# Enhanced Akt3 kinase activity reduces atherosclerosis in hyperlipidemic mice in a gender-dependent manner

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**Akt3 is one of the three members of the serine/threonine protein kinase B (AKT) family, which regulates multiple cellular processes. We have previously demonstrated that global knockout of Akt3 in mice promotes atherogenesis in a macrophage-dependent manner. Whether enhanced Akt3 kinase activity affects atherogenesis is not known. In this study, we crossed atherosclerosis-prone ApoE$^{-/-}$ mice with a mouse strain that has enhanced Akt3 kinase activity (Akt3$^{nmf350}$) and assessed atherosclerotic lesion formation and the role of macrophages in atherogenesis. Significant reduction in atherosclerotic lesion area and macrophage accumulation in lesions were observed in ApoE$^{-/-}$/Akt3$^{nmf350}$ mice fed a Western-type diet. Experiments using chimeric ApoE$^{-/-}$ mice with either ApoE$^{-/-}$/Akt3$^{nmf350}$ bone marrow or ApoE$^{-/-}$ bone marrow cells showed that enhanced Akt3 activity specifically in bone marrow-derived cells is atheroprotective. The atheroprotective effect of Akt3$^{nmf350}$ was more pronounced in male mice. In line with this result, the release of the pro-inflammatory cytokines IL-6, MCP1, TNF-α, and MIP-1α was reduced by macrophages from male but not female ApoE$^{-/-}$/Akt3$^{nmf350}$ mice. Levels of IL-6 and TNF-α were also reduced in atherosclerotic lesions of ApoE$^{-/-}$/Akt3$^{nmf350}$ male mice compared to ApoE$^{-/-}$ mice. Macrophages from male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice were also more resistant to apoptosis *in vitro* and *in vivo* and tended to have more pronounced M2 polarization *in vitro*. These findings demonstrated that enhanced Akt3 kinase activity in macrophages protects mice from atherosclerosis in hyperlipidemic mice in a gender-dependent manner.**

The serine/threonine protein kinases B (Akt) regulate numerous cellular processes including cell growth, differentiation, proliferation, apoptosis as well as glucose and lipid metabolism (1). The Akt family comprises three isoforms (Akt1, Akt2, and Akt3). Analysis of individual Akt isoforms' deletion in mice showed that they have distinct functions in atherosclerosis. Mice lacking Akt1 developed severe coronary atherosclerosis due to the enhanced expression of pro-inflammatory genes in the artery wall, and endothelial cell and macrophage apoptosis (2). Lack of Akt2 suppresses the ability of macrophages to undergo M1 polarization, reducing both early and advanced atherosclerosis (3). Our previous studies using Akt3 deficient mice on an ApoE$^{-/-}$ background demonstrated exacerbated atherogenesis, which was associated with increased macrophage foam cell formation (4, 5).

Atherosclerosis is a chronic inflammatory vascular disease, with a well-known sexual dimorphism in terms of incidence and complications. Inflammatory cytokines play a key role in regulating immune response and the pathogenesis of autoimmune disease and atherosclerosis (6, 7). Gender differences were observed in both immune and inflammatory responses (8–13). It was also reported that the cytokine production by cells is gender dependent (14, 15). This evidence indicated that cytokines release during immune and inflammatory response may contribute to gender differences in atherosclerosis. Pro-inflammatory cytokines, such as Interleukin-6 (IL-6), Tumor necrosis factor-alpha (TNF-α), Interleukin-1beta (IL-1β), Monocyte chemoattractant protein-1 (MCP-1), and Macrophage inflammatory protein-1 alpha (MIP-1α), play a significant role in the inflammatory response of atherosclerosis (16–19). While our previous results revealed that Akt3 deficiency promotes macrophage foam cell formation (4), the effect of Akt3 on macrophage pro-inflammatory cytokines release has not been studied.

In the current study, we sought to determine whether enhanced Akt3 activity in mice reduces atherosclerosis to test whether the effect is gender dependent and to identify the potential mechanism. To this end, we generated ApoE$^{-/-}$/Akt3$^{nmf350}$ mice. Akt3$^{nmf350}$ mice have a missense mutation, D219 V, in the Akt3 gene that results in enhanced Akt3 kinase activity (20). We detected a significant reduction of the atherosclerotic lesion area, macrophage accumulation, and apoptosis in ApoE$^{-/-}$/Akt3$^{nmf350}$ mice compared to ApoE$^{-/-}$ mice. Using chimeric mice with Akt3$^{nmf350}$ expressed in bone marrow cells we showed that the effect depends on bone marrow cells presumably macrophages. The reduction of atherosclerosis was more pronounced in male mice. We detected several differences in the phenotype of Akt3$^{nmf350}$ macrophages that can contribute to atheroprotection. Importantly, in line with the difference in atherogenesis between

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male and female ApoE$^{-/-}$/Akt3$^{nmf350}$ mice, we detected a reduced pro-inflammatory cytokine release by macrophages collected from male but not female Akt3$^{nmf350}$ mice, reduced apoptosis of macrophages from male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice *in vitro* and *in vivo*. Taken together, our findings demonstrate the gender-dependent protective function of Akt3 in atherosclerosis.

## Results

### *Enhanced Akt3 kinase activity reduces atherosclerosis in hyperlipidemic ApoE$^{-/-}$ mice in a gender-dependent manner*

To study the effect of enhanced kinase activity of Akt3 on atherogenesis, we subjected age- and sex-matched ApoE$^{-/-}$/Akt3$^{nmf350}$ and ApoE$^{-/-}$ mice to a Western diet for 15 weeks. *En face* analysis of the aortas revealed a 71.6% decrease in the atherosclerotic lesion area in ApoE$^{-/-}$/Akt3$^{nmf350}$ mice as compared to ApoE$^{-/-}$ controls (Fig. 1, *A* and *B*). To evaluate the effect of gender on the reduction of atherosclerotic lesion area, data from male and female mice were analyzed separately. A significant reduction was observed in both male and female ApoE$^{-/-}$/Akt3$^{nmf350}$ mice (Fig. 1*C*). The reduction was more pronounced in male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice (78.6%) *versus* female ApoE$^{-/-}$/Akt3$^{nmf350}$ mice (53.5%) compared to ApoE$^{-/-}$ mice (Fig. 1*D*). Lesion areas in cross-sections of the aortic sinus were also significantly reduced in ApoE$^{-/-}$/Akt3$^{nmf350}$ mice (28%, Fig. 1*E*). A significant reduction in the size of atherosclerotic plaques was observed over a distance of 720 μm within the aortic sinus area (Fig. 1*F*). Again, a significant reduction was observed in both male and female mice, and the effect tended to be more pronounced in male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice (34%) *versus* females (21%) (Fig. 1, *G* and *H*). The plasma cholesterol levels were similar between ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice both on the chow diet and after 15 weeks of western diet feeding (Fig. S1*A*). While total plasma triglycerides of ApoE$^{-/-}$/Akt3$^{nmf350}$ mice were significantly higher than that of ApoE$^{-/-}$ mice on the chow diet, after 15 weeks of western diet feeding the total plasma triglycerides were similar between these two groups (Fig. S1*A*), suggesting that effect of Akt3 activity on atherosclerosis may be independent of plasma lipids. Distribution of cholesterol in lipoprotein fractions was similar between these two groups of mice fed with a chow diet, except for a small but statistically significant increase of HDL level in ApoE$^{-/-}$/Akt3$^{nmf350}$ mice on Western diet (Fig. S1*B*). Further analysis of this data revealed a statistically significant reduction of plasma LDL/VLDL levels specifically in male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice fed a Western diet, compared to ApoE$^{-/-}$ mice, but not in female mice (Fig. S1*C*). Plasma-free fatty acid and glucose levels were similar between the two genotypes in male mice and in female mice on the Western diet (Fig. S1*D*).

