Bone marrow transplantation in mice as a tool to study M2 macrophage activation in atherogenesis

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Hematopoietic Akt2 restoration enhances foam cell formation but does not affect atherosclerosis in Akt2/LDL receptor double knockout mice

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AKT serine/threonine Kinase 2 (Akt2) plays a key role in insulin signaling and cardiovascular disease. LDL receptor knockout (LDLr KO) mice lacking Akt2 are glucose intolerant, but atherosclerosis susceptibility is not affected or even slightly decreased. Disruption of Akt2 function specifically in bone marrow-derived cells leads to a dramatic reduction in atherosclerotic lesion development in LDLr KO mice, indicating a potent pro-atherogenic effect of hematopoietic Akt2. The contribution of Akt2 in bone marrow-derived cells to the effects on atherosclerosis susceptibility in Akt2/LDLr double KO (dKO) mice, however, is currently unclear.

In this study, we restored bone marrow Akt2 in Akt2/LDLr dKO mice by transplanting functional Akt2 containing LDLr KO bone marrow into Akt2/LDLr dKO recipients. Akt2/LDLr dKO recipients transplanted with Akt2/LDLr dKO bone marrow served as controls. In line with a pro-atherogenic role for macrophage Akt2, enhanced foam cell formation was observed in the peritoneal cavity of Akt2/LDLr dKO mice transplanted with LDLr KO BM accumulation (3 weeks WTD, +88.0 %, $P<0.05$; 5 weeks WTD, +59.5%, $P=0.07$). Surprisingly, bone marrow Akt2 restoration in Akt2/LDLr dKO mice did not affect glucose tolerance or atherosclerosis development. The null effect on atherosclerosis can likely be explained by the fact that the pro-atherogenic increase in foam cell formation was counteracted by a beneficial change in the inflammatory status, since Akt2 restoration suppressed LPS-induced M1 macrophage polarization (evidenced by -77.8% iNOS/Arg1 ($P<0.001$) expression, -20.9% TNF-alpha ($P<0.01$) secretion, and +90% IL-10 ($P<0.001$) secretion).

In conclusion, restoration of Akt2 in Akt2/LDLr dKO macrophages suppresses M1 polarization and increases macrophage foam cell formation, but does not affect atherosclerosis susceptibility of in Akt2/LDLr dKO mice reconstituted with Akt2 positive bone marrow.

Introduction

Protein kinase B (PKB or Akt) is a family of serine/threonine-specific protein kinases, discovered by Staal and colleagues in 1977.[1] Akt plays critical roles in various cellular processes, including proliferation, apoptosis, migration, angiogenesis and glucose metabolism.[2-6] Three highly homologous isoforms have been identified: Akt1, Akt2, and Akt3. Each isoform has a distinct tissue distribution and function.[7-10] Whereas Akt1 is ubiquitously expressed and plays a key role in the modulation of cell survival,[8,9,11] Akt3 is predominantly expressed in the brain and is essential for postnatal brain development.[10] In addition, Akt2 is highly expressed in insulin-responsive tissues and plays a crucial role in the regulation of insulin signaling.[12-14] Akt2 deficiency in mice induces insulin resistance and a type 2 diabetic phenotype.[13-15] Although type 2 diabetes is generally associated with an increased risk for the development of cardiovascular disease,[16] Akt2 deficiency does not augment atherosclerosis development in the LDL receptor knockout background.[15-21] Thus, Akt2 is likely to exert an anti-atherosclerotic function that compensates for the pro-atherogenic effects of the glucose intolerant phenotype.

Macrophages play an essential role in all stages of atherosclerotic lesion development and atherosclerosis susceptibility is influenced by the phenotype of these immune cells. Grossly, two distinct types of macrophages can be distinguished: 1) pro-inflammatory M1 macrophages and 2) anti-inflammatory, healing M2 macrophages. Importantly, Akt2 deficiency is known to skew macrophages to a phenotype hallmarked by: 1) attenuated inflammatory cytokine production,[22] 2) impaired migration capability,[23] and 3) reduced foam cell formation.[17] In line with this pro-atherogenic function of Akt2, disruption of Akt2 function specifically in bone marrow-derived cells reduces atherosclerotic lesion development in LDLr KO mice.[17,23] The contribution of macrophage

Akt2 to atherosclerosis susceptibility in glucose intolerant Akt2/LDLr double KO (dKO) mice, however, is currently unclear.

Insulin resistance and glucose intolerance both affect macrophage activation and polarization. Whereas some studies have established that high glucose levels induce an M1 macrophage phenotype, Chenming and colleagues reported that peritoneal exudate macrophages and bone marrow-derived macrophages from streptozotocin-induced diabetic mice exhibit a more M2-like macrophage phenotype.[24] This phenotype could also be induced in vitro by exposing macrophages to high glucose levels in the medium. Moreover, the macrophages from diabetic mice showed an impaired inflammatory response to LPS/IFN-γ stimulation, which could be reversed by insulin treatment. Importantly, similar observations were made after treatment with an Akt inhibitor, underlining a role for Akt in macrophage polarization. To elucidate whether the atheroprotective potential of macrophage Akt2 remains present under insulin resistant conditions, the current study was aimed at investigating the effects of hematopoietic Akt2 on atherosclerotic lesion development in glucose intolerant Akt2/LDLr dKO mice by reconstituting these mice with Akt2 positive bone marrow.

