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VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced NASH model in mice

Arumugam Velayudham, Angela Dolganiuc, Michael Ellis, Jan Petrasek, Karen Kodys, Pranoti Mandrekar, and Gyongyi Szabo

Liver Center, Department of Medicine, University of Massachusetts Medical School, Worcester, MA

Abstract

Non-alcoholic fatty liver disease (NAFLD) and its advanced stage, non-alcoholic steatohepatitis (NASH), are the most common causes of chronic liver disease in the United States. NASH features the metabolic syndrome, inflammation, and fibrosis. Probiotics exhibit immunoregulatory and anti-inflammatory activity. We tested the hypothesis that probiotic VSL#3 may ameliorate the methionine-choline-deficient (MCD) diet-induced mouse model of NASH. MCD diet resulted in NASH in C57Bl6 mice compared to methionine-choline supplemented (MCS) diet feeding evidenced by liver steatosis, increased triglycerides, inflammatory cell accumulation, increased TNF α , and fibrosis. VSL#3 failed to prevent MCD-induced liver steatosis or inflammation. MCD diet, even in the presence of VSL#3, induced upregulation of serum endotoxin and expression the TLR4 signaling components, including CD14 and MD2, MyD88 adaptor, and NF- κ B activation. In contrast, VSL#3 treatment ameliorated MCD diet-induced liver fibrosis resulting in diminished accumulation of collagen and α -SMA. We identified increased expression of liver PPARs and decreased expression of pro-collagen and matrix metalloproteinases in mice fed MCD+VSL#3 compared to MCD diet alone. MCD diet triggered up-regulation of TGF β , a known pro-fibrotic agent. In the presence of VSL#3 the MCD diet-induced expression of TGF β was maintained, however, the expression of Bambi, a TGF β pseudoreceptor with negative regulatory function, was increased.

In summary, our data indicate that VSL#3 modulates liver fibrosis but does not protect from inflammation and steatosis in NASH. The mechanisms of VSL#3-mediated protection from MCD diet-induced liver fibrosis likely include modulation of collagen expression and impaired TGF β signaling owed to modulation of TGF β signaling.

Keywords

Tumor Necrosis Factor- α ; α -smooth muscle actin; peroxisome proliferator activated receptors; toll-like receptor 4 signaling; TGF β /Bambi

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States (1, 2). The advanced stage of NAFLD, non-alcoholic steatohepatitis (NASH), features liver inflammation and fibrosis, and has a strong association with the metabolic syndrome, including insulin resistance, dyslipidemia and

obesity (1–4). The complexity and the chronology of pathophysiological events leading to development of NAFLD/NASH are not fully understood. Among mechanisms of inflammation, tumor necrosis factor α (TNF α) appears to play a critical role in both insulin resistance and hepatic inflammatory cell recruitment in NAFLD/NASH (3–5). Furthermore, activation of nuclear factor κ B, a master regulator of inflammation, has been demonstrated in non-alcoholic fatty livers (3,6). Based on the similarities in the pathologic changes in the liver in NASH and alcoholic steatohepatitis, it has been proposed that endotoxin from gut-derived gram-negative bacteria and LPS-sensing machinery, including TLR4/MyD88 pathways, may play a role in the pathogenesis of NASH (7). Both fat metabolism and inflammation are regulated by peroxisome proliferator-activated receptors (PPAR) (1–3) suggesting that inflammation is a complex process regulated at different levels during development of NAFLD and its progression to NASH.

NASH can lead to liver fibrosis and cirrhosis (1). Recent studies suggest that most “cryptogenic” cirrhosis is the result of previously undiagnosed NASH (8); however, there are no known determinants of progressive liver damage at the cellular or molecular level and therapeutic modalities that could prevent progression of NASH are yet to be developed.

