Liver X receptor activation enhances CVB3 viral replication during myocarditis by stimulating lipogenesis

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Received 5 May 2014; revised 30 March 2015; accepted 13 May 2015; online publish-ahead-of-print 21 May 2015

Time for primary review: 24 days

Aims
Viral myocarditis (VM) is severe cardiac inflammation that can result in sudden death or congestive heart failure in previously healthy adults, with no effective therapy. Liver X receptor (LXR) agonists have both anti-inflammatory and lipid-lowering properties. This study investigates whether LXR agonist T0901317 may modulate viral replication and cardiac inflammation during VM.

Methods and results
(i) Adult mice were administered T0901317 or vehicle with the onset of inflammation during CVB3 virus myocarditis or (ii) treated 2 days prior to CVB3 infection. Against what we expected, T0901317 treatment did not alter leucocyte infiltration after CVB3 infection; yet pre-administration with T0901317 resulted in increased mortality upon CVB3 infection, higher cardiac viral presence, and increased cardiomyocyte damage when compared with the vehicle. Furthermore, we show a correlation of fatty acid synthase (FAS) and sterol regulatory element-binding protein 1c (SREBP-1c) with CVB3 viral load in the heart and that T0901317 is able to enhance the cardiac expression of FAS and SREBP-1c. Finally, we show in vitro that T0901317 is able to exaggerate CVB3-mediated damage of Vero cells, whereas inhibitors of FAS and the SREBP-1c reduce the viral presence of CVB3 in neonatal cardiomyocytes.

Conclusion
LXR agonism does not modulate cardiac inflammation, but exacerbates virus-mediated myocardial damage during VM by stimulating lipid biosynthesis and enhancing CVB3 replication.

Keywords
Myocarditis • Viral replication • Inflammation • Liver X receptors • Lipids

1. Introduction
Viral myocarditis (VM) is inflammation of the heart due to common viruses and is an important cause of cardiac failure and sudden death in previously healthy children and adults. The heterogeneity in patient clinical presentation makes for difficult and time-consuming diagnosis, with no specific immune-modulatory therapy recommended as current guidelines only endorse management of ventricular dysfunction using the guidelines for heart failure. VM is the combination of adverse inflammation and uncontained viral infection in the heart; therefore, effective therapy needs to target both aspects of the disease. The CVB3 virus is a common causative agent in human myocarditis belonging to the Picornaviridae and is a non-enveloped, single-stranded positive RNA virus. While virus–host interactions are numerous and complex, it is becoming evident that the viral replicative cycle requires a certain amount of lipid availability in order to be successful. Non-enveloped viruses such as the CVB3 predominantly release progeny by membrane disruption causing extensive cellular damage, but transient lipid envelopment followed by exit through the secretory route has also been described. Resident leucocytes, such as macrophages, raise the alarm to viral presence and are the first-line defence in the heart; yet, in order to clear infected cardiomyocytes and limit viral injury, they cause collateral damage in the process. Targeting the high presence of macrophages either through depletion or reduction of...
pro-inflammatory macrophages has proved to be beneficial on cardiac injury and subsequent disease outcome.

Liver X receptors (LXRs) belong to the family of nuclear hormone receptors of transcription factors that are critical in the regulation of cholesterol, fatty acids, and glucose homeostasis as well as inflammation.9 While LXRβ is expressed ubiquitously, LXRα is expressed highly in liver, spleen, intestine, heart, and macrophages.10 LXRα is able to regulate the expression of important cholesterol transporters (e.g. ATP-binding cassette transporter), which mobilize cholesterol from peripheral tissues back to the liver, so-called reverse cholesterol transport. This has led to promising results in preclinical studies where LXR agonists attenuate lesion formation and cholesterol absorption in mouse models of atherosclerosis.11 However, this comes at the expense of enhanced accumulation of lipids in the liver as administration of synthetic receptor agonists results in unfavourable hypertriglyceridaemia and hepatic steatosis,12 which limits the clinical use of synthetic LXR agonists. Mechanistically, LXRs regulate a wide array of downstream genes—such as the ligendipenic fatty acid synthase (FAS) and the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c).13 Finally, LXRα can modulate immune and inflammatory responses in macrophages via the transrepression of NfκB genes.14 As a result of these anti-inflammatory properties, LXRα agonists are being heralded for the treatment of multiple diseases ranging from dermatitis,15 chronic obstructive pulmonary disease,16 as well as atherosclerosis.17 In light of the lack of therapies for VM and the reported anti-inflammatory function of LXR agonists, we hypothesized that LXR agonism would be beneficial during VM.

