets were present. For each mouse, lesions of the aortic sinus were visualized and quantified in four sections separated by 40  $\mu\text{m}$ , stained with Oil Red O, and counterstained with Gill's hematoxylin (Fisher Scientific). Histological analysis was performed on all three cusps of subsequent aortic sinus sections from all mice in each group and experimental study [i.e., at least 14 sections per group, selected to be representative of the average lesion size for each mouse (nearest to the mean lesion area that had been determined previously for that individual mouse)]. Lesion areas comprised the entire intima, including lipid cores and fibrotic components. Masson's trichrome stain was used to identify collagen within the aortic sinus section, and the percentage of lesion area stained blue was calculated by digital image analysis. GFP+ BM-derived cell infiltration was monitored using a Rainbow Radiance 2100 laser scanning confocal system with a Nikon TE2000-U inverted microscope (Bio-Rad-Zeiss). EC staining was performed with rat anti-mouse CD31 (clone MEC 13.3; BD Pharmingen) followed by Alexa 647 goat anti-rat IgG (Invitrogen) and nuclear staining with 4',6-diamino-phenylindole (DAPI). The total area occupied by GFP+ cells was assessed in each of the three valve cusps individually for all mice by quantitative fluorescence using Image J (NIH Imaging) and Image Pro Plus 3DS (Media Cybernetics) software.

### Primary cell culture

For each experiment, two adult mice (12–14 weeks of age) per group were euthanized to obtain BM and aortas for primary cell culture. BM macrophages were cultured as described previously (10). After 5–7 days of culture, cells were harvested with Versene (Gibco), pooled for each mouse, counted, and seeded at  $\sim 2 \times 10^6$  cells per well onto six-well plates. Aortas were minced and digested for either EC or SMC isolation using a protocol adapted from previous studies (14, 15). Briefly, for EC isolation, the minced aortas were digested for 1 h at 37°C, 5% CO<sub>2</sub> in HBSS (Invitrogen) containing 1 mg/ml collagenase type II (Invitrogen). Cells were separated from debris and incompletely digested tissue with cell strainers, washed, and stained with rat anti-mouse CD31 (Pharmingen), followed by magnetically activated cell sorting using goat anti-rat IgG-conjugated paramagnetic beads. Isolated ECs were then cultured in ECM-2 medium (Clonetics) on fibronectin-coated 12-well tissue culture plates (one aorta per well) and expanded to one 6-well plate per mouse in three passages. The purity of the cells was >90% as determined by staining for mouse CD31 and by the uptake of Alexa Fluor 488-labeled acetylated low density lipoprotein (Molecular Probes, Eugene, OR). Similarly, to obtain vascular SMCs, aortas were excised, minced, and incubated at 37°C, 5% CO<sub>2</sub> in high-glucose DMEM containing 2 mg/ml collagenase/dispase (Roche), 0.5 mg/ml



**Fig. 2.** Atherosclerotic lesion formation upon HFD feeding. The extent of atherosclerosis was assessed in ApoE<sup>-/-</sup> (circles) and BgApoE<sup>-/-</sup> (squares) mice after 12 (left) or 16 (right) weeks of consuming a HFD. A: En face aortic lesion area was expressed as a fraction of total area. B: Aortic sinus lesion area (reported as  $\mu\text{m}^2$ ) was calculated as a mean of four 10  $\mu\text{m}$  sections for each mouse. Values for individual mice are shown; symbols with error bars indicate means  $\pm$  SD (n = 14–16 mice per group).

soybean trypsin inhibitor (Sigma), and 0.2 mg/ml elastase II-A (Sigma). After 2 h, cells were separated by centrifugation at 200 g, seeded into 25 cm<sup>2</sup> cell culture flasks (one per aorta) in P-STIM medium (BD Biosciences), fed every 3 days, and split when subconfluent. All SMCs and ECs were expanded for three to four passages before seeding at  $\sim 2.5 \times 10^5$  cells per well onto six-well plates for the monocyte chemoattractant protein 1 (MCP-1) experiments. Cells were allowed to adhere, serum-deprived for 24 h, and incubated in the absence or presence of phorbol ester (PMA) for 6 or 24 h, respectively. Transcripts for MCP-1 and GAPDH as a loading control were detected by Northern blotting. Total RNA was prepared from cells after 6 h of incubation using the Trizol reagent (Invitrogen), and 5  $\mu$ g samples were separated on 1% agarose-formaldehyde gels, transferred to nylon membranes, and hybridized to <sup>32</sup>P-labeled specific DNA probes. Conditioned medium was harvested after 24 h to determine secreted MCP-1 protein level using a BD OptEIA mouse ELISA set.