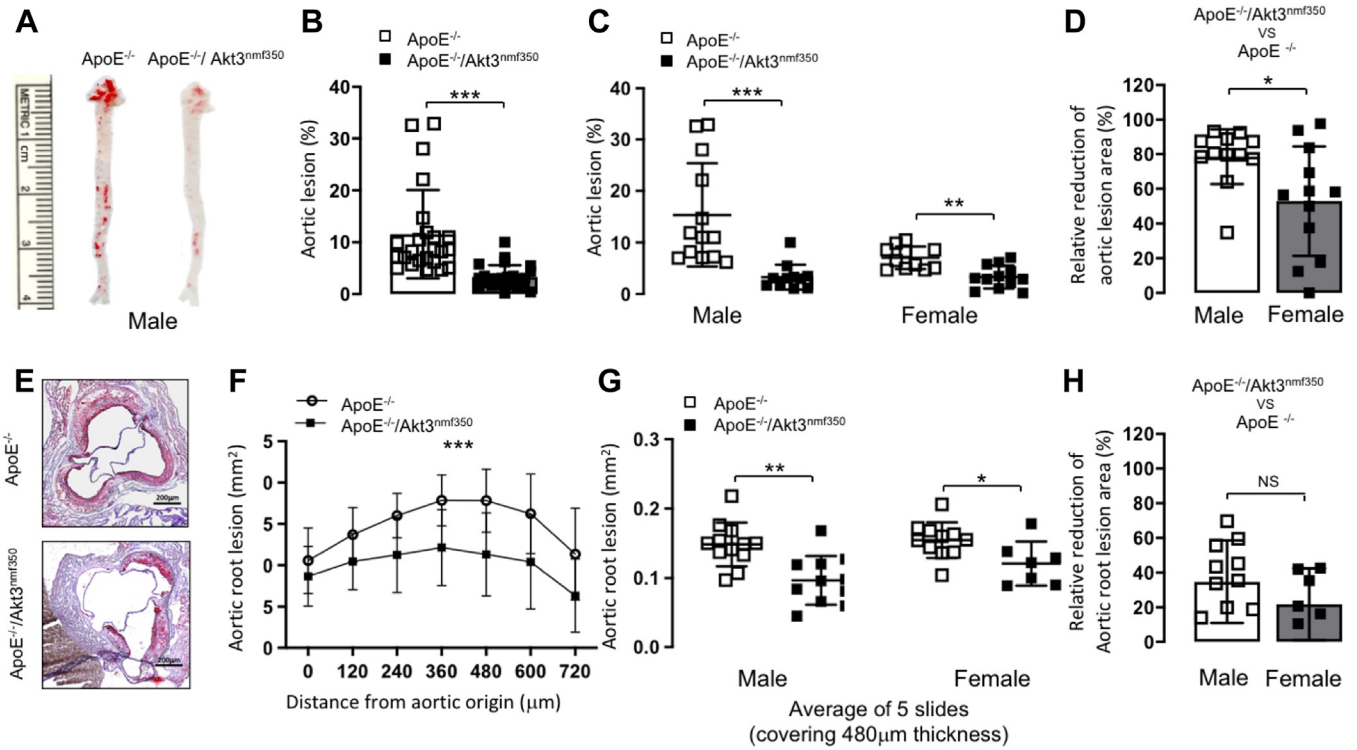


**Figure 1. Increased Akt3 kinase activity is associated with reduced atherosclerosis in ApoE$^{-/-}$ mice fed a Western diet.** *A*, *en face* oil red O staining of whole aortas isolated from male ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice after 15 weeks on a Western diet. *B*, quantification of lesion areas of aortas (Percentage of atherosclerotic lesion to total aortic surface area. Both genders. n = 24. *t* test, $p < 0.0001$). *C*, quantification of lesion areas detected in male or female aortas (n ≥ 12. *t* test, male $p < 0.0001$; female $p = 0.0012$). *D*, comparison between male and female lesion area of aortas after normalization to male or female ApoE$^{-/-}$, respectively (n = 12. *t* test, $p = 0.0226$) *E*, representative image of oil red O staining of cross-sections of aortic sinus. *F*, quantification of lesion areas of cross-sections from different layers throughout the studied region of aortic root (n ≥ 18. Two-way ANOVA, $p < 0.0001$). *G*, quantification of aortic root lesion areas detected in male and female mice (n ≥ 7. *t* test, male $p = 0.0014$; female $p = 0.0346$). *H*, comparison between male and female aortic root lesion areas after normalization to male or female ApoE$^{-/-}$, respectively (n ≥ 6. *t* test, $p = 0.2192$). Data are reported as means ± SD. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; NS, not significant.

Thus, the enhanced kinase activity of Akt3 suppresses atherogenesis in hyperlipidemic mice, and the atheroprotective effect is more pronounced in male mice. Although this suppression appeared to be independent of plasma glucose, FFA levels, and plasma lipid profiles, the significant reduction of plasma LDL/VLDL levels in male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice fed Western diet, but not in female, may at least partially contribute to the gender differences.

### *Enhanced Akt3 kinase activity in bone marrow-derived cells is atheroprotective*

Our previous studies demonstrated that lack of Akt3 specifically in macrophages promotes atherogenesis (4, 5). Thus, we next investigated whether enhanced Akt3 kinase activity specifically in bone marrow-derived cells such as macrophages contributes to reduced atherogenesis in ApoE$^{-/-}$/Akt3$^{nmf350}$-mice. We generated ApoE$^{-/-}$ chimeric mice with either ApoE$^{-/-}$/Akt3$^{nmf350}$ bone marrow or ApoE$^{-/-}$ bone marrow cells, fed them a Western diet, and performed atherogenesis analysis. The areas of atherosclerotic lesions in the aortas were significantly decreased in chimeric mice with ApoE$^{-/-}$/Akt3$^{nmf350}$ bone marrow (80% decrease, Fig. 2, *A* and *B*). To evaluate whether the atheroprotective effect of enhanced macrophage Akt3 activity is gender-dependent, data from male and female mice were analyzed separately. A statistically significant reduction was observed in male mice. The reduction in female mice did not reach statistical significance (Fig. 2, *C* and *D*). Lesion areas in cross-sections of the aortic sinus were also reduced in chimeras with ApoE$^{-/-}$/Akt3$^{nmf350}$ bone marrow as compared with ApoE$^{-/-}$ bone marrow controls (Fig. 2*E*). A significant reduction in the size of atherosclerotic plaques was observed over a distance of 720 μm within the aortic sinus area (Fig. 2*F*). When genders were analyzed separately, a significant decrease in lesion area in the aortic sinus was again observed in male chimeras with ApoE$^{-/-}$/Akt3$^{nmf350}$ bone marrow (49% decrease) but not in female mice (Fig. 2, *G* and *H*). These findings strongly suggest that the increased Akt3 activity in bone marrow derived cells, presumably macrophages, is atheroprotective, with effect being more pronounced in males.