The human gut microflora is important in regulating host immune homeostasis (9). *In vivo* administration of VSL#3, a probiotic preparation of live, freeze-dried bacteria containing eight bacterial species [*S. salivarius* subsp. *thermophilus*, *Bifidobacterium* (*B. breve*, *B. infantis*, *B. longum*), *L. acidophilus*, *L. plantarum*, *L. casei*, *L. delbrueckii* subsp. *Bulgarius*], attenuated liver steatosis in ob/ob mice that present characteristics of NAFLD (10). Although the cellular and molecular basis of the action of probiotics is yet to be understood, some liver-related beneficial effects of VSL#3 treatment were found in a small cohort of patients with NAFLD (11).

Based on the features of NAFLD/NASH in humans, which include steatosis and liver inflammation followed by fibrosis (12), and based on the suggested anti-inflammatory properties of VSL#3 in animal models of chronic inflammation (10,13), we entertained the hypothesis that probiotic treatment with VSL#3 will ameliorate MCD-induced NASH by modulation of liver inflammation and/or fibrosis.

Materials and Methods

Animals and Experimental Protocol

The study obeyed IACUC regulations at the University of Massachusetts Medical School. Female C57BL/6 mice were fed a methionine-choline-deficient (MCD) diet or a methionine-choline-supplemented (MCS) diet; a group of MCD diet-fed mice also received VSL#3 (the protocol is detailed in Supplemental Materials). The MCD diet feeding represents an animal model of NAFLD/NASH, which reproduces several aspects of human diseases, such as liver steatosis, inflammation and fibrosis (14–16).

Preparation of serum and tissue, histopathological analysis, biochemical assays and cytokine detection were described previously (17,18) and detailed in the Supplemental Materials.

Electrophoretic Gel Mobility Shift Assay (EMSA) was performed using 5 μ g of nuclear protein; other proteins were quantified in **Western Blot**, as we described previously (17,18), and detailed in Supplemental Materials.

RNA analysis

Total RNA was extracted from liver tissue and mRNA analysis was performed using quantitative real-time PCR (qPCR) as we previously described (17,18). All specific mRNA

levels were corrected for the 18S internal control results from the same sample. The specific PCR primer sequences used in this study are listed in Table 1.

Statistical Analysis

Statistical significance for *in vivo* tests was determined using non-parametric Kruskal-Wallis and Mann-Whitney tests. Data are presented as mean $\pm$ SE; a *p* value ≤ 0.05 was employed as the statistical threshold of significance.

Results

VSL#3 treatment prevents fibrosis but not fatty liver and inflammation in the MCD-diet induced model of NASH

Administration of a methionine-choline-deficient (MCD) diet to C57Bl6 mice resulted in a classical pathophysiological picture of NASH compared to methionine-choline supplemented (MCS) diet feeding: first, disturbed lipid metabolism was suggested by micro- and macrovesicular steatosis (Fig 1A) and increased liver triglyceride levels (Fig 1B); second, inflammation occurred as indicated by multiple foci of inflammatory cell accumulations in the livers (Fig 1A) and by increased in serum (data not shown) and liver TNF α (Fig 1C); and, third, liver fibrosis occurred, as indicated by increased accumulation of collagen (Fig 1F) and α -SMA (Fig 1G). These changes induced by the MCD diet feeding lead to increased liver-to-body weight ratio (Fig 1D) and liver damage, as suggested by the elevated serum ALT (Fig 1E).

Co-administration of the probiotic VSL#3 had no significant effect on MCD diet-induced disturbance of fat metabolism as it failed to prevent steatosis (Fig 1A) or to protect from accumulation of liver triglycerides (Fig 1B). VSL#3 also failed to prevent the development of the inflammatory component of NASH as suggested by the lack of protection from inflammatory cell recruitment (Fig 1A) or elevation of MCD diet+VSL#3-induced TNF α increase (Fig 1C) compared to the MCD diet alone. VSL#3 treatment also failed to protect from MCD diet-induced liver injury (Fig 1E, 1D).