### Statistical analysis

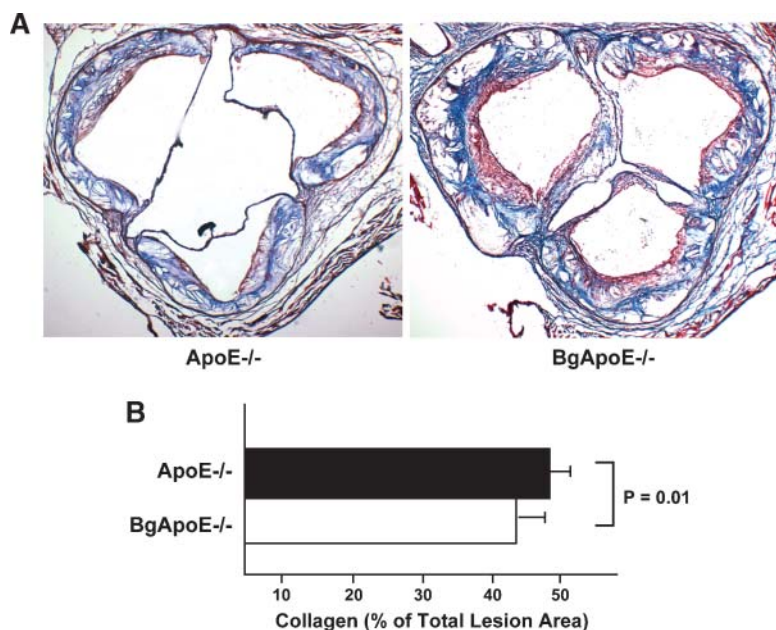
All results are expressed as means  $\pm$  SD except the lesion data, for which individual values are shown. Statistical differences of the extent of atherosclerosis between experimental groups were analyzed using the nonparametric ANOVA on rank tests in end point studies and the two-factor ANOVA for time course experiments. The analysis of factor level effects was done with the Holm-Sidak test of pairwise multiple comparisons. Total plasma cholesterol changes over time were analyzed using two-factor repeated-measures ANOVA. All other analyses used a two-tailed, unpaired *t*-test. All statistical analysis was done with the use of the SigmaStat 3.5 statistics package (SPSS, Inc.). A value of *P* < 0.05 was considered significant.

## RESULTS

### In ApoE<sup>-/-</sup> mice fed a HFD, expression of the beige mutation leads to the exacerbation of atherosclerosis

Double mutant BgLDLr<sup>-/-</sup> mice display increased atherosclerosis and a distinct plaque morphology compared with LDLr<sup>-/-</sup> mice (9, 10). To verify that this beige pheno-

type was disease-related and not an LDLr<sup>-/-</sup> phenotype, the progression of atherosclerosis and changes in lesion morphology were assessed in ApoE<sup>-/-</sup> and BgApoE<sup>-/-</sup> mice. In two independent studies, mice were fed a proatherogenic high-fat, high-cholesterol diet (HFD) for 12 and 16 weeks, respectively, to accelerate disease progression. As expected, plasma cholesterol levels were greatly increased in both groups fed the HFD. In contrast to earlier results with BgLDLr<sup>-/-</sup> mice, fasting plasma cholesterol levels in BgApoE<sup>-/-</sup> mice were significantly higher compared with those in ApoE<sup>-/-</sup> mice at each time point, except the 4 week time point in the 12 week HFD study (Fig. 1). Yet, a comparison of the extent of atherosclerotic lesions in the entire aortas in the 12 week study revealed significantly smaller lesion areas in BgApoE<sup>-/-</sup> mice. After 16 weeks, a larger percentage of total aorta was covered with plaques, but the extent of the lesions was similar in both groups of mice (Fig. 2A), suggesting that the rate of lesion progression was different in the two groups. Consistent with this idea, aortic sinus lesions, which typically develop earlier than arterial plaques, were comparable in all mice at 12 weeks, but by 16 weeks they were increased significantly in BgApoE<sup>-/-</sup> mice (Fig. 2B). A characteristic of these lesions in the double mutant mice was the increased presence of lipid cores and a sparser and less uniform collagen matrix, as revealed by Masson's trichrome staining (Fig. 3A). To quantify this difference after 16 weeks of HFD consumption, lesional collagen levels were normalized to percentage of total lesion area and found to be decreased significantly compared with ApoE<sup>-/-</sup> mice (Fig. 3B), suggesting a less stable and/or more advanced lesion phenotype. These findings imply that although lesion development at earlier time points was delayed in high-fat-fed ApoE<sup>-/-</sup> mice, the beige mutation increases the extent of atherosclerosis in both LDLr<sup>-/-</sup> and ApoE<sup>-/-</sup> mice. Nevertheless, the effect on lesion morphology was different between the two models.