2. Methods

2.1 Animal experiments

Mice were maintained in an open animal facility (University of Leuven, Belgium). Male C3H/HeN-Hsd mice (4–6 weeks old) were administered T0901317 via the chow ad libitum, as described.18 The approximate daily dose of T0901317 was 50 mg/kg/day. To study the effect of LXR agonist in VM, two experiment designs were performed: (i) adult mice were administered T0901317 or vehicle with the onset of inflammation caused by CVB3 infection (at Day 5) or (ii) treated 2 days prior to CVB3 infection. To induce VM, mice were injected intraperitoneally with 105 CCID50 of CVB3 as previously described.19 Mice were anaesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg) once via intraperitoneal injection, and blood was taken for routine clinical diagnostic assessment of HDL, TG, and cholesterol levels as well as CVB3 levels. The number of CVB3 copies is expressed as the log10 value of the absolute number, per micro-liter plasma. After cervical dislocation, organs were excised, weighed, and taken for further histological and molecular analyses.

2.2 Histological analysis

Hearts were fixed in 1% paraformaldehyde and processed for further standard histological stainings [e.g. haematoxylin and eosin (H&E) as well as Oil Red O staining]. Paraffin-embedded sections (4 μm) were immunolabelled with anti-CD45 antibody (Phar malign), anti-CD3 (Dako), anti-CD68 (SantaCruz), and anti-LXRα (R&D systems), and quantified per myocardial area. Mosaic images at ×5 magnification were acquired using a Zeiss Axiosvert 200M equipped with an AxioCam HRC digital camera. Subsequently, each image was processed with Fiji distribution of the well-known ImageJ software suite and analysed with the colour deconvolution tool.20 Using the threshold command provided in the software applied on the different channels, myocardial necrosis was determined as a percentage of necrotic cardiac area per total cardiac area and, using the same methodology, immune cells infiltration was calculated as a percentage of CD45+ area per total myocardial area, or CD3/CD68+ cells per mm². Morphometric analysis was performed by a person unaware of the experimental treatment.

2.3 Molecular analysis

RNA was extracted from heart tissues using the RNeasy® Kit (Qiagen GmbH, Hilden, Germany). For quantitative real-time PCR analysis, 1 μg of total RNA was reverse-transcribed into cDNA, using the IQ SYBR-green supermix (Bio-Rad, Veenendaal, The Netherlands) in a MyIQ iCycler (Bio-Rad). Primers were designed to span exon–exon junctions where possible and can be found in Table 1.

<table>
<thead>
<tr>
<th>Gene (species)</th>
<th>5′–3′ primer</th>
<th>3′–5′ primer</th>
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<td>CVB3</td>
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<tr>
<td>FAS (mouse)</td>
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<td>FAS (human)</td>
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<td>CTTCGCGACACCCTGACGA</td>
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<tr>
<td>GAPDH (mouse)</td>
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<td>CTCTCTTTGTCCAGTGCTTGT</td>
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<td>GAPDH (human)</td>
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<td>GAGAAAATTCTTGGAAAGG</td>
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<tr>
<td>SREBP-1c (mouse)</td>
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<td>SREBP-1c (human)</td>
<td>TGGCGGAAGGGCTTCTCTT</td>
<td>CTCGACATCATATGTGTC</td>
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<tr>
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<td>SRF</td>
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<td>GCCACTCTCTTGCTGACTCC</td>
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</tr>
<tr>
<td>TLR3</td>
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All gene expression data are normalized for GAPDH. Troponin T levels were measured using a high sensitivity ELISA (Roche Diagnostics).

