Loss of two Akt isoforms in hematopoietic cells diminished monocyte and macrophage survival and reduces atherosclerosis in *Ldl-receptor* null mice

Vladimir R. Babaev[1], **Lei Ding**[1], **Youmin Zhang**[1], **James M. May**[1,2], **Stephen A. Ramsey**[5], **Kasey C. Vickers**[1,3], and **MacRae F. Linton**[1,4]

[1]Atherosclerosis Research Unit, Division of Cardiovascular Medicine, Department of Medicine, Vanderbilt University School of Medicine, 2220 Pierce Avenue, Nashville, TN 37232-6300

[2]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine

[3]Molecular Physiology and Biophysics, Vanderbilt University School of Medicine

[4]Department of Pharmacology, Vanderbilt University School of Medicine

[5]Oregon State University, School of Electrical Engineering and Computer Science, Department of Biomedical Sciences, 208A Dryden Hall, Corvallis, OR 97331.

Abstract

Objective: Macrophages express three Akt isoforms, Akt1, Akt2 and Akt3, which display isoform-specific functions but may be redundant in terms of Akt survival signaling. We hypothesize that loss of two Akt isoforms in macrophages will suppress their ability to survive and modulate the development of atherosclerosis.

Approach and Results: To test this hypothesis, we reconstituted male *Ldl*-receptor null (*Ldlr*$^{-/-}$) mice with double *Akt2/Akt3* knockout hematopoietic cells expressing only the Akt1 isoform (Akt1^{only}). There were no differences in body weight and plasma lipid levels between the groups after 8 weeks of the Western diet, however Akt1^{only}→*Ldlr*$^{-/-}$ mice developed smaller (57.6% reduction) atherosclerotic lesions with more apoptotic macrophages than control mice transplanted with wild-type (WT) cells. Next, male and female *Ldlr*$^{-/-}$ mice were reconstituted with double *Akt1/Akt2* knockout hematopoietic cells expressing the Akt3 isoform (Akt3^{only}). Female and male Akt3^{only}→*Ldlr*$^{-/-}$ recipients had significantly smaller (61 and 41%, respectively) lesions than the control WT→*Ldlr*$^{-/-}$ mice. Loss of two Akt isoforms in hematopoietic cells resulted in markedly diminished levels of white blood cells, B-cells and monocytes, and compromised viability of monocytes and peritoneal macrophages compared to WT cells. In response to LPS, macrophages with a single Akt isoform expressed low levels of inflammatory cytokines, however Akt1^{only} macrophages were distinct in expressing high levels of anti-apoptotic *Il10* compared to WT and Akt3^{only} cells.

Corresponding authors: Vladimir R. Babaev, Ph.D. or MacRae F. Linton, M.D., Vanderbilt University School of Medicine, 2220 Pierce Avenue, Nashville, Tennessee 37232-6300, Phone: (615) 936-1450, vladimir.babaev@vanderbilt.edu or macrae.linton@vanderbilt.edu.

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Conclusions: Loss of two Akt isoforms in hematopoietic cells, preserving only a single Akt1 or Akt3 isoform, markedly compromises monocyte and macrophage viability and diminishes early atherosclerosis in $Ldlr^{-/-}$ mice.

Graphical Abstract
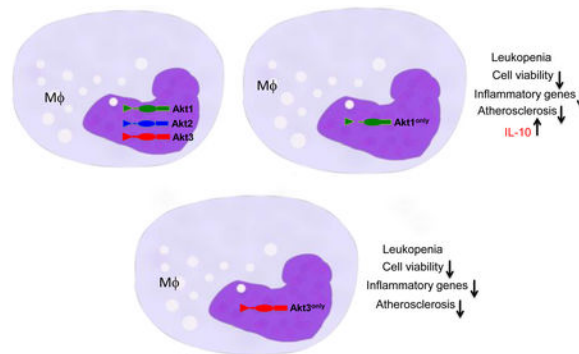
Keywords

Atherosclerosis; Macrophages; Akt signaling; Apoptosis; Monocytopenia

Introduction

Atherosclerosis is a chronic inflammatory disease that arises from an imbalance in lipid metabolism and a maladaptive immune response driven by the accumulation of cholesterol-laden macrophages in the artery wall [1]. Macrophages and macrophage-derived foam cells are the major cell type of early atherosclerotic lesions, and they play crucial roles in every stage of atherosclerosis [2]. Therefore, changes in the ability of blood monocytes or macrophages to respond to inflammatory or pro-apoptotic stimuli may significantly modulate atherogenesis.

Akt is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and migration [3, 4]. Akt has three isoforms, Akt1, Akt2 and Akt3, that share similar domain structure with 80% sequence homology [5], which are largely redundant with respect to Akt signaling but exhibit distinct isoform-specific functions [6]. Studies with Akt isoform-specific knockout mice demonstrated that each isoform gives a distinct phenotype. For example, mice lacking *Akt1* have increased perinatal mortality and reduced body weight [7, 8]. In contrast, *Akt2* deficient mice display normal growth and a diabetes-like syndrome [9], whereas *Akt3* knockout mice exhibit impaired brain development and reduced brain volume [10]. In regard to atherogenesis, loss of *Akt1* in apolipoprotein E deficient ($Apoe^{-/-}$) mice leads to severe atherosclerosis [11]. *Akt3* deficiency in macrophages of $Apoe^{-/-}$ mice promoted foam cell formation and atherosclerosis [12]. Knockout of *Akt2* in LDL-receptor null mice has been reported to impair glucose tolerance and induce more complex atherosclerotic plaques compared to controls [13]. Finally, we and others have shown that loss of macrophage *Akt2* suppresses their ability to undergo M1 polarization resulting in impaired recruitment and migration, which reduces both early and advanced atherosclerosis

[14, 15]. Together these results demonstrate isoform-specific effects of Akt in macrophage biology and atherogenesis.

In contrast to inactivation of a single Akt isoform, which is generally well tolerated and does not impact mouse survival, loss of two Akt isoforms produces detrimental effects in mice. For example, double *Akt1/Akt3* deficient mice were embryonic lethal (at 11-12 day) producing severe developmental defects in the cardiovascular and nervous systems [16]. There was also a clear dose-dependent effect with combined *Akt1* and *Akt3* deficiency indicating that both isoforms are required for embryo development and mouse survival [16]. Similarly, *Akt1/Akt2* knockout mice displayed a rather severe phenotype with dwarfism, impaired skin development, delayed bone development, reduced adipogenesis, and early lethality after birth [17]. In contrast, *Akt2/Akt3* knockout mice survive both the embryonic and postnatal periods, but they later develop insulin intolerance and a reduction in body weight compared to wild-type mice [18]. Moreover, the presence of a single functional allele of Akt1 ($Akt1^{+/-} Akt2^{-/-} Akt3^{-/-}$) in mice appears to be sufficient for embryonic development and postnatal survival [18]. This data is consistent with the notion that Akt1 is the principal pro-survival isoform [11, 19, 20]. Further studies are necessary to clarify the impact of the different Akt isoforms on cell viability and atherosclerosis.

Beyond the impressive impact of total loss of two Akt isoforms on mouse survival, the PI3K/Akt pathway plays critical roles in the viability of hematopoietic cells. For example, Akt signaling promotes both the maturation and the survival of thymocytes [21, 22] and peripheral B-cells [23]. Therefore, mice reconstituted with *Akt1/Akt2* knockout hemotopoietic cells expressing the Akt3 isoform (Akt3[only]) had developmental defects of thymocytes [24, 25], whereas follicular B cells exhibited a profound survival defect in a competition assay against wild-type B cells in vivo [23]. Similarly, the *Akt1/Akt3* knockout, leaving only a single Akt2 isoform, interferes with the differentiation of thymocytes [25]. In addition, hematopoietic stem cells with a single Akt3 isoform exhibited decreased ROS production and survival [26]. There is tremendous interest in developing inhibitors of PI3K/Akt pathway for the treatment of cancer and to modulate antitumor responses [27]; therefore, it is crucial to understand the impact of reduced Akt expression by hematopoitic cells, including macrophages, on the development of atherosclerosis. Importantly, it remains unknown whether mouse blood monocytes and macrophages have a threshold effect for Akt protein levels in survival. For comparison, the Akt3 isoform accounts for only ~25% of total macrophage Akt content and has little impact on macrophage viability [12]; whereas Akt2 is the predominant isoform in mouse macrophages [14] and the Akt1 isoform is crucial for their survival [11]. Since monocyte/macrophages are key cells in atherosclerosis[1], it would be important to elucidate the impact of Akt protein reduction to a single isoform in monocytes/macrophages on atherogenesis.

Here we generated chimeric *Ldlr*$^{-/-}$ mice reconstituted with hematopoietic cells expressing a single Akt1 or Akt3 isoform. These mice had drastically lower levels of white blood cell counts and blood monocytes compared to control mice transplanted with WT hematopoietic cells. Loss of two Akt isoforms in hematopoietic cells was detrimental for the viability of monocytes/macrophages and resulted in dramatic reductions in the extent of early atherosclerosis.

Material and Methods

The authors declare that all additional data that support the findings of this study are available in the online-only version as Supplemental Data.