**Fig. 3.** Characterization of aortic sinus atherosclerosis after 16 weeks of HFD. **A:** Aortic sinus lesion morphology in ApoE<sup>-/-</sup> and BgApoE<sup>-/-</sup> mice. Sections were stained with Masson's trichrome (blue stain) for collagen-rich extracellular matrix. Unstained (white) areas within the cusps of the heart sinus are lipid cores and are larger in the BgApoE<sup>-/-</sup> lesions. Original magnification, 50 $\times$ . **B:** Collagen content is reported as percentage staining per total aortic sinus lesion area (means  $\pm$  SD) in 14 ApoE<sup>-/-</sup> mice (closed bar) and 14 BgApoE<sup>-/-</sup> mice (open bar).

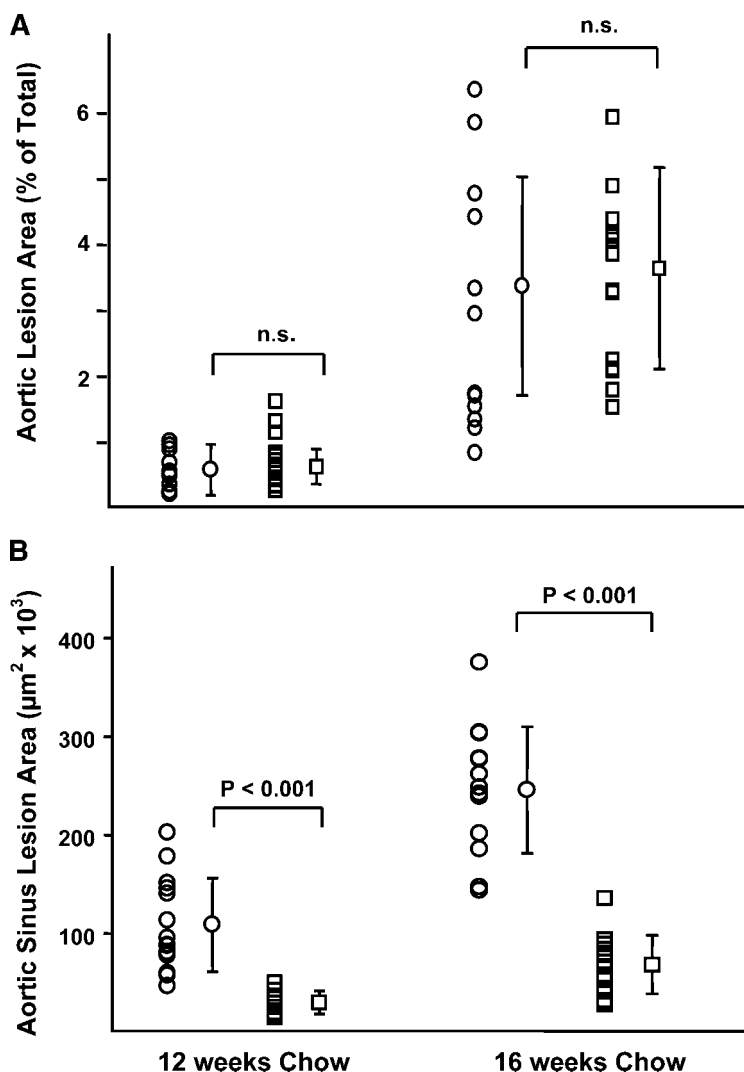
### The beige mutation is atheroprotective in chow-fed ApoE<sup>-/-</sup> mice

A given treatment can selectively modulate the development of atherosclerotic lesions throughout the vasculature, resulting in differences in atherosclerotic outcomes at various sites at risk (16). Moreover, atherosclerosis in mice can progress at different rates even at the same anatomical site. Therefore, to understand the effect of single mutations on atherosclerosis, the extended course of disease initiation and progression should be evaluated (17). Because our analyses of the plaques in these HFD studies suggested that the rate of lesion progression differed in ApoE<sup>-/-</sup> and BgApoE<sup>-/-</sup> mice, the studies were repeated with mice fed a chow diet to reduce the overall rate of disease progression. Atherosclerosis was assessed in these chow-fed groups of mice at 12 or 16 weeks after initiation of the study (i.e., at 20–22 or 24–26 weeks of age). First, no differences in total plasma cholesterol were observed between ApoE<sup>-/-</sup> and BgApoE<sup>-/-</sup> mice in either of the two studies. In the 12 week study, both groups of mice fed the chow diet maintained average plasma cholesterol levels of 425–500 mg/dl. Both groups of mice had slightly higher cholesterol levels during the 16 week chow diet,