### 2.4 Cardiac cell fraction and FACS analysis

Immediately after euthanasia of the mice, the heart was dissected out, the atria removed and placed in PBS, for isolation of the immune cell fraction. Briefly, the ventricles were minced into 1 mm pieces with a sterile surgical blade, and placed in an enzymatic solution (125 U/mL of collagenase type XI, 60 U/mL of hyaluronidase type I-s, 60 U/mL of DNase1, and 450 U/mL of collagenase type I, Sigma-Aldrich) for 1 h at 37°C. After trituration, the single cell suspension was underlaid with Histopaque solution, in order to separate the immune from the non-immune fraction. The interphase (immune fraction) was rinsed with PBS, and cell pellets were either lysed for further molecular analysis or leucocyte analysis by FACS analysis. Leucocyte subpopulations were defined as follows: neutrophils (CD45+, CD11b+, Ly6G+), monocytes (CD45+, CD11b+, Ly6G+), pro-inflammatory monocytes (CD45+, CD19−, CD11b+, F4/80−, Ly6C+), macrophages (CD45+, CD11b+, Ly6G−, F4/80+) CD4+ T-helper lymphocytes (CD45−, CD3+, CD4+), cytotoxic lymphocytes (CD45−, CD3+, CD8+), and B lymphocytes (CD45−, B220+). For FACS analysis, the following antibodies were used: anti-CD45 (Biolegend, clone 30-F11), F4/80 (Biolegend, clone BM8), CD45R/B220 (Bioscience, clone RA3-6B2), Ly6G (BD, clone 1A8) or CD11b (BD, clone M1/70), Ly6C (Miltenyi, 1G7.G10), CD3 (Bioscience, clone 145-C11), CD4 (BD, clone RM4-5), and CD8 (BD, clone 53-6.7).

### 2.5 Isolation of adult cardiomyocytes

Adult mouse cardiac cells were extracted by enzymatic digestion (collagenase I, Worthington, UK). The hearts were dissected and immediately perfused with calcium-free Tyrode solution via a Langendorff perfusion system. After enzymatic digestion, calcium was slowly reintroduced. Freshly isolated, adult cardiomyocytes were lysed directly in RNA lysis buffer for further molecular analysis.

### 2.6 In vitro experiments

Vero-E6 cells (African green monkey kidney epithelial cells; ATCC CRL-1586) were maintained in Dulbecco's modified Eagle medium (11966, Gibco, Belgium) enriched with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics.

HL-1 cells were grown in Claycomb Medium™ (JRH Bioscience, Lenexa, KS, USA) supplemented with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA), 0.1 mM norepinephrine, 2 mM l-glutamine (Invitrogen Corporation), and antibiotic/antimycotic solution (complete medium). All culture dishes and flasks were precoated with 0.5% fibronectin (BD Biosciences, Breda, The Netherlands) in 0.02% gelatin (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Neonatal rat ventricular cardiac myocytes were isolated by enzymatic dissociation of 1- to 2-day-old Lewis rat pups, plated on gelatinized six-well plates, and cultured as previously described. Cells were grown till 90% confluence and placed for 4 h in 2% FCS-supplemented medium prior to stimulation. Stimulation was with LXR agonist (TO-901317, Sigma-Aldrich, 1 μM) and/or the FAS inhibitor (C75, Sigma-Aldrich, 50 μM), and/or the SREBP inhibitor [25-hydroxycholesterol (25-HC), Sigma-Aldrich, 50 μM] or LXR agonist (GW3965, Sigma-Aldrich, 1 μM).