In contrast, co-administration of VSL#3 improved the progression of MCD diet-induced liver fibrosis as indicated by minimal evidence of collagen, identified by trichrome and sirius red staining (Fig 1F) in MCD+VSL#3 diet-fed mice compared to MCD diet alone. Further, the increased α -SMA expression in the livers of MCD diet-fed animals was significantly diminished by VSL#3-treatment as indicated by immunohistochemical staining (Fig 1G). Consistent with the histology staining results, western blot analysis revealed increased collagen (Fig 1F) and α -SMA (Fig 1G) protein levels in the liver of MCD but not of MCS or MCD+VSL#3 treated mice. These results demonstrated that VSL#3 has a beneficial effect in the MCD-induced model of NASH. Our data also suggested that VSL#3 attenuated the fibrosis, but not the inflammation and liver damage in MCD diet-induced steatohepatitis.

VSL#3 treatment inhibits expression of type I collagen and matrix metalloproteinases

Collagen deposition and fibrosis are the result of stellate cell activation in the liver (15,19). Recent evidence suggests that Peroxisome Proliferator-Activated Receptors (PPAR) may have anti-fibrotic effects (20,21). PPARs, especially PPAR α and PPAR γ isoforms, not only play an important role in non-alcoholic fatty liver disease through regulation of fat and glucose metabolism but also regulate stellate cell (SC) activity (20,21). The effect of probiotics on PPAR activity is largely unknown. Based on our findings of anti-fibrotic effects of VSL#3, we hypothesized that VSL#3 treatment may modulate liver PPAR activity.

Investigation of liver mRNA levels revealed significantly increased PPAR α (Fig 2A) and PPAR γ (Fig 2B) levels in VSL#3-treated mice compared to MCD diet feeding alone. While the PPAR activation and DNA binding were comparable in MCD and MCD+VSL#3-treated mice (Fig 2C), the antibody supershift indicated a predominant presence of PPAR α and RXR, and to a lesser extent of PPAR γ , in MCD- and MCD-VSL#3-induced PPAR activation (Fig 2D). Downstream in the activation pathway, the mRNA levels of the Peroxisome Proliferator-Activated Receptor- γ Coactivator 1 α (PGC-1 α), a transcriptional co-activator of PPAR (22), was significantly increased by VSL#3 treatment (Fig 2E).

Administration of MCD diet increased mRNA levels of pro-collagen 1 α and this response was attenuated in the presence of VSL#3 treatment (Fig 3A). Changes in pro-collagen I- α 1 mRNA levels closely mirrored changes in collagen protein levels and correlated with collagen expression detected by trichrome and Sirius red staining (Fig 1F).

Changes in collagen expression also correlated with the expression of matrix metalloproteinases (MMP) that play an important role in hepatic fibrosis (23). Stellate cells are important sources of MMP in the liver (23–24) and PPAR activators modulate MMP expression (25). We, thus, followed our finding of the elevated PPAR activity in the liver of MCD+VSL#3-compared to MCD-fed animals (Fig 2) and predicted that first, the MCD diet will elevate liver MMP levels, and second, VSL#3 treatment will modulate liver MMP levels. Indeed, we found a significant induction of MMPs, including MMP-2 and MMP-9, in livers of animals on MCD diet (Fig 3B,C). Confirming our hypothesis, co-administration of VSL#3 significantly attenuated the MCD diet-induced liver expression of both MMP-2 and MMP-9 (Fig. 3B,C).

Diet-induced steatohepatitis is associated with increased serum endotoxin levels, increased expression of the TLR4 receptor complex, and hyper-responsiveness to LPS stimulation

Previous studies suggested that gut-derived endotoxin plays a key role in development and progression of NASH (1–3, 5–7). Endotoxin is a potent activator of liver parenchymal and non-parenchymal cells, of which Kupffer cells and stellate cells govern the development of NASH (26). Further, probiotic bacteria prevent hepatic damage and maintain colonic barrier function in a mouse model of sepsis (13). We identified a moderate but statistically significant increase in serum LPS levels in mice with the MCD diet-induced steatohepatitis compared to MCS control diet-fed mice (Fig 4). However, VSL#3 treatment did not significantly affect serum endotoxin levels compared to the MCD diet alone (Fig 4). These data were in agreement with our findings that elevated serum (data not shown) and liver TNF α (Fig 1C) and ALT (Fig 1E) levels were not impacted by VSL#3 treatment, since the endotoxin is a major stimulator of the TNF α -producing immune cells and TNF α plays a key role in liver damage during NASH (26,27).