### *Macrophage accumulation and apoptosis are reduced in aortic root lesions of ApoE$^{-/-}$/Akt3$^{nmf350}$ mice*

Macrophage accumulation and subsequent foam cell formation play a key role in atherosclerotic plaque formation. We next evaluated the macrophage content within the atherosclerotic lesion areas. We observed a marked (57.3%) reduction of macrophage content in plaques of the aortic sinus from ApoE$^{-/-}$/Akt3$^{nmf350}$ mice compared to ApoE$^{-/-}$ mice (Fig. 3*A*).

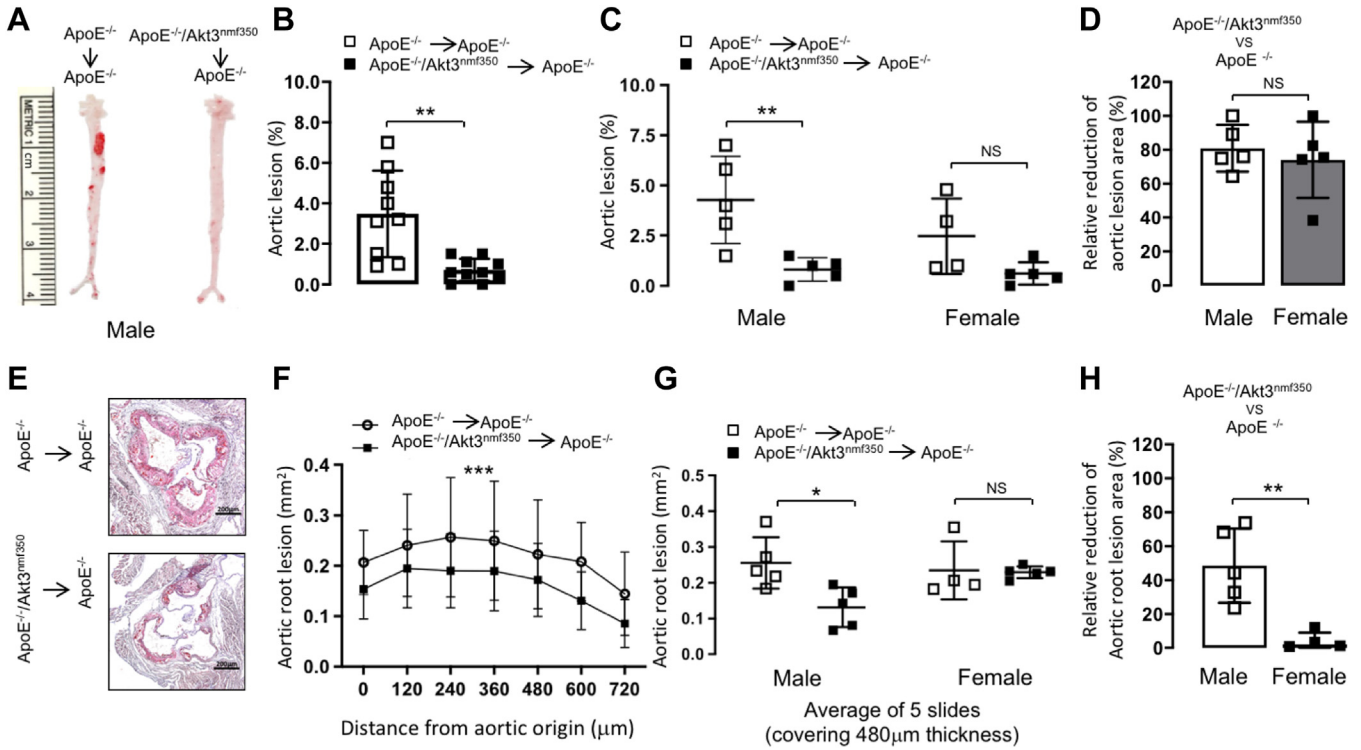


**Figure 2. Atherosclerotic lesions are reduced in ApoE$^{-/-}$ chimeras with ApoE$^{-/-}$/Akt3$^{nmf350}$ bone marrow.** *A*, *en face* oil red O staining of whole aortas isolated from male ApoE$^{-/-}$ chimeras with male ApoE$^{-/-}$ or ApoE$^{-/-}$/Akt3$^{nmf350}$ bone marrow. *B*, quantification of lesion areas of aortas isolated from ApoE$^{-/-}$ chimeras (Percentage of atherosclerotic lesion to total aortic surface area. Both genders. n ≥ 9. *t* test, $p = 0.0016$). *C*, quantification of lesion areas detected in male and female ApoE$^{-/-}$ chimera aortas (n ≥ 4. *t* test, male $p = 0.0159$; female $p = 0.0476$). *D*, comparison of male and female lesion area of aortas after normalized to male or female ApoE$^{-/-}$ chimeras, respectively (n = 5. *t* test, $p = 0.7460$). *E*, representative images of oil red O staining of cross-sections of the aortic sinus isolated from ApoE$^{-/-}$ chimeras. *F*, quantification of lesion areas of cross-sections from different layers throughout the studied region of aortic sinus (n = 9. Two-way ANOVA, $p = 0.0001$). *G*, quantification of aortic sinus lesion areas detected in male and female ApoE$^{-/-}$ chimera mice (n ≥ 4. *t* test, male $p = 0.0159$; female $p = 0.4127$). *H*, comparison between male and female aortic sinus lesion area after normalization to male or female ApoE$^{-/-}$ chimeras, respectively (n = 5. *t* test, $p = 0.0079$). Data are reported as means ± SD. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; NS, not significant.