### 2.7 FAS activity

Cells were grown till 90% confluence and placed for 4–6 h in 2% FCS-supplemented medium prior to stimulation with TO901317 (1 μM) alone or with the CVB3 [multiplicity of infection (MOI) = 0.3] overnight. For FAS activity, a fixed amount of cell homogenate (10 μg protein) was used and 20 μL of reaction mix [10 mM NADPH, 25 mM acetyl-CoA, 25 mM malonyl-CoA, and 166 μM [2–14C]malonyl-CoA (60 μCi/mmol); PerkinElmer, Zaventem, Belgium] was added, and samples were incubated for 15 min at 37°C. Reactions were stopped by the addition of 1 mL of ice-cold 1 M HCl/methanol (6:4, v/v). Fatty acids were extracted with petroleum benzine, and incorporation of [2–14C] malonyl-CoA was analysed by scintillation counting.

### 2.8 Cytopathogenic effect assay

Eighteen hours before infection with CVB3, the inhibitors (FAS inhibitor, SREBP inhibitor, or LXR agonist) were added on top of the cells only 1 h before infection (i.e. 1 h after treatment with the inhibitors). The final concentration of the FAS inhibitor (C75) was 30 μM; of the SREBP inhibitor (25-HC) was 50 μM; and of LXR agonist (T09) was 1 μM. At 90% confluence, cells were infected with CVB3 (Nancy strain) with an MOI of 0.5. Eight hours later, the virus-infected medium was removed, cells were washed two times with culture medium, and fresh culture medium was put on the monolayer. Seventy-two hours after infection, the cytopathogenic effect (CPE) was scored under light optical microscopic imaging (Zeiss) at ×10 magnification. A CPE score of 0 = no CPE observed; 1 = a few apoptotic cells, no necrotic zones; 2 = CPE present, however, less than the proportion of healthy cells; 3 = proportion of CPE equals that of healthy cells; 4 = proportion of CPE exceeds that of healthy cells; and 5 = complete CPE, necrosis, clustering, and empty zones with debris.

### 2.9 Ethics

Experiments were performed according to the guidelines for the care and use of laboratory animals approved by the institutional animal committee of the University of Leuven conform to the European Commission guidelines (project license 067/2008, 243/2013).

### 2.10 Statistics

Data are expressed as mean ± SEM. An unpaired Student’s t-test was used when comparing two groups, which passed the normality test. For multiple group comparisons, an ANOVA test was used with Bonferroni post hoc analysis. Survival analysis was performed using the Mantel–Haenzel log-rank test. For the statistical analysis of Troponin T levels, a non-parametric Mann–Whitney test was performed, as data did not pass the normality test. For the correlation analysis of FAS and SREBP vs. CVB3 levels, FAS and SREBP levels were normalized to a housekeeping gene (GAPDH) and expressed as a fold change of the average sham group value. CVB3 levels were normalized to GAPDH, but not normalized to sham. A linear regression with Spearman or Pearson correlation coefficient was calculated. P-values of <0.05 were considered statistically significant. The Prism software was used as a statistics platform.