Based on these findings, we speculated that VSL#3 treatment would fail to prevent the activation of endotoxin-triggered inflammatory activation in the MCD diet-induced NASH model. LPS induces activation of the pro-inflammatory cascade and cellular activation via the Toll-like receptor 4 complex expressed on most of liver cells, including non-parenchymal (Kupffer and stellate cells), immune inflammatory cells, and hepatocytes (28). Thus, increased expression of the components of the TLR4 receptor complex, including TLR4, its co-receptors, CD14, MD-2, and the common TLR adapter MyD88 may increase cellular responses to LPS (28,29). We found that RNA levels of CD14 (Fig 5A), MD-2 (Fig 5B) and MyD88 (Fig 5C) were significantly upregulated after MCD or MCD+VSL#3 diet feeding compared to the control MCS diet. Further, MD-2 (Fig 5B) mRNA levels were increased by VSL#3 treatment while there was no significant change in the mRNA levels of TLR4 (Fig 5E) between mice on the different diets. These results suggested that MCD diet-

induced upregulation of the TLR4 co-receptors, CD14 and MD-2, and molecules involved in TLR4 downstream signaling, such as MyD88, may sensitize livers with steatohepatitis to increased responsiveness to LPS.

Activation of TLR4 triggers downstream signaling that culminates in activation of nuclear transcription factors (28,29). Among those, NF- κ B pathway plays a key role in activation of Kupffer cells and stellate cells during liver diseases (26). We found that baseline activation of NF- κ B was statistically similar in all analyzed groups (Fig 5E). Based on the fact that NASH is a multi-hit disease (1–3, 5–7) we further employed an exogenous LPS administration strategy to reveal the physiological relevance of our above-described findings in the LPS-sensing receptor complex. LPS challenge resulted in significantly higher NF- κ B nuclear translocation and DNA binding in the livers of MCD diet-fed mice compared to MCS control diet-fed mice (Fig 5E). Furthermore, VSL#3 administration augmented the LPS-induced NF- κ B activation in MCD diet-fed mice (Fig 5E). These results suggested that MCD diet-induced steatohepatitis activated pro-inflammatory cytokine induction pathways and VSL#3 treatment failed to attenuate the exaggerated pro-inflammatory activation in response to LPS.

VSL#3 modulates TGF β signaling pathways

Our data suggested so far that VSL#3 failed to ameliorate inflammation, yet prevented fibrosis in the MCD diet-induced model of NASH. While inflammation has been suggested as a prerequisite for development of fibrosis (7,12,26), the chronology and inter-dependence of inflammation and fibrosis are yet to be fully understood in NASH. More recently, a link between pro-inflammatory and pro-fibrogenic signals was suggested (26). Seki et al indicated that during liver inflammation, LPS downregulates the TGF β pseudoreceptor, Bambi, to sensitize the SC to TGF β -induced signals from inflammatory cells in a TLR4/MyD88-NF- κ B-dependent manner, thus modulating liver fibrosis (26). Based on our data, we predicted that the MCD diet-induced NASH could modulate TGF β expression and/or signaling due to endotoxemia and LPS-receptor-mediated signaling, similar to changes seen in other inflammation models (26). We further hypothesized that VSL#3 treatment could modulate the MCD diet-induced changes in expression of Bambi, the TGF β pseudoreceptor, and thus disrupt the pro-fibrotic TGF β signaling pathway, despite of an on-going inflammation. As shown in fig 6A, TGF β RNA levels were increased by MCD diet, suggesting that the MCD-triggered TGF β could contribute to stellate cell activation and collagen production (Fig 1, Fig 3). In the presence of VSL#3 during MCD diet, TGF β levels showed a decreasing trend that was not statistically significant (Fig 6A). However, we found that Bambi was significantly upregulated in the presence of VSL#3 treatment compared to MCD diet alone (Fig 6B). These data suggested that VSL#3 treatment promoted the expression of the TGF β pseudoreceptor Bambi that could arrest the stellate cells in a quiescent state.