but mean values remained between 500 and 675 mg/dl throughout the study (see supplementary Fig. 1). This indicates that the beige mutation had no impact on plasma cholesterol levels in chow diet-fed ApoE<sup>-/-</sup> mice. En face lipid staining of the entire length of the aorta was minimal (compare Figs. 4A and 2A), and no differences between the two groups of mice at either 12 or 16 weeks were observed (Fig. 4A). In spite of a significant increase in the en face lesion area between 12 and 16 weeks ( $P < 0.001$ ), there were only minimal Sudan IV-stained lesions visible on the luminal surface of the aortas of all mice. BgApoE<sup>-/-</sup> mice developed significantly less extensive aortic sinus lesion area than the ApoE<sup>-/-</sup> animals after both 12 and 16 weeks of chow, demonstrating a protective effect of the beige mutation under conditions of moderate hypercholesterolemia and minimal disease (Fig. 4B). This result was in sharp contrast to our reported effects of the beige mutation in LDLr<sup>-/-</sup> mice fed a HFD.

### Expression of the beige mutation in BM-derived cells is not sufficient for atheroprotection in chow-fed ApoE<sup>-/-</sup> mice

Because less disease was observed in the heart sinus lesions of chow-fed BgApoE<sup>-/-</sup> mice that was not associated

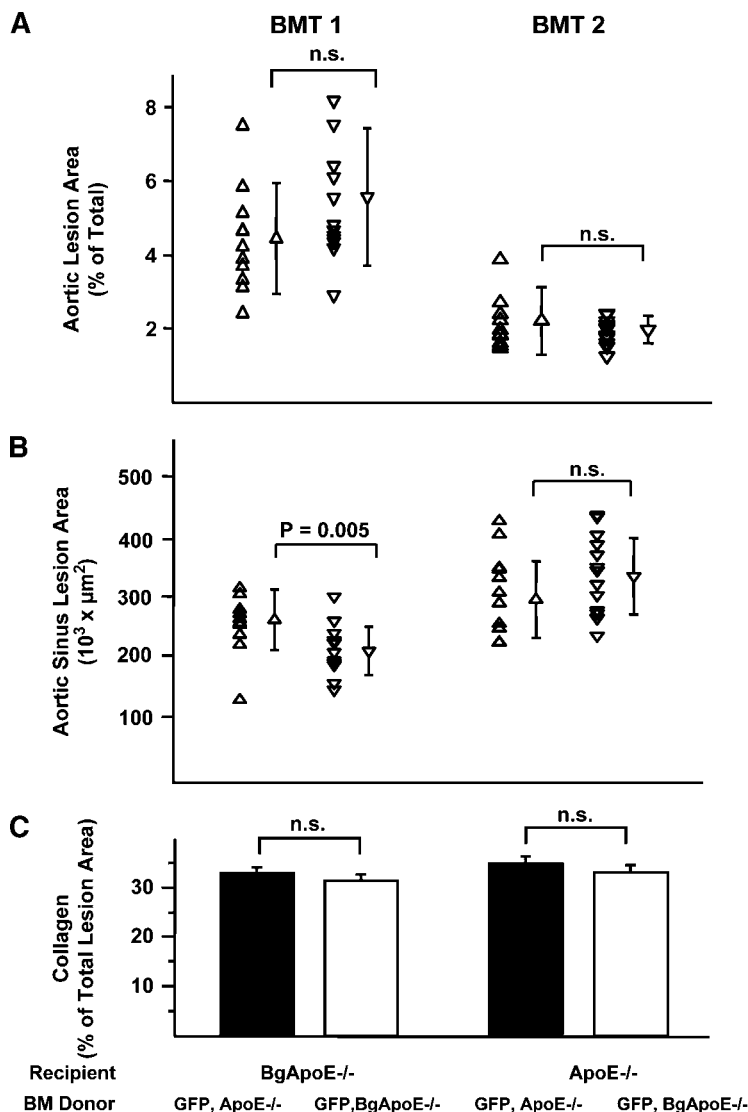


**Fig. 4.** Decreased atherosclerotic lesion formation in BgApoE<sup>-/-</sup> mice fed chow. A: En face aortic lesion area as a fraction of total area. B: Mean aortic sinus lesion area of four 10 µm sections per mouse. Shown are individual values for ApoE<sup>-/-</sup> (circles) and BgApoE<sup>-/-</sup> (squares) mice after 12 (left) or 16 (right) weeks of consuming the chow diet; symbols with error bars indicate means ± SD (n = 12–15 mice per group).