### 3. Results

#### 3.1 LXRs up-regulation during VM

Seven days after CVB3 infection, there is significant infiltration of leucocytes within the myocardium, comprising cells of both the innate and adaptive immune systems (Figure 1A). While there is a significant increase in infiltrating lymphocytes (T- and B-cells), macrophages and pro-inflammatory monocytes are the most prominent leucocytes within the heart after 1 week (Figure 1B). Macrophages express high levels of LXRα and LXRβ, whereas both T- and B-cells express low levels of both receptors (Figure 1C). Neutrophils express low levels of both LXR receptors when compared with macrophages, yet uniquely express more LXRβ than LXRα. Upon CVB3 infection, there is a significant up-regulation of LXRα but not of LXRβ in whole cardiac tissue (Figure 1D). Furthermore, in ex vivo adult cardiomyocytes, both LXRα and LXRβ are up-regulated after viral infection (Figure 1E). Finally, histological analysis shows the expression of LXRα in virus-infected cells.
Figure 1 LXRα is up-regulated upon CVB3 infection in vivo. (A) Representative flow cytometry plots of the leucocyte cardiac infiltration before and after CVB3 infection, identifying the major leucocyte subpopulations. (B) Quantification of the cardiac leucocyte infiltrate demonstrates that while all leucocyte populations increased due to CVB3 infection, macrophages and monocytes constitute the largest subpopulation in the heart (n = 5). (C) Gene expression analysis of LXRα and LXRβ in isolated leucocyte populations demonstrates that macrophages expressed both receptors in high levels when compared with the other subpopulation (n = 3). (D) Whole-heart expression of LXRα but not LXRβ increased significantly due to VM, (E) whereas in isolated adult cardiomyocytes both receptors were up-regulated due to viral infection (n = 8). (F) Immunohistochemical analysis revealed expression of LXRα strongly on infiltrating cells (black arrows), but also on cardiomyocytes (red arrows) during viral infection. *P < 0.05; **P < 0.01. Scale bar represents 50 μm.
cardiac tissue when compared with non-infected hearts (Figure 1F). In non-infected mice, only a mild signal is present, which increases significantly after CVB3 infection (black arrows, Figure 1F). Interestingly, LXRα staining is present in cardiomyocytes bordering the infiltrating immune cells after CVB3 infection (red arrows).

3.2 LXR activation does not reduce cardiac inflammation upon CVB3 infection in vivo
Administration of LXR agonist T0901317 (T09, daily 50 mg/kg) or vehicle commenced with the onset of inflammation at Day 5 and the effect on leucocyte infiltration was assessed (Figure 2A). We performed immunohistochemistry for CD45+ leucocytes and confirmed the onset of leucocyte influx at Day 5 (Figure 2B and C). Leucocyte infiltration continued to increase significantly at Day 11 after infection; yet the amount of cardiac inflammation did not alter after LXR agonist treatment. Further analysis of the T-lymphocytic (CD3+) and monocyctic (CD68+) cardiac population showed no differences due to T0901317 treatment (see Supplementary material online, Figure S1A and B). Histological analysis of the heart (via deconvolution of a H&E staining of the heart) showed the cardiomyocyte necrosis at Day 11, but T0901317 administration did not affect the amount of necrosis in the heart (see Supplementary material online, Figure S1C). While CVB3 levels in the heart were highest at Day 5, suggesting that the peak in viraemia precedes the inflammatory phase, cardiac viral levels did not significantly differ between T0901317 treatment and vehicle at Day 11 (Figure 2D). Similarly to what has been previously reported, we observed significant hepatic steatosis with LXR agonist treatment (Figure 2E). Lipid synthesis is regulated by the key enzyme complex FAS23 and its transcription factor SREBP-1c,24 established gene targets of LXRs. Finally, cardiac expression of target genes FAS and SREBP-1c

Figure 2  LXR activation does not protect against adverse cardiac inflammation upon CVB3 infection in vivo. (A) Experimental design: 5 days after CVB3 infection, in conjunction with the onset of cardiac inflammation, mice were randomized to normal chow (vehicle; n=12) vs. LXR agonist-enriched chow (T09-treated, n=12). (B) Representative images of CD45 (pan leucocyte marker) staining in the heart at baseline, Day 5, and Day 11 (T09- and vehicle-treated). (C) Quantification of the CD45+ stainings shows that no differences were seen in the CD45+ cardiac presence at Day 11 due to T09 treatment. (D) CVB3 viral levels in the hearts were highest at Day 5, but not significantly increased due to T09 treatment. (E) T09 treatment increases significantly the liver weights. (F) Viral infection of the heart results in increased FAS and SREBP-1c expression at Day 5 and Day 11, which is significantly higher in T09-treated mice. *P < 0.05; ***P < 0.001. Scale bar represents 1000 μm.
steadily increased at Days 5 and 11 after viral infection, which was significantly enhanced with LXR agonist treatment (Figure 2F).