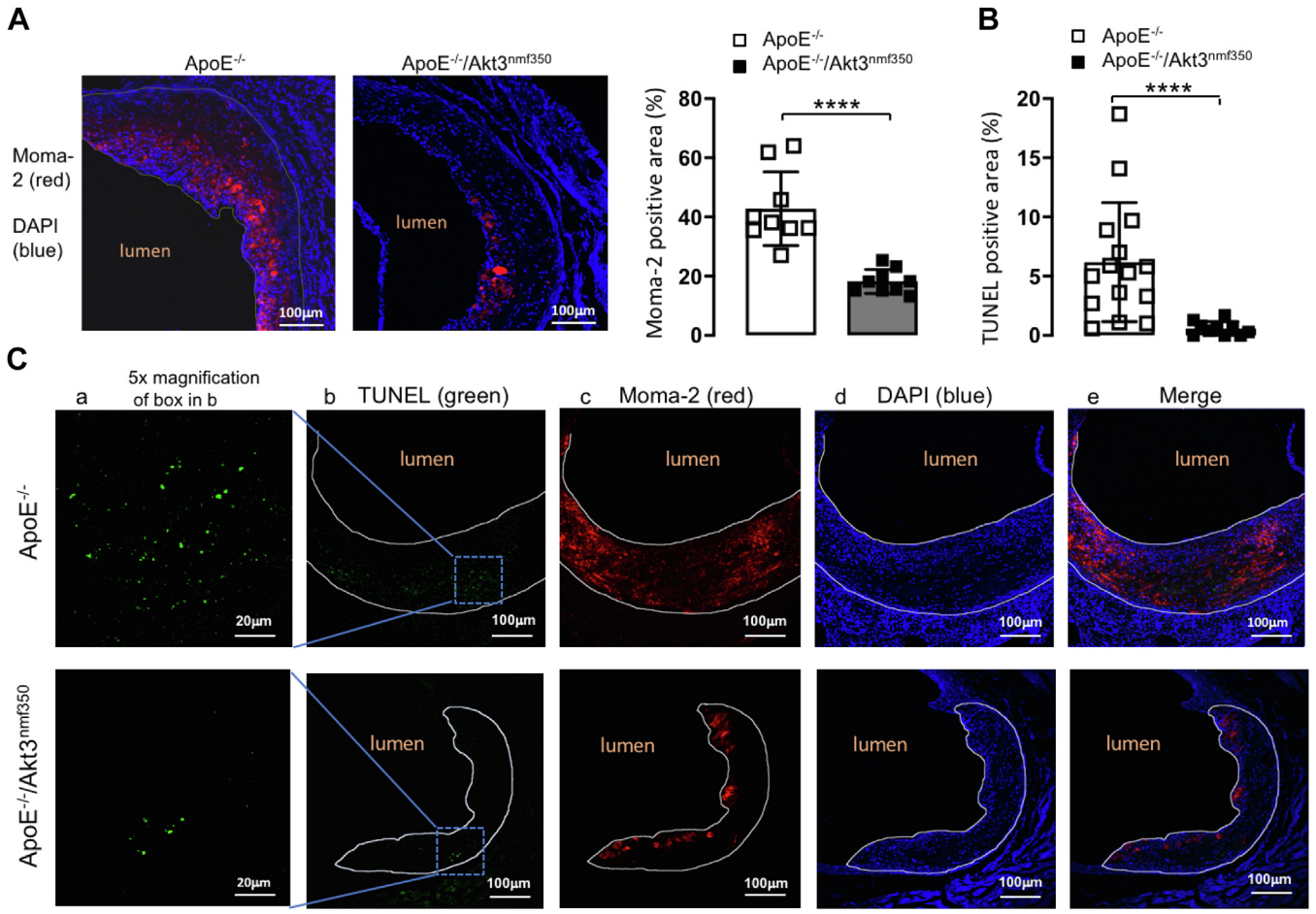


**Figure 3. Macrophage accumulation and in-situ TUNEL staining of aortic root lesions.** *A*, macrophage marker Moma-2 fluorescent staining (*left panel*) and quantification (relative to lesion areas, *right panel*) of aortic sinus sections from ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice after 15 weeks of western diet (n = 9. *t* test, $p < 0.0001$). *B*, quantification of TUNEL staining positive area (relative to lesion area) of aortic sinus sections from ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice after 15 weeks of western diet (n ≥ 10. *t* test, $p < 0.0001$). *C*, TUNEL staining (*green*), macrophage marker Moma-2 staining(*red*) and DAPI staining (*blue*) of aortic sinus sections from ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice. Data are reported as means ± SD. ****$p \leq 0.0001$.

The distribution of macrophages in the lesions was different as well with macrophages in lesions of ApoE$^{-/-}$/Akt3$^{nmf350}$ mice being located mostly on the luminal side of the plaque.

Macrophage apoptosis contributes significantly to atherogenesis with effects opposite in early and advanced lesion stages. Reduced macrophage apoptosis is proatherogenic in early atherosclerotic plaque formation but leads to reduced lesion size in advanced atherosclerotic lesions (21). TUNEL staining of advanced aortic root lesions revealed that fewer apoptotic cells were present in aortic root lesions of ApoE$^{-/-}$/Akt3$^{nmf350}$ mice (90% reduction) compared to ApoE$^{-/-}$ mice (Fig. 3, *B* and *C*).

To test the effect of Akt3 on macrophage apoptosis *in vitro*, macrophages isolated from ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice were either cultured in conditions of nutritional deprivation, or treated with ac-LDL in the presence or absence of ACAT1 inhibitor K604 for 48 h. The percentage of apoptotic cells in macrophages isolated from ApoE$^{-/-}$/Akt3$^{nmf350}$ mice was reduced in every condition, compared to macrophages isolated from ApoE$^{-/-}$ mice (Fig. 4, *A* and *B*). Further analysis of data from male and female mice showed 79.5%, 81.3%, and 76.9% (nutritional deprivation, ac-LDL and ac-LDL plus K604 treatment, respectively) reductions of apoptotic cells in macrophages isolated from male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice compared to those from male ApoE$^{-/-}$ mice. 46.9%, 68.8%, and 63.6% (nutritional deprivation, ac-LDL, and ac-LDL plus K604 treatment, respectively) reductions of apoptotic cells were observed in macrophages isolated from female ApoE$^{-/-}$/Akt3$^{nmf350}$ mice compared to those from female ApoE$^{-/-}$ mice. Interestingly, the resistance to apoptosis was significantly more pronounced in macrophages of male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice, than in macrophages from ApoE$^{-/-}$/Akt3$^{nmf350}$ female mice (Fig. 4*A*), in line with atherogenesis data. Thus, macrophage Akt3 protects cell from apoptosis *in vitro* and *in vivo* in a gender-dependent manner.

To further investigate the mechanism of the atheroprotective function of Akt3 in macrophages, we assessed cell migration in thioglycolate elicited peritoneal macrophages (MPM) and bone marrow-derived macrophages (BMDM) *in vitro*. As shown in Figure 4, *C* and *D*, ApoE$^{-/-}$/Akt3$^{nmf350}$ MPM demonstrated greater migratory activity toward the 10% serum containing media than ApoE$^{-/-}$ MPM. Similar results were obtained in BMDM (Fig. 4*E*). Whether the increased cell migratory capability of macrophages in ApoE$^{-/-}$/Akt3$^{nmf350}$