Animal Procedures:

Mice homozygous for *Akt1* (stock# 004912) [8] and *Akt2* (stock# 006966) [9] knockout mutations (both on C57BL/6 background), recipient *Ldlr*^{-/-} (on C57BL/6 background) and C57BL/6 mice were purchased from the Jackson Laboratories. *Akt3* knockout mice [10] (on C57BL/6 background) were provided by Dr. Morris J. Birnbaum, from Perelman School of Medicine, University of Pennsylvania. Mice were maintained in microisolator cages on a rodent chow diet containing 4.5% fat (PMI 5010, St. Louis, MO) or a Western type diet containing 21% milk fat and 0.15% cholesterol (Teklad, Madison, WI). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Institutional Animal Care and Usage Committee. The mouse experiments were designed, performed and reported using approaches consistent with the recommendations of the ATVB Council Statement[28].

Fetal liver cell (FLC) transplantation:

FLC were isolated on day 14-16 of gestation and the gender of the embryos were determined by PCR as described [29]. Recipient *Ldlr*^{-/-} or C57BL6 mice were lethally irradiated (9Gy) and transplanted (4×10^6) with FLCs as described[30].

Analysis of Serum Lipids and Aortic Lesions:

Serum total cholesterol and triglyceride levels were determined after overnight fasting by the enzyme method. All measurements were performed according to the guidelines for experimental atherosclerosis studies described in the AHA Statement [31]. Aortas were flushed through the left ventricle and the entire aorta was dissected for en face analysis as described [32]. Cryosections of the proximal aorta were analyzed using an Imaging system KS 300 (Kontron Electronik GmbH).

Peritoneal macrophage isolation and reagents.

To analyze peritoneal macrophages, we reconstituted male C57BL6 mice with male WT, Akt1[only] or Akt3[only] FLC. Eight weeks post-transplantation, thioglycollate-elicited peritoneal macrophages were isolated and cultured. Two days later, macrophages were used in vitro experiments

Western blotting.

Cells were lysed in a lysis buffer (Cell Signaling Technology, Danvers, MA) containing protease and phosphatase inhibitors. Proteins were measured with the DC Protein assay kit (Bio-Rad Laboratories) and resolved by NuPAGE Bis-Tris electrophoresis and transferred onto nitrocellulose membranes (Amersham Bioscience). Blots were probed with primary rabbit antibodies and secondary goat anti-rabbit horseradish peroxidase-conjugated

antibodies (Sigma). Proteins were visualized with ECL western blotting detection reagents (GE Healthcare) and quantified by densitometry using ImageJ software (NIH).

Immunocytochemistry:

Serial 5-micron cryosections of the proximal aorta were fixed with 2% paraformaldehyde-PBS and stained with the appropriate antibodies or non-immune rabbit or rat IgG were used as a negative control. Double staining of macrophages with MOMA-2 and cell nuclei with DAPI with analysis of nucleus numbers in macrophage area as previously described [33].

RNA isolation and real-time PCR.

Total RNA was isolated and relative quantitation of the target mRNA was performed as described [33] using the gene expression assays that were normalized with 18S ribosomal RNA as an endogenous control.

Flow Cytometry:

Blood cells were stained with a cocktail of antibodies against lineage markers and Ly-6C . Then the cells were treated with Lysing buffer (BD Pharm, cat#555899), washed and analyzed using FACS DiVa v6.1 software (BD Biosciences) in the Research Flow Cytometry Core Laboratory, Veterans Administration Medical Center.

Apoptosis assessment.

Cultured cells in Laboratory-Tek chambers (Nalge Nunc International) or 5-micron cryosections from the proximal aorta were fixed in 4% paraformaldehyde in PBS, treated with 3% citric acid and apoptotic cells were detected by the *in situ* cell death detection kit (Roche Applied Science). TUNEL-positive (TUNEL+) cells were counted in 4 different sections of each aorta as described[32]. For in vitro analysis, peritoneal macrophages were seeded in Laboratory-Tek chambers (Nalge Nunc International), then treated with BSA or 0.5M PA-BSA with or without a mouse recombinant Il-10 (100ng/ml; R&D System) for 24 hours or human Ox-LDL (100mg/ml) or Ac-LDL (100mg/ml) with ACAT inhibitor Sandoz 58035 (2mg/ml) for 48 hours. Apoptotic cells were detected by an Alexa Fluor488 Annexin V/Dead Cell Apoptosis kit (Life Technologies, catalog number V13241). The number of TUNEL+ cultured cells were counted as a percentage of the total number of cells in at least four separate fields (containing ≈1,000 cells) from duplicate chambers.

Statistical Analysis:

Data are provided as Means ± SEM. For the comparison of two groups, data were initially tested for normal distribution and equal variance. If the data were normally distributed, a Student *t* test was performed. For nonparametric data the Mann–Whitney rank-sum test was used. To compare multiple groups, one way ANOVA and Kruskal-Wallis one way ANOVA on Ranks were used. For one way ANOVA, when the data met the assumption of homogeneity of variances, we used Tukey's post hoc test. In the case of Kruskal-Wallis analysis, the Games Howell post hoc test was run. If the data were not normally distributed, the nonparametric Kruskal–Wallis one-way ANOVA on ranks was performed. $P<0.05$ was

considered significant. All statistical analyses were performed using SigmaPlot 14.0 a SPSS Statistics Premium 22 (IBM, Armonk, NY) or GraphPad Prism 7.

Results

Mice with a single Akt1 isoform in hematopoietic cells have reduced atherosclerosis.

Because mice with double knockouts of Akt isoforms have significant neonatal mortality, we used a fetal liver cell (FLC) transplantation approach [29] to generate mice chimeric for loss of two Akt isoforms in hematopoietic cells. First, Akt2$^{-/-}$ and Akt3$^{-/-}$ mice were crossed, then their littermates were intercrossed and FLCs were collected on day 16-18. Next, ten-week-old male *Ldlr*$^{-/-}$ mice were lethally irradiated and transplanted with male WT (control group; n=10) or *Akt2/3* knockout FLC expressing only Akt1 (Akt1^{only}; n=10) FLC. Four weeks after transplantation, the recipients were challenged with the Western diet for 8 weeks. At sacrifice, thioglycollate-elicited peritoneal macrophages were isolated from the recipient mice. Western bot analysis demonstrated that macrophages did not express Akt2 or Akt3, preserving only the Akt1 isoform (Figure 1A). Importantly, there were no statistically significant differences between the groups of recipient mice in body weight, serum total cholesterol or triglyceride levels (Table 1A). In contrast, analysis of atherosclerotic lesions in the aortic sinus (Figure 1B) demonstrated that Akt1^{only}→*Ldlr*$^{-/-}$ mice developed smaller (57.6% reduction) atherosclerotic lesions than control WT→*Ldlr*$^{-/-}$ mice (Figure 1C). Compared to WT→*Ldlr*$^{-/-}$ mice, Akt1^{only}→*Ldlr*$^{-/-}$ mice also had a smaller area of the lesions occupied by macrophages as determined by staining with the anti-macrophage antibody, MOMA-2 (Fig 1D) and fewer nuclei in the MOMA-2-positive area (Figure 1E) analyzed as previously described [34]. In addition, Akt1^{only}→*Ldlr*$^{-/-}$ mice had increased numbers of apoptotic TUNEL-positive cells in the atherosclerotic lesions (Figure 1F). Double staining for TUNEL and macrophage marker CD68 verified the presence of apoptotic cells in the atherosclerotic lesions that are macrophage in origin (Figure I of Supplemental Data). Since this marker may be expressed by smooth muscle cells after lipid loading [35], we used another specific marker of macrophages, MOMA2 (Figure SII). Similar double staining of WT macrophages with CD68 and DAPI combined with antibodies to Akt1 or Akt3 revealed that macrophages expressed both Akt1 and Akt3 isoforms but did not stain with the isotype control (Figure SIII). Staining of serial aortic sections of mice reconstituted with Akt1^{only} hematopoietic cells demonstrated that macrophages in the atherosclerotic lesions contained only Akt1 but not Akt2 and Akt3 isoforms (Figure SIV). Thus, reduction of Akt to a single Akt1 isoform in hematopoietic cells significantly suppresses early atherosclerotic lesion formation.

Female and male mice with a single Akt3 isoform in hematopoietic cells have reduced atherosclerosis.