with differences in plasma cholesterol, BMT studies were performed to evaluate the role of beige BM-derived cells in the observed atheroprotection. As mice developed modest lesions after consuming a chow diet for 12 and 16 weeks (which was <26 weeks of age) in our previous studies, this time atherosclerosis was quantified at 20 weeks after the mice recovered from BMT (at 32–34 weeks of age). The experimental design of the BMT 1 study involved examination of the beige mutation expressed in all cells except BM-derived cells. Lethally irradiated BgApoE<sup>-/-</sup> mice received BM cells from either GFP,ApoE<sup>-/-</sup> mice (treated mice) or GFP,BgApoE<sup>-/-</sup> mice (control mice), and all mice entered the study protocol 4 weeks later. Total plasma cholesterol levels did not change appreciably during this study, and no significant differences in fasting plasma cholesterol levels were observed between the two groups over time ( $P = 0.4$ ) (see supplementary Fig. II). The experimental design of the BMT 2 study involved the examination of the beige mutation in only BM-derived cells. In this study, a significant difference over time was observed between the ApoE<sup>-/-</sup> recipient groups with higher plasma cholesterol levels in mice expressing the beige

mutation exclusively in BM-derived cells (treated mice) ( $P < 0.001$ ). In this study, however, the distribution of total cholesterol within the major lipoprotein fractions was similar in pooled plasma samples from both ApoE<sup>-/-</sup> recipient groups (see supplementary Fig. II). This indicates that expression of the beige mutation in only BM-derived cells affected total plasma cholesterol levels but not cholesterol distribution.

Consistent with our observations in the chow diet studies, all BMT mice had minimal visible lesions in their aortas at 20 weeks, as assessed by staining of the en face aortas for neutral lipids. Lesion areas were similar between the recipient groups in each BMT study (Fig. 5A). As shown in Fig. 5B, BgApoE<sup>-/-</sup> mice in BMT 1 reconstituted with both ApoE<sup>-/-</sup> and BgApoE<sup>-/-</sup> BM developed considerably fewer atherosclerotic lesions in the aortic sinus than their ApoE<sup>-/-</sup> recipient counterparts ( $P < 0.001$ ). These results suggested that a determining factor in plaque development was not the genotype of the BM donor but the genotype of the recipient. Moreover, mice in BMT 1 developed significantly more extensive en face aortic lesions compared with mice in BMT 2 ( $P < 0.001$ ). Whereas



**Fig. 5.** Comparison of atherosclerosis in bone marrow transplantation (BMT) studies BMT 1 and BMT 2. Atherosclerotic lesions were analyzed in mice reconstituted with green fluorescent protein (GFP), ApoE<sup>-/-</sup> (triangles) and GFP,BgApoE<sup>-/-</sup> (inverted triangles) bone marrow (BM) in BMT 1 (left) and BMT 2 (right). A: En face aortic lesion area was expressed as a fraction of total area. Values for individual mice are shown. B: Mean aortic sinus lesion area (reported as μm<sup>2</sup>) of four 10 μm sections per mouse. Values for individual mice are shown. In A and B, symbols with error bars indicate means ± SD. C: Collagen content is reported as percentage staining per total aortic sinus lesion area (means ± SD) in mice receiving GFP,ApoE<sup>-/-</sup> (closed bars) or GFP,BgApoE<sup>-/-</sup> (open bars) BM (n = 14–16 mice per group).

