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

Ren, B.

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Enhanced atherosclerotic lesion development in LDL receptor knockout mice lacking Upstream Stimulating Factor 1 (Usf1) in bone marrow-derived cells

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Upstream Stimulatory Factor 1 (Usf1), a ubiquitous transcription factor associated with familial combined hyperlipidemia, regulates the expression of genes involved in lipid metabolism. Previous studies showed that mice lacking Usf1 developed a beneficial cardiometabolic profile. In this study, we investigated the atherogenic effect of hematopoietic Usf1 in low-density lipoprotein receptor (LDLr) knockout (KO) mice.

Bone marrow from Usf1 KO mice and wild-type mice was transplanted into male LDLr KO mice. After 8 weeks recovery on a regular chow diet, the mice were challenged with a pro-atherogenic Western-type diet for 20 weeks. Specific deletion of hematopoietic Usf1 also protected against diet-induced obesity. However, opposed to total-body Usf1 deficiency, deletion of hematopoietic Usf1 in LDLr KO mice led to a significant increase in atherosclerotic lesion size (130%, $p<0.05$). The increased atherosclerosis susceptibility coincided with increased neutrophil counts in the circulation (200%, $p<0.01$) and elevated VLDL cholesterol levels (162%, $p<0.05$). Interestingly, the phenotype induced by hematopoietic Usf1 deficiency in LDLr KO mice is likely attributed to an impaired clearance of VLDL by white adipose tissue (WAT). The mRNA expression of Peroxisome proliferator activated receptor gamma, Lipoprotein lipase and VLDL receptor, key players in regulation of VLDL clearance, were downregulated in WAT of Usf1 KO bone marrow recipients (-41.7%, $p<0.05$; -30.1%, $p<0.05$; and -49.4%, $p<0.005$; respectively) and associated with a decreased lipid content in WAT.

Taken together, these results suggest that hematopoietic ablation of Usf1 does not account for the beneficial effects of global Usf1 deletion.

Introduction

Upstream Stimulatory Factor 1 (Usf1) is a ubiquitously expressed transcription factor that plays an important role in lipid metabolism.[1] Usf1 regulates the expression of many genes involved in lipid metabolism, including apolipoprotein A2 (*apoA2*),[2] *apoA5*,[3] *apoC3*,[4] *apoE*,[5] hepatic lipase (*HL*),[6] ATP-binding cassette transporter 1 (*ABCA1*),[7,8] and fatty acid synthase (*FASN*).[9-11] Two forms of the Usf protein have been identified, which are referred to as Usf1 and Usf2, respectively.[12] The Usf proteins form hetero- and (less common) homo-dimers and bind to the E-box motif. Although the *Usf* genes are ubiquitously expressed in mammalian cells, the relative abundance of the *Usf1* and *Usf2* gene products varies among cell types.[13] Importantly, the function of Usfs is modulated in a cell-specific manner.[14] Variants of Usf1 have been associated with familial combined hyperlipidemia (FCHL), characterized by increased serum total cholesterol, triglycerides or both.[11,15] Whole-body or liver-specific over-expression of human *Usf1* significantly decreased total plasma cholesterol levels in C57BL/6J mice, while triglycerides tended to be slightly higher.[16] Conversely, Laurila *et al.* showed that deletion of Usf1 in C57Bl/6J mice led to elevated plasma total cholesterol, primarily in high density lipoprotein (HDL) particles, and decreased very low density lipoprotein (VLDL) triglycerides.[17]

In addition to its role in lipid metabolism, there are indications that Usf1 might also modulate the immune response. A correlation was found between *Usf1* and IL-6 on transcriptional level in the liver of transgenic mice overexpressing human *Usf1*.[13,16]Moreover, downregulation of *Usf1* in the RAW 264.7 macrophage cell line upregulates mitochondrial uncoupling protein 2 (*UCP2*) which suppresses the production of pro-inflammatory mitochondria-derived reactive oxygen species (mtROS).[18] In agreement, global Usf1 deficiency also led to lower circulating inflammatory cytokines in mice.[17] Collectively, these data imply that Usf1 might play an important role in lipid

metabolism and the immune response. However, only limited research on the link between Usf1 and atherosclerosis has been described.[17,19-21] Recent studies by Laurila and colleagues showed that low-density lipoprotein (LDL) receptor (r) knockout (KO) mice lacking Usf1 display remarkably decreased susceptibility to atherosclerotic lesion development.[17] However, the mechanisms underlying the reduction of atherosclerosis induced by Usf1 deficiency are poorly defined. For instance, it is not known whether Usf1 merely affects blood lipid levels or whether it also modulates atherosclerosis susceptibility by impacting the immune system. Bone marrow-transplantation (BMT) allows to specifically delete Usf1 in BM-derived leukocytes. The aim of the current study was, therefore, to specifically assess the role of Usf1 in immune cells and the consequences for atherosclerosis development. Hereto, BM from Usf1 KO mice was transplanted into male LDLr KO mice and atherosclerosis susceptibility was determined after 20 weeks challenge with a pro-atherogenic Western-Type Diet (WTD).

Material and methods

Animals and bone marrow transplantation

LDLr KO mice (C57Bl/6J background) purchased from the Jackson Laboratories, were maintained and bred under standard laboratory conditions at the Gorlaeus Laboratories in Leiden, the Netherlands. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was 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.

Usf1 KO and wild-type (WT) littermates (both C57BL/6J background) were bred at the National Institute for Health and Welfare, and University of Helsinki, in compliance with the Finnish government guidelines. Experiments were conducted in conformity with the Finnish regulations and the European parliament Directive 2010/63/EU. Other details regarding Usf1 KO mice are available in a recent report.[17]

Bones were harvested from Usf1 KO and WT mice and transported to Leiden in Dulbecco's modified Eagle medium (DMEM). Within 36 hours of collection of the bones, bone marrow (BM) was isolated. LDLr KO mice recipients (male, approx. 12 weeks old) were transplanted with either Usf1 KO BM or WT BM. BMT was performed by intravenous tail vein injection of 5×10^6 cells into the recipients, one day after lethal irradiation (Röntgen, 8 Gy). The recipients were allowed to recover for 8 weeks on a chow diet (RM3; Special Diet Services). Subsequently, the mice were fed a pro-atherogenic WTD, containing 0.25% cholesterol, 15% cocoa butter and 1% corn oil (SDS, Sussex, UK). After 20 weeks of WTD feeding, the mice were sacrificed. In short, the mice were anaesthetized using a mix of xylazine, ketamine and atropine. Blood was collected by retro-orbital bleeding (for flow cytometric analysis and testing on a veterinary haematology analyzer (Sysmex)) or by tail cut (for lipid analysis). Subsequently, the animals were perfused with PBS, and the heart and other organs were collected for further research. Erythrocytes in the blood were lysed with erythrocyte lysis buffer (0.15 mol/L NH_4Cl, 10 mmol/L $NaHCO_3$, 0.1 mmol/L EDTA, pH 7.3) and subsequently the white blood cells were used for flow cytometric analysis.

Plasma lipid determination

After 4 hours fasting, blood was collected via tail sampling in potassium-EDTA microvette CB 300 tubes (Sarstedt, Nümbrecht, Germany), and centrifuged with 2,000 rpm at 4°C for 5 minutes to separate out the plasma. Free cholesterol and total cholesterol levels were determined in plasma as previously described.[22] Furthermore, plasma was used for lipoprotein profile analysis using fast protein liquid chromatography (FPLC) using a high-resolution size-exclusion chromatography Superose 6 HR column (3.2 × 30 mm; Smart-System, Pharmacia, Uppsala, Sweden).