In the next set of experiments, we examined whether the presence of a single Akt3 isoform in hematopoietic cells has an impact on atherogenesis in vivo. Ten-week-old female *Ldlr*$^{-/-}$ mice were lethally irradiated and transplanted with female WT(n=10) or Akt3^{only}(n=10) FLC, and, four weeks later, the recipient mice were challenged with the Western diet for 8 weeks. At sacrifice, the analysis of proteins extracted from peritoneal macrophages demonstrated that macrophages of Akt3^{only}→*Ldlr*$^{-/-}$ mice contained only the Akt3 isoform

(Figure 2A). No differences in the ratio of Akt3/β-actin were noted between WT and Akt3^{only} macrophages (0.63 ± 0.08 vs. 0.53 ± 0.02, respectively, p=0.29). Moreover, we compared *Akt1* and *Akt3* gene expression levels in WT, Akt1^{only} and Akt3^{only} macrophages and found no compensatory increase in the levels of the preserved gene (Figure SV). There were no statistically significant differences between recipient mice of different groups in body weight, serum total cholesterol or triglyceride levels (Table 1B). However, mice reconstituted with Akt3^{only} FLC had markedly smaller (61% reduction) atherosclerotic lesions in the aortic sinus than control WT→*Ldlr*$^{-/-}$ mice (Figure 2B,C). Akt3^{only}→*Ldlr*$^{-/-}$ mice had a significantly smaller area of the lesions occupied by macrophages (Figure 2D), with fewer nuclei in the MOMA-2+ area (Figure 2E) and an increased number of apoptotic TUNEL-positive cells in the atherosclerotic lesions (Figure 2F) than WT→*Ldlr*$^{-/-}$ mice. Thus, female *Ldlr*$^{-/-}$ mice with Akt3^{only} hematopoietic cells had dramatically suppressed early atherosclerosis.

Next, to elucidate whether these findings were gender specific, twenty-four-week-old male *Ldlr*$^{-/-}$ mice were lethally irradiated and transplanted with male FLCs expressing WT (n=9) or Akt3^{only}(n=11). Four weeks post-transplantation, the recipient mice were challenged with the Western diet for 8 weeks. Again, there were no statistically significant differences in body weight and serum lipid levels between the two groups of recipients (Table 1C). Interestingly, the 24-week old male WT→*Ldlr*$^{-/-}$ mice developed larger atherosclerotic lesions than the 10-week old male or female recipients used in the previous experiments, despite spending a similar amount of time on the Western diet. This allowed us to analyze the extent of atherosclerosis in the aortas en face (Figure 3A), which showed that male *Ldlr*$^{-/-}$ mice reconstituted with Akt3^{only} FLC had a 75% reduction in atherosclerotic lesion area versus control WT→*Ldlr*$^{-/-}$ mice (Figure 3C). In addition, Akt3^{only}→*Ldlr*$^{-/-}$ mice had smaller (41%) atherosclerotic lesions in the aortic sinus (Figure 3D), less MOMA2-positive area (Figure 3E), fewer nuclei in the MOMA-2+ area and more TUNEL-positive cells in the atherosclerotic lesions (Figure 3F) than WT→*Ldlr*$^{-/-}$ mice. Again, double staining of atherosclerotic lesions of mice transplanted with Akt3^{only} bone marrow cells revealed that macrophages expressed only the Akt3 isoform (Figure SVI). Together these results demonstrate that both female and male *Ldlr*$^{-/-}$ mice reconstituted with Akt3^{only} hematopoietic cells have significantly suppressed early atherosclerotic lesions compared to the same gender of control WT→*Ldlr*$^{-/-}$ mice.

Mice with a single Akt isoform in hematopoietic cells have low levels of white blood cells, B-cells and monocytes, and increased monocyte apoptosis.

To evaluate the impact of Akt1^{only} or Akt3^{only} isoforms in hematopoietic cells of recipient mice on peripheral blood leukocytes, blood cells were analyzed by multicolor flow cytometry. Remarkably, both groups of Akt1^{only}→*Ldlr*$^{-/-}$ and Akt3^{only} →*Ldlr*$^{-/-}$ mice had similar changes in blood cells: dramatic reductions of white blood cell counts (Figure 4A) and B-cells (Figure 4B), and significant increases (>2-fold) in neutrophils (Figure 4C) compared to control WT→*Ldlr*$^{-/-}$ mice. In addition, mice reconstituted with Akt1^{only} and Akt3^{only} hematopoietic cells exhibited substantial reductions (38 and 52%, respectively) in blood monocyte numbers (Figure 4C) with equally diminished levels of inflammatory Ly-6C^{hi}/CCR2-positive and patrolling Ly-6C^{lo}/CX3CR1-positive subsets (Figure 4E,F).

Thus, expression of only a single Akt1 or Akt3 isoform in hematopoietic cells induced leukopenia with dramatically reduced numbers of white blood cells, B-cells and monocytes, whereas neutrophil numbers were increased.

Our flow cytometry analysis also detected an increase in total blood apoptotic cells in Akt1^{only}→$Ldlr^{-/-}$ and Akt3^{only}→$Ldlr^{-/-}$ recipients compared to control WT→$Ldlr^{-/-}$ mice (Figure 4G). There were no differences in neutrophil apoptosis (Figure 4H), but the levels of apoptotic monocytes were significantly increased in mice expressing only the Akt1 or Akt3 isoform in hematopoietic cells (Figure 4I). To further elucidate this phenomenon, freshly isolated WT, Akt1^{only} and Akt3^{only} bone marrow cells and peritoneal macrophages were analyzed by flow cytometry. Again, there was a statistically significant increase of apoptosis in Akt3^{only} cells versus Akt1^{only} bone marrow and peritoneal macrophages compared to control WT cells (Figure 5A, B).

Next, blood monocytes were isolated from the recipient mice, seeded in culture chambers and treated with control BSA or a lipotoxic pro-apoptotic factor palmitic acid (PA) complexed to BSA to induce ER stress [36]. Analysis of apoptosis by Annexin V staining demonstrated that all of these cells had similar levels of apoptosis after incubation with BSA, but PA treatment significantly increased apoptosis in a step-shaped manner in WT, Akt1^{only} and Akt3^{only} monocytes, respectively (Figure 5C,D). Thus, Akt3^{only} monocytes have significantly higher susceptibility to apoptosis than Akt1^{only} cells.

Macrophages with a single Akt1 or Akt3 isoform are less resistant to ER stress.

Since Akt signaling plays a pro-survival role preventing cells from undergoing apoptosis[3] and the threshold of Akt protein levels is important for cell survival under stressed conditions[37], we examined Akt phosphorylation in a short-term response to insulin treatment and under conditions of long-lasting ER stress. Compared to WT, Akt3^{only} cells had comparable levels of Akt phosphorylation in response to insulin treatment (Figure SVII) indicating that short-term Akt signaling is essentially preserved in these cells. In contrast, when WT and Akt3^{only} peritoneal macrophages were incubated in the presence of lipotoxic PA-BSA for several hours, the PA-induced stress gradually weakened Akt signaling in WT cells; whereas, cells expressing the Akt3^{only} isoform exhibited less p-Akt than WT cells at baseline and in response to PA-BSA (Figure 6A,B). Correspondingly, the Akt down-stream target and anti-apoptotic member of the BCL-2 family, p-Bad S^{136}, was also drastically reduced in Akt3^{only} macrophages (Figure 6A). Similar results were seen in macrophages expressing the Akt1^{only} isoform, which exhibited slightly lower levels of Akt signaling at every time point of ER stress than WT cells (Figure SVIII). Thus, ER stress exhausts anti-apoptotic Akt signaling more rapidly in Akt3^{only} macrophages than in WT and Akt1^{only} cells.

Next, we compared the ability of WT, Akt1^{only} and Akt3^{only} macrophages to resist pro-apoptotic stimuli. Treatment with BSA had no impact on apoptosis and there were no differences between the groups (Figure 6A). In contrast, PA-BSA significantly increased apoptosis in all four groups but to a significantly greater extent in Akt1^{only} macrophages than WT cells and cells with a single isoform deletion (Figure 6A,B). Similarly, BSA alone did not induce apoptosis in any of the different groups (Figure SIX), whereas PA-BSA

treatment induced less apoptosis in WT, $Akt1^{-/-}$ and $Akt2^{-/-}$ macrophages than Akt3[only] macrophages, indicating that loss of two Akt isoforms markedly decreases cell survival (Figure 6C). These Akt3[only] macrophages also showed an enhanced sensitivity to different pro-apoptotic stimuli such as oxidized LDL or free cholesterol loading with acetylated LDL in combination with an ACAT inhibitor (Figure 6D). A direct comparison of cells with only a single Akt isoform demonstrated that Akt3[only] macrophages have the lowest levels of survival compared to Akt1[only] and especially WT cells (Figure 6E). Together these data demonstrate that under conditions of ER stress, loss a single Akt isoform alone is dispensable for macrophage survival, and the loss of two Akt isoforms markedly compromises cell survival, with higher levels of resistance to apoptosis in Akt1[only] cells compared to Akt3[only] macrophages.

In response to LPS, Akt1[only] macrophages express high levels of Il-10 that likely increases their survival.