Material and Methods

Animals

Low-density lipoprotein receptor knockout (LDLr KO) mice and Akt2/LDLr double KO (Akt2/LDLr dKO) mice were obtained from Jackson laboratory (Bar Harbor, ME, USA). All mice used in the current study were bred and maintained at the Gorlaeus Laboratories in Leiden, the Netherlands. The studies were approved by the Dutch Ethics Committee and regulatory authority at Leiden University. The animal experiments were carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Bone marrow transplantation (BMT)

The endogenous BM of female Akt2/LDLr dKO recipient mice (13-15 weeks old) was destroyed by total-body X-ray irradiation (9-Gy) 1 day before the bone marrow transplantation (BMT). Subsequently, five-million BM cells, freshly isolated from LDLr KO mice or Akt2/LDLr dKO mice was transplanted into the recipient mice via tail vein injection. After BMT, recipient mice were maintained on antibiotic water (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate and 2.5 g/L sugar) and chow diet (RM3; Special Diet Services) for 8 weeks. Thereafter, the mice were challenged with Western-type diet (WTD, Special Diet Services, product code 824171) for 3 or 5 weeks to induce atherosclerosis development. At sacrifice, the mice were anaesthetized by an anaesthetic mixture containing xylazine (12.5 mg/kg), ketamine (100 mg/kg) and atropine (125 µg/kg) and subjected to total body PBS perfusion before organ collection.

Oral glucose tolerance test (OGTT)

The oral glucose tolerance test was performed in the recipient mice at 4 weeks on WTD after a 16-hours fasting period. Glucose levels of tail blood were determined by using a manual ACCU-CHEK Compact glucose monitor (Roche Diagnostics, Almere, the Netherlands).

Blood glucose levels before glucose administration were determined as baseline (t=0). Immediately, the mice were administered 2 gr/kg glucose by oral gavage using a 10% Beta-D (+)-glucose (Sigma-Aldrich, catalog number: G8270) solution in PBS. Additional blood samples were taken at 15, 30, 45, 60, 90 and 120 minutes after glucose administration.

Serum Cholesterol assay

Plasma total cholesterol and free cholesterol levels were determined using a standard enzymatic colorimetric assay (chemicals from Sigma Aldrich, USA or Roche Diagnostics, Mannheim, Germany) as previously described.[25]

Haematology Analysis

An automated haematology analyzer (XT-2000iV, Sysmex Corporation, Japan) was used to analyse leukocytes and quantify macrophage foam cell formation in the peritoneum of the Akt2/LDLr KO BMT recipients as described previously.[26]

Histological Analysis of Atherosclerosis

Sections of the aortic root were made by a Leica CM3050S cryostat. Subsequently, the sections were stained with Oil red-O for neutral lipids, with Masson's Trichrome (HT15-1, 4, Sigma Aldrich) for collagen and with MOMA-2 (Research Diagnostics Inc) for macrophages as described previously.[27] The atherosclerotic plaque size and composition were determined using Leica Qwin Imaging software (Leica Ltd, Cambridge, UK).

Cell culture

Bone marrow-derived macrophages (BMDMs) from LDLr KO and Akt2/LDLr dKO mice were generated by culturing bone marrow cells with 20 ng/mL M-CSF (eBioscience, catalog number: 14-8983-80) for 7 days. To induce foam cell formation, BMDMs were incubated with 20 µg/mL copper-oxidized LDL (oxLDL) for 24 hours.[28] To prime M1 or M2 macrophages, BMDMs were incubated for 24 hours with 100 ng/mL LPS (Escherichia coli0111:B4; Sigma Aldrich, catalog number: L2630-10MG) or 20ng/mL IL-4 (Peprotech, catalog:214-14). BMDMs that were treated with PBS instead of oxLDL, LPS or IL-4 served as control. Macrophage culture supernatants were collected and stored at -20°C until further analysis.

Analysis of gene expression by quantitative PCR (qPCR)

Total RNA was isolated using the guanidinium thiocyanate method.[29] cDNA was synthesized using a RevertAid M-MuLV enzyme (Fermentas, Burlington, Canada). The mRNA expression levels were measured on a 7500 Fast Real-Time PCR system (ABI PRISM 7500; Applied Biosystems, Foster City, CA) using SYBR green technology (Applied Biosystems). The average cycle threshold (CT) of *36B4* (acidic ribosomal phosphoprotein P0) and *RPL27* (Ribosomal Protein L27) were used as housekeeping gene expression control values.