Hepatic lipid extraction

Total lipids were extracted from liver samples using the Bligh & Dyer method that was described previously,[23] and dissolved in 2% Triton X-100. The cholesterol and triglyceride content in the homogenate were measured and divided by the protein content as determined using a BCA assay,[24] and expressed as "µg lipid/mg protein".

Adipose tissue lipid content quantification

Paraffin embedded sections (5 µm) from gonadal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) of the transplanted LDLr KO recipients were prepared and stained by hematoxylin and eosin. The lipid–droplet-positive area as percentage of the total WAT and BAT area was qualified using Image J software (version 1.47).[25]

Sysmex haematology analyser and flow cytometry

Blood leukocyte counts were analysed using an automated Sysmex XT-2000iV Veterinary Haematology analyser (Sysmex Corporation). Fluorescent activated cell sorting (FACS) analysis was performed on a FACS Canto II apparatus (BD Biosciences, Mountain View, CA) to detect cell surface markers on blood cells. The antibodies anti-Ly6C, anti-Ly6G and anti-CD11b were all from eBioscience, Ltd. Nile red (Sigma-Aldrich, USA) and used to detect lipid-rich leukocytes. Data were analysed using FlowJo Software v10 (TreeStar Inc).

Atherosclerotic lesion analysis in aortic root

Hearts were fixed in 4% Shandon Zinc Formal-Fixx (Thermo Fisher Scientific, 9990245) for 24 hours, and subsequently embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, USA) until further processing. Cryosections (7 µm) at the level of the aortic sinus were obtained using a Leica CM3050s cryostat. Lipid-rich atherosclerotic plaques were stained with Oil Red O. Plaque area (in μm^2) quantitation was performed using a Leica image analysis system (Leica Ltd, Cambridge, UK).

mRNA expression analysis by real time PCR

Total RNA was isolated from liver, gonadal white adipose tissue and interscapular brown adipose tissue samples obtained at sacrifice after 20 weeks WTD challenge. cDNA were synthesized using RevertAid M-MuLV reverse transcriptase (Thermo Scientific, USA) according to the manufacturer's protocol (Thermo Scientific, USA). Quantitative gene expression was measured on a 7500 Fast Real-Time PCR system (Applied Biosystems, CA) using SensiMix SYBR green (GC biotech B.V., The Netherlands) technology. The average expression of the housekeeping genes *β-actin*, *RPL27* and

36B4 was used as a reference for calculation of the relative expression of the genes of interest. The primer sequences are shown in Table 1.

Table 1 qPCR primer sequences.

Primer sequences used for qPCR		
Gene	Orientation of primers	Sequences
36B4	Forward primer	CTGAGTACACCTTCCCACTTACTGA
	Reverse primer	CGACTCTTCCTTTGCTTCAGCTTT
ABCA1	Forward primer	AGAGCAAAAAGCGACTCCACATAGAA
	Reverse primer	CGGCCACATCCACAACTGTCT
ApoA	Forward primer	ATGTGTCCCAGTTTGAATCCTCCT
	Reverse primer	TTTCTCCAGGTTATCCCAGAAGTCC
β-actin	Forward primer	AACCGTGAAAAGATGACCCAGAT
	Reverse primer	CACAGCCTGGATGGCTACGTA
CD68	Forward primer	TGCCTGACAAGGGACACTTCGGG
	Reverse primer	GCGGGTGATGCAGAAGGCGATG
FASN	Forward primer	GGCGGCACCTATGGCGAGG
	Reverse primer	CTCCAGCAGTGTGCGGTGGTC
HMG-coA	Forward primer	CGAGCCACGACCTAATGAAGAATG
	Reverse primer	TGCATCACTAAGGAACTTTGCACC
LPL	Forward primer	CCCCTAGACAACGTCCACCTC
	Reverse primer	TGGGGGCTTCTGCATACTCAAA
LRP	Forward primer	CTTCTGGTGGCTGGCGTGGTG
	Reverse primer	CATCCGCTGGTGCTGGAAGCC
MTTP	Forward primer	TCTCACAGTACCCGTTCTTGGT
	Reverse primer	GAGAGACATATCCCCTGCCTGT
PPARg	Forward primer	AAGCCCTTTGGTGACTTTATGGAGCC
	Reverse primer	TGCAGCAGGTTGTCTTGGATGTCC
RPL27	Forward primer	CGCCAAGCGATCCAAGATCAAGTCC
	Reverse primer	AGCTGGGTCCCTGAACACATCCTTG
SCD1	Forward primer	TACTACAAGCCCGGCCTCC
	Reverse primer	CAGCAGTACCAGGGCACCA
SR-B1	Forward primer	AAACAGGGAAGATCGAGCCAGTAG
	Reverse primer	CGTAGTGAAGAACCTGGGGCAT
UCP1	Forward primer	CCAAGCTGTGCGATGTCCATGTACA
	Reverse primer	AAACATGATGACGTTCCAGGACCCG
Usf1	Forward primer	AGTTGGGAGATACAAAGTCCTCCG
	Reverse primer	TGCACTGTTCCCTCTTCGGTT
VLDLr	Forward primer	TGGAGATGAAGACTGTGCGG
	Reverse primer	CGAAGTCAGACTCAGCACACG

Statistical analysis

All values are expressed as means ± SEM. Differences between the groups were statistically analysed with an unpaired Student's T-test or two-way ANOVA using GraphPad Prism software (GraphPadSoftware Inc., San Diego, California, USA). Welch correction was applied in case of unequal variances in the dataset. A two-sided P value lower than 0.05 was considered as statistically significant.

Results

Hematopoietic Usf1 deficiency lowers body weight gain in LDLr KO mice

Total body deletion of Usf1 leads to a beneficial metabolic profile in C57BL/6 mice associated with leanness, increased lipolysis and improved insulin sensitivity compared to their WT littermates.[17] To investigate whether hematopoietic Usf1 deficiency influences body weight gain of the mice, changes in body weight were monitored throughout the study. Mice lacking Usf1 in bone marrow-derived cells gained less weight compared to mice that received WT BM (Figure 1A). The 2 groups started to diverge at week 18 after BMT (10^{th} week on WTD) (Figure 1B). At sacrifice (20^{th} week on WTD), LDLr KO mice with Usf1 KO BM were 2.5 g (8.5%) lighter in weight compared to mice transplanted with WT BM (Figure 1C).

Interestingly, *Usf1* mRNA expression in the gonadal white adipose tissue of LDLr KO mice reconstituted with Usf1 KO BM was 44% lower compared to the mice that received WT BM ($p<0.05$; Figure 1D). The decrease was likely not the result of a reduced macrophage content of the adipose tissue as no significant difference was found in the expression of the macrophage marker *CD68* between the 2 groups ($p=0.11$; figure 1E). The reduced *Usf1* expression in white adipose tissue was associated with 30% lower expression of lipoprotein lipase (*LPL*, $p<0.05$; Figure 1F) and 48% lower very-low density lipoprotein receptor (*VLDLr*, $p<0.05$; Figure 1G) expression, genes responsible for VLDL-TG-derived fatty acid uptake, and 42% lower Peroxisome proliferator-activated receptor-γ (*PPARg*, $p<0.05$; Figure 1H) expression, which is an important transcriptional factor regulating *LPL* and *VLDLr* expression. The expression of intracellular lipolysis related genes, including hormone-sensitive lipase (*Hsl*), adipose triglyceride lipase (*Atgl*), and the glucose transporter type 4 (*Glut4*) and lipid droplet-associated protein (*Plin*) were not changed (data not shown). Collectively, these data indicate decreased uptake of VLDL-TG-derived fatty acids by white adipose tissue upon deletion of Usf1 in bone marrow-derived cells. Therefore, lipid content of WAT was measured in paraffin-embedded gonadal WAT. In line with the body weight and gene expression data, a decreased WAT lipid content was observed in the Usf1 BMT mice compared to the WT mice, (-7%, $p<0.05$; Figure 1I). Morphological examination also confirmed a small decrease in adipocyte cell size (Figure 1J).