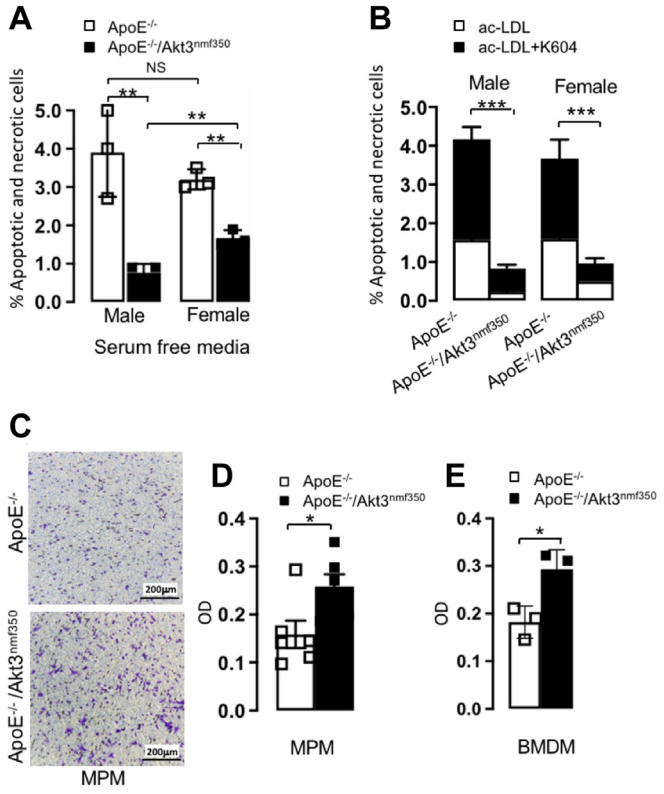


**Figure 4. Akt3$^{nmf350}$ macrophages demonstrate reduced apoptosis and higher migratory capability compared to the control group.** Peritoneal macrophages were isolated from ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice. *A*, macrophages were incubated with serum-free culture media and apoptosis was detected 48 h after treatment using Annexin V-FITC and propidium iodide (PI) staining (n = 3. *t* test, male: $p$ = 0.0099; female: $p$ = 0.0014; ApoE−/−/Akt3nmf350 male vs female: $p$ = 0.00742). *B*, macrophages were incubated with ac-LDL (100 mg/ml) in the presence or absence of ACAT1 inhibitor K604 (2 mM) and apoptosis was detected 48 h after treatment using Annexin V-FITC and propidium iodide (PI) staining (n = 3). *C*, peritoneal macrophages (MPM) were cultured in serum-free cell culture media overnight, then cells were allowed to migrate toward cell culture media containing 10% FBS for 7 h, then the migrated cells were stained (*purple*). *D* and *E*, MPM and bone marrow-derived macrophages (BMDM) were cultured in serum-free cell culture media overnight, then cells were allowed to migrate toward cell culture media containing 10% FBS for 7 h, then the migrated cells were stained and staining quantified (MPM: n = 6. *t* test $p$ = 0.026; BMDM: n = 3, $p$ = 0.0239). Data are reported as means ± SD. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$.

mice contributes to the reduced cell accumulation and apoptotic cells in atherosclerotic lesion areas needs further studies.

Macrophage polarization plays a crucial role in atherosclerotic lesion progression. To test whether the increased Akt3 activity in macrophages affects macrophage polarization, we collected bone marrow cells (BMDM) from wild-type (WT) and Akt3$^{nmf350}$ male mice and then induced M1 polarization *in vitro* by LPS/IFN-γ or M2 polarization by IL-4/IL-13. Western blotting was then performed on cell lysates for M1 cell marker iNOS (inducible nitric oxide synthase) and for M2 cell marker Arginase-1 (Fig. S2*A*). iNOS levels in M1 macrophages were similar between these two groups, while the level of Arginase-1 was 1.5-fold higher in M2 macrophages from Akt3$^{nmf350}$ male mice as compared to WT male mice (Fig. S2*B*). These data suggests that increased Akt3 activity in macrophages promotes alternative activation of macrophages (M2) which may contribute to the atherosclerotic lesion reduction in Akt3$^{nmf350}$ male mice.

Taken together, these results further support the significant contribution of macrophages to reduced atherosclerosis in ApoE$^{-/-}$/Akt3$^{nmf350}$ mice.

### *Enhanced Akt3 kinase activity affects pro-inflammatory cytokines release from MPMs in a gender-dependent manner*

Compelling evidence demonstrated the importance of inflammation and macrophage cytokines secretion in atherogenesis (16–19, 22–25). Gender differences in cytokine production were also reported (26–31). We analyzed the release of inflammatory cytokines from WT and Akt3$^{nmf350}$ resident murine peritoneal macrophages (MPMs) *in vitro*. We observed a significant decrease in the release of pro-inflammatory cytokines IL-6, TNF-α, MCP1, and MIP-1α from male Akt3$^{nmf350}$ resident macrophages as compared to control WT macrophages, but this decrease was not observed in female mice (Fig. 5*A*). There were no differences of pro-inflammatory cytokines MIP-1β and IL-1β release between these two groups (Fig. 5*B*). The release of anti-inflammatory cytokines TGF-β2 and IL-10 were also similar between these two groups (Fig. 5*C*). In contrast, we detected a significant increase in IL-6 and TNF-α production by MPM and BMDM macrophages from AKT3 knockout mice (Fig. S5), further demonstrating that AKT3 suppresses inflammatory cytokine production in macrophages. These data are in line with *in vivo* results on atherosclerosis and may at least partially explain the gender difference in the effect of enhanced Akt3 activity on atherogenesis. To get further insights into the mechanism of the reduction of the proinflammatory cytokines production in male Akt3$^{nmf350}$ macrophages, we analyzed the cytokine gene expression in WT and Akt3$^{nmf350}$ resident peritoneal macrophages. We did not observe any statistically significant difference of mRNA levels for IL-6, TNF-α, MCP1, and MIP-1α in macrophages from Akt3$^{nmf350}$ mice compared to WT macrophages (Fig. S3). These results indicate that increased Akt3 activity suppresses proinflammatory cytokines production at post-transcriptional levels.

The transcription factor NF-kB induces the expression of a number of pro-inflammatory cytokines and chemokines (32). To test whether increased Akt3 kinase activity in Akt3$^{nmf350}$ macrophages affects NF-kB (P65) activation, we collected bone marrow cells from WT and Akt3$^{nmf350}$ male mice, stimulated with 100 ng/ml LPS and 10 ng/ml IFN-gamma for M1 polarization or 10 ng/ml IL-4 and 10 ng/ml IL-13 for M2 polarization. The relative abundance of NF-kB (P65) in nuclear extracts was analyzed. There were no significant differences in NF-kB (P65) levels in M0 and M2 macrophages between these two groups, but there was a mild but statistically significant increase in P65 activity in male Akt3$^{nmf350}$ M1 macrophages (Fig. S4*A*). In agreement with this result, the production of nitric oxide (NO) a marker of macrophage activation was also moderately increased in male Akt3nmf350 M1 macrophages (Fig. S4*B*).