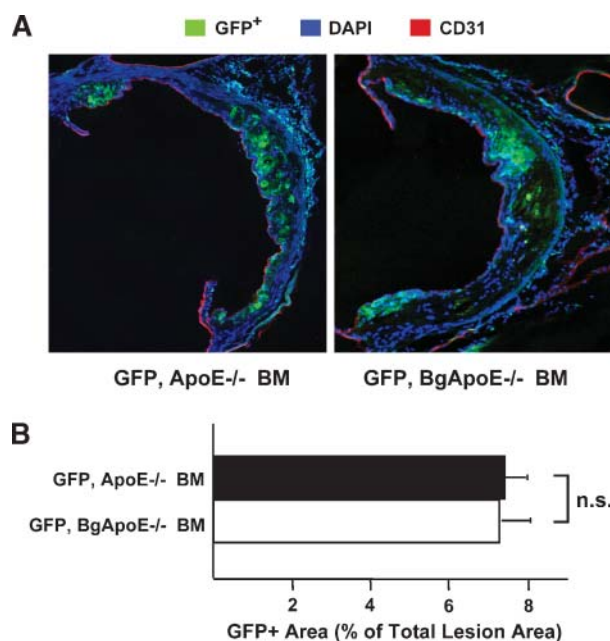
expressing the beige mutation exclusively in BM-derived cells (treated group in BMT 2) had no significant effect, a marked decrease in aortic sinus atherosclerosis was observed in BgApoE<sup>-/-</sup> mice receiving BgApoE<sup>-/-</sup> BM compared with the same mice receiving ApoE<sup>-/-</sup> BM (BMT 1). Histological characterization performed on aortic sinus sections selected based on their size (nearest to the mean lesion area of each mouse) revealed similar collagen staining in all BMT mice. When normalized to percentage of total lesion area, there were no significant differences in collagen content between mice reconstituted with ApoE<sup>-/-</sup> or BgApoE<sup>-/-</sup> BM in either BMT study (Fig. 5C), suggesting that leukocyte-specific beige mutation induced no changes in plaque stability. Together, these BMT studies clearly indicated that expression of the beige mutation by cells of BM origin was necessary but not sufficient for the less severe disease phenotype.

To further detail the effect of macrophage expression of the beige mutation in ApoE<sup>-/-</sup> mice, infiltration of BM-derived cells and localization of GFP+ cells was observed in aortic sinus lesions of all ApoE<sup>-/-</sup> recipient mice (BMT 2) by confocal microscopy. In both control and treated mice, GFP+ BM-derived cells were distributed uniformly throughout the lesion, with a somewhat higher density in the subluminal area (Fig. 6A). When normalized to percentage of total lesion area, no quantitative differences were detectable between the ApoE<sup>-/-</sup> mice reconstituted with ApoE<sup>-/-</sup> or BgApoE<sup>-/-</sup> BM within the plaques

(Fig. 6B). Thus, expression of the beige mutation solely in BM-derived cells led to no adverse qualitative changes in lesion morphology in ApoE<sup>-/-</sup> mice, such as increased accumulation of infiltrated leukocytes.

### MCP-1 levels in primary cell cultures are affected by the expression of the beige mutation

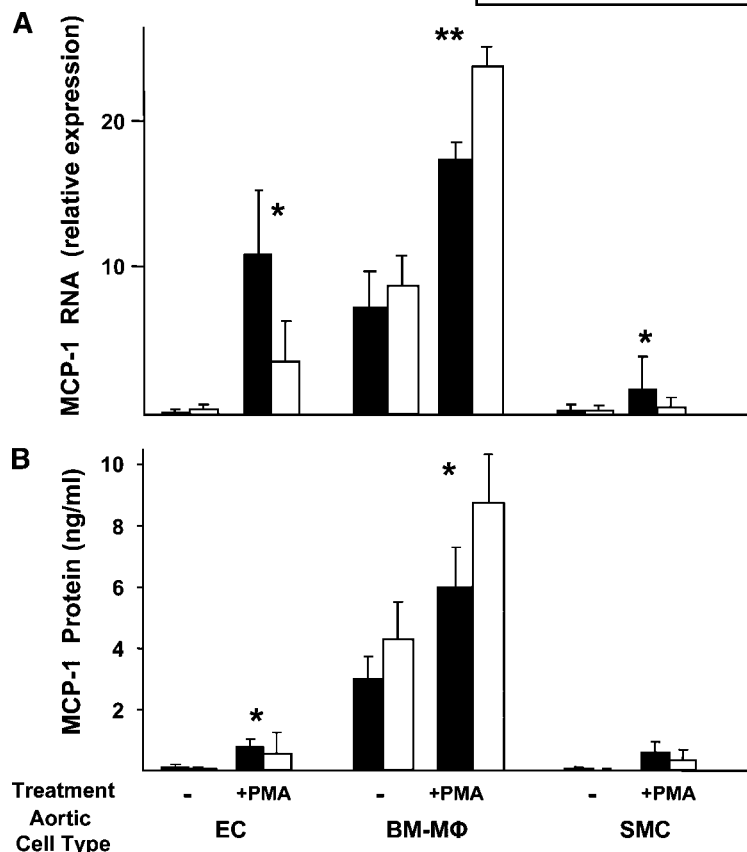
Expression of the beige mutation quantitatively reduced disease severity in chow-fed ApoE<sup>-/-</sup> mice, and BMT experiments indicated that macrophages, as well as additional cell types, were involved in this atheroprotective effect. Because the beige mutation can alter protein secretion, this raises the possibility that the mutation might cause dissimilar release of inflammatory mediators by the different cell types implicated in the progression of atherosclerotic disease. To characterize in vitro the contribution of BM-derived macrophages, SMCs, and ECs to the atherosclerosis-related inflammatory processes in BgApoE<sup>-/-</sup> mice, primary cell cultures were established from aortas and BM from ApoE<sup>-/-</sup> and BgApoE<sup>-/-</sup> mice. Expression of MCP-1 in quiescent and PMA-stimulated cells was analyzed at both the RNA and protein levels. Baseline transcript for MCP-1 and secreted cytokine protein levels in cultured ECs and SMCs were minimal but comparable between ApoE<sup>-/-</sup> and BgApoE<sup>-/-</sup> mice (Fig. 7). Upon stimulation with PMA, there was an attenuated induction of MCP-1 expression in cells from BgApoE<sup>-/-</sup> mice, suggesting that the beige mutation altered the ability of these cells to secrete MCP-1 in response to proinflammatory stimuli, which may explain in part the less severe phenotype in chow-fed mice. In contrast, cultured BM macrophages from both genotypes expressed MCP-1 at the baseline level, and this was increased in macrophages from BgApoE<sup>-/-</sup> mice. Compared with BM macrophages from ApoE<sup>-/-</sup> mice, BgApoE<sup>-/-</sup> macrophages displayed a significantly enhanced responsiveness to stimulation with PMA (Fig. 7). These results suggested that expression of the beige mutation in macrophages in combination with ECs and SMCs led to a moderation of the proinflammatory environment that may account for the observed protection against plaque development in chow-fed BgApoE<sup>-/-</sup> mice.