Plasma cholesterol is elevated in LDLr KO mice reconstituted with Usf1 KO bone marrow

Deletion of Usf1 in bone marrow-derived cells of LDLr KO mice resulted in a significant increase in total cholesterol and free cholesterol levels (+36%, $p<0.01$ and +26%, $p<0.05$ respectively; Figure 2A-B) after 20 weeks on WTD, while this effect was not observed on chow diet (data not shown). Determination of the lipoprotein distribution pattern showed that the increase in plasma cholesterol levels on WTD could be attributed to increased VLDL cholesterol levels (+60%, $p<0.05$; Figure 2C and D). Moreover, a trend towards higher plasma triglycerides was observed in LDLr KO mice with Usf1 KO BM on WTD (+17%, $p=0.12$; Figure 2E). No effect of BM Usf1 deletion was found on plasma glucose levels in the LDLr KO recipients (data not shown).

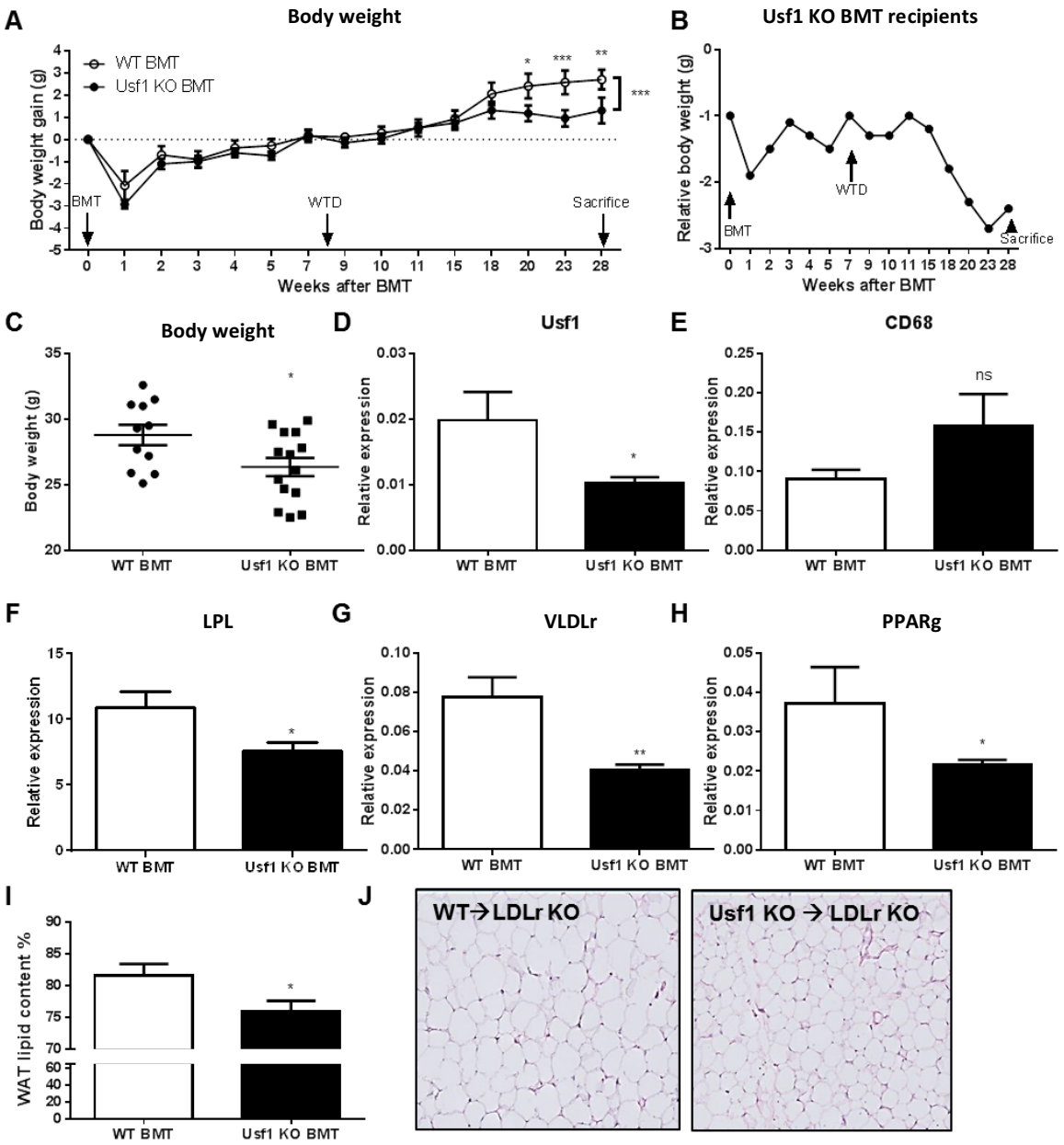

Figure 1 Deletion of hematopoietic Usf1 in LDLr KO mice attenuates body weight gain and adiposity.

LDLr KO mice received 5×10^{6} bone marrow cells intravenously from either WT or Usf1 KO mice after 8 Gy X-ray lethal irradiation. The mice were allowed to recover for 8 weeks on chow diet, and were then fed WTD diet for 20 weeks to induce atherosclerosis. A) Bodyweight gain of LDLr KO recipients reconstituted with WT bone marrow (open circles "o") or Usf1 KO bone marrow (closed circles "●") from 0-28 weeks after BMT. 2-way ANOVA was used to analyze the statistical significant difference in time. B) Relative body weight of LDLr KO recipients with Usf1 KO BM corrected by the bodyweight of recipients with WT, the distinctive bodyweight difference starts on week 15 (7 weeks WTD feeding). C) Bodyweight of LDLr KO recipients at sacrifice. D-H) Relative mRNA expression of *Usf1*, *CD68*, *LPL*, *VLDLr* and *PPARg* in inguinal adipose tissue of LDLr KO mice with WT bone marrow (open bar) or Usf1 KO bone marrow (closed bar). I) Lipid content of gonadal WAT in LDLr KO recipients with WT bone marrow (open bar) or Usf1 KO bone marrow (closed bar). J) Representative histology photographs of hematoxylin/eosin stained paraffin sections of gonadal white adipose tissue (original magnification10X).*P<0.05 **P<0.01 as compared to LDLr KO mice reconstituted with WT bone marrow (n=7-14).