Discussion

Our study shows that VSL#3 treatment prevents fibrosis in the methionine-choline deficient diet-induced NASH model without significant attenuation of the ongoing steatohepatitis. This observation supports the concept that *in vivo* fibrosis and steatohepatitis can be regulated independently (30,31) and points to a potentially new therapeutic application of VSL#3.

The current view on the mechanisms and progression of NASH favors a model in which steatosis and then steatohepatitis are induced as a result of fatty acid overload and inflammation, leading to subsequent activation of stellate cells that produce collagen and lead to liver fibrosis (1–3, 5–7,26). The key component in the mechanisms of fibrosis in the

liver is the activation of stellate cells that are the primary source of α -SMA and collagen deposition (14,20,26). Stellate cell activation is induced by multiple insults, including TNF α , and TGF β (4,5,7,26). We identified increased TNF α production in MCD diet-induced NASH, which remained elevated in MCD+VSL#3 treated mice, in agreement with studies from Ewaschek (13) and Hart (32). TNF α modulates SCs activation via a mechanism that involves inhibition of PPAR expression and its binding to the peroxisomal proliferator response element (PPRE) (33). We found an increase in PPAR mRNA levels in the livers of MCD+VSL#3-treated mice compared to MCD diet alone. While there were no significant differences in the levels of PPAP activity or in composition of the PPAR complex, we identified that VSL#3 treatment during MCD diet lead to an increase in PGC-1 α and a decrease in Coll1 α , which are targets of PPARs (34,35). Taken together, these changes suggested a role for PPARs in the anti-fibrotic effects of VSL#3. However, we identified that MCD diet upregulated TGF β , a known stellate cell activator (26). Because TGF β regulates collagen production (30,36), increased TGF β production could contribute to the MCD-induced fibrosis. VSL#3 inhibited fibrosis and, importantly, triggered the production of Bambi, a transmembrane protein highly similar to TGF β receptors (37). In contrast to regular TGF β R, the intracellular domain of Bambi is short and lacks a serine/threonine-kinase domain that is essential for transducing TGF β signals; thus Bambi functions as a pseudo-receptor and acts as a negative regulator of TGF β signaling pathway (26,37). To date the fine mechanisms of Bambi regulation are not fully understood. However, several authors reported that the BMP family, that also includes Bambi (37), is regulated via NF κ B-dependent mechanisms (26,38–40). We report increased NF κ B activity and elevated expression of Bambi in MCD diet-fed VSL#3-treated group compared to MCD-diet alone controls. Further, Bambi RNA changes mirror the protein levels and Bambi expression is restricted to stellate cells of the liver (26). Thus, in the presence of VSL#3, high levels of Bambi could prevent TGF β -induced signals, and control the unrestricted activation of stellate cells by on-going inflammation. These data are in agreement with those of Seki et al, who showed that downregulation of Bambi mRNA and protein expression, and subsequent sensitization to TGF β signals is mediated by MyD88/NF- κ B-dependent pathway and occurs with on-going liver inflammation (26).

We identified increased expression of the components of the signaling pathway initiated by LPS via TLR4, including CD14, MyD88 and NF- κ B during MCD-induced NASH and these were further exacerbated in MCD+VSL#3-treated mice, thus the increased serum LPS levels in the MCD-diet-fed mice are likely to contribute to the sustained inflammation. In light of these findings, and taking into consideration the significantly higher levels of serum ALT in VSL#3-exposed animals compared to MCD diet-fed controls, it is possible that VSL#3 treatment not only failed to inhibit but also augmented MDC diet-induced inflammation; such conclusion could not be reached due to the imprecise nature of histological scoring. VSL#3 has TLR2 and TLR9-stimulating capacity (41); both TLR2 and TLR9 share the MyD88-dependent signaling pathway with TLR4 (28,29). We did not identify changes of TLR2 levels (data not shown), however the increased expression of MyD88 in VSL#3-exposed animals could accommodate signaling via TLR2, TLR4 or TLR9. We also acknowledge that the presence/processing of VSL#3 *in vivo* is needed in order to achieve anti-fibrotic effects; in this context the effects of VSL#3 on gut, the gut microbiota-liver relationship, the detailed composition of VSL#3-derived microbial products and their specific interactions with stellate cells at biochemical and mechanistic levels remain the subject of future research.