**Fig. 6.** BM-derived cell infiltration in the aortic sinus of ApoE<sup>-/-</sup> recipient mice. **A:** Aortic sinus sections from ApoE<sup>-/-</sup> mice reconstituted with either GFP, ApoE<sup>-/-</sup> (left) or GFP, BgApoE<sup>-/-</sup> (right) BM. Sections were stained with anti-CD31 [red; endothelial cells (ECs)] and 4',6-diamino-phenylindole (DAPI) (blue; nuclei). Original magnification, 100 $\times$ . **B:** GFP-positive areas are reported as percentage GFP per total aortic sinus lesion area (means  $\pm$  SD) in ApoE<sup>-/-</sup> mice reconstituted with GFP, ApoE<sup>-/-</sup> (closed bar) and GFP, BgApoE<sup>-/-</sup> (open bar) BM (n = 16 mice per group).

## DISCUSSION

Plaque vulnerability, defined as the propensity of atherosclerotic plaques to disrupt, together with superimposed thrombosis often lead to acute coronary events in humans. Plaque composition rather than size is the major determinant of plaque disruption. More vulnerable lesions are characterized by thin fibrous caps, large lipid accumulations, large numbers of macrophages, and depletion of SMCs (18–20). Atherosclerotic plaques in mice are regarded as resistant to rupture, and the relevance of mouse studies to model the final events precipitated by plaque disruption of human atherosclerotic lesions is controversial (21, 22). Nevertheless, a previous report from



**Fig. 7.** Induction of monocyte chemoattractant protein 1 (MCP-1) in primary cell cultures. Serum-starved BM-derived macrophages (MΦ), aortic smooth muscle cells (SMCs), and aortic ECs isolated from ApoE<sup>-/-</sup> (closed bars) and BgApoE<sup>-/-</sup> (open bars) mice were incubated for 6 h (A) and 24 h (B) in the presence or absence of 50 nM phorbol ester (PMA), as indicated. A: Transcripts for MCP-1 and GAPDH were detected by Northern blotting. MCP-1 gene expression levels were quantified by laser densitometry and are reported relative to GAPDH (means ± SD; n = 3–6). B: MCP-1 protein concentrations were determined by ELISA in the conditioned medium (means ± SD; n = 3–4). Significant differences among groups are denoted with asterisks: \* *P* < 0.05, \*\* *P* < 0.005.