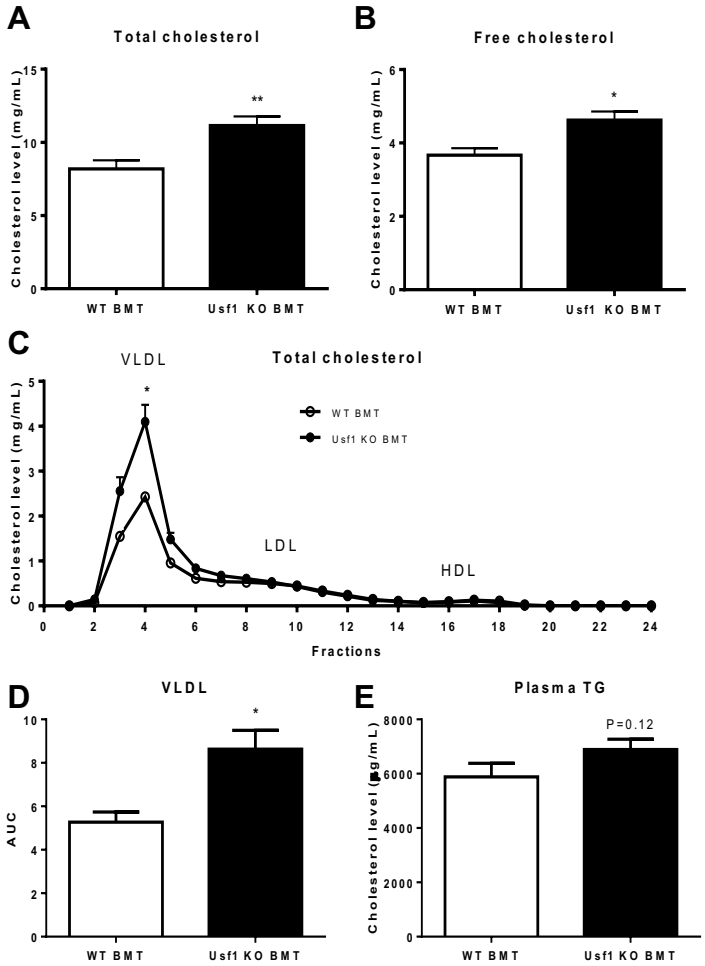

Figure 2 Deletion of hematopoietic Usf1 in LDLr KO mice induces plasma cholesterol levels.

After 20 weeks WTD feeding, plasma from LDLr KO mice reconstituted with WT bone marrow or Usf1 KO bone marrow was collected subsequently to 4 hours fasting. A) Total plasma cholesterol and B) free plasma cholesterol levels of LDLr KO recipients of WT bone marrow (open bars) or Usf1 KO bone marrow (closed bars). C) Plasma samples were fractionated with FPLC size-exclusion chromatography and cholesterol levels were measured from these fractions. Fractions 2-6 represent VLDL, fractions 7-14 LDL, fractions 15-19 HDL. Open circles (○) represent LDLr KO recipients with WT bone marrow, closed circles (●) represent LDLr KO recipients with Usf1 KO bone marrow. D) Area under the curve of cholesterol content in VLDL particles from recipients with WT bone marrow (open bar) or with Usf1 KO bone marrow (closed bar). E) Plasma triglycerides levels on of recipients. *P<0.05, **P<0.01 as compared to mice with WT bone marrow (n=4-5).

Deletion of hematopoietic Us1 in LDLr KO mice increased hepatic cholesteryl ester accumulation, but kept unaffected the mRNA expression of genes involved in hepatic lipid metabolism

In order to investigate if the increased plasma cholesterol in BM-specific Usf1 KO mice was associated with an altered hepatic lipid metabolism, the lipid content and mRNA expression levels of genes involved in lipid homeostasis were determined. BM-specific Usf1 deletion led to increased Oil red O staining for neutral lipids (Figure 3A) in the liver after 20 weeks WTD feeding, accompanied by increased cholesteryl ester accumulation evidenced by quantitative analysis after Bligh and Dyer extraction (+72%, $p<0.05$; Figure 3B), while free cholesterol and triglycerides were not affected ($p>0.05$; figure 3C). This is in contrast to the total body Usf1 knockout mice which were protected against hepatic steatosis, in line with their overall beneficial metabolic phenotype.[17]

Despite the observed increase in cholesteryl ester accumulation in the liver of LDLr KO mice lacking Usf1 in bone marrow-derived cells, the liver expression of genes involved in lipid synthesis, *i.e.* 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMG-coA reductase*), microsomal triglyceride transfer protein (*MTTP*), stearoyl-CoA desaturase-1 (*SCD1*), *FASN* and the cholesterol esterification enzyme acyl-CoA:cholesterol acyltransferase (*ACAT*) were not affected (Supplementary figure 1A). Furthermore, while total body Usf1 deletion in mice leads to increased plasma HDL levels,[17] BM-specific Usf1 deletion did not affect HDL cholesterol. Not surprisingly, genes involved in HDL

metabolism, *i.e. ApoA1* and *ABCA1* were not affected in the liver of Usf1 KO BMT recipients compared to WT BMT recipients (Supplementary figure 1B). Also no effects were observed on the expression of genes involved in cholesterol clearance *i.e.* low-density lipoprotein receptor-related protein 1 (*LRP1*) and scavenger receptor class B type I (*SR-BI*) (Supplementary figure 1C). Moreover, the hepatic expression of *Usf1* was unchanged (p>0.05; Supplementary figure 1D). However, a 2.7-fold increase in expression was found of the Kupffer cell marker *CD68* in livers of LDLr KO mice reconstituted with Usf1 KO BM as compared to controls (p<0.001; Figure 3D), suggesting increased hepatic inflammation induced by the augmented cholesteryl ester accumulation in the liver.

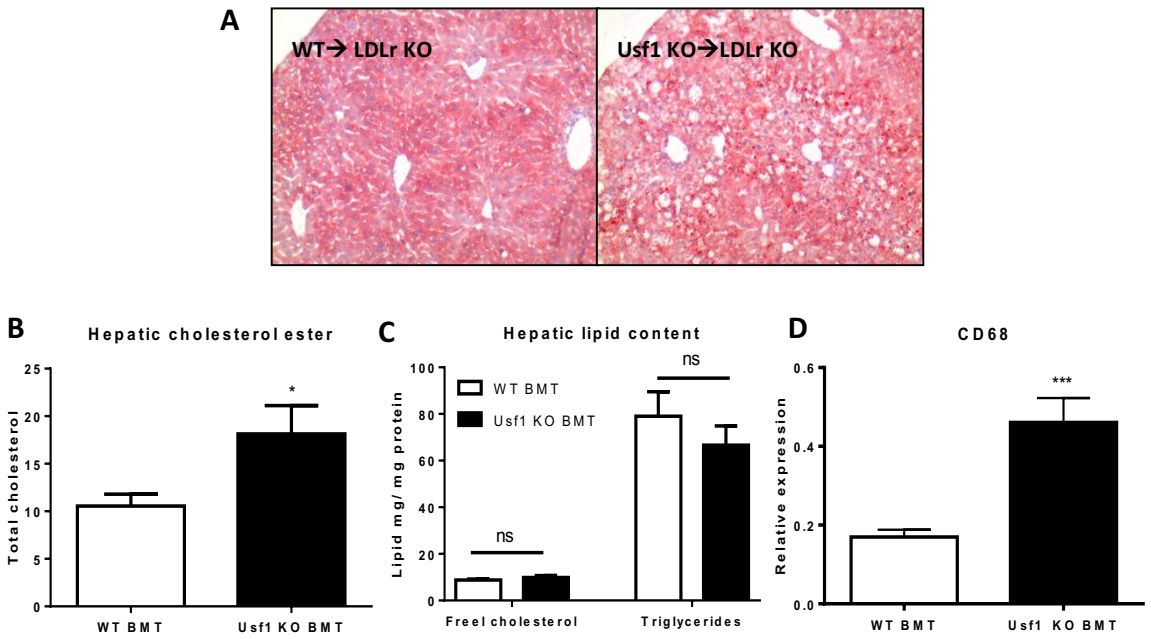

Figure 3 Effect of hematopoietic Usf1 deficiency on hepatic lipid accumulation after 20 weeks WTD feeding.

A) Representative images of Oil red O and H&E stained sections (original magnification 10x). Lipid was extracted from livers and the content of B) cholesteryl ester, C) free cholesterol and triglyceride were measured and normalized for protein level. D) Relative mRNA expression of *CD68* in liver. Open bars represent the recipients of WT bone marrow, closed bars represents the recipients of Usf1 KO bone marrow. *P<0.05; ***P<0.001 as compared to WT BMT livers (n=8).