Cytokine analysis and nitric oxide assay

The supernatants of the BMDM cell cultures were thawed and the content of the cytokines TNF-α, IL-6, and IL-10 were determined according to the manufacturer's' instructions (all ELISA kits were purchased from BD Biosciences, United States). The nitric oxide (NO) concentration in the supernatants was determined using the Griess method according to the manufacturer's protocol (Sigma-Aldrich).

Statistical analysis

All the values are expressed as means ± SEM. The statistical significant differences between the groups were tested using the unpaired Student's t-test or two-way ANOVA with GraphPad Prism software (GraphPad Software Inc., San Diego, California, USA). A Welch correction was applied to the t-test in case of unequal variances in the dataset. A two-sided P value lower than 0.05 was defined as statistically significant.

Results

Bone marrow Akt2 restoration does not affect blood glucose levels and glucose tolerance of Akt2/LDLr dKO mice

Total body Akt2 deficiency in LDLr KO mice leads to impaired glucose tolerance.[18] To establish the contribution of bone marrow Akt2 to the regulation of glucose metabolism in Akt2/LDLr KO mice, blood glucose levels were determined and a glucose tolerance test was performed at 12 weeks after BMT, when mice were fed a WTD for 4 weeks. Restoration of bone marrow Akt2 did not impact on fasting blood glucose levels after either 2 or 4 weeks of WTD feeding ($p>0.05$; figure 1A-B). Upon oral glucose administration both Akt2/LDLr dKO mice transplanted with Akt2/LDLr dKO BM or LDLr KO BM showed a rapid increase in blood glucose levels. In both groups the peak glucose value was reached at 30 minutes after the glucose bolus injection (Akt/LDLr dKO BM vs. LDLr KO BM: 18.3 ± 0.7 mM vs 19.2 ± 0.5 mM, $p<0.05$ compared to basal glucose levels; figure 1C). It is worth to note that, 120 minutes after oral glucose administration, the plasma glucose levels in both groups failed to get back to basal levels ($p<0.01$ compared to basal glucose levels, figure 1C), supporting the glucose intolerant phenotype of the Akt2/LDLr dKO recipient mice. Conclusively, we observed no difference in glucose handling between the two experimental groups, indicating that restoration of bone marrow Akt2 in Akt2/LDLr dKO mice does not influence the glucose intolerant phenotype of the recipient mice.

Bone marrow Akt2 restoration does not affect atherosclerosis development in Akt2/LDLr dKO mice

To assess the effect of Akt2 restoration in bone marrow-derived cells on atherosclerosis susceptibility of Akt2/LDLr dKO mice, the atherosclerotic plaque size in the aortic root was determined after 3 and 5 weeks of WTD feeding. At both time points the lesions were primarily composed of macrophage foam cells with no deposited collagen (Figure 2A). Surprisingly, Akt2/LDLr dKO recipients transplanted with LDLr KO BM containing functional Akt2 developed atherosclerotic lesions of a similar size as Akt2/LDLr dKO transplanted controls, both after 3 weeks WTD challenge (Akt2/LDLr dKO BM vs. LDLr KO BM: $119 \times 10^3 \pm 14 \times 10^3$ μm^2 vs. $125 \times 10^3 \pm 21 \times 10^3$ μm^2, $p>0.05$; figure 2B and 2C) and after 5 weeks WTD feeding (Akt2/LDLr dKO BM vs. LDLr KO BM: $154 \times 10^3 \pm 14 \times 10^3$ μm^2 vs. $169 \times 10^3 \pm 24 \times 10^3$ μm^2, $p>0.05$; figure 2B and 2C). Thus, in contrast

to the previously published results showing that deletion of Akt2 in bone marrow-derived cells of LDLr KO mice is athero-protective,[17,23] restoration of Akt2 in glucose intolerant Akt2/LDLr dKO mice did not increase atherosclerotic lesion development.

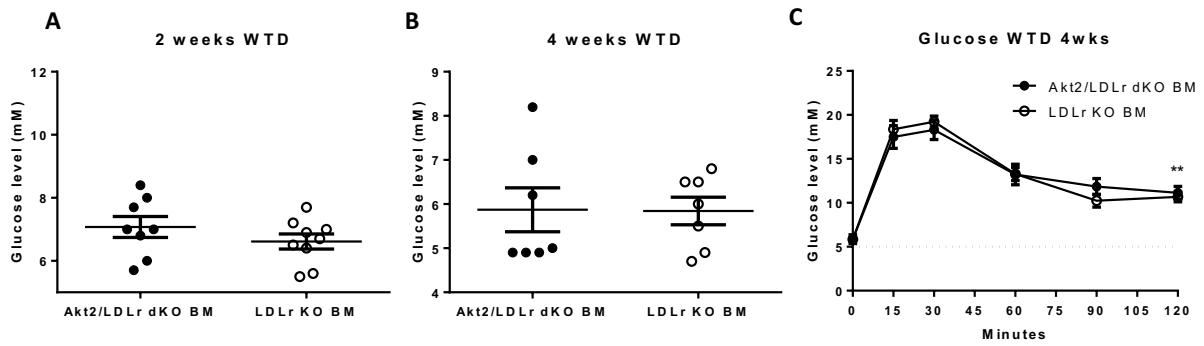

Figure 1 Restoration of Akt2 in bone marrow of Akt2/LDLr double KO mice does not alter recipient's blood glucose levels and glucose tolerance.

Akt2/LDLr dKO mice received LDLr KO bone marrow (BM) or Akt2/LDLr dKO BM. The fasting blood glucose levels were measured after A) 2 weeks Western-type diet (WTD) or B) 4 weeks WTD challenge. C) Glucose tolerance test by oral gavage of 2 g/kg glucose in fasted recipient mice is shown at 4 weeks WTD. **p<0.01, compared to the basal glucose concentration. (n=7-9).

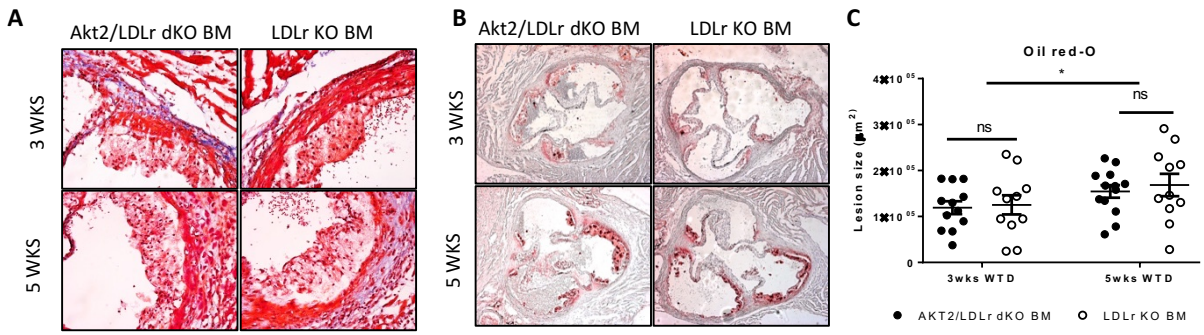

Figure 2 Restoration of bone marrow Akt2 does not affect atherosclerosis development in the aortic root of Akt2/LDLr dKO mice

A) Masson's Trichrome stained atherosclerotic lesions in the aortic root of Akt2/LDLr dKO mice transplanted with Akt2/LDLr dKO or LDLr KO bone marrow (BM) after 3 and 5 weeks Western-type diet. B) Representative pictures of Oil red-O stained atherosclerotic lesions in the aortic root and C) quantification of the atherosclerotic lesion size. ns, p>0.05; *p<0.05; (n=11-12).

Bone marrow Akt2 restoration leads to increased foam cell formation in the peritoneal cavity of Akt2/LDLr dKO mice

Akt2 has been shown to influence macrophage migration.[30-32] Therefore, next we investigated the leukocyte populations in the peritoneal cavity of Akt2/LDLr dKO recipients after 3 weeks and 5 weeks WTD challenge. The total number of peritoneal leukocytes and macrophages in Akt2/LDLr dKO mice transplanted with LDLr KO bone marrow containing functional Akt2 were similar as in Akt2/LDLr dKO BM transplanted controls (figure 3A and B). This indicates that bone marrow Akt2 restoration likely had no impact on overall leukocyte and macrophage recruitment into the peritoneal cavity of Akt2/LDLr dKO mice. Interestingly, the counts of macrophage foam cells were increased upon restoration of bone marrow Akt2 in Akt2/LDLr dKO mice (3 weeks WTD, +88.0 %, p=0.04; 5 weeks WTD, +59.5 %, p=0.07; figure 3C). This increase in foam cell number could not be attributed to changes in plasma cholesterol levels, as mice transplanted with reconstituted BM

Akt2 showed similar plasma cholesterol levels as compared to control mice, both on chow diet and after a WTD challenge (table 1). Moreover, the plasma lipoprotein distribution profile was also not altered upon restoration of Akt2 in bone marrow-derived cells of Akt2/LDLr dKO mice (figure 4).

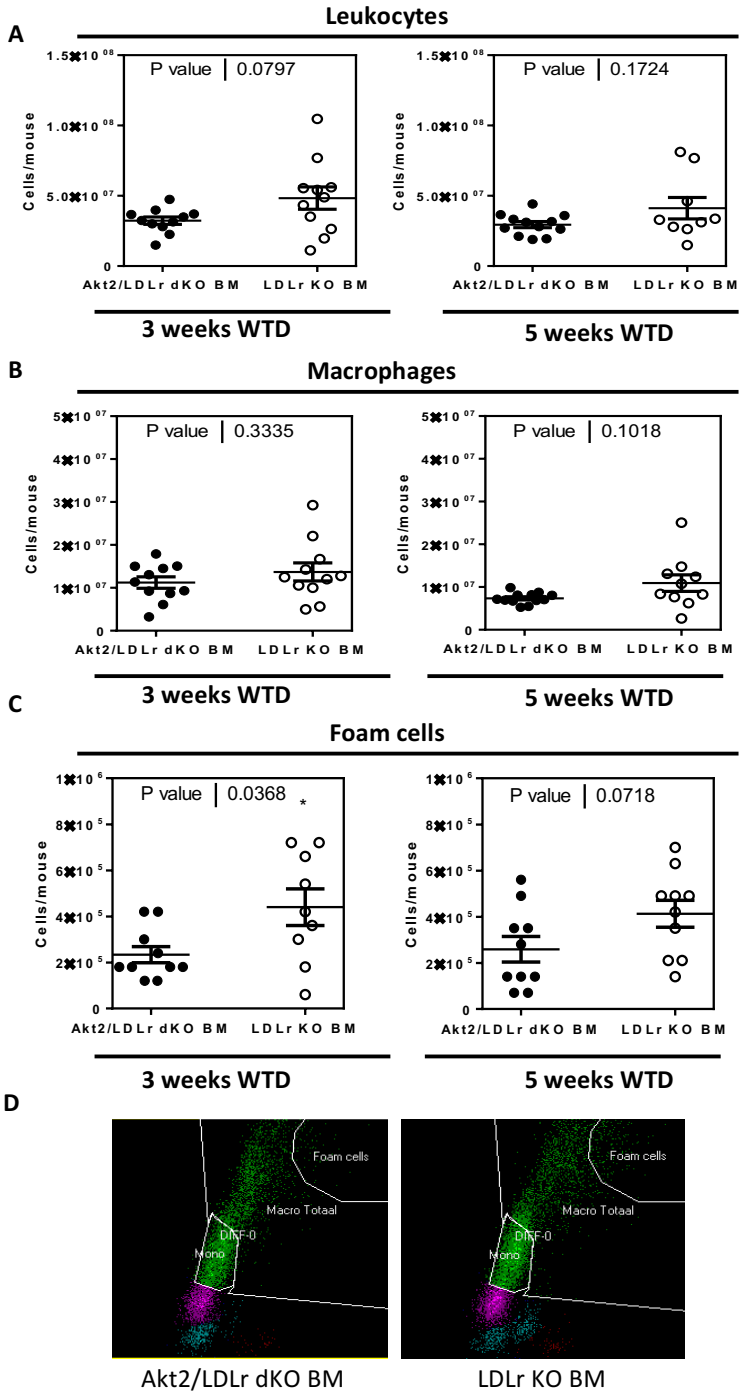

Figure 3 Restoration of bone marrow Akt2 in Akt2/LDLr dKO mice induces foam cell formation in the peritoneal cavity.

Peritoneal leukocytes were harvested from Akt2/LDLr dKO recipients transplanted with LDLr KO BM and Akt2/LDLr dKO BM after 3 weeks or 5 weeks Western-type diet (WTD) feeding. A) total leukocyte counts, B) macrophage counts and C) foam cell counts were determined by using a Sysmex hematology analyzer. D) Example picture of the hematology analysis (from the 3 weeks WTD challenge group). *p<0.05. n=11-12.

Table 1 Restoration of bone marrow Akt2 does not impact plasma cholesterol levels in Akt2/LDLr dKO mice.

	Chow diet		WTD		
mg/mL	FC	TC		FC	TC
Akt2/LDLr dKO BM	0.75 ± 0.05	2.39 ± 0.12	3 weeks	3.26 ± 0.19	9.43 ± 1.10
			5 weeks	2.65 ± 0.16	7.35 ± 0.68
LDLr KO BM	0.77 ± 0.03	2.45 ± 0.10	3 weeks	2.68 ± 0.44	7.32 ± 1.89
			5 weeks	2.51 ± 0.24	7.23 ± 0.60

Plasma was collected by tail bleeding of Akt2/LDLr dKO recipients that received LDLr KO BM and Akt2/LDLr dKO BM while on chow diet and at sacrifice after 3 and 5 weeks Western-type diet (WTD) feeding. The free cholesterol and total cholesterol levels were determined. (n=6-12).

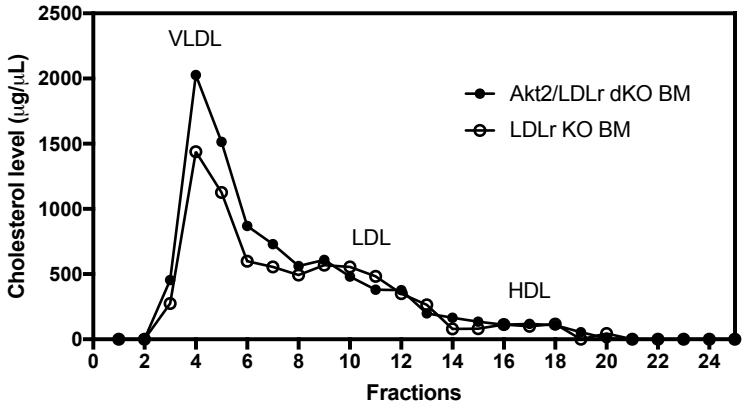

Figure 4 Restoration of bone marrow Akt2 does not impact on plasma cholesterol distribution among the different types of lipoproteins in Akt2/LDLr dKO mice.

Plasma was collected by tail-bleeding of Akt2/LDLr dKO recipients that received LDLr KO BM or Akt2/LDLr dKO BM at sacrifice after 3 weeks Western-type diet (WTD) feeding. Cholesterol lipoprotein distribution was determined by FPLC. (n=6-12 of pooled plasma).

Akt2 promotes foam cell formation in LDLr deficient macrophages by regulating the expression of cholesterol influx and efflux genes

To gain further insight into the mechanisms underlying the increased foam cell formation of LDLr KO macrophages with functional Akt2 *in vivo*, an *in vitro* foam cell formation assay was performed using LDLr KO and LDLr/Akt2 dKO BMDMs. After 24 hours of oxLDL incubation, LDLr KO macrophages showed significantly more cellular lipid accumulation as evidenced by more extensive Oil red-O staining compared to Akt2/LDLr dKO macrophages (figure 5A). The mRNA expression of *SR-A* and *CD36*, the main receptors for oxLDL uptake,[33] was significantly higher in LDLr KO macrophages compared to Akt2/LDLr dKO macrophages (*SR-A*, +60.7%, p<0.001; figure 5B; *CD36*, +24.8%, p<0.001, figure 5C) after oxLDL stimulation. The expression of low-density lipoprotein receptor-related protein 1 (*LRP1*, 1.16 ± 0.05 vs 0.87 ± 0.13, p>0.05, figure 5D) was not affected and the expression of *VLDLr*, responsible for the uptake of unmodified apoE-containing lipoproteins,[34,35] was decreased (-59.1%, p<0.05, figure 5E) in LDLr KO macrophages compared to Akt2/LDLr dKO macrophages. In addition, the expression of the cholesterol efflux genes *ABCA1* and *ABCG1* was decreased in LDLr KO macrophages compared to Akt2/LDLr dKO controls (*ABCA1*, -39.0%, p<0.05, figure 5F; *ABCG1*, -40.5%, p<0.01, figure 5G). In summary, these results suggest that LDLr KO macrophages with functional Akt2 are more susceptible to oxLDL-induced foam cell formation due to augmented uptake of oxLDL and decreased cellular cholesterol efflux.

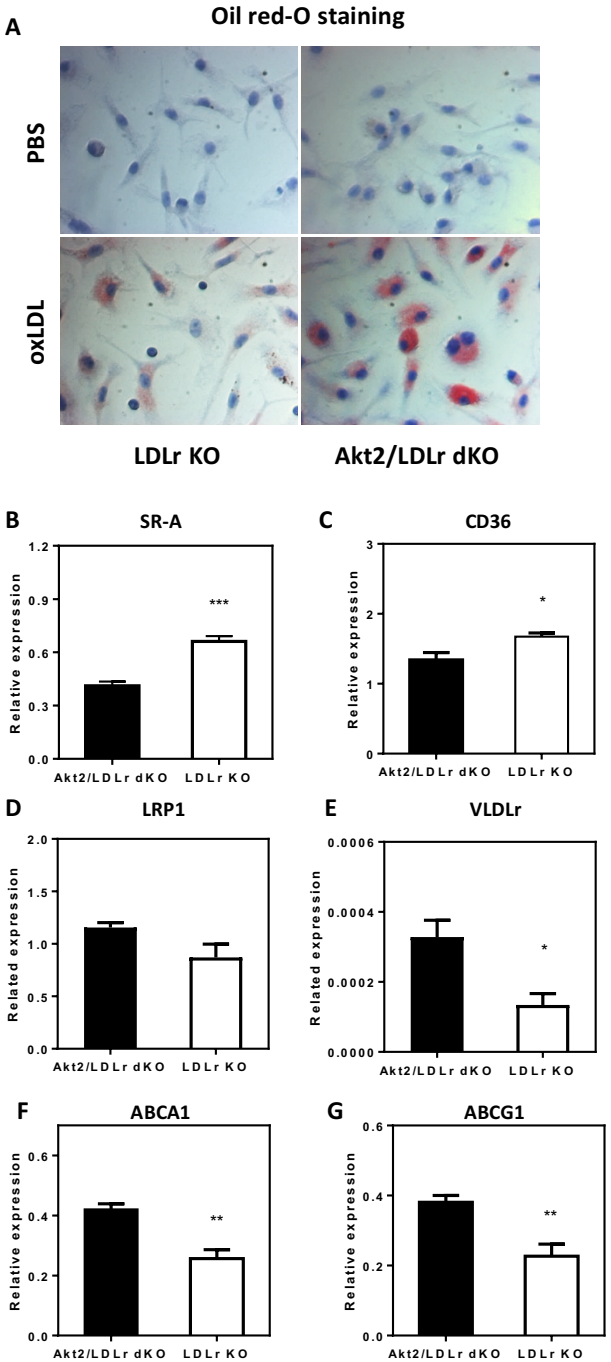

Figure 5 Akt2 presence increases susceptibility of LDLr KO macrophages to oxLDL-induced foam cell formation.