Akt1 and Akt3 isoforms exert different functions in macrophages [11][12]. Previously we have shown that loss of Akt1 but not Akt2 significantly suppressed Il-10 gene expression in macrophages [14] indicating that the Akt1 isoform may play a role in regulation of the gene. Moreover, a recent report specifies that IL-10 is an important anti-inflammatory cytokine that mediates metabolic reprogramming of macrophages [38]. To examine whether loss of two Akt isoforms impacts cytokine expression, WT, Akt1[only] and Akt3[only] macrophages were treated with LPS and analyzed by real-time PCR. Both *Akt1* and *Akt3* macrophages showed markedly reduced expression of pro-inflammatory genes including *Tnfα*, *Il12a*, *Il6*, *Nfkb1 (p50)* and *Rela (p60)* (Figure SIX). However, Akt1[only] macrophages expressed significantly higher (3-fold) levels of *Il10* gene than WT and Akt3[only] cells (Figure 6F). To test whether IL-10 is facilitating a pro-survival effect in mouse macrophages, we treated WT, Akt1[only] and Akt3[only] peritoneal macrophages with BSA, a mouse recombinant IL-10 and PA-BSA alone or together with IL-10 for 24 hours. Interestingly, IL-10 alone had no impact on apoptosis but when combined with PA it dramatically reduced levels of apoptosis in every type of macrophage (Figure 6G). Importantly, Akt1[only] macrophages were significantly more protected from apoptosis via incorporation of exogenous IL-10 than WT and Akt3[only] cells (73.6% vs. 31.8 and 43.5% reduction, respectively). Thus, IL-10 protects macrophages from apoptosis and high levels of *Il-10* gene expression in Akt1[only] macrophages likely contributes to their survival advantage over Akt3[only] cells.

Discussion

The viability of monocyte/macrophages and their survival in the artery wall plays a crucial role in the pathogenesis of atherosclerotic lesions [39]. Liu and co-workers[19] were the first to demonstrate that constitutive activation of Akt1 is vital for the survival of human macrophages and that inhibition of Akt signaling induced apoptosis in human and mouse macrophages[32]. Macrophages express three Akt isoforms, Akt1, Akt2 and Akt3, which are likely redundant with regard to Akt signaling. Recent studies have reported that mice with hematopoietic cells expressing only the *Akt3* isoform exhibited low viability of B-, T-cells and stem cells [23-26]. Based on these data, we hypothesized that marked reduction of Akt to a single isoform may compromise the viability of monocytes and macrophages, which would

be expected to alter atherosclerosis. To test this hypothesis, we generated mice expressing Akt1[only] or Akt3[only] in hematopoietic cells. This novel approach allowed us to compare the impact of expression of the individual Akt1 and Akt3 isoforms on macrophage viability and atherosclerosis. Remarkably, both groups of mice reconstituted with Akt1[only] or Akt3[only] hematopoietic cells exhibited dramatic reductions in white blood cell counts and monocytopenia. In addition, monocytes and macrophages of these mice had increased sensitivity to apoptotic stimuli. As a result, male *Ldlr*^{−/−} mice reconstituted with Akt1[only] FLC had significantly smaller atherosclerotic lesions compared to control mice with WT cells. Similarly, male and female *Ldlr*^{−/−} mice transplanted with Akt3[only] FLC had reduced atherosclerotic lesions compared to control WT→*Ldlr*^{−/−} mice. Thus, the reduction of Akt protein to a single isoform compromised monocyte and macrophage viability and this significantly diminished early atherosclerosis.

A striking finding of our studies is the fact that mice expressing only the Akt1 or Akt3 isoform in hematopoietic cells had lower levels of white blood cells but increased numbers of neutrophils. Recently Chen and co-authors [40] have shown that *Akt2* knockout mice exhibited a 3-fold increase in blood neutrophils due to their defects in migration, and release of O_2. In our experiments, *Akt2* was deleted in both groups of Akt1[only] or Akt3[only] hematopoietic cells, and this may explain the increased number of neutrophils. The decreased number of white blood cells in these mice has been reported to be due in part to a profound survival defect in B-cells [23] as well as developmental defects of thymocytes [24, 25] and stem cells [26]. In addition, we report here for the first time that the reduction of Akt protein to a single isoform in hematopoietic cells significantly lowered levels of blood monocytes, both Ly-6C^{hi} and Ly-6C^{low} subsets. These monocytes were less viable than WT cells in response to increased ER stress. Importantly, Akt1[only] monocytes were more resistant to apoptosis than Akt3[only] cells (Figure 5C-F), and these data support the concept that Akt1 is the principal pro-survival isoform in macrophages [11, 19, 20].

Monocyte/macrophages are central cells of the innate immune system, and they play a key role in the initiation and progression of atherosclerosis [2]. There is a strong correlation between blood monocyte count and atherosclerosis [41] suggesting that monocytopenia may significantly suppress atherogenesis. Indeed, Smith et al. [42] first demonstrated that *Apoe* null mice deficient in macrophage colony-stimulating factor (M-CSF), which have very low numbers of blood monocytes, developed significantly smaller atherosclerotic lesions than control mice. Later, Rajavashisth and co-workers [43] revealed that *Ldlr*^{−/−} mice homozygous for *M-CSF* gene deficiency (op/op) have dramatically reduced atherosclerotic lesions (~0.3% of control lesions), whereas heterozygous mice had lesions < 1% in size of controls. They highlighted the fact that a 2-fold decrease in M-CSF expression reduced lesion size approximately 100-fold. Similarly, *Ldlr*^{−/−} mice deficient for monocyte chemoattractant protein-1 developed smaller atherosclerotic lesions compared to control *Ldlr*^{−/−} mice [44]. Consistently, genetic deficiency [45] and pharmacological inhibition [46] of the chemokine receptor CX3CR1 in *Apoe*^{−/−} mice significantly decreased atherosclerosis. Similarly, combined loss of CCL2, CX3CR1, and CCR5 in *Apoe*^{−/−} mice significantly reduced circulating monocytes and markedly suppressed (90%) atherosclerosis [47]. Moreover, depletion of monocytes from the circulation using clodronate (dichloromethylene bisphosphonate) considerably reduced plaque formation in rabbits [48]. Finally, Stoneman et

al [49] utilized transgenic mice expressing diphtheria toxin in CD11b-positive cells to demonstrate that depletion of monocytes profoundly reduced early atherosclerotic lesions. Together these data indicate that genetic reduction as well as chemical depletion of blood monocytes markedly suppresses atherosclerosis. Thus, the dramatic reduction of blood monocytes in Akt1^{only}→$Ldlr^{-/-}$ and Akt3^{only}→$Ldlr^{-/-}$ mice likely contributes to the suppression of atherosclerosis seen in these mice.

Since the loss of two Akt isoforms in hematopoietic cells impairs lymphocyte survival and increases neutrophils in the blood, we cannot conclude that compromised viability of monocyte/macrophages is the only mechanism responsible for the reduction atherosclerosis in $Ldlr^{-/-}$ mice reconstituted with Akt1^{only} and Akt3^{only} FLC. Future studies will be required to evaluate the impact of expression of a single Akt isoform by neutrophils, T- and B-cells on atherosclerosis. Here we demonstrated that both monocytes and macrophages expressing a single Akt isoform had increased apoptosis under conditions of ER stress, whereas loss of either *Akt1* or *Akt2* alone in macrophages did not compromise cell survival. A potential limitation of the in vitro macrophage apoptosis studies is the small number of mice (n=3/group) used; however, hundreds of peritoneal macrophages were studied per mouse and the responses to different pro-apoptotic factors (Figure 6B-E) were very similar and consistent for the different genotypes of macrophages (WT, Akt1$^{-/-}$, Akt2$^{-/-}$, Akt1^{only} and Akt3^{only}). Furthermore, these results are consistent with the finding that further depletion of Akt3 protein in Akt1/2 knockout mouse embryonic fibroblast spontaneously induced apoptosis [37], supporting the notion that Akt is critical for cell survival. Together, our results provide strong *in vitro* and *in vivo* evidence that a reduction of total Akt content in monocytes and macrophages markedly reduces their resistance to apoptosis. Dramatic reductions in atherosclerotic lesion area of mice reconstituted with Akt1^{only} or Akt3^{only} hematopoietic cells were coupled with finding fewer macrophages and more apoptotic cells that support the current concept [50] that an inverse relationship exists between macrophage apoptosis and lesion size in early atherosclerotic lesions. By showing that reduced macrophage Akt protein levels promote increased sensitivity to apoptosis and that this suppresses early atherosclerosis, our data demonstrate that Akt protein content is crucial for macrophage survival in the highly stressed environment of atherosclerotic lesions.

We have previously reported that mouse peritoneal macrophages have significantly higher levels of Akt1 and Akt2 isoforms than Akt3 [14]. However, it remains unknown whether macrophage-derived foam cells of atherosclerotic lesions may have differential expression of the individual Akt isoforms compared to other macrophages, such as bone marrow-derived or peritoneal macrophages. To examine this possibility, we analyzed publicly available microarray gene (mRNA) expression datasets that utilized laser capture micro-dissection and examined gene expression in CD68-positive macrophages of atherosclerotic lesions of Reversa mice [51]. These mice are LDL receptor deficient and express an apolipoprotein B100-only allele, but they also harbor a floxed microsomal triglyceride transfer protein gene ($Ldlr^{-/-}$ $Apob^{100/100}Mttp^{fl/fl}Mx1Cre^{+/+}$) that allows reversal of the hyperlipidemia and atherosclerosis by conditional deletion of MTP [33, 52]. Surprisingly, expression of the Akt3 isoform was significantly higher in macrophage-derived foam cells of both control mice (Supplemental Figure XIA) and in mice with deletion of the floxed *Mttp* gene in the liver (Figure XIB) than expression of Akt1 or Akt2 isoforms. In contrast, the expression of Akt1

and Akt2 isoforms were significantly higher than Akt3 expression in bone marrow-derived macrophages [53] (Figure XIC). A potential limitation of these results with bone marrow-derrived macrophages is the small sample size, but there was a large difference between the groups with little variation within each group and this pattern of Akt isoform expression is consistent with our previous results for peritoneal macrophages [14]. Most importantly, our results show differential expression of Akt isoforms in macrophage-derived foam cells of atherosclerotic lesions with increased levels of Akt3 compared to the expression pattern seen in bone marrow-derived and peritoneal macrophages.

Previously, Arranz and co-workers [54] demonstrated that Akt isoforms play opposing roles in macrophage polarization with the Akt1 isoform promoting the M2 phenotype and Akt2 initiating the M1 phenotype. More recently, we have shown that *Akt2* null macrophages are skewed to the M2 phenotype with decreased expression of pro-inflammatory genes and reduced migration, and these changes suppress atherogenesis [14]. In the current study, we verified that the Akt3 isoform expression alone in macrophages has no impact on cell polarization. In response to LPS, both Akt1^{only} and Akt3^{only} macrophages exhibited significantly lower levels of inflammatory gene expression. However, Akt1^{only} macrophages expressed markedly increased levels of *Il-10* gene compared to WT and Akt3^{only} macrophages. Moreover, IL-10 protects cells from apoptosis, including astrocytes [55], myeloid progenitor cells [56] and cardiomyocytes [57]. Here we demonstrated that recombinant IL-10 significantly reduces levels of apoptosis in monocytes and macrophages. These findings may explain, at least in part, pro-survival advantages of Akt1^{only} cells over Akt3^{only} macrophages.

In conclusion, we generated chimeric *Ldlr*$^{-/-}$ mice expressing a single Akt1 or Akt3 isoform in hematopoietic cells. These mice had low levels of white blood cell counts and monocyte numbers. In addition, they also exhibited increased apoptosis in blood monocytes, in freshly isolated peritoneal macrophages and bone marrow cells. In conditions of ER stress, loss of two Akt isoforms in hematopoietic cells was detrimental for the viability of monocytes and macrophages. These changes in viability of monocyte/macrophages resulted in dramatic reductions in the extent of early atherosclerosis. Furthermore, our findings may have important implications for the development of new therapeutic approaches. In recent years, the PI3K/Akt pathway has emerged as one of the most promising targets of anticancer drug therapy [58], and a number of clinical trials with inhibitors of Akt are currently underway in cancer patients [59]. Therefore, our current data predict a potential unanticipated impact of therapeutic inhibition of Akt signaling on monocyte/macrophage survival that is likely to reduce atherogenesis, which may have important clinical relevance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations:

Akt	protein kinase B
FLC	fetal liver cells
LDLR	LDL-receptor

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Highlights:

- Akt protein reduction to a single Akt1 or Akt3 isoform in blood monocyte and macrophage markedly compromises their survival under conditions of ER stress.

- *Ldlr*$^{-/-}$ mice reconstituted with Akt1^{only} or Akt3^{only} hematopoietic cells develop low white blood cell counts and monocytopenia, which dramatically reduces early atherosclerosis.

- Akt1^{only} macrophages expressed significantly higher levels of *Il10* gene, which may contribute to their survival advantages over Akt3^{only} cells.
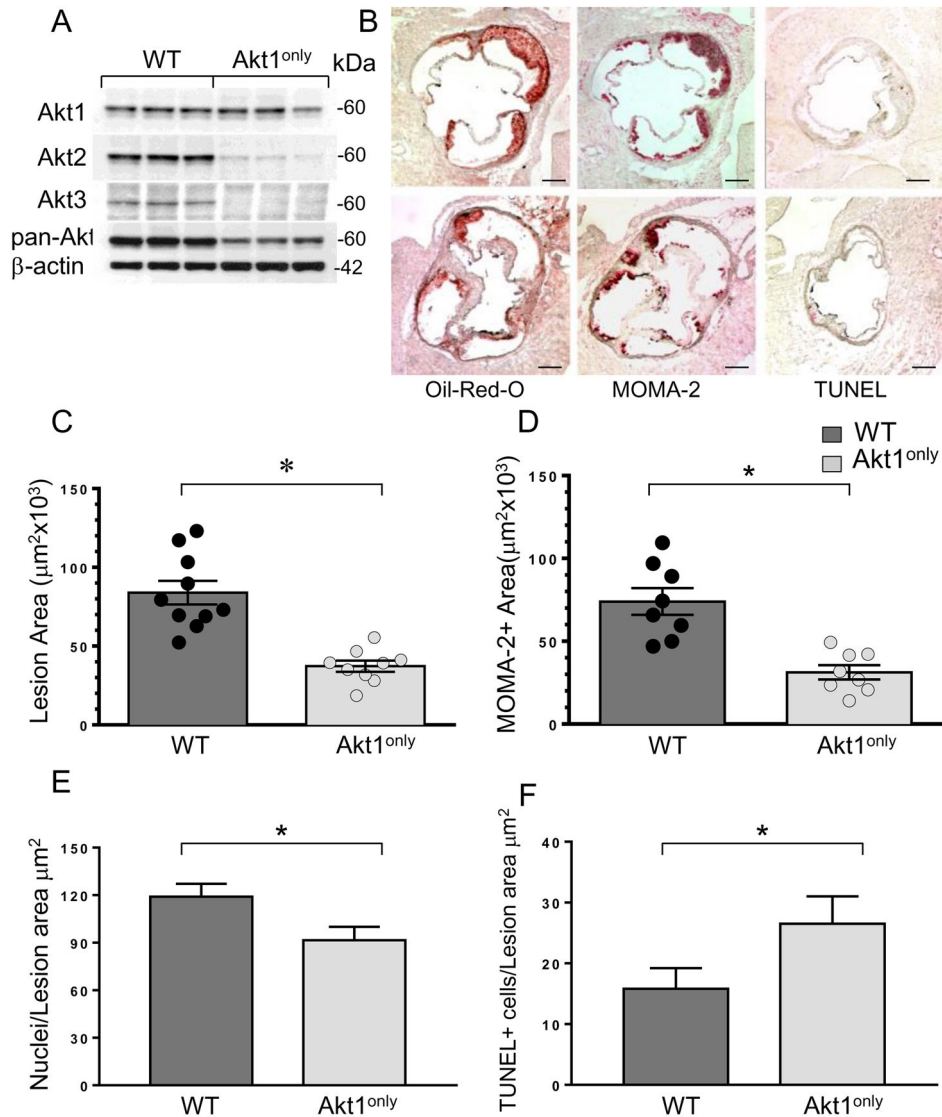
Figure 1. Reconstitution of Akt1^{only} hematopoietic cells into male Ldlr$^{-/-}$ mice significantly decreases Akt protein levels in macrophages and reduces early atherosclerosis.

(**A**) Double *Akt2/Akt3* knockout macrophages preserve only the Akt1 isoform. Peritoneal macrophages were isolated from WT→*Ldlr$^{-/-}$* and *Akt1^{only}*→*Ldlr$^{-/-}$* mice (n=3/group), proteins were extracted from cells and analyzed by Western blot;

(**B**) Detection of atherosclerotic lesions in the aortic sinus of WT→*Ldlr$^{-/-}$* (top panel) and *Akt1^{only}*→*Ldlr$^{-/-}$* mice (bottom panel); Serial aortic cryosections were stained to detect lipids by Oil-Red-O stain, reveal macrophages using antibody to MOMA-2 or identify apoptotic cells by TUNEL assay; scale bars, 200µm;

(**C-F**) The extent of atherosclerotic lesions (C), MOMA-2-positive area (D), numbers of nuclei in MOMA-2+ area (E) and TUNEL+ cells (F) in atherosclerotic lesions of WT→*Ldlr$^{-/-}$* and *Akt1^{only}*→*Ldlr$^{-/-}$* mice FLC; Graphs represent data (mean ± SEM, ∗p<0.05 by Mann-Whitney Rank Sum and ∗p<0.05 by t-tests).
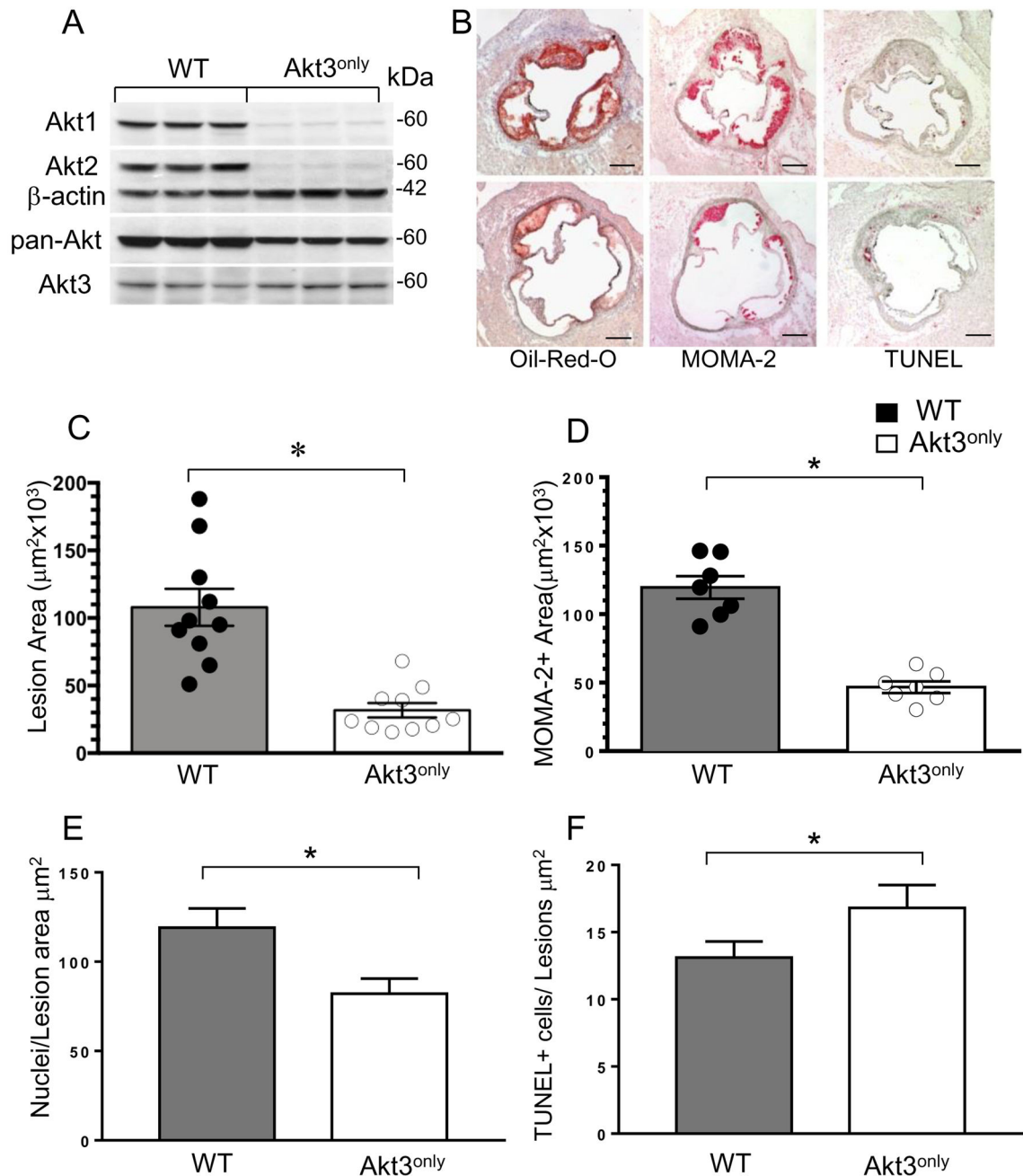
Figure 2. Reconstitution of Akt3^{only} hematopoietic cells into female Ldlr$^{-/-}$ mice significantly decreases Akt protein levels in macrophages and diminishes atherosclerotic lesions.

(**A**) Double *Akt2/Akt3* knockout macrophages expressed the Akt3 isoform. Peritoneal macrophages were isolated from WT→*Ldlr$^{-/-}$* and *Akt3^{only}*→*Ldlr$^{-/-}$* mice (n=3/group), proteins were isolated from cells and analyzed by Western blot.

(**B**) Detection of atherosclerotic lesions in the aortic sinus of WT→*Ldlr$^{-/-}$* (top panel) and *Akt3^{only}*→*Ldlr$^{-/-}$* mice (bottom panel); Serial sections were stained with Oil-Red-O, MOMA-2 or TUNEL assay; scale bars, 200μm.

(**C-F**) The percent of atherosclerotic lesions (C), macrophage area (D), numbers of nuclei (E) and apoptotic cell numbers (F) in atherosclerotic lesions of female $Ldlr^{-/-}$ mice reconstituted with WT or $Akt3^{only}$ FLC; Graphs represent data (mean ± SEM, *p<0.05 by Mann-Whitney Rank Sum and *p<0.05 by t-tests).
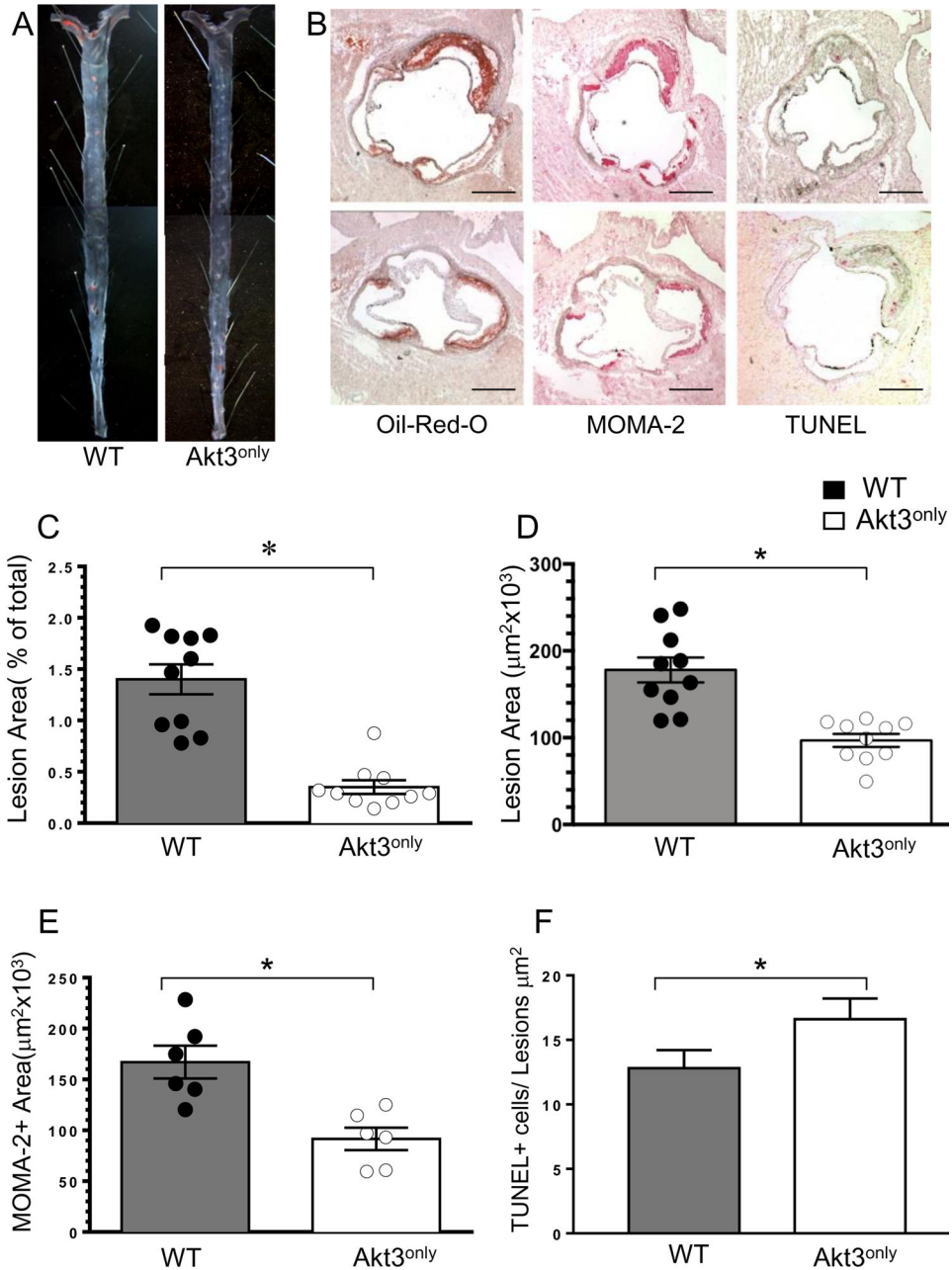
Figure 3. Reconstitution of Akt3^{only} hematopoietic cells into male Ldlr$^{-/-}$ mice reduces atherosclerosis

(**A**) Detection of atherosclerotic lesions in pinned out aorta *en face* of WT→*Ldlr*$^{-/-}$ and Akt3^{only}→*Ldlr*$^{-/-}$ mice; Aortas were stained with Sudan IV, pin size 10μm.

(**B**) Detection of atherosclerotic lesions, MOMA-2+ area and apoptotic cells in aortic sinus of WT→*Ldlr*$^{-/-}$ (top) and Akt3^{only}→*Ldlr*$^{-/-}$ mice (bottom); Aortic sections were stained with Oil-Red-0, MOMA-2, or TUNEL assay; Scale bars, 200μm.