Hematopoietic Usf1 does not affect brown adipose tissue of LDLr KO recipients

Activated brown adipose tissue (BAT) efficiently takes up fatty acids released from triglyceride-rich lipoproteins (TRL) such as chylomicrons and VLDL upon lipolysis of their core triglycerides leading to rapid clearance of the generated chylomicron and VLDL remnants by the liver.[26, 27] Mice with total-body Usf1 deletion display elevated uptake of TRL-derived fatty acids by BAT, and reduced BAT lipid content as well as smaller brown adipocyte size due to enhanced BAT thermogenesis.[17] This effect was independent of *UCP1* expression, the specific uncoupling protein of BAT, which was not changed due to the global Usf1 deletion.[17] In the current study, mRNA expression of *UCP1* in BAT of LDLr KO mice was also not altered upon deletion of Usf1 in bone marrow-derived cells. (p>0.05; Supplementary figure 2A). More importantly, in contrast to the total body Usf1 KO mice, the lipid content of BAT was not affected by BM Usf1 deletion (p>0.05, Supplementary figure 2B), suggesting a smaller contribution of BAT, if any, to the reduced bodyweight of the LDLr KO mice lacking hematopoietic Usf1.

Circulating neutrophil and monocyte counts are increased in LDLr KO mice lacking hematopoietic Usf1

High fat, high cholesterol feeding not only leads to hepatic inflammation, but also to augmented systemic inflammatory markers in mice.[28-30] Global Usf1 deficiency previously showed protection against low-grade systemic inflammation, a condition associated with metabolic disturbances.[17] To explore whether deletion of Usf1 in bone marrow-derived cells also affected the systemic inflammatory status under WTD feeding conditions, the circulating leukocyte profile was assessed using flow cytometry and haematological analysis (Sysmex). After 20 weeks of WTD feeding, a trend towards higher total white blood cell (WBC) counts was observed (+19%, p=0.09; Figure 4A). The observed trend to increased WBC counts was attributed to significantly higher amounts of circulating neutrophils (+63%, p<0.01; Figure 4B), and monocytes (+45%, p<0.05; Figure 4B), but not lymphocytes (data not shown). The results were confirmed by flow cytometric analysis. Compared to the WT controls, Usf1 KO transplanted mice showed an increase in the total amount of circulating $CD11b^+$ cells (+68%, p<0.05; Figure 5A-B); increased $CD11b^+Ly6G^+$ neutrophils (+100%, p<0.01; Figure 5C-D); and a small trend towards an increase in $CD11b^+Ly6C^{hi}$ pro-inflammatory monocytes (+23%, p=0.11; Figure 5E-F), but no difference in $CD11b^+Ly6C^{low}$ patrolling monocytes (p>0.05, Figure 5E-F).

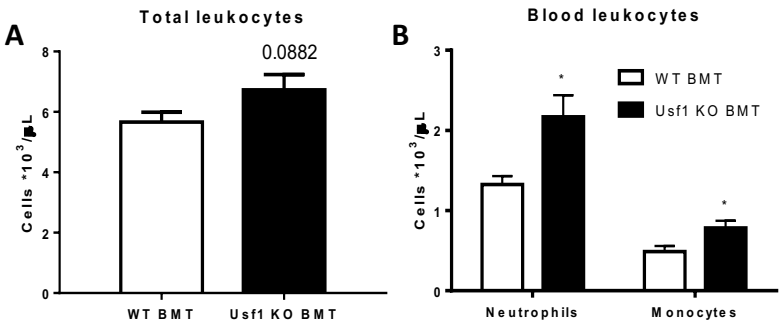

Figure 4 Effect of hematopoietic Usf1 deficiency on circulating leukocytes in LDLr KO mice.

After 20 weeks of WTD feeding, the circulating leukocyte profile was assessed using a hematological analyzer. A) Total leukocyte counts, B) Neutrophil and monocyte counts. Open bars represent LDLr KO mice reconstituted with WT bone marrow, closed bars represent mice with Usf1 KO bone marrow. *P<0.05 as compared to WT BMT mice (n=11-16).

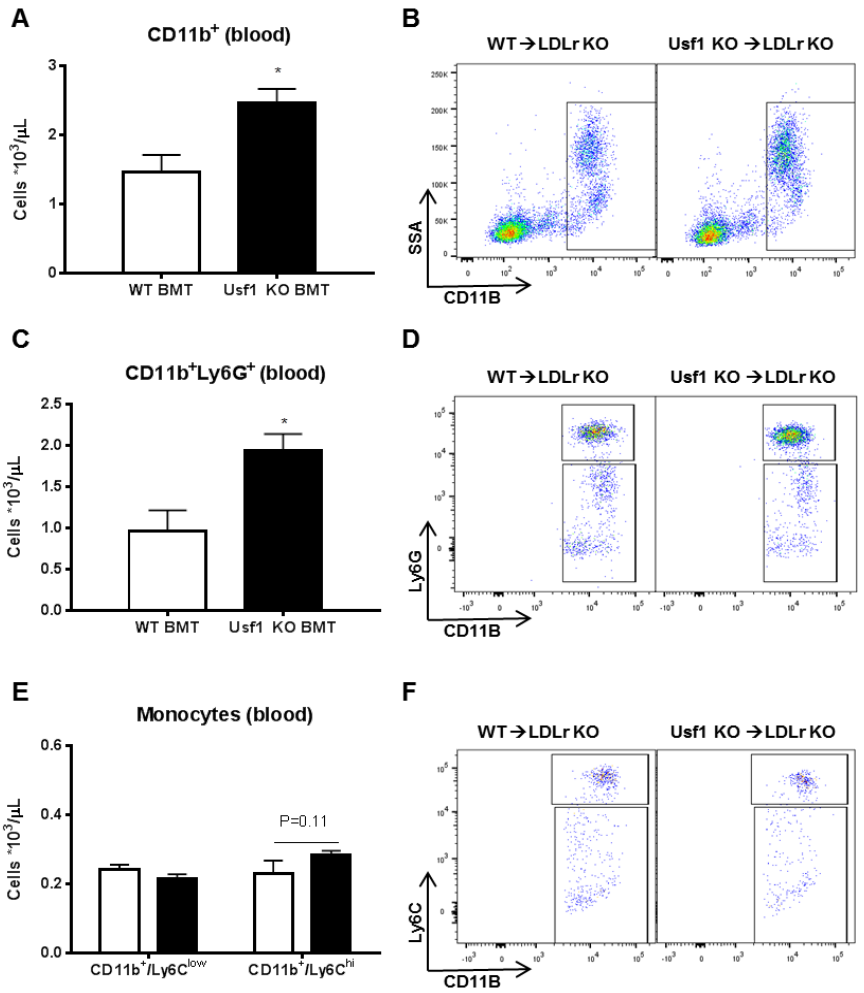

Figure 5 Flow cytometric analysis of blood cells in LDLr KO mice with hematopoietic Usf1 deficiency.

Circulating leukocytes in LDLr KO recipients were analysed by flow cytometry after 20 of weeks WTD feeding. A) Absolute numbers of $CD11b^+$ cells, C) $CD11b^+/LY6G^+$ neutrophils and E) $CD11b+/Ly6C^{low}$ anti-inflammatory monocytes and $CD11b^+/Ly6C^{high}$ pro-inflammatory monocytes were analyzed in the LDLr KO recipient mice with WT bone marrow (open bars) or Usf1 KO bone marrow (closed bars). B, D, F) Representative flow cytometric plots. *P<0.05; as compared to WT BMT mice (n=6).