In summary, our data indicate that VSL#3 modulates liver fibrosis but does not protect from inflammation and steatosis in NASH. Within the limitations of the animal model (14,15,18,42), our current working model takes into consideration a role for the endotoxin/TLR4/MyD88 pathway, but also acknowledges the differential contributions of TNF α –, NF-

κ B-, PPAR- and TGF β /Bambi-mediated activation pathways towards development of inflammation and fibrosis during NASH (Fig 7). Our results suggest that, at least in NAFLD/NASH model, the benefit of the VSL#3 treatment on fibrosis may occur even in the absence of significant changes in markers of inflammation and fat in the liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

α-SMA	alpha-smooth muscle actin
Bambi	Bone morphogenic protein and activin membrane-bound inhibitor
ip	intraperitoneal
LPS	lipopolysaccharide
MCD	methionine-choline-deficient
MCS	methionine-choline-supplemented
MMP	matrix metalloproteinase
MyD88	myeloid differentiation primary response gene 88
mRNA	messenger ribonucleic acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NF-κB	Nuclear factor kappa B
PGC-1α	Peroxisome Proliferator-Activated Receptor- γ Coactivator 1 α
PPAR	Peroxisome Proliferator-Activated Receptor
PPRE	peroxisome proliferator-response element
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TGFβ	transforming growth factor beta
SC	stellate cells

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Fig. 1.

VSL#3 treatment failed to prevent MCD diet-induced liver injury.

Mice were fed MCS or MCD diet for 10 weeks; VSL#3 was administered for the last 9 weeks of the MCD diet. Liver-to-body weight ratio was determined. Data are shown from 6 mice per experimental group.

Liver histology was assessed after hematoxylin and eosin staining of liver tissue (A, representative picture, magnification 100X; the * indicates inflammatory foci); liver/body ratio (D), liver triglycerides (TG) (B), liver TNF α (C), and serum ALT (E) were determined from n=6/group.

(F) Liver sections were analyzed for collagen expression with trichrome (top panel) and Sirius Red (medium panel) staining; liver collagen I protein content was quantified in Western blot using equal amounts of total liver proteins from each animal; one representative blot and densitometric analysis from n=6/group (bottom panel) are shown. (G) Liver sections were analyzed for α -SMA expression using immunohistochemistry (top panel) staining; liver α -SMA protein content was quantified in Western blot using equal amounts of total liver proteins from each animal; one representative blot and densitometric analysis from n=6/group (bottom panel) are shown.

Fig. 2.

VSL#3 induces expression of peroxisome proliferator-activated proteins.

The expression of liver peroxisome proliferator-activated receptor (PPAR)- α (A), PPAR- γ (B), and PPAR- γ coactivator 1 α (PGC-1 α) (E) were assessed using quantitative PCR. Data are shown as fold increase of MCD or MCD+VSL#3 group over control MCS diet with 6 mice/group.

(C) Equal amounts of nuclear proteins were analyzed in EMSA for binding to the PPAR response element (PPRE). One sample was pre-incubated with cold PPRE oligonucleotide prior to EMSA as specificity control (C); a representative EMSA gel (top) and the densitometric analysis from 6 mice/group (bottom panel) are shown.

(D) Equal amounts of nuclear proteins were analyzed pre-incubated with anti-PPAR α , -PPAR γ or -RXR antibodies and subjected to EMSA for binding to the PPAR response element (PPRE); a cold competition control (Comp) was included as above. A representative

EMSA gel (top) and the densitometric analysis from 6 mice/group (bottom panel) are shown.