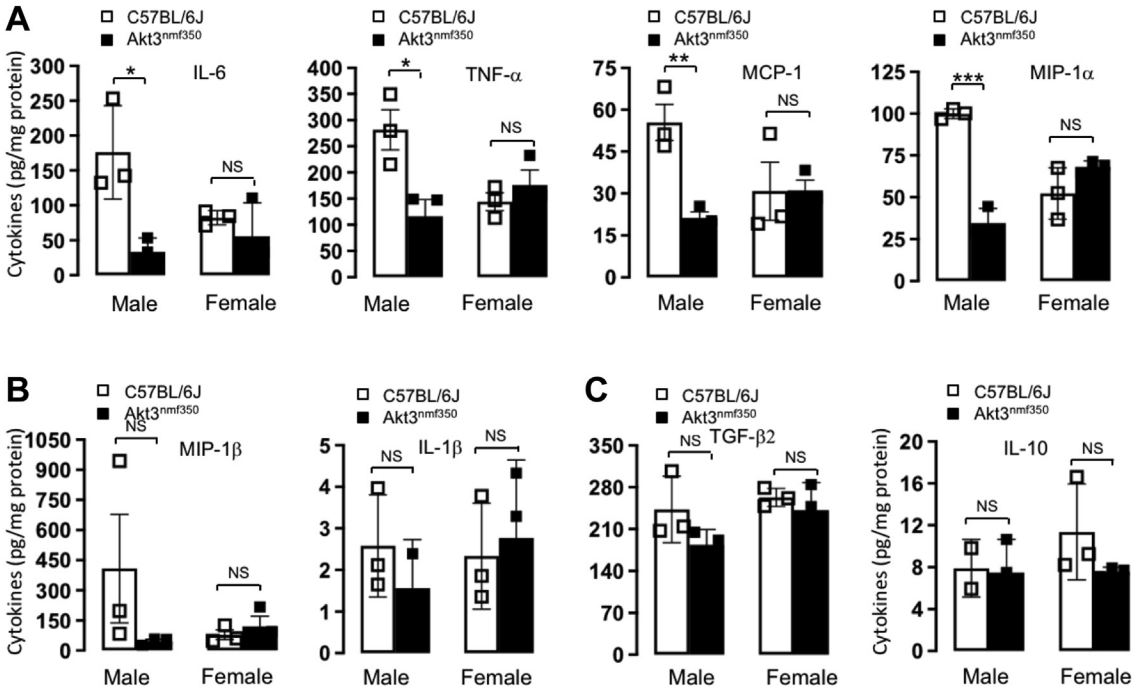


**Figure 5. Enhanced Akt3 activity in peritoneal resident macrophages of male mice is associated with reduced pro-inflammatory cytokines release.** Resident peritoneal macrophages collected from wild-type and Akt3$^{nmf350}$ mice fed with a chow diet were cultured in serum-free media overnight. The cell culture media was analyzed for indicated pro-inflammatory and anti-inflammatory cytokines by ELISA. *A* and *B*, pro-inflammatory cytokines of IL-6 (*t* test, male: $p = 0.0241$, female: $p = 0.4004$), TNF-a (*t* test, male: $p = 0.0300$, female: $p = 0.4005$), MCP-1 (*t* test, male: $p = 0.0075$, female: $p = 0.9781$), MIP-1a (*t* test, male: $p = 0.0002$, female: $p = 0.1538$), MIP-1b (*t* test, male: $p = 0.2508$, female: $p = 0.4903$), and IL-1b (*t* test, male: $p = 0.4249$, female: $p = 0.7556$) levels in cell culture media. *C*, anti-inflammatory cytokine TGF-b2 (*t* test, male: $p = 0.1686$, female: $p = 0.4872$) and IL-10 (*t* test, male: $p = 0.8939$, female: $p = 0.2277$) levels in cell culture media. The data are reported as means ± SD. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; NS, not significant.

### *Reduced levels of pro-inflammatory cytokines TNF-α and IL-6 observed in aortic sinus lesion area in ApoE$^{-/-}$/Akt3$^{nmf350}$ male mice*

IL-6 and TNF-α are involved in the progression of atherosclerosis and have been detected in atherosclerotic lesions (33, 34). To evaluate the effect of Akt3 kinase activity on the level of IL-6 and TNF-α in atherosclerotic lesions, we performed an immunohistochemical analysis in advanced aortic sinus lesions of ApoE$^{-/-}$/Akt3$^{nmf350}$ male mice. We found a significant reduction of IL-6 and TNF-α positive area in aortic sinus sections from ApoE$^{-/-}$/Akt3$^{nmf350}$ mice compared to ApoE$^{-/-}$ mice (76% and 89.1%, respectively) (Fig.e 6, *A* and *B*) in agreement with *in vitro* data.

## Discussion

While genetic ablation of Akt3 leads to macrophage-dependent accelerated atherosclerosis in hyperlipidemic ApoE$^{-/-}$ mice (4), the effect of increased Akt3 kinase activity on atherogenesis is unknown. In this study, we used Akt3$^{nmf350}$ mice characterized by increased Akt3 kinase activity (20) on ApoE$^{-/-}$ background to demonstrate macrophage-dependent atheroprotective effect of enhanced activity of Akt3. We further showed gender dependence of the effect and identified mechanisms that likely underlay the phenotype.

Enhanced Akt3 kinase activity in hyperlipidemic ApoE$^{-/-}$ mice suppresses the development of atherosclerosis both in the aorta and the aortic sinus. Using ApoE$^{-/-}$ chimeric mice with ApoE$^{-/-}$/Akt3$^{nmf350}$ bone marrow, we also demonstrated the atheroprotective role of enhanced Akt3 activity strongly depends on its expression in bone marrow-derived cells, presumably macrophages. Macrophage accumulation, macrophage apoptosis, and pro-inflammatory cytokines production were reduced in the lesions of ApoE$^{-/-}$/Akt3$^{nmf350}$ mice. *In vitro* macrophages isolated from ApoE$^{-/-}$/Akt3$^{nmf350}$ mice also showed the higher capability of cell migration and increased alternative M2 polarization. Alternative polarization of macrophages is known to be associated with the resolution of inflammation and may contribute to reduced atherogenesis in ApoE$^{-/-}$/Akt3$^{nmf350}$ mice. There were only moderate differences in plasma cholesterol and no differences in plasma FFA and glucose between these two groups, indicating that the atheroprotective function of enhanced Akt3 kinase activity is likely independent of these atherogenic factors. These results extend our previous observation on the proatherogenic role of Akt3 deficiency in hyperlipidemic mice (4).

Furthermore, we now show that the atheroprotective effect of increased Akt3 activity is gender dependent, being more pronounced in male mice. This result was reproduced in mice with a whole-body expression of Akt3$^{nmf350}$ and in mice with Akt3$^{nmf350}$ expressed only in bone marrow-derived cells, supporting the key role of these cells in the phenotype. Interestingly, we observed a moderate decrease in LDL/VLDL cholesterol levels specifically in male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice fed a high-fat diet. While this reduced cholesterol may contribute to increased atheroprotection of male ApoE$^{-/-}$/Akt3$^{nmf350}$ mice as compared to females, our studies in bone marrow chimeras suggest that this contribution is not critical.