### 3.3 LXR activation predisposes mice to increased mortality upon viral infection with CVB3

To investigate the effect of LXR activation prior to VM, adult male mice were administered LXR agonist T0901317 or vehicle 2 days prior to infection with the CVB3 virus (Figure 3A). Under these experimental conditions, increased mortality was observed in T09-treated mice (Figure 3B, 5/17) when compared with vehicle-treated mice (0/14, \( P = 0.03 \)). Histological analysis of the heart showed no differences in CD45\(^+\) leucocytes (Figure 3C), CD68\(^+\) monocytes (see Supplementary material online, Figure S1A), or CD3\(^+\) lymphocyte infiltration (see Supplementary material online, Figure S1B), nor difference in cardiomyocyte necrosis (see Supplementary material online, Figure S1D).

Further pathological examinations revealed that mortality was related to enhanced inflammation and necrosis of the small and large intestine (data not shown). Interestingly, CVB3 gene expression in the cardiac tissue was significantly higher in T09-treated mice when compared with mice treated with vehicle (Figure 3D). Similarly to the previous experimental design, we observed hepatic steatosis in the mice treated with LXR agonist (Figure 3E). We found that both lipogenic factors, FAS and SREBP-1c, were significantly increased in the cardiac tissue of T09-treated mice (Figure 3F). To determine whether there was increased myocardial damage due to the increased viral load, we measured levels of circulating Troponin T (Figure 3G). Mice receiving LXR agonist had higher levels of Troponin T when compared with vehicle-treated mice (\( n = 5 \) for vehicle-treated vs. \( n = 3 \) for T0901317-treated mice, respectively, \( P = 0.036 \)). Furthermore, gene expression of natriuretic peptides, ANP and BNP, in cardiac tissue, were significantly higher in the LXR agonist-treated group (\( n \geq 6 \) per group, Figure 3H). Finally, gene expression of myosin heavy chain beta (MHC\(_{B} \)), serum response factor (SRF), and IL-6, markers of cardiomyocyte stress, were significantly higher in mice treated with T0901317 (Figure 3I).

![Figure 3](https://academic.oup.com/cardiovascres/article-abstract/107/1/78/514331) LXR activation predisposes mice to increased mortality upon viral infection with CVB3. (A) Experimental design: 2 days prior to CVB3 infection, mice were randomized to normal chow (vehicle, \( n = 17 \)) vs. LXR agonist-enriched chow (T09-treated; \( n = 14 \)). (B) Survival curve shows significant difference between the two groups (\( P = 0.03 \)). (C) Representative images of CD45 staining in the heart Day 11 (T09- and vehicle-treated). No differences were seen in the leucocytic cardiac presence at Day 11 due to T09 treatment. (D) CVB3 viral levels in the hearts are significantly increased in the LXR chow group (\( P = 0.03 \)). (E) T09 treatment increases significantly the liver weights resulting in hepatic steatosis. (F) T09-treated resulted in increased FAS and SREBP-1c expression at Day 11. (G) LXR agonism increases levels of circulating troponin T (\( n = 5 \) and 3 for vehicle- and T09-treated mice, respectively, \( P = 0.036 \)). (H) Cardiac gene expression of the cardiac hormones ANP and BNP are significantly higher when mice are treated with T09. (I) LXR agonism increases the cardiac expression of cardiomyocyte stress markers MHC\(_{B}\), SRF, and IL-6 (\( n \geq 6 \)). *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \). Scale bar represents 1000 \( \mu m \).
3.4 Infection with CVB3 causes dyslipidaemia