Macrophages generated from Akt2/LDLr dKO BM and LDLr KO BM were treated with 20µg/mL oxLDL for 24 hours. A) Oil red-O visualized foam cell formation, B-G) mRNA expression of genes involved cholesterol metabolism were determined using qPCR. *p<0.05; **p<0.01; ***p<0.001. (n=5).

Akt2 suppresses LPS-induced M1 macrophage polarization in LDLr KO macrophages

M2 macrophages are known to be more susceptible to foam cell formation as compared to M1 macrophages.[36] To investigate if the increased foam cell formation of LDLr KO macrophages as compared to Akt2/LDLr dKO macrophages is linked to the macrophage phenotype, a polarization assay was performed using BMDMs.

Upon LPS exposure, LDLr KO macrophages with functional Akt2 showed a decreased mRNA expression ratio of the M1 marker *iNOS* over the M2 marker *Arg1* (-77.8%, p<0.001, figure 6A), indicating a less M1-like macrophage phenotype. In support, the nitric oxide (NO) production by

LDLr KO macrophages was also significantly lower, compared to Akt2/LDLr dKO macrophages (-32.6%, p<0.001, figure 6B). Furthermore, the production of pro-inflammatory cytokines was evaluated as a reflection of macrophage function. In line with the observed reduction of the M1-like phenotype, LDLr KO macrophages displayed a less pronounced pro-inflammatory cytokine profile upon LPS stimulation, as the production of pro-inflammatory cytokines TNF-α and IL-6 was reduced (TNF-α, -20.9%, figure 6C; IL-6, -24.0%, figure 6D), whereas the production of anti-inflammatory cytokine IL-10 was increased (+89.1%, figure 6E) in LDLr KO macrophages as compared to Akt2/LDLr dKO macrophages. In support of the more M2-like phenotype, IL-4 stimulation induced a significant increase in Arg1 expression (+297%, p<0.001, figure 6F). These data clearly showed that Akt2 presence in LDLr KO macrophages leads to a less M1, and more M2-like macrophage phenotype, which might be linked to the increased susceptibility to foam cell formation that these macrophages show.

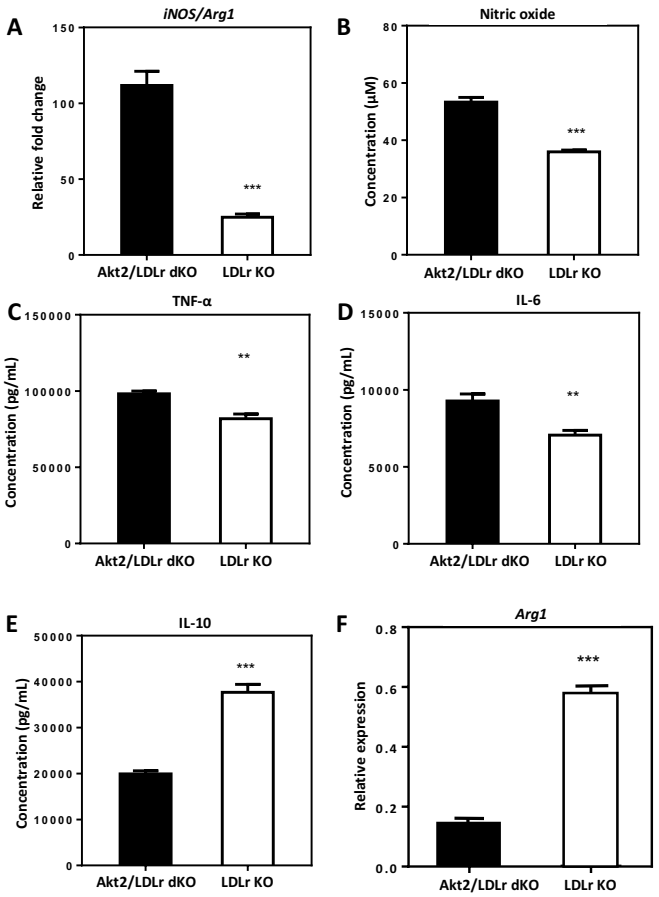

Figure 6 The presence of Akt2 suppresses the inflammatory response in LDLr KO macrophages upon LPS stimulation.

BMDMs generated from Akt2/LDLr dKO and LDLr KO bone marrow were treated with 100ng/mL LPS for 24 hours to promote M1 polarization, A) the mRNA expression ratio of iNOS/Arg1, B) nitric oxide production, and cytokine production of C) TNF-α, D) IL-6 and E) IL-10 were determined. BMDMs generated from Akt2/LDLr dKO and LDLr KO bone marrow were treated with 20ng/mL IL-4 for 24 hours to promote M2 polarization, F) the relative mRNA expression of Arg1 was determined. **p<0.01; ***p<0.001. (n=6).