Fig. 3. VSL#3 limits the expression of MCD diet-induced matrix metalloproteinase in the liver. The liver RNA levels of liver procollagen I- α 1 (A), matrix metalloproteinase (MMP)-2 (B), and MMP-9 (C), and the 18S control were analyzed using qPCR. Data are shown as fold increase of MCD or MCD+VSL#3 group over control MCS diet, all adjusted to 18S internal controls, with 6 mice/group.

Fig. 4. VSL#3 failed to protect from MCD diet-induced endotoxemia. Mice were fed MCD or MCD diet for 10 weeks; VSL#3 was administered for the last 9 weeks of the MCD diet. Serum levels of endotoxin were analyzed at the end of the 10-week feeding period using a Limulus Amebocyte Lysates assay. Mean $\pm$ SE data from 6 mice/group are shown.

Fig. 5.

VSL#3 augments MCD diet-induced modulation of the LPS signaling complex. The liver mRNA levels of CD14 (A), MD-2 (C), MyD88 (C), toll-like receptor (TLR) 4 (D), and 18S were analyzed using qPCR. Data are shown as fold increase of MCD or MCD +VSL#3 group over control MCS diet, all adjusted to corresponding 18S housekeeping controls, with 6 mice/group.

(E) Mice were fed MCS or MCD diet for 10 weeks; VSL#3 was administered for the last 9 weeks of the MCD diet. At the end of the 10 week-feeding period, the animals were challenged with LPS (0.5mg/kg body weight, i.p. for 1.5 hours). Liver nuclear extracts were analyzed for NF-κB binding activity in EMSA using specific radioisotope-labeled

oligonucleotides; 20x excess of unlabelled oligonucleotide was used for cold competition (Comp). A representative gel is shown on the top and the densitometric analysis from 6 mice/group is shown on the bottom of each panel. * represents $p < 0.01$ compared to the saline group with the same diet feeding.

Fig. 6.

VSL#3 modulates TGF β pathway

The liver RNA levels of TGF β (A), Bambl (B) and 18S were analyzed using qPCR. Data are shown as fold increase of MCD or MCD+VSL#3 group over control MCS diet, all adjusted to corresponding 18S housekeeping controls, with 6 mice/group.

Fig. 7.
Hypothetical model of the effects of VSL#3 on MCD-induced NASH model.

Table 1

qPCR primers.

Target gene	Forward primer (5'→3')	Reverse primer (5'→3')
18S	gta acc cgt tga acc cca tt	cca tcc aat cgg tag tag cg
TNF α	cac cac cat caa gga ctc aa	agg caa cct gac cac tct cc
TLR4	gcc ttt cag gga att aag ctc c	aga tca acc gat gga cgt gta a
CD-14	gga agc cag aga aca cca tc	cca gaa gca aca gca aca ag
MD-2	gac gct gct ttc tcc cat a	cat tgg ttc ccc tca gtc tt
MyD88	aga aca gac aga cta tcg gct	cgg cga cac ctt ttc tca at
PPAR α	aac atc gag tgt cga ata tgt gg	agc cga ata gtt cgc cga aag
PPAR γ	gga aga cca ctc gca ttc ctt	tcg cac ttt ggt att ctt gga g
PGC1 α	aga cgg att gcc ctc att tga	tgt agc tga gct gag tgt tgg
TGF- β 1	att cct ggc gtt acc ttg	ctg tat tcc gtc tcc ttg gtt
MMP-2	ttt gct cgg gcc tta aaa gta t	cca tca aac ggg tat cca tct c
MMP-9	tgc cca ttt cga cga cga c	gtc cag gcc gaa tag gag c
Procollagen-I	gct cct ctt agg ggc cac t	cca cgt ctc acc att ggg g
Bambi	aaa act tca gac ggg tgt gg	tgg tgc tgg aga aat cac ag