Hematopoietic Usf1 deficiency in LDLr KO mice aggravates intracellular lipid accumulation in blood leukocytes

Nile red was used to quantify the cellular neutral lipid content of circulating blood cells using flow cytometry.[31, 32] In agreement with the elevated VLDL-cholesterol levels, an increased percentage of foamy leukocytes with a high nile red intensity were observed in Usf1 KO BMT mice compared to WT BMT controls on WTD (Figure 6A), indicating an induction of the amount of lipid-laden cells in the blood stream.

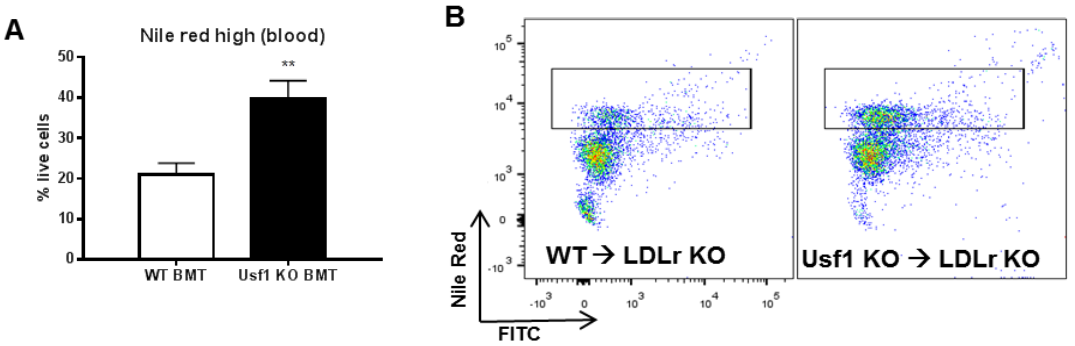

Figure 6 LDLr KO mice with hematopoietic Usf1 deficiency display aggravated intracellular lipid accumulation in blood.

After 20 weeks WTD feeding, A) Percentage of nile red positive circulating leukocytes. Open bars represent recipients of WT bone marrow, and closed bars recipients of Usf1 KO bone marrow. **P<0.01 (n=6) B) Representative flow cytometric plots of nile red staining of circulating leukocytes. (n=6).

Usf1 deficiency in bone marrow-derived cells of LDLr KO mice leads to increased atherosclerosis susceptibility

Since Usf1 deletion in bone marrow-derived cells of LDLr KO mice was associated with increased VLDL cholesterol and the appearance of more lipid-rich inflammatory cells in the circulation, we next assessed the effect of hematopoietic Usf1 deficiency on atherosclerosis susceptibility in the LDLr KO recipients. As expected, after 20 weeks WTD feeding, larger aortic root atherosclerotic lesions were found in the Usf1 KO BMT mice compared to the WT BMT controls (+31%, p<0.05; Figure 7A-B). Total cholesterol levels correlated well to atherosclerotic lesion sizes (p<0.05; figure 7C), indicating the elevated VLDL cholesterol is likely responsible for the increased susceptibility to atherosclerosis of the hematopoietic Usf1 KO mice.

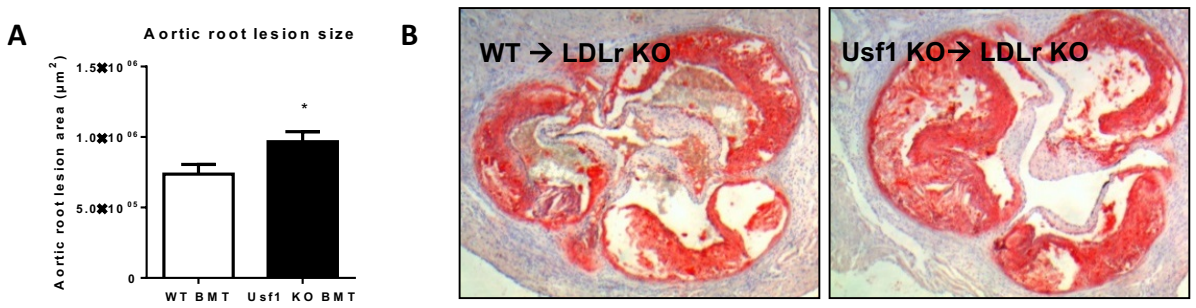

Figure 7 Bone marrow Usf1 deficiency increases atherosclerosis susceptibility of LDLr KO mice.

After 20 weeks of WTD feeding, atherosclerotic lesion development was assessed. A) Atherosclerotic lesion size in the aortic root of LDLr KO mice reconstituted with either WT bone marrow (open bars) or Usf1 KO bone marrow (closed bars). B) Representative aortic root atherosclerotic lesion stained for neutral lipids with Oil red O. *P<0.05 as compared to WT BMT mice (n=16-18).

Discussion

In the current study, we show that deletion of Usf1 in hematopoietic cells protects LDLr KO mice against diet-induced obesity, but leads to increased levels of cholesterol in circulating VLDL particles and elevated counts of lipid-laden inflammatory leukocytes in the circulation culminating into an increased susceptibility to atherosclerosis.

A recently published study by Laurila *et al.* showed that total body Usf1 KO mice are also protected against diet-induced obesity.[17] However, in contrast to our findings upon selective deletion of Usf1 in bone marrow-derived cells, total body Usf1 KO mice displayed a beneficial cardiometabolic lipid profile with decreased VLDL-triglycerides, and elevated HDL-cholesterol and were protected against atherosclerotic lesion development. In this context, it is important to note that the activity and function of Usfs is cell-type dependent.[14] The most pronounced tissue effect in the global KO mice was ascribed to BAT, although the contribution of other tissues could not be ruled out. Specific deletion of Usf1 in bone marrow-derived cells, as expected, only minimally affected the expression of *Usf1* in livers of the Usf1 KO BMT mice, while expression in BAT was not affected.

Global Usf1 deletion protects mice against the development of diet-induced obesity by increasing the activation of BAT.[17] Interestingly, in the current study we found that specific deletion of Usf1 in bone marrow-derived cells also protected LDLr KO mice from diet-induced obesity, suggesting that also bone-marrow dependent mechanisms could account for the reduced body weight of the global knockouts.[17] In contrast to total-body Usf1 KO mice, in our BM-specific Usf1 KO model, BAT lipid content was not changed nor was the expression of *UCP1* altered, suggesting that brown fat activation is not a causative factor in the lean phenotype observed in the Usf1 KO BMT mice. To fully exclude BAT activity as a causative factor in hematopoietic Usf1 deficiency-induced protection against weight gain, more comprehensive measurements of BAT activity, including measurements of BAT oxygen consumption and uptake of lipids and glucose, would have to be performed.