(**C-F**). The percent of atherosclerotic lesions in pinned out aortas (C), the extent of lesion area (D), MOMA-2+ area (E) and number of TUNEL+ cells (F) in aortic sinus of male *Ldlr*

[−/−] mice reconstituted with WT or *Akt3* FLC; Graphs represent data (mean ± SEM, ∗*p*<0.05 by Mann-Whitney Rank Sum and **p*<0.05 by t-tests).
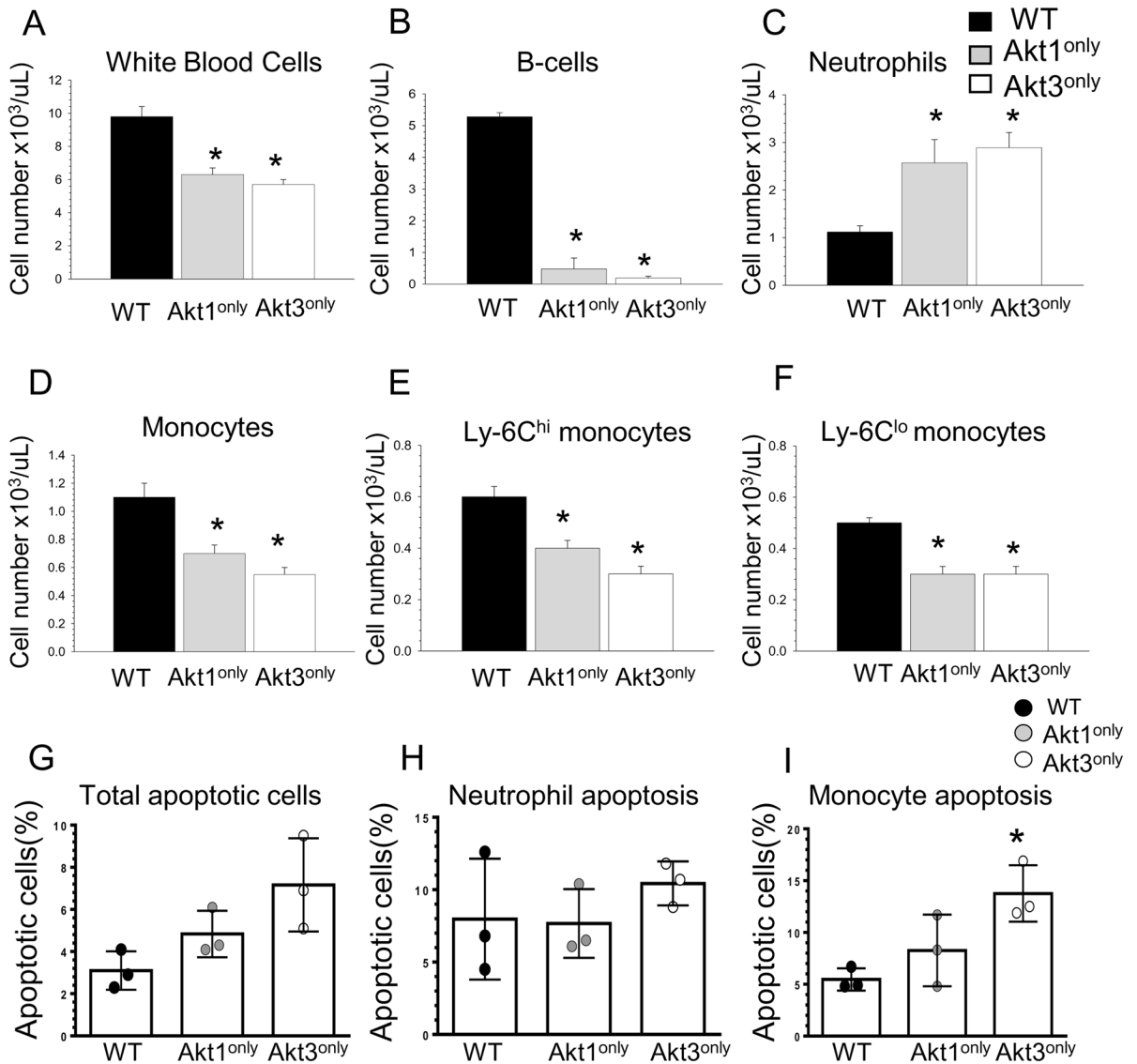
Figure 4. Mice with a single Akt isoform in hematopoietic cells have low levels of white blood cells, B-cells and monocytes but increased numbers of neutrophils and augmented monocyte apoptosis.

(A-F); Blood cells were collected from retro-orbital sinus of mice reconstituted with WT, Akt1^{only} or Akt3^{only} FLC (n=4/group). Cells were incubated with antibodies to CD19, CD11b, Ly-6G, Ly-6C and CCR2, and then analyzed by multicolor flow cytometry. Graphs represent data (mean ± SEM, *p<0.05 compared to WT cells by One Way Analysis of Variance (Tukey test). The experiment was repeated three times.

(G-I) Total number of apoptotic cells (G), apoptotic neutrophils (H) and monocytes (I) in blood of mice transplanted with WT, Akt1^{only} and Akt3^{only} FLC. Cells were isolated from mice (n=3/group) and incubated with antibodies to CD11b, Ly-6G and then treated with Annexin V Alexa Flour 647 to detect apoptotic cells. The experiment was repeated two times. Graphs represent data (mean ± SEM), *p<0.05 compared to WT cells by One Way ANOVA.
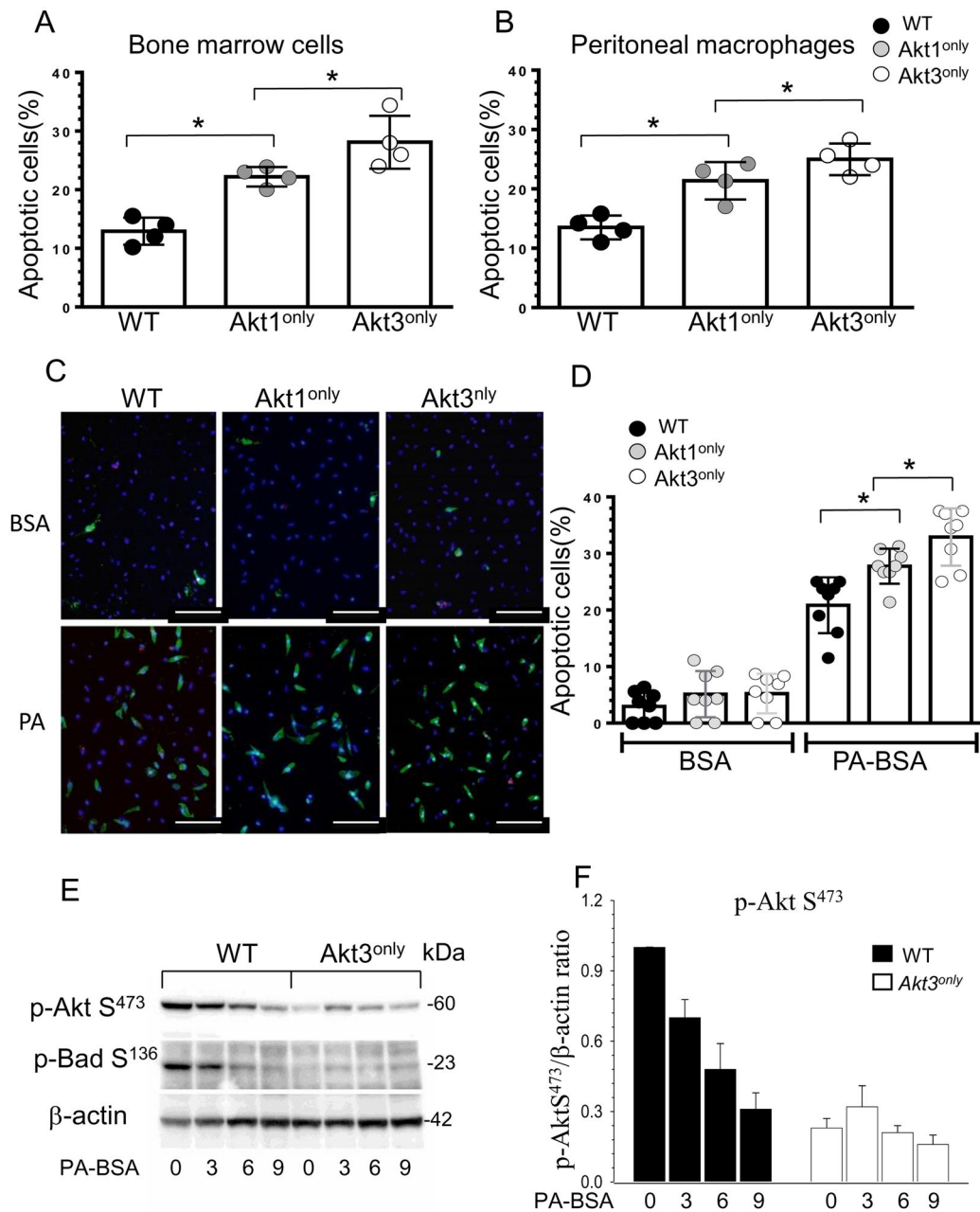
A Bone marrow cells

B Peritoneal macrophages

● WT
● Akt1only
○ Akt3only

C WT Akt1only Akt3nly

BSA

PA

D

● WT
● Akt1only
○ Akt3only

BSA PA-BSA

E

WT Akt3only kDa

p-Akt S473 -60
p-Bad S136 -23
β-actin -42

PA-BSA 0 3 6 9 0 3 6 9

F p-Akt S473

■ WT
□ Akt3only

PA-BSA 0 3 6 9 0 3 6 9

Figure 5. Blood Akt1only or Akt3only monocytes are more sensitive to ER stress than WT cells(A,B)

Apoptotic cells in bone marrow and peritoneal macrophages freshly isolated from *Ldlr*−/− mice reconstituted with WT, Akt1only and Akt3only FLC; cells were incubated with Alexa Fluor 647 Annexin V antibody and analyzed by flow cytometry; *$p < 0.05$ compared to WT cells by One Way Analysis of Variance (Tukey test).