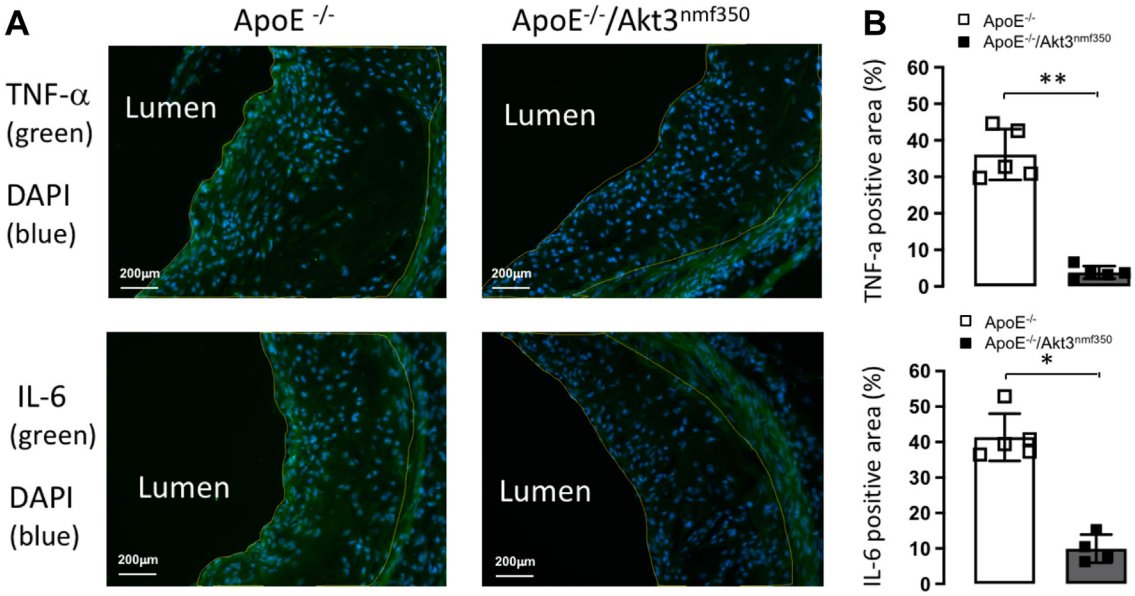


**Figure 6. Reduced TNF-a and IL-6 levels are detected in atherosclerotic lesion areas in mice with enhanced Akt3 kinase activity.** Fluorescent staining (A) and quantification (B) of TNF-alpha (n = 5. *t* test, $p = 0.0079$) and IL-6 ($ApoE^{-/-}$, n = 5; and $ApoE^{-/-}/Akt3^{nmf350}$, n = 4. *t* test, $p = 0.0159$) contents in atherosclerotic lesion area of aortic sinus cross-sections from $ApoE^{-/-}$ and $ApoE^{-/-}/Akt3^{nmf350}$ male mice. *A*, representative images of cross-sections of the aortic sinus stained with TNF-alpha or IL-6. Five animals were analyzed for each group. One cross-section was used for each animal. Three to five fields were analyzed for each cross-section. TNF-alpha and IL-6 positive areas and the total lesion area of each cross-section were measured using ImageJ software. The percentage of the total positive area over the total lesion area of each section was calculated and analyzed by the Mann–Whitney U test with GraphPad Prism9 and reported as means ± SD. Non-immune antibodies were used as control. *, $p \leq 0.05$; **, $p \leq 0.01$.

The uptake of modified lipoproteins by macrophages in atherosclerotic lesions, resulting in lipids accumulation and subsequent foam cell formation, is a key event in atherosclerotic lesion development (35). We have previously shown that a lack of Akt3 promotes macrophage foam cell formation (4). In the present study, we did not observe any differences in macrophage foam cell formation and cholesteryl ester synthesis between macrophages from $Akt3^{nmf350}$ and wild type, or $ApoE^{-/-}/Akt3^{nmf350}$ and $ApoE^{-/-}$ mice (data not shown). This finding suggests that while Akt3 is necessary for inhibition of CE synthesis, the increased Akt3 activity is not sufficient to further suppress CE synthesis.

Macrophage apoptosis is a key determinant of atherosclerotic lesion progression. In the advanced stage of atherosclerosis, the efferocytosis is suppressed, and apoptotic cells cannot be cleared promptly; thus, the apoptosis of foam cells promotes atherosclerosis (21). While Akt3 overexpression was shown to protect several types of cells, including cancer cells, from apoptosis (36–38), whether enhanced Akt3 activity protects macrophages from apoptosis is unknown. In this study, we detected reduced macrophage apoptosis in lesions of $ApoE^{-/-}/Akt3^{nmf350}$ and then demonstrated *in vitro* that macrophages isolated from $ApoE^{-/-}/Akt3^{nmf350}$ mice exhibit significant resistance to apoptosis. Interestingly, macrophages from male mice seem to be more resistant in line with results of atherogenesis studies indicating that this phenotype may contribute to gender difference in atheroprotection of enhanced Akt3 kinase activity.

We also found higher migratory ability of macrophages from $ApoE^{-/-}/Akt3^{nmf350}$ mice compared to macrophages from $ApoE^{-/-}$ mice. Whether the increased cell mobility promotes macrophage egress from lesion area, resulting in the reduced macrophage content within the atherosclerotic lesion area in $ApoE^{-/-}/Akt3^{nmf350}$ mice is not clear and needs further investigation.

Peripheral macrophages produce pro-inflammatory cytokines such as MCP-1, Il-6 and TNF-α, which are crucial for immune cell recruitment, immune response, and atherosclerosis development (39). Macrophages also express anti-inflammatory cytokines such as IL-10, which play crucial roles in the resolution of inflammation (40–43). Production of cytokines is also known to be affected by sex. Proatherogenic cytokines, including IL-6, TNF-α, and IL-1β, are significantly higher in men than in women (15, 44–46). The effect of Akt3 on macrophage cytokines production is not known. We observed that macrophages from Akt3-deficient mice produced significantly more IL-6 and TNF-α *in vitro*. In contrast, we found that increased activity of Akt3 in macrophages was associated with significantly reduced production of pro-inflammatory cytokines IL-6, TNF-α, MCP-1, and MIP-1α *in vitro*. This reduction was prominent in male but not in female mice. We also confirmed lower levels of IL-6, TNF-α in atherosclerotic lesions of male $ApoE^{-/-}/Akt3^{nmf350}$ mice. Looking for the mechanism of cytokine regulation we found that expression of IL-6, TNF-α, MCP-1, and MIP-1α genes were not changed in $Akt3^{nmf350}$ macrophages, indicating that the mechanism of regulation is at post-transcriptional levels. Activation of NF-kB pathway known to promote proinflammatory cytokine gene expression at transcriptional level. In line with gene expression results we found no reduction in NF-kB activity in M1 macrophages derived from male $Akt3^{nmf350}$ mice. Considering the critical role of macrophage

cytokine production in atherosclerosis, it is plausible that the difference in cytokine plays a key role in the gender dependence of the atheroprotective effect of enhanced Akt3 kinase.

Taken together our studies demonstrated the novel atheroprotective function of increased activity of macrophage Akt3 kinase, revealed the gender-dependent nature of the effect, and identified potential mechanisms of the gender dependence.

## Experimental procedures

### Mice

C57BL/6J, homozygous ApoE$^{-/-}$ and Akt3$^{nmf350}$ mice were purchased from The Jackson Laboratory. Then double homozygous ApoE$^{-/-}$/Akt3$^{nmf350}$ mice were created from a crossbreed between ApoE$^{-/-}$ and Akt3$^{nmf350}$ mice. We used sex-, age-, and genetic background-matched 8 to 12 weeks old WT and Akt3$^{nmf350}$ mice, or ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice in our experiments. Atherosclerosis was induced by feeding of 8-week-old ApoE$^{-/-}$ and ApoE$^{-/-}$/Akt3$^{nmf350}$ mice with Western diet, which contains 0.2% cholesterol and provides 42% calories as fat (TD88137, Harlan Teklad), for 15 weeks. We performed all procedures according to protocols approved by the Cleveland Clinic IACUC.