Adipose tissue mass can grow as a result of the expansion of the number of adipocytes (hyperplasia) or growth of the size of existing cells (hyperthrophy). Knockdown of *Usf1* in 3T3-L1 cells, a murine adipocyte model, by small interfering RNA (siRNA) represses adipogenesis,[10] indicating that Usf1 also has direct effects on adipogenesis. Notably, in our study, after challenge with WTD for 20 weeks, *Usf1* mRNA expression was largely decreased in adipose tissue of Usf1 KO BMT mice as compared to WT BMT controls. By performing a BMT with GFP-expressing BM Yuyama *et al*. previously showed that up to 16.7% of the adipocytes in mice challenged with a high fat diet for 7 weeks were derived from BM progenitors.[10] The decreased *Usf1* expression in adipose tissue of Usf1 KO transplanted LDLr KO mice is thus likely not only the consequence of deletion of Usf1 in adipose tissue macrophages (ATMs), but also in adipocytes from BM-origin. The decreased *Usf1* expression in adipose tissue coincided with lower *VLDLr* expression. VLDLr deficiency protects against obesity by lowering adipose tissue mass, which is associated with smaller adipocyte size due to a reduction in intracellular lipid droplet deposition.[33] Analysis of the lipid content of WAT in mice with BM Usf1 deletion showed a small decrease (-7%) in lipid content, but this is probably not sufficient to explain the overall 8.5% lower bodyweight. The lean body weight phenotype is thus likely also the consequence of an impaired expansion of the number of adipocytes, which is in line with the previously published role of Usf1 in adipogenesis.[10]

In contrast to total-body Usf1 KO mice that display a beneficial cardiometabolic profile,[17] in the current study we showed that selective deletion of Usf1 in bone marrow-derived cells in LDLr KO mice led to increased plasma levels of pro-atherogenic VLDL-cholesterol while HDL-cholesterol was not affected. No effects were observed on the hepatic expression of genes involved in VLDL clearance (*LRP1* and *SR-BI*) or VLDL synthesis (*HMG-coA reductase*, *MTTP*), suggesting that the observed augmented VLDL cholesterol levels are unlikely the result of an altered hepatic uptake.

Besides the liver, white adipose tissue is an important organ for cholesterol storage.[34] Adipocytes in rodents are a significant site for cholesterol synthesis and storage,[35] and, in obese subjects

adipose tissue stores up to 50% of the total body cholesterol.[34,36] Conversely, LDLr KO mice lacking adipose tissue display severe hyperlipidemia due to impaired plasma cholesterol clearance.[37] Similarly, our mice lacking Usf1 in bone marrow-derived cells remained lean and displayed increased plasma VLDL-cholesterol levels upon WTD feeding. Notably, WAT of LDLr KO mice reconstituted with Usf1 KO BM showed a decreased lipid content compared WAT of mice transplanted with WT BM, indicating reduced adipose tissue lipid deposition. Therefore, we hypothesized that an impaired VLDL-TG-derived fatty acid clearance capacity of the adipose tissue upon hematopoietic Usf1 deletion might be a causative factor in the observed elevation of circulating cholesterol levels, likely by impairing hepatic VLDL remnant clearance. LPL and VLDLr are two key proteins involved in VLDL clearance by white adipose tissue.[38] Adipocytes can remove VLDL particles directly from the circulation via the VLDLr.[39,40] or after hydrolysis of the particles' core triglycerides to free fatty acids by LPL, generating a VLDL remnant particle.[41] The expression of the *VLDLr* and *LPL* is regulated by PPAR-γ activation.[42] Yuyama *et al.* previously showed that knockdown of *Usf1* in the adipocyte cell line 3T3-L1 downregulated *PPARg* expression in this cell type.[10] Notably, *PPARg* expression was decreased in adipose tissue of LDLr KO mice transplanted with Usf1 KO BM. In support of the decreased activity of PPAR-γ in adipose tissue of LDLr KO transplanted with Usf1 KO BM, both the expression of the *VLDLr* and *LPL* were decreased. We speculate that deletion of Usf1 in bone marrow-derived cells might lead to elevated plasma VLDL levels through inhibition of VLDL clearance by adipose tissue through impairment of the PPAR-γ-VLDLr/LPL axis.

The excessive amounts of VLDL in the plasma due to impaired VLDL clearance by adipose tissue, could cycle back to the liver,[43] leading to increased hepatic cholesteryl ester accumulation. Indeed, cholesterol deposition in livers of LDLr KO recipients reconstituted with Usf1 KO BM was increased. Interestingly and in agreement with our findings, Jones and colleagues previously showed that conditional deletion of PPAR-γ in adipose tissue protects mice from high fat diet-induced obesity, and stimulates lipid accumulation in the liver.[44] The augmented cholesterol deposition in livers of LDLr KO mice transplanted with Usf1 KO BM coincided with a dramatic increase in hepatic *CD68* expression compared to WT transplanted controls, suggesting augmented hepatic inflammation.[45,46] Moreover, augmented systemic inflammation was found as evidenced by the observed increase in neutrophil counts and the trend towards increased pro-inflammatory monocyte counts in the circulation upon hematopoietic Usf1 deletion.

Besides inducing lipid accumulation in the liver, high levels of VLDL and VLDL remnant cholesterol also rapidly give rise to lipid droplet formation in monocytes, both in humans and in mice.[47,48] Consistently, BM-specific deficiency of Usf1 in LDLr KO mice led to increased counts of foamy monocytes in the circulation. Lipid-rich monocytes in blood are predictive markers for the development of atherosclerosis[49] and VLDL and remnants were reported to be the best predictor of aortic root atherosclerosis in the LDLr KO model.[50] In agreement, we observed larger atherosclerotic lesions in the aortic sinus of the Usf1 KO BMT mice after 20 weeks WTD feeding, correlating with the augmented serum cholesterol in the circulation of these animals. Interestingly, in contrast to the observed 1.3-fold increase in atherosclerotic plaque area in LDLr KO mice transplanted with Usf1 KO BM, Laurila *et al.*[17] recently reported that total body Usf1/LDLr double KO mice exhibit a 4-fold decrease in atherosclerotic plaque size after 20 weeks of WTD feeding in *en face* aortic sections, which is in line with the improved cardiometabolic lipid profile in these animals. Importantly, this observation was supported by a 45% reduction in atherosclerotic plaque area in humans being homozygous for an allele which induces 18 % decrease in *usf1* expression.[17,51] Thus, the beneficial metabolic effects caused by global Usf1 deficiency in mice and

humans are able to overcome the detrimental effects of Usf1 in bone marrow-derived cells as shown in the current study (Figure 8).

In conclusion, our study revealed a potential role of hematopoietic Usf1 in VLDL metabolism, obesity and atherosclerosis development and highlights the importance of studying tissue-specific effects of gene modifying strategies in animal models.

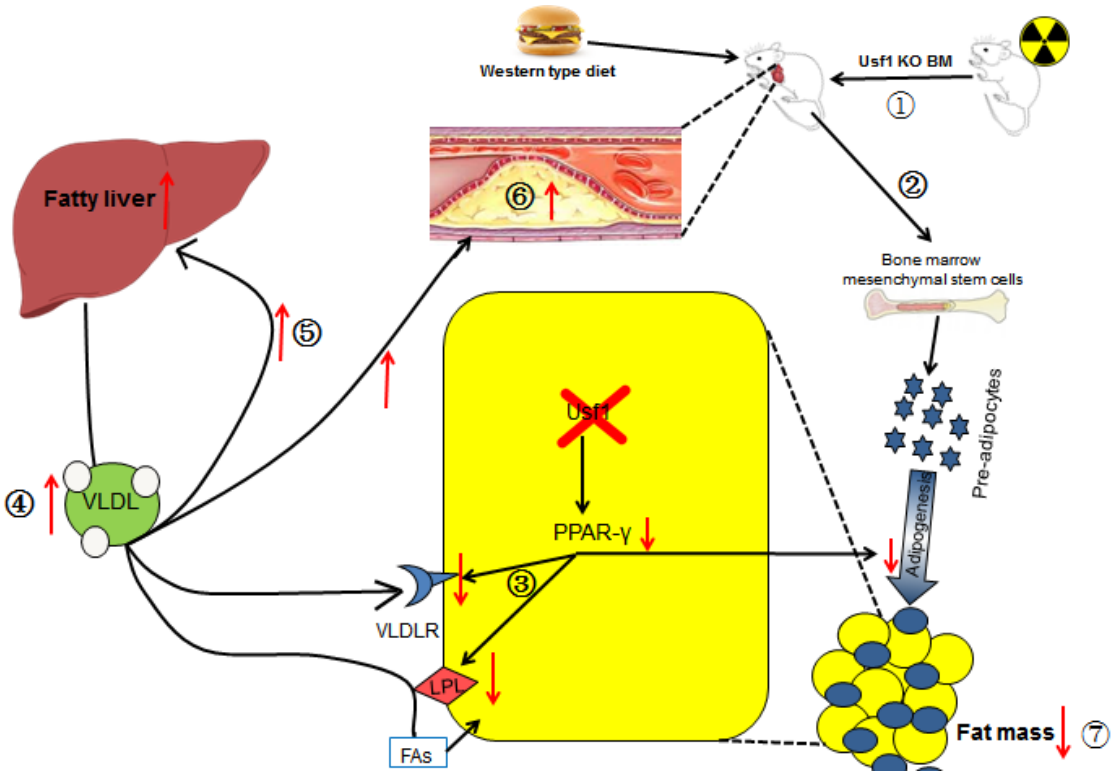

Figure 8 Effect of hematopoietic Usf1 on VLDL metabolism, adiposity, and atherosclerosis susceptibility.