our laboratory documented changes in plaque composition that define the double mutant BgLDLr<sup>-/-</sup> mouse, which resemble the characteristics of a more stable type of human lesion (10). Therefore, the present study was designed to identify features/mechanisms that control plaque stability in mice with the beige phenotype. Our results show that expression of the beige mutation in ApoE<sup>-/-</sup> mice led to divergent effects, dependent on diet and the duration of the study. With a chow diet, minimal aortic lesions were observed and only small areas of the aortic valve were covered with plaques. Under these dietary conditions, we found a dramatic decrease in aortic sinus lesion progression in chow-fed BgApoE<sup>-/-</sup> mice (Fig. 4). When fed a HFD for 12 weeks, lesion areas of en face aortas were smaller in BgApoE<sup>-/-</sup> mice, whereas the extent of aortic sinus lesions was similar in both groups of mice. After consumption of the proatherogenic diet for 16 weeks, BgApoE<sup>-/-</sup> mice displayed more extensive aortic sinus lesions than ApoE<sup>-/-</sup> mice and rather undesirable changes (e.g., lower collagen content and larger lipid cores) in plaque composition (Figs. 2, 3). At this time point, lesion areas in both groups of mice were substantially larger than plaque areas observed in the 16 week study with BgLDLr<sup>-/-</sup> mice. Because atherosclerotic lesions in the aortic sinus are visible at earlier stages of the disease and appear to progress faster than in the aorta, and because feeding ApoE<sup>-/-</sup> mice a diet enriched in fat greatly accelerates lesion development (11), these results could be attributed to different effects of the beige mutation in early versus advanced stages of the disease.

Atherosclerotic lesion development is a chronic inflammatory process that occurs in distinct phases, with early atherogenesis characterized by endothelial activation and expression of proinflammatory cytokines and with lesion progression the result of leukocyte infiltration, macrophage activation, and macrophage-derived foam cell formation (23, 24). Hence, our data could also imply that leukocyte recruitment and/or infiltration are severely impaired in BgApoE<sup>-/-</sup> mice. If this is the case, expression of the beige mutation exclusively in BM-derived cells would be expected to delay the progression of the disease. Using mouse chimeras with GFP expression in BM-derived cells, we quantified leukocyte infiltration and found no differences between chow-fed ApoE<sup>-/-</sup> recipient mice reconstituted with GFP, ApoE<sup>-/-</sup> or GFP, BgApoE<sup>-/-</sup> BM. Moreover, no atheroprotective effect was observed when the beige mutation was absent in non-BM-derived cells (Fig. 5). Because aortic sinus lesions were considerably smaller in both groups of mice in the inverse BMT experiment in which BgApoE<sup>-/-</sup> mice were reconstituted, we concluded that host-derived non-BM cells, including ECs and vascular SMCs, were most likely decisive in early events of lesion progression. Nevertheless, aortic sinus atherosclerosis in BgApoE<sup>-/-</sup> mice receiving BgApoE<sup>-/-</sup> BM was reduced substantially compared with that in the same mice receiving ApoE<sup>-/-</sup> BM (BMT 1), indicating that BM-derived cells are also involved in the atheroprotective effect of the beige phenotype.

It is widely accepted that there is an enhanced production and release of inflammatory mediators in atheroscle-



rotic lesions. Macrophage infiltration followed by the secretion of growth factors and chemokines that further promote cell accumulation in lesions play an important role in disease development and progression. Moreover, cytokines, chemokines, adhesion molecules, and matrix metalloproteinases can influence several biologic processes that regulate the stability of the plaque and its resistance to rupture (19). All cell types present in the atherosclerotic plaque can be both a source and a target of cytokines (25); therefore, inflammatory and/or adhesive changes induced by the beige mutation could be implicated in the attenuated atherosclerosis development under conditions of moderate hyperlipidemia. MCP-1 and its cognate receptor, chemokine receptor 2, play important roles in the recruitment of blood monocytes, the precursors of the lipid-laden foam cells, and studies with knockout mice demonstrated their role in the development of atherosclerotic lesions (26, 27). MCP-1 is produced by macrophages, ECs, and vascular SMCs. We observed that secretion of MCP-1 by cultured BM-derived macrophages, vascular SMCs, and aortic ECs was altered significantly by the beige mutation. Non-BM BgApoE<sup>-/-</sup> cells exhibited less responsiveness to stimulation with PMA compared with ApoE<sup>-/-</sup> controls. Because ECs play a major role in early atherogenesis, if secretion of MCP-1 is reduced at this stage, the balance between proinflammatory and anti-inflammatory responses may be tipped toward atheroprotective effects and could lead to diminished growth of plaques.

In summary, we have demonstrated that BgApoE<sup>-/-</sup> mice had markedly reduced development of atherosclerotic lesions when fed a chow diet. Although the specific mechanisms by which the beige mutation reduces atherosclerosis remain to be established, these data indicate the participation of both BM-derived and endothelial and smooth muscle beige cells in modulating atherogenic events during early lesion development induced by moderate hyperlipidemia in chow-fed ApoE<sup>-/-</sup> mice. ■■

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