Discussion

Previous studies have shown that disruption of Akt2 function specifically in bone marrow-derived cells induces an anti-inflammatory M2 macrophage phenotype, and leads to a dramatic reduction in atherosclerotic lesion development in LDLr KO mice. [17, 18] In the current study we investigated the relative contribution of macrophage Akt2 on atherosclerosis susceptibility in Akt2/LDLr double KO (dKO) mice. We show that restoration of Akt2 in bone marrow-derived cells of Akt2/LDLr dKO mice does not impact atherosclerotic lesion development, despite an observed increase in

macrophage foam cell formation. This lack of effect on lesion development can likely be explained by the observation that Akt2 restoration in macrophages also reduced the macrophage polarization towards the pro-inflammatory M1 macrophage phenotype, and correspondingly lowered the production of pro-inflammatory cytokines.

Total body Akt2 loss is known to lead to glucose intolerance in LDLr KO mice.[17,18] We here show that restoration of bone marrow Akt2 in Akt2/LDLr dKO mice does not change the blood glucose levels nor the glucose intolerant phenotype of these mice, suggesting that macrophage Akt2 is not involved in the regulation of glucose homeostasis. In line with this, *Babaev et al.* also found that deletion of bone marrow Akt2 in LDLr KO mice did not affect glucose levels.[23] These data indicate that bone marrow Akt2 is not a decisive factor for the observed glucose intolerant phenotype of mice with a total-body Akt2 deletion. Indeed, Akt2 maintains glucose homeostasis mainly by regulating hepatic glucose production and skeletal muscle insulin resistance, cell types that are not affected by bone marrow transplantation.[37-39]

Previous studies have demonstrated that bone marrow Akt2 plays a pro-atherogenic role in non-diabetic LDLr KO mice, as evidenced by reduced atherosclerotic lesion development upon bone marrow-specific deletion of Akt2.[17,23] In our study, we did not observe any impact of bone marrow Akt2 restoration on atherosclerotic lesion size in glucose intolerant Akt2/LDLr dKO mice fed a Western-type diet. Despite the lack of effect on lesion size, Akt2 restoration in bone marrow did promote macrophage foam cell formation in these mice. This increased susceptibility to macrophage foam cell formation of mice with functional bone marrow Akt2 could not be attributed to changes in the plasma cholesterol levels or lipoprotein distribution. However, *in vitro* studies showed that the increase in macrophage foam cell formation is likely to be caused by an increased influx of oxidized LDL together with a decreased cholesterol efflux as evidenced by an increased mRNA expression of the LDL cholesterol uptake receptors *SR-A1* and *CD36* and a decreased expression of cholesterol efflux pumps *ABCA1* and *ABCG1*. In agreement with our study, Rotllan *et al.* also found that macrophage Akt2 promotes foam cell formation on a wildtype background.[17]

Macrophage susceptibility to foam cell formation is influenced by the macrophage phenotype, with M2 macrophages being more prone to become foam cells as compared to M1 macrophages.[36] Indeed, in line with the increased foam cell formation, LDLr KO macrophages with functional Akt2 showed a suppressed M1 macrophage phenotype in response to LPS stimulation compared to LDLr KO macrophages lacking Akt2. This result is in contrast to previous findings showing that Akt2 deficiency leads to a more M2-like macrophage phenotype after LPS stimulation, compared to WT macrophages.[22,23,40] Several differences between our current study and the previous studies can be distinguished, such as the duration of the LPS stimulation period, the LPS concentration used, strain variabilities (LDLr KO vs WT),[41,42] macrophage heterogeneities (bone marrow-derived macrophages vs peritoneal macrophages), and culture medium compositions. Hence, further research into this topic is warranted.

It should be noted that both the previous studies on macrophage polarization[22,23,40] and the bone marrow transplantation experiments of Rotllan[17] and Babaev *et al.*[23] applied Akt2 deficient cells with a functional LDLr. For bone marrow transplantation-based atherosclerosis studies, this is a commonly used strategy as it prevents the requirement of extensive cross-breeding to generate LDLr KO donors lacking the gene of interest. However, although previous studies have shown that the presence or absence of the LDLr in bone marrow-derived cells only minimally affects

atherosclerosis per se,[43,44] it cannot be excluded that upon interaction with the gene of interest absence or presence of the LDLr does affect macrophage function and atherosclerotic lesion development. Therefore, further analysis is warranted to confirm this possible interaction between LDLr and Akt2. Understanding the underlying mechanisms of a possible gene interaction is beyond the scope of our current study. However, the difference in the LPS-induced phenotype of Akt2 deficient macrophages in the LDLr KO background in our current study as compared to the wildtype background in earlier studies, might prove a motivation for initiating further research.

Taken together, our study showed that: 1) bone marrow Akt2 is not a decisive factor in Akt2-regulated glucose metabolism, and 2) bone marrow Akt2 promotes macrophage foam cell formation but does not affect atherosclerosis development in Akt2/LDLr dKO mice, which might be due to the counteracting anti-inflammatory effects of the Akt2-induced suppressed M1-like macrophage phenotype.

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