The in vivo experiments suggested that LXR agonism during viraemia affects mortality, warranting further exploration. Analysis of CVB3 levels in the plasma showed that, at Day 2, there was a rapid rise in CVB3 genome copies, which were significantly lower at Day 4, and almost cleared out of the circulation by Day 7 (Figure 4A). The spike in plasma CVB3 levels coincided with the increased circulating enzymes ALT and AST, indicative of liver damage (Figure 4B). These data suggest that CVB3 virus undergoes a rapid expansion in the liver and is subsequently released into the plasma in order to enter other organs such as the pancreas, spleen, and heart. Lipids in the circulation, such as triglycerides, cholesterol, and high-density lipoprotein, did not increase at Day 2, but rose from Day 4 onwards and increased significantly at Day 7 after infection (Figure 4C–E). Remarkably, this did not correlate with the time of liver damage but with significant increases in heart weight (Figure 4F) caused by infiltrating leucocytes and oedema at Day 7 (Figure 4G). Furthermore, CVB3 levels in the heart started to increase significantly at Day 2 and continued to rise, plateauing at Day 7 (Figure 4H). The association of lipid availability in the heart and viral replication was substantiated by the significant correlation of cardiac viral levels and FAS expression (Figure 4I) as well as SREBP-1c expression (Figure 4J). Finally, Oil Red O staining of the lipid content in the heart demonstrated increased lipid droplets in the heart, and also in the areas of leucocyte infiltration and cardiomyocyte damage (Figure 4K). This was not observed in sham hearts (Figure 4K) or in remote areas of the heart where no infection was visible (data not shown).

3.5 LXR activation increases lipogenesis facilitating viral replication and cytolysis

We further explored the levels of FAS and SREBP-1c in vivo and in vitro during viral infection and with T0901317 treatment. In vivo expression levels of FAS (Figure 5A) increased in the heart from Day 4 onwards and significantly at Day 7, in line with the viraemic phase of VM and prior to the onset of inflammation. To confirm that LXR agonism affects FAS protein levels, we investigated whether FAS enzymatic activity was affected in Vero cells, a monkey kidney epithelial cell line often used to measure viral replication (Figure 5B). LXR agonist T0901317 alone induced a significant increase in FAS activity ($P = 0.04$). Exposure of Vero cells to the CVB3 virus resulted in an increase, though not significant, in FAS activity ($P = 0.2$), which was significant when combined with LXR agonism ($P = 0.02$). In vivo expression levels of SREBP-1c increased in the heart from Day 4 onwards and significantly at Day 7, similarly to FAS levels (Figure 5A). Furthermore, in vitro stimulation with T0901317 (1 μM) of Vero cells significantly increased FAS levels (see Supplementary material online, Figure S2A), which was significantly increased.

Figure 4 Infection with CVB3 causes liver damage and dyslipidaemia. (A) Plasma levels of CVB3 2, 4, and 7 days after CVB3 infection show a peak in CVB3 levels at Day 2 in the circulation (minimum of $n = 5$ per group). (B) AST/ALT plasma levels at 2, 4, and 7 days after CVB3 infection show significant increases at 2 days after CVB3. (C) Plasma triglyceride levels increase significantly after CVB3 infection at Day 7 alone, which is mirrored in (D) cholesterol levels and (E) HDL cholesterol levels. (F) Heart weight only increases significantly from Day 7 onwards due to inflammation and oedema. (G) Representative images of an H&E staining of the heart at different sham and Day 7 following viral infection. (H) CVB3 levels in the hearts increase significantly at Day 2, yet reaches a plateau at Day 7. (I) Correlation between cardiac CVB3 levels and FAS during the course of VM. (J) Correlation between cardiac CVB3 levels and SREBP-1c during the course of VM. (K) Representative images of Oil Red O stainings of hearts with or without CVB3 infection, showing an increased lipid-richness in the inflamed area of the heart. *$P < 0.05$; **$P < 0.01$; ***$p < 0.0001$. Scale bar represents 1000 μm (G) or 20 μm (K).
Liver X receptor activation enhances CVB3 viral replication during myocarditis