### Bone marrow transplantation

Eight-week-old male and female recipient ApoE$^{-/-}$ mice were lethally irradiated with a single dose of whole-body irradiation (900 rad) on the day of transplantation. Bone marrow cells from the 8-week-old male and female donor ApoE$^{-/-}$ or ApoE$^{-/-}$/Akt3$^{nmf350}$ mice were isolated. Each recipient ApoE$^{-/-}$ mouse received 1 x $10^7$ bone marrow cells from sex- and age-matched donor by tail vein injection. After 4 weeks of the chow diet, the mice were fed with a Western diet for 12 weeks and then used for atherosclerotic lesion analysis. The bone marrow and tail tissue of each animal were used to confirm bone marrow reconstitution by PCR analysis and DNA sequencing.

### Atherosclerotic lesion analysis

Mice fed a Western diet were euthanized by anesthetic overdose, perfused with 10 ml PBS and 10 ml 4% paraformaldehyde. For *en face* analysis, the entire aorta from the heart, extending 5 to 10 mm after bifurcation of the iliac arteries and including the subclavian right and left common carotid arteries, was removed, dissected, stained with oil red O, and evaluated for lesion development by morphometry of scanned images using ImageJ software. For the aortic sinus analysis, serial cryosections (10 μm thickness) were taken from the region of the proximal aorta through the aortic sinuses and stained with oil red O, hematoxylin (Baglione and Smith, 2006). Morphometry of lesions was made using ImageJ software (NIH).

### Immunohistochemistry

Aortic sinus cryosections were stained with macrophage biomarker Moma-2, cytokines IL-6 or TNF-α. Sections stood for at least 30 min at room temperature before staining, followed by fixation with acetone at room temperature. Then slides were blocked with 10% (v/v) normal goat serum in 5% BSA/TBS, followed by incubation with primary antibody (Moma-2, ab33451, Abcam; IL-6, ab179570, Abcam; or TNF- α, 11,948, Cell Signaling) 4 °C overnight, and washed in TBS. Slides then were incubated with secondary antibody (Rhodamine red-x-conjugated-goat anti-rat-IgG, 112–295–003, Jackson ImmunoResearch; Alexa-488 conjugated goat anti-rabbit-IgG H + L, 111–545–003, Jackson ImmunoResearch; Alexa-488 conjugated goat anti-rabbit-IgG H + L, 111–545–003, Jackson ImmunoResearch, respectively) and then washed in TBS. Slides were then mounted with a mounting solution containing DAPI (Fluoromount-G, with DAPI, 00–4959–52, Invitrogen), and inspected using Leica confocal microscope.

### Aortic sinus sections TUNEL assay

Aortic sinus cryosections were first stained with Moma-2. After incubation with a secondary antibody for Moma-2, slides were washed with PBST, followed by TUNEL staining (TACS2 TdT Fluorescein *in situ* apoptosis detection kit, 4812–30-K, Trevigen). All procedures were performed following the manufacturer's protocol.

### Trans-well macrophage migration assay

Thioglycollate elicited murine peritoneal macrophages (MPM) or bone marrow-derived macrophages (BMDM) from ApoE$^{-/-}$ or ApoE$^{-/-}$/Akt3$^{nmf350}$ mice were used for cell migration assay using QCM Chemotaxis cell migration assay (EDM Millipore, Cat# ECM508). Macrophages were suspended and diluted to 1 × $10^6$ cells/ml in serum-free cell culture media. 300 μl of 1 × $10^6$ cells/ml cells were added per well, and incubated at 37 °C overnight. Then 500 μl of cell culture media containing 10% FBS was added into each lower well and incubated at 37 °C for 7 h. Then the inserts were taken out, the inner surface membrane was wiped with cotton swabs and cells migrated to the underside of the insert membrane were stained with crystal violet solution. Then the stain was extracted and Optical Density (OD) was measured at 560 nm on a microplate reader to assess the number of migrated cells.

### Macrophages polarization assay

Bone marrow cells were collected from wild-type (WT) and Akt3$^{nmf350}$ male mice. Cells were cultured in DMEM-F12 containing 5% FBS, 1% penicillin/streptomycin, and 25 mg/ml M-CSF for 5 days. On day 6, cells were stimulated with 50 ng/ml GM-CSF. On day 7, M1 macrophages were generated by overnight stimulation with LPS (100 ng/ml) and IFN-γ (10 ng/ml). M2 macrophage polarization was induced by overnight stimulation with IL-4 (10 ng/ml) and IL-13 (10 ng/ml). Western blot was performed on cell extracts with anti-iNOS (inducible nitric oxide synthase) primary antibody for M1 cells and anti-Arginase-1 for M2 cells (Fig. S2*A*). Quantification results were obtained using ImageJ.

### Macrophage NO production and NF-kB activity assays

Nuclear extracts of M0, M1 and M2 male macrophages were used for NF-kB (P65) DNA binding activity assay (Cayman chemical company, NF-kB (P65) Transcription Factor Assay kit. Cat# 10007889). For NO production assay, cell culture supernatants were collected from M0, M1 and M2 cell culture and NO production was detected using Nitric Oxide (total) detection kit (Enzo, Cat# ADI-917–020) according to the manufacturer's protocol.

### Measurement of macrophage inflammatory cytokines production

Murine resident peritoneal macrophages were isolated and seeded in 48-well plate and cultured overnight. Cells were washed 5 times with PBS and cultured for 8 h in 225ul RPMI with 1% BSA. Media was collected, spun at 16000 rpm for 5 min, transferred to a fresh tube, and frozen at −80 °C. Cells were washed with PBS and lysed with 15ul Tris Lysis buffer with protease/phosphatase inhibitors. Cytokine concentrations were determined from cell media supernatant with a custom 11-cytokines mouse U-PLEX panel and U-PLEX TGF-β Combo Mouse panel (Meso Scale Diagnostics).

### Statistical analysis

Data are presented as mean ± SD unless otherwise specified. The statistical significance of differences was evaluated using the Student's test, Mann-Whitney U test, or two-way ANOVA with Bonferroni's post-hoc test. Significance was accepted at the level of $p < 0.05$.

## Data availability

All data are contained within the manuscript.

*Supporting information*—This article contains supporting information.

*Author contributions*—L. Z., L. D., O. C., T. V. B., and E. A. P. methodology; L. Z. and E. A. P. writing–original draft. L. Z. and J. A. investigation. J. A. performed macrophage cytokine release experiments. E. A. P. project administrartion.

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*Abbreviations*—The abbreviations used are: AKT, serine/threonine protein kinase B; IL-6, Interleukin-6; IL-1β, Interleukin-1beta; iNOS, inducible nitric oxide synthase; MIP-1α, Macrophage inflammatory protein-1 alpha; MCP-1, Monocyte chemoattractant protein-1; TNF-α, Tumor necrosis factor-alpha.

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