(**C,D**) Detection and the percent of apoptotic monocytes after treatment with BSA or PA-BSA overnight. Monocytes were isolated from blood of *Ldlr*−/− mice reconstituted with Akt1only, Akt3only or WT FLC, seeded in culture chambers and, two days later, treated with BSA or a pro-apoptotic stress factor, PA-BSA in the presence of 3% fetal bovine serum and

10nM of mouse macrophage colony-stimulating factor overnight, then apoptotic cell numbers were analyzed using Alexa Flour 488 Annexin V/Dead cell apoptosis kit. Graphs represent data (mean ± SEM) obtained from the same (n=8/group) mice; *p<0.05 compared to WT cells treated with PA by One Way Analysis of Variance, Dunnett's method); Scale bar is 50mm.

(**E,F**) ER stress inhibits Akt signaling faster in Akt3[only] macrophages than in WT cells. WT, Akt3[only] peritoneal macrophages were treated with BSA or 0.5M PA-BSA for 0, 1, 3 and 6 hours. Extracted proteins were used for analysis of Akt and BadS[136] signaling. Graphs represent data (mean ± SEM) of three experiments.

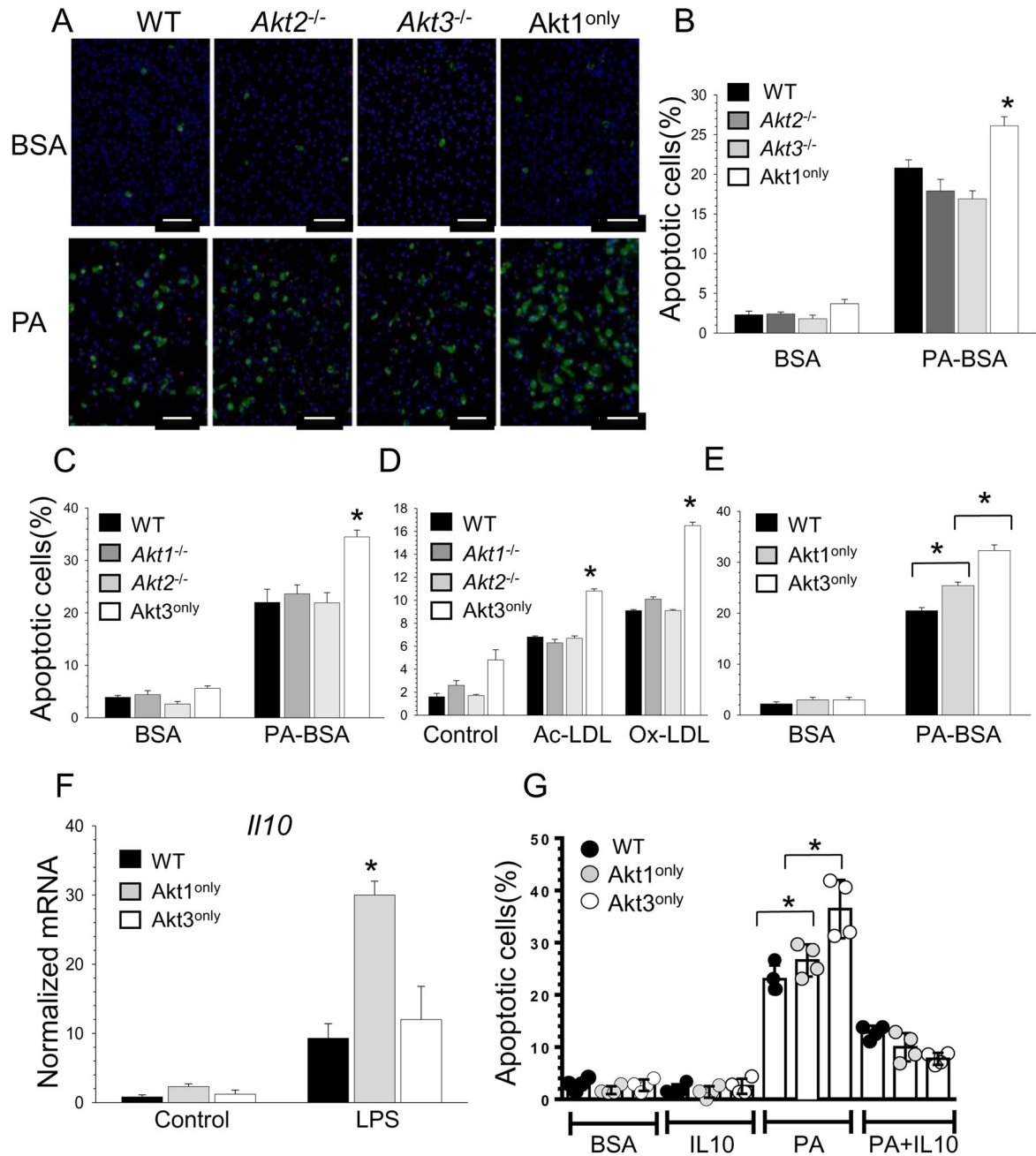**Figure 6. Akt1only and Akt3only macrophages are more sensitive to pro-apoptotic cells stimuli than WT and a single Akt isoform knockout cells, and Il-10 suppresses apoptosis in macrophages.**

(**A,B**) WT, *Akt2*−/−, *Akt3*−/− or Akt1only peritoneal macrophages were isolated from recipient mice (n=4/group) and two days later treated with BSA or 0.5mM PA-BSA overnight. Apoptotic cell numbers were detected by Alexa Flour 488 Annexin V/Dead cell apoptosis kit. Graphs represent data (mean ± SEM; *p<0.05 compared to WT cells treated with PA-BSA by One Way Analysis of Variance); Scale bar is 50mm.

(**C-D**) The percent of apoptosis in WT, *Akt1*−/−, *Akt2*−/− or Akt3only macrophages after treatment with BSA or PA-BSA overnight, or incubated with human Ac-LDL (100mg/ml)

plus an ACAT inhibitor Sandoz 58035 (2mg/ml) or with Ox-LDL (100mg/ml) for 48 hours. Graphs represent data (mean ± SEM) obtained from the same number (n=3/group) of mice, *p<0.05 compared to WT cells treated with PA-BSA, Ac-LDL or Ox-LDL by One Way Analysis of Variance.

(**E**) Akt1^{only} macrophages are more resistant to apoptosis than Akt3^{only} cells

(**F**) WT, Akt1^{only} *and* Akt3^{only} macrophages were treated with LPS (20ng/ml) for 6 hours and gene expression levels were measured by real-time PCR. Graphs represent data (mean ± SEM) obtained from the same number (n=3/group) of mice (*p<0.05 compared to WT cells treated with LPS by One Way Analysis of Variance).

(**G**) Macrophages were isolated from mice reconstituted with WT, Akt1^{only} *and* Akt3^{only} FLC and, after two days in culture, were treated with BSA or a mouse recombinant Il-10 (100ng/ml) or 0.5M PA-BSA with or without of IL-10 for 24 hours. Apoptotic cells were detected by Alexa Flour 488 Annexin V. Graphs represent data (mean ± SD) obtained from the same number (n=4/group) of mice (*p<0.05 compared to WT cells treated with PA-BSA by One Way Analysis of Variance).

Table 1.

Body weight (BW), total serum cholesterol (TC) and triglyceride (TG) levels in $Ldlr^{-/-}$ mice reconstituted with WT and male Akt1^{only} (A), female and male Akt3^{only} (B,C) hematopoietic cells after 8 weeks of the Western diet.

Recipient Mice	BW (g)	TC (mg/dl)	TG (mg/dl)
A. Male FLC→male $Ldlr^{-/-}$ mice WT (n=10)	25.7+1.1	880±39	197±5
Akt1^{only} (n=10)	23.2±0.6	872±48	201±24
p =	0.17	0.87	0.90
B. Female FLC →female $Ldlr^{-/-}$ mice WT (n=10)	21.5±0.4	673±42	229±7
Akt3^{only} (n=10)	21.2±0.5	660±33	200±23
p =	0.58	0.82	0.62
C. Male FLC→male $Ldlr^{-/-}$ mice WT (n=9)	29.3±0.9	906±76	237±16
Akt3 ^{only} (n=11)	27.4±0.7	835±48	216±15
p =	0.46	0.46	0.37

Data are (Mean ± SEM). The number of recipient mice in each group is indicated by n;

[*] p, there are no statistically significant differences between the groups.