1) Bone marrow transplantation deletes Usf1 in all bone marrow-derived cells of LDLr KO recipients. 2) WTD feeding induces the generation of novel adipocytes of which a fraction is derived from bone marrow progenitors lacking Usf1, contributing to decreased adipose *Usf1* expression in the Usf1 KO BMT mice. 3) Adipose Usf1 positively regulates the adipose tissue ability to take up plasma VLDL-TG-derived fatty acids through transcriptional regulation of VLDLr and LPL expression via PPAR-γ. 4) Clearance of VLDL remnant is impaired due to reduced *Usf1* expression in adipose tissue, leading to elevated plasma VLDL-cholesterol 5) Increased cycling of VLDL-cholesterol to the liver contributes to increased hepatic lipid accumulation in Usf1 BMT mice. 6) Increased plasma VLDL-cholesterol promotes atherosclerosis susceptibility in LDLr KO recipients. 7) Reduced lipid deposition and impaired adipogenesis co-lead to the decreased body weight gain and adiposity. The black lines and arrows indicate the normal pathways. The red cross means knockout and red arrows indicate the effects of hematopoietic Usf1 deficiency.

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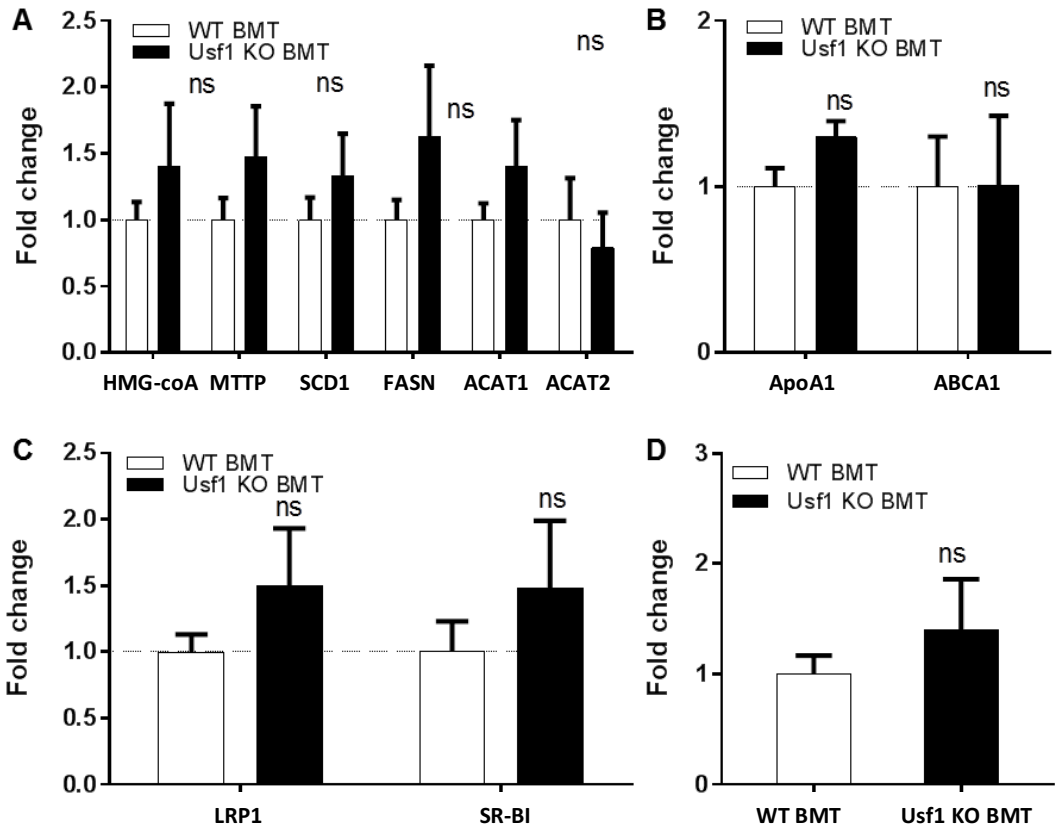
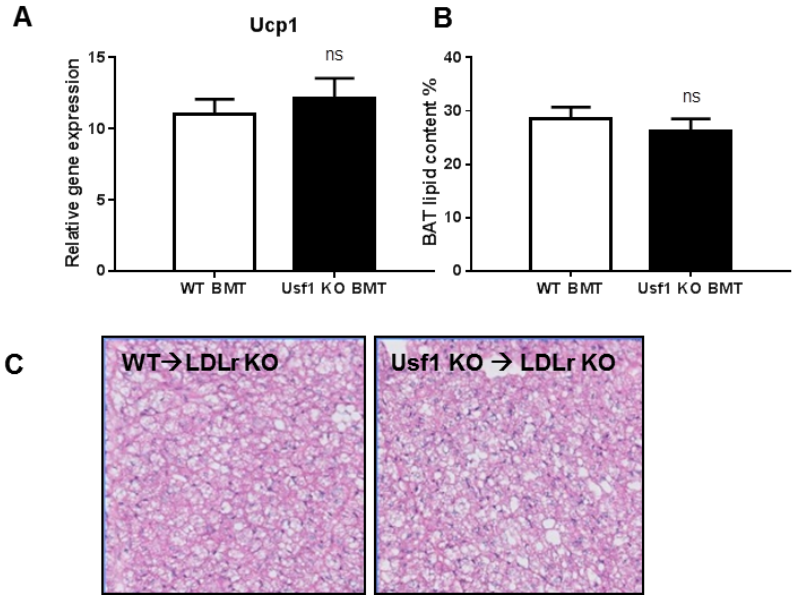
Supplementary material



Supplementary Figure 1 Hepatic lipid metabolism was not affected in LDLr KO mice lacking Usf1 in bone marrow-derived cells.

After 20 weeks WTD feeding, livers from LDLr KO mice reconstituted WT bone marrow or Usf1 KO bone marrow were collected for mRNA expression analysis. A) Relative mRNA expression of genes involved in VLDL metabolism, B) genes involved in HDL metabolism, C) genes involved in cholesterol clearance, and D) hepatic *Usf1* were analyzed. Open bars represent livers from mice reconstituted with WT bone marrow (n=8), closed bars represents mice transplanted with Usf1 KO bone marrow (n=8).



Supplementary Figure 2 Brown adipose tissue (BAT) morphology in LDLr KO mice reconstituted with either WT or Usf1 KO bone marrow after 20 weeks Western-type diet feeding.

A) mRNA expression of *UCP1*, B) lipid content in BAT was analyzed (n=9-16); ns indicates non-significant difference. C) Representative histology photographs of hematoxylin-stained paraffin sections of intercapular adipose tissue (original magnification 10×).

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