**Figure 5** LXR activation increases lipid availability enhancing viral replication. (A) The gene expression of FAS and SREBP-1c trends to increase in vivo from Day 4 onwards during the course of VM and is significant at Day 7 ($n=6$ per group). (B) In vitro exposure of Vero cells to T09 increased FAS activity when in the presence of the CVB3 virus ($n=3$). (C) LXR agonism by T09 enhances the CPE caused by CVB3 infection in Vero cells while treatment with the FAS or SREBP-1c inhibitors reduced the cytolytic activity of CVB3. Only the SREBP-1c inhibitor could blunt the increase in the CPE of CVB3 by T09 ($n=3$). (D) LXR agonism by T09 enhances the CPE caused by CVB3 infection in cardiac HL-1 cells while treatment with the FAS and SREBP-1c inhibitors reduced the cytolytic activity of CVB3 ($n=6$). (E) Correlation between CVB3 levels and FAS during CVB3 infection of primary cardiomyocytes. (F) Correlation between CVB3 levels and SREBP-1c during CVB3 infection of primary cardiomyocytes. (G) CVB3 viral load during CVB3 infection of primary cardiomyocytes ($n=3$). Cellular CVB3 levels were only reduced in the presence of both C75 and 25-HC. (H) In primary cardiomyocytes, pattern recognition receptors MDA5 and TLR3 are up-regulated upon CVB3 infection ($n=3$). Cellular antiviral machinery is not activated when cells are pretreated with either FAS or SREBP-1c inhibitors. *$p<0.05$, **$p<0.01$, ***$p<0.001$, $p<0.05$, $p<0.01$, $p<0.001$ when compared with CVB3 only for MDA5, $p<0.05$, $p<0.01$, $p<0.001$ when compared with CVB3 only for TLR3; T09, LXR agonist; C75, FAS inhibitor; 25-HC, SREBP-1c inhibitor.
reduced back to baseline levels, by administration of the potent synthetic FAS inhibitor C75 (1 μM), as well as the SREBP-1c inhibitor 25-HC (1 μM). T0901317 also significantly increased the expression of SREBP-1c in vitro (see Supplementary material online, Figure S2B). The FAS inhibitor was not able to blunt LXR-induced increases in SREBP-1c gene expression, which could be achieved by the specific SREBP-1c inhibitor, suggesting that the effects of LXR agonism are mainly mediated via SREBP-1c. These data confirm the activation of FAS and SREBP-1c by LXR agonist, the specificity of the respective inhibitors. Finally, Vero cells were pretreated with the FAS and SREBP-1c inhibitors alone or in combination with LXR agonist T0901317 and exposed to the CVB3 virus, and cytolytic activity was assessed. The CVB3 virus caused extensive cellular damage, which was exaggerated when the cells were pre-incubated with LXR agonist (Figure S3 and see Supplementary material online, Figure S2A). The enhanced cytolytic effect by T0901317 was blunted when SREBP-1c was inhibited but not when FAS was inhibited, in line with maintained stimulation of SREBP-1c gene expression under these conditions (see Supplementary material online, Figure S2B). Interestingly, both FAS and SREBP-1c inhibition were sufficient to significantly reduce the CPE of the CVB3 virus in Vero cells in the absence of T0901317 treatment (Figure S3C and see Supplementary material online, Figure S2D). Finally, in order to validate the antiviral mechanism of FAS and/or SREBP-1c inhibition, we performed a cytopathogenic assay on HL-1 cells (Figure 5D and see Supplementary material online, Figure S3A). Similarly to Vero cells, CVB3 induced cellular damage, which was slightly but not significantly enhanced by T0901317 treatment. Pretreatment with either the selective FAS inhibitor C75, or the SREBP-1c inhibitor 25-HC, reduced the cellular damage caused by the CVB3 virus in the presence of T0901317, but only when both were present was there a significant decrease (n = 3, P < 0.01). To demonstrate that altering lipid metabolism directly affects viral load, primary neonatal rat cardiomyocytes were infected with CVