Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A mouse model of humanized liver shows a human-like lipid profile, but does not form atherosclerotic plaque after western type diet

Gulce Sari [a], Eric J. Meester [b, f], Leonie C. van der Zee [c], Kristiaan Wouters [d], Jeanine R. van Lennep [c], Maikel Peppelenbosch [a], Andre Boonstra [a], Kim Van der Heiden [b], Monique M.T. Mulder [c], Thomas Vanwolleghem [a, e, *]

[a] Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, the Netherlands
[b] Department of Biomedical Engineering, Thoraxcenter, Erasmus University Medical Center, Rotterdam, the Netherlands
[c] Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, the Netherlands
[d] Department of Internal Medicine, CARIM School for Cardiovascular Diseases, MUMC+, Maastricht, the Netherlands
[e] Laboratory of Experimental Medicine and Pediatrics, Faculty of Medicine and Health Sciences, University of Antwerp and Department of Gastroenterology and Hepatology, Antwerp University Hospital, Antwerp, Belgium
[f] Department of Radiology & Nuclear Medicine, Erasmus University Medical Center, Rotterdam, the Netherlands

ARTICLE INFO

Article history:
Received 22 December 2019
Accepted 11 January 2020
Available online xxx

Keywords:
Human liver chimeric mouse model
Atherosclerosis
High fat diet
Lipoprotein
Cardiovascular disease

ABSTRACT

Mouse models are a crucial and often used tool to provide insight into the underlying mechanisms of human atherosclerosis. However, mice profoundly differ from humans in lipoprotein synthesis and metabolism, key factors in atherosclerotic plaque formation. Mouse models often require genetic and dietary modifications to mimic human pathophysiology, shifting from a high-density lipoprotein to an low-density lipoprotein dominant lipoprotein profile. We examined the suitability of mice with a humanized liver as a model for lipoprotein studies and studies on plaque formation, given the central role of hepatocytes in lipoprotein synthesis and metabolism. Our results show a progressive humanization of the mouse liver and a humanized lipoprotein profile. However, no atherosclerotic plaque formation was observed in the studied time frame, despite presence of functional macrophages and application of a high cholesterol western-type diet. The humanized-liver mouse model therefore might require further modifications to induce atherosclerosis, yet seems a valuable model for *in vivo* studies on lipoprotein metabolism.

1. Introduction

Cardiovascular disease (CVD) accounts for 31% of deaths worldwide [1–4], and most cardiovascular events are a result of atherosclerosis. Atherosclerosis is a lipid driven disease in which lipids accumulate in the vessel wall. Once in the vessel wall, oxidized lipids are engulfed by macrophages which in turn differentiate into foam cells that trigger further plaque progression and can eventually result in cardiovascular events. Serum lipoproteins are circulating particles carrying different amounts of cholesterol, hydrophobic lipid molecules and triglycerides [5] between organs and are inevitably involved in lipid metabolism and related diseases. Disturbances in lipoprotein metabolism therefore play a crucial role in atherosclerotic plaque formation [6]. Animal models of atherosclerosis that recapitulate human lipoprotein metabolism are therefore essential tools to assess novel drugs or to study the pathophysiology. However, mice and humans differ in fundamental aspects of lipoprotein synthesis and metabolism.

The main differences in human and rodent lipoprotein physiology are a circulating lipoprotein profile dominant in high-density lipoprotein (HDL) in mice opposed to a low-density lipoprotein (LDL) dominant profile in humans, a lack of cholesteryl ester transfer protein (CETP) expression and lipoprotein (a), and a different bile acid composition [7–9]. As a consequence, mice do not develop atherosclerosis and require genetic, dietary and/or mechanical modifications to be optimally applied as disease

Abbreviations: CVD, cardiovascular diseases; HDL, high density lipoproteins; HFD, high fat diet; LDL, low density lipoproteins; NOG, NOD/Shi-scid/IL-2Rγ^{null}; TK, The herpex simplex virus thymidine kinase; uPA, urokinase type plasminogen; VLDL, very low density lipoproteins.

* Corresponding author.
E-mail addresses: g.sari@erasmusmc.nl (G. Sari), t.vanwolleghem@erasmusmc.nl (T. Vanwolleghem).

https://doi.org/10.1016/j.bbrc.2020.01.067

models. There are several genetically modified inbred mouse models available, each with their pros and cons, for example low-density lipoprotein receptor knockout (ldlr$^{-/-}$), apolipoprotein E knockout (ApoE$^{-/-}$) and C57Bl/6 mice carrying a mutation in protein convertase subtilisin/kexin type 9 (Pcsk9) gene [7]. Although these models have their merits and are often used, they lack a human-like lipid profile. Experimental results can therefore not always be readily translated to the human setting and testing of lipid lowering drugs is difficult. Humanization of the liver can be a promising alternative to genetically modified models to mimic human lipoprotein synthesis and metabolism.

Therefore, as an alternative to genetic models, we here examine the humanization of the lipoprotein profile and metabolization pathways by transplanting human hepatocytes in mice with a genetic (urokinase type plasminogen overexpression) or inducible (thymidine kinase expression) liver damage [10–14] on an immunodeficient background. These mice have been shown to have a human-like liver metabolization of xenobiotics and have been applied for liver infectious disease studies, such as viral hepatitis and malaria [15–18]. In the present study, our aim was to investigate if human liver chimeric mice have a serum lipoprotein profile that can be applied as a model for atherosclerosis studies. After demonstrating humanization of the lipoprotein profile, we further examined whether atherosclerotic plaques developed upon high fat feeding. Despite 10-weeks of high fat diet, humanization of the lipoprotein profile, and presence of functional macrophages no atherosclerotic plaques could be observed in the aortic vasculature or carotid bifurcations.

2. Methods

2.1. Ethical permissions

All animal studies were approved by the animal ethics committee of the Erasmus University Medical Center (EMC 3019(141-12-11)) and Maastricht University Medical Center (2018–011) and conducted according to Dutch national guidelines.

2.2. Mouse origins and genotyping

The herpes simplex virus thymidine kinase (TK)/NOD/Shi-scid/IL-2Rγ^{null}v(NOG) and urokinase-type plasminogen activator (uPA)/NOG mice embryos were provided by dr. Suemizu, Central Institute for Experimental Animals, Kawasaki, Japan [19]. Mice were bred at the Central Animal Facility of the Erasmus Medical Center and offspring zygosity was determined using a copy number duplex qPCR performed on phenol-chloroform-isoamyl alcohol (Sigma-Aldrich, St. Louis, MO, USA)-extracted genomic mouse DNA from toe snips. The TaqMan Genotyping master mix (Life technologies, Carlsbad, CA, USA) with the TaqMan uPA genotyping assay (Mm00422051_cn; Life Technologies) or custom designed primer-probe mix F: CGATTCGCCGCGTTTACG, R: CGCCGCCCTGCAGATA and probe: [6FAM]CCGCACCGTATTGGCAA[BHQ1] targeting TK gene and Tert gene references mix (Life technologies) were used according to the manufacturer's protocol.

Ldlr$^{-/-}$ mice were obtained from the breeding colony of the Maastricht University Medical Center research facilities. Serum samples of HFD Ldlr$^{-/-}$ mice were a kind gift from Dr. Menno Hoekstra, Leiden Academic Centre for Drug Research, Leiden University.

2.3. Human hepatocyte transplantation

TK+ and uPA-homozygous mice were anesthetized and transplanted with 0.5×10^6 to 2×10^6 viable cryopreserved human hepatocytes (Lonza, Basel, Switzerland, and Corning, Corning, NY, USA) via intrasplenic injection as previously described [16]. At day −7 and −5 before transplantation, TK + mice received an intraperitoneal ganciclovir injection to initiate liver damage [19]. Engraftment level and transplantation success were determined using a human albumin ELISA as previously described (Bethyl laboratories, Montgomery, TX, USA) [16].

2.4. Animal housing and diet

Mice were fed *al libitum* with pelleted rodent normal chow (Rat and Mouse Breeder and Grower, CRM(P), Special Diet Services, Essex, UK). For the high fat diet (HFD), animals were fed with food containing 0,3% cholesterol (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) up to 10 weeks. Ldlr$^{-/-}$ mice were fed with gamma irradiated rodent chow from Ssniff (V1534-703, Soest, Germany). For the hydragel analysis, positive control serum sample was obtained from ldlr$^{-/-}$ mice after the Western-type diet (diet W) containing 0.25% cholesterol and 15% fat from Special Diet Services (Witham, Essex).

2.5. Determining plaque presence ex vivo

Mice were euthanized via CO_2 inhalation, after which their blood was collected via cardiac puncture. Afterwards, the vasculature was flushed with 1x phosphate buffered saline (PBS) via the left ventricle, followed by excision of the aorta and carotid arteries. The arteries were cleaned from connective tissue and stained for lipids (Oil red O (ORO)) to evaluate plaque presence. The arteries were subsequently embedded in tissue-tec and stored at −80 °C for histological analysis. 5 μm cryosections of the vasculature were immunohistochemically stained with anti-CD68 (1:100 Biorad, MCA1957) to assess if macrophages, which are the most prevalent inflammatory cell in plaque, were present in the vasculature.

2.6. Hydragel analysis of lipoproteins

Hydragel analysis of lipoprotein profiles of serum samples was performed using Hydragel 7 Lipoprotein(e) (Sebia Inc., Georgia, USA) using a HYDRASYS System (Sebia Inc., Gergia, USA). Briefly, samples were loaded into wells of ready-to-use agarose gels (0,8 g/dL) and electrophoresis was performed using buffered strips. Samples were run at 160V for 20–25 min. Dried gels were stained with a Sudan Black solution provided by the manufacturer, washed with 75% ethanol and the migration pattern and lane density of the samples were calculated. Area under the curve (AUC) calculation was performed using GraphPad Prism 5 for Windows (v.5.01, 2007) software.

2.7. Fast protein liquid chromatography (FPLC)

Fractionation of the lipoproteins was analyzed using FPLC. FPLC gel filtration was performed using a Tricorn Superdex 200 10–300 GL and a Tricorn Superose 6 10–300 GL size exclusion column in series (GE Healthcare) on FPLC system (Pharmacia, 1983).

2.8. RNA isolation, cDNA synthesis and qPCR analysis

RNA isolation was performed using whole liver tissues stored in RNA*later* RNA stabilization reagent (Qiagen, Hilden, Germany) with RNeasy mini Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was generated by using PrimeScript™ reverse transcriptase master mix (Takara Bio Inc., Kusatsu, Japan) according to manufacturer's protocol. Human specific CTEP gene expression was measured using custom designed primers (F:

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CAGATCAGCCACTTGTCCAT and R: CAGCTGTGTGTTGATCTGGA) with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Human specific GAPDH was applied as a housekeeping control gene.

2.9. FITC-labelled Escherichia coli (E.coli) phagocytosis assay

Splenocytes were isolated from $uPA^{+/+}$ mice and $ldlr^{-/-}$ mice spleen using 100 μm cell strainer. Cells were counted using Trypan blue staining and 2×10^8 cells were incubated with 200 μg/ml FITC labelled *E.coli* (Glycotope, Germany) either at 4° or 37° for 45 min. Following incubation, free or unbound *E.coli* was removed by washing and cells were stained with Aqua-Amcyan, CD45-APC (30F11, eBioscience48-0451-82), CD11c-APC-Cy7 (N418, Biolegend 117324), CD11b-PE-Cy7 (M1/70, eBioscience 25–011282) and F4/80-APC (BM8, eBioscience 17-4801-82) and measured using a multi-color flow cytometer (Canto II) for detection of splenic macrophages and elimination of dendritic cells as described previously [20] (for gating strategy see Supp. Fig.1). After gating on splenic macrophages, the ratio of the FITC-positive and −negative cell population was analyzed to document their phagocytic activity using FlowJo (v.10, Tree Star Inc.).

2.10. Statistical analysis

GraphPad Prism 5.01 for Windows (GraphPad Software, Inc., San Diego California USA) was used for statistical analysis and illustrations. Spearman r was applied for the statistical analysis of the association, and $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Human liver chimeric NOG mice adopt a human-like serum lipoprotein profile

To demonstrate humanization of the serum lipoprotein profile of human liver chimeric mice, we analyzed serum samples before and after human hepatocyte transplantation (Fig. 1A) with agarose gel electrophoresis and subsequent visualization with Sudan black. Lipoproteins are separated based on their size dependent migration differences in this assay (HDL-fast, LDL-slow and VLDL −intermediate migration). In addition, the requirement of small sample volumes, allows us to analyze the lipoprotein profile of the same animal at multiple time points. As shown in Fig. 1B, human liver chimeric NOG mice demonstrate merged VLDL and LDL bands with increased intensity compared to non-transplanted mice. To examine the constituents of the merged peaks, we analyzed larger volumes (>150 μL) of serum samples obtained at sacrifice using FPLC. Fig. 1C shows a representative example of cholesterol and triglyceride concentrations per FPLC fraction. Elutes with high cholesterol concentrations correspond to HDL, the elutes with high triglyceride concentrations correspond to VLDL, while LDL has intermediate levels of both cholesterol and triglycerides. FPLC data could therefore confirm that both LDL and VLDL are abundantly present in humanized mouse sera, suggesting a humanization of the lipoprotein profile. We then compared human and humanized mouse serum lipoprotein profiles by analyzing the ratio of VLDL + LDL to HDL, as the former are predominantly present in human sera, while the latter is predominantly present in wild type mouse serum. We therefore calculated the area under the curve (AUC) of the respective lipoprotein peaks after gel electrophoresis. The AUC ratio of (VLDL + LDL)/HDL is significantly higher in

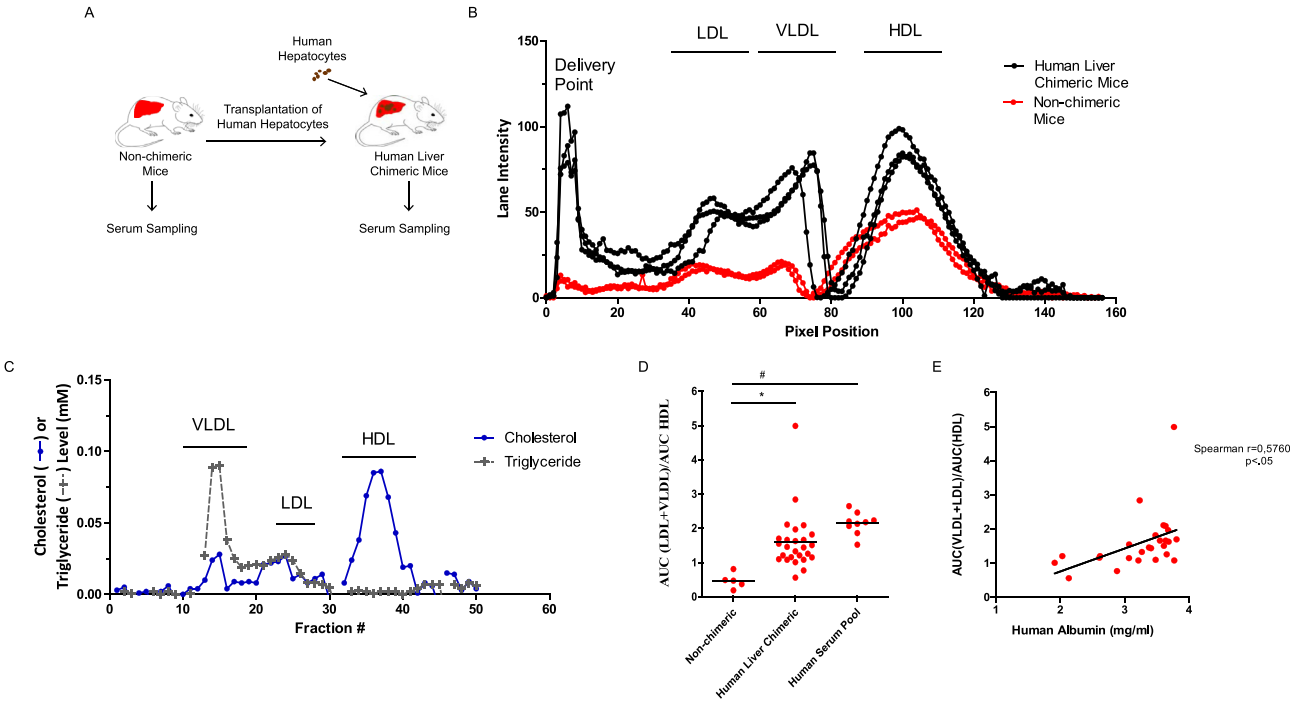

Fig. 1. Human liver chimeric mice adopt a human-like lipoprotein profile during progressive liver repopulation by human hepatocytes. **A.** Schematic representation of the experiments. **B.** Sudan black lane intensity on agarose gel electrophoresis blots of chimeric and non-chimeric mouse serum samples. **C.** Triglyceride (grey line) and cholesterol (blue line) levels in FPLC fractions of a humanized mouse serum sample. VLDL and LDL peaks can be discerned based on their different elution profiles during FPLC. **D.** Humanization of the mouse serum lipoprotein pool. As HDL predominates the lipoprotein pool in mouse serum, humanization was expressed by combining LDL and VLDL peaks, divided by the HDL peak in serum agarose gel electrophoresis studies (AUC(LDL + VLDL)\AUC(HDL)) (*$p < 0.01$ and & $p < 0.0001$). **E.** This AUC ratio correlates with mouse serum human albumin levels ($p < 0.05$; Spearman $r = 0.5760$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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transplanted compared to non-transplanted mice, approaching the ratio observed for human serum (Fig. 1D) (* and #p < 0.01). In addition, the AUC ratio of (VLDL + LDL)/HDL positively correlates with serum human albumin levels in human hepatocyte transplanted mice (Fig. 1E**),** which is an indicator of human hepatocyte engraftment success (p < 0.05). Overall these data demonstrate that successful repopulation of the liver with human hepatocytes leads to a progressive humanization of the mouse serum lipoprotein profile.

3.2. Despite functional macrophages and a high fat diet, human liver chimeric mice are not prone to atherosclerotic plaque formation

A human like lipoprotein profile is the prerequisite for atherosclerotic plaque formation in animal models of atherosclerosis. We therefore explored predilection zones in the vasculature (aortic arch and carotid artery bifurcations) of humanized mice for atherosclerotic plaques. However, no plaques were detected after 14 weeks of normal chow diet (data not shown). Atherosclerotic mouse models often require at least 8–10 weeks of HFD (HFD, 0.3% cholesterol), resulting in high LDL levels before atherosclerotic plaques can be detected. We therefore fed humanized mice a HFD (0.3% cholesterol) for up to 10 weeks (Fig. 2A). This HFD resulted in a significant increase in total serum cholesterol levels (Fig. 2B), characterized by an increase of all lipoprotein levels on gel electrophoresis studies (normal chow serum vs week 8 HFD serum and normal chow serum vs week 10 HFD serum, p < 0.05; Fig. 2C).

Besides higher LDL and VLDL levels, macrophages are essential for plaque formation. Hence, we next examined the phagocytic capacity of macrophages isolated from uPANOG mice. We compared them to macrophages of ldlr$^{-/-}$ mice as a positive control, plaque formation can be observed in these mice due to high serum LDL levels. Isolated splenocytes from uPANOG mice were able to phagocytose FITC-labelled *E.coli*, although the ratio of FITC positivity was 20–30% less than the splenic macrophages of ldlr$^{-/-}$ mice (Fig. 3). Despite the increases in total cholesterol and LDL and VLDL levels, no lipid accumulations (via ORO staining) or CD68 $^{+}$ expressing cells (a marker of macrophages) were observed in the mouse vasculature within the tested time window (Fig. 2D and 2E).

4. Discussion

We and others have shown that the human liver chimeric

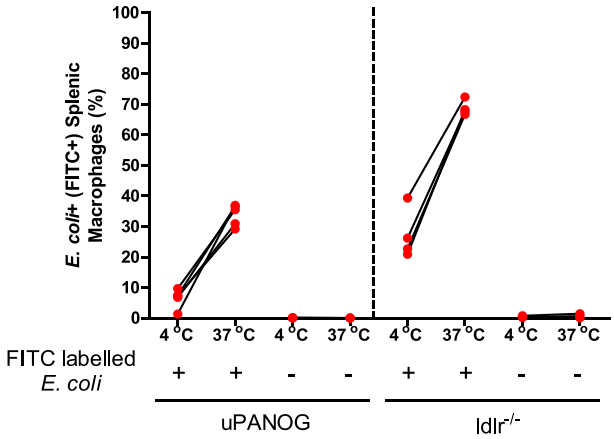

Fig. 3. Splenic macrophages of uPA ± NOG mice phagocyte *E.coli* to a lesser extent compared to those of ldlr$^{-/-}$ mice. Splenic macrophages were incubated for 45 min at 4 °C or 37 °C with or without FITC-labelled *E.coli* and analyzed by FACS. Each dot shows an individual mouse. N = 4 for both mouse strains.

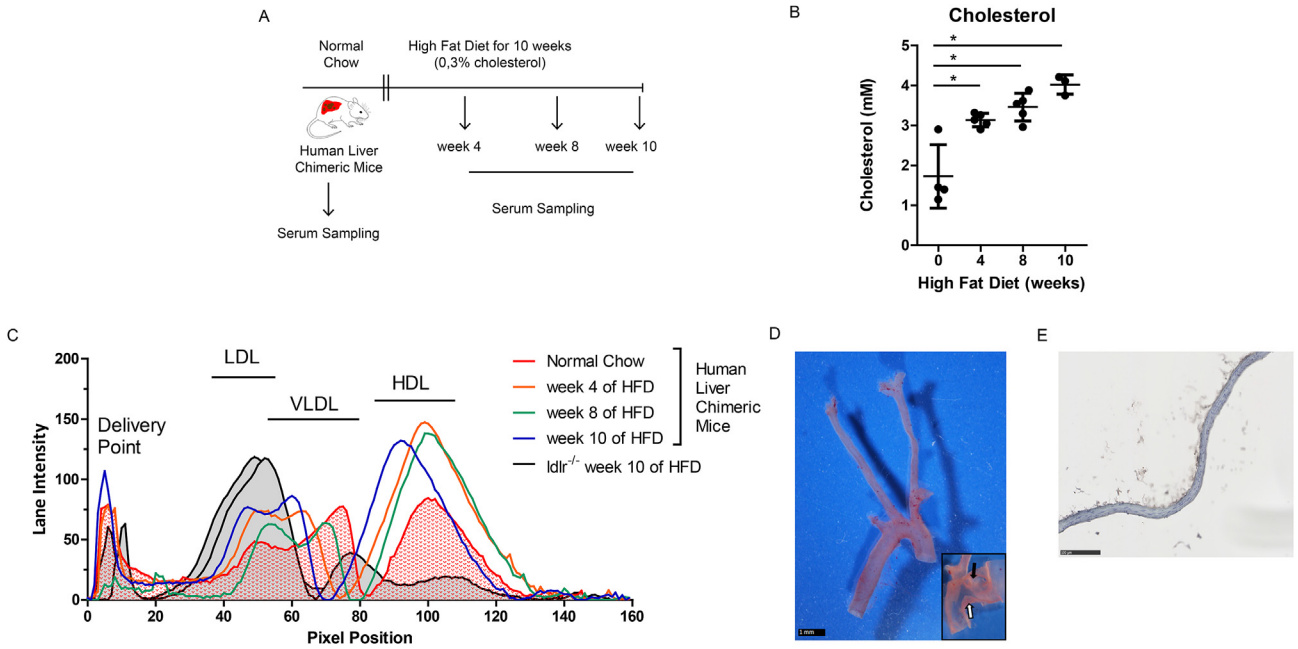

Fig. 2. Human liver chimeric mouse serum cholesterol levels increase following high fat diet (HFD) without significant plaque formation. **A.** Schematic representation of the experiments. **B.** Serum cholesterol levels of human liver chimeric mice during HFD. **C.** Sudan black lane intensity per pixel position after agarose gel electrophoresis of serum samples (n = 6) of human liver chimeric mice (n = 4, red/orange/green/blue lines) and ldlr$^{-/-}$ mice (n = 2, black line and grey surface area) fed with either normal chow (red line and dotted surface area) or HFD (all other mice). **D.** Perfused and ORO stained artery of a human liver chimeric mouse after 10 weeks of HFD. The inset shows a zoomed image of the arterial lumen after a longitudinal incision. The arrows indicate common locations of plaque formation: the black arrow indicates the location between two branching arteries of the aorta, the white arrow indicates the inner curve of the aorta. No ORO stained tissue is visible in the arteries. **E.** CD68 staining, as a marker of macrophages, of the aortic arch of a human liver chimeric mouse after 10 weeks of HFD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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mouse model is a promising and informative model in many research areas, including viral hepatitis and drug metabolism studies [15–18]. In the present study we assessed the human liver chimeric mouse as a candidate model for lipoprotein related disease studies. As is known, the mouse serum lipoprotein pool consists of HDL predominantly [21]. As shown in Fig. 1, repopulation of the mouse liver after transplantation of human hepatocytes leads to higher serum VLDL and LDL levels. Furthermore, this humanization of serum lipoproteins correlates with serum human albumin levels, a marker of successful and progressive liver humanization.

Inflammation and a disturbed lipid metabolism are crucial in atherosclerotic plaque initiation and progression. Most animal models mimic the human pathophysiology by interventions like a genetic modification such as ApoE or ldlr knock outs, PCSK9 knock-in and/or dietary modifications next to the genetic modification [7]. Different physiological mechanisms underlie this murine resistance to atherosclerosis: a different composition of mouse bile acid and salts, a lack of CETP expression and formation of lipoprotein (a), and also higher serum HDL to LDL ratios [7–9]. Because the human liver chimeric mouse model has humanized lipoprotein ratios, we hypothesized that the model might have the potential to be applied in atherosclerosis research without any genetic modifications altering the lipid metabolism. It is important to stress that in addition to humanized lipoprotein ratios and the presence of high amounts of oxidized LDL the presence, migration and also phagocytocytic capacity of macrophages are other key steps in atherosclerotic plaque formation [22],. As both uPANOG and TKNOG mice have an immunodeficient, IL-2Rγ^{null} background, we first demonstrated a preserved −albeit reduced− *E. c*oli phagocytic activity of splenic macrophages compared to ldlr$^{-/-}$ mice. Secondly, we found a HFD diet induced increase in serum total cholesterol levels, which is comparable to ApoE−/− but lower than ldlr$^{-/-}$ mouse fed with HFD [23,24] accompanied by elevations in LDL and VLDL fractions, but also an increase in serum HDL. These lipid changes did not however result in an accumulation of plaques in the aortic vasculature after a period of 10 weeks. The latter would normally suffice to induce plaque formation in ldlr$^{-/-}$ mice [25].

Several reasons might explain why our model does not demonstrate atherosclerotic plaque formation, despite having a humanized lipoprotein profile, functional macrophages and a substantial period of high fat diet. First, while LDL and VLDL increase, HDL shows a similar increase after HFD. The HDL increase might be reflecting the response of remaining mouse hepatocytes in chimeric livers to an increase in dietary cholesterol and provide anti-inflammatory signals that prevent plaque formation [26–29]. Second, the observed increase in LDL and VLDL is still lower than that of the ldlr$^{-/-}$ control mouse strain, which is prone to plaques. Third, Kupffer cell derived CETP governs the composition of circulating human lipoproteins, but is not expressed in mice so that mice carry the serum cholesterol in HDL [21]. Liver humanized mice harbor only human hepatocytes, and no CETP mRNA was detected in chimeric mouse livers (data not shown). Finally, other inflammatory cells which are not present in NOG mice might be required for inflammatory crosstalk and plaque initiation.

To conclude, our data show that along with the humanization of the liver, a progressive humanization of the serum lipoprotein profile is induced in human liver chimeric mice. This model therefore offers unique opportunities for translational studies on lipoprotein metabolism. Furthermore, having the advantage of harboring differentiated human hepatocytes, without any genetic changes, this model promises to be a tool for pharmacological studies of novel lipid lowering drugs that specifically act at the hepatocyte level. In addition, the model might be applied to study the roles of other inflammatory cells in the formation of atherosclerotic plaques via adoptive cell transfer experiments. Importantly, high fat diet and the presence of functional macrophages were found to be insufficient to initiate atherosclerotic plaque formation within the tested time window. These data suggest that extra factors or modifications are required for atherosclerotic plaque formation such as other immune cells, or longer durations of HFD.

Financial support

This study was supported by a 2014 MRace Pilot Grant from the Erasmus University Medical Center.

Declaration of competing interest

The authors declare no commercial relationships that might pose a conflict of interest in connection with the submitted manuscript.

Acknowledgements

The authors thank Dr. Menno Hoekstra, Leiden Academic Centre for Drug Research, Leiden University. Authors also would like to thank Gertine W. van Oord for the great technical assistance and help during the sample collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.01.067.

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Please cite this article as: G. Sari et al., A mouse model of humanized liver shows a human-like lipid profile, but does not form atherosclerotic plaque after western type diet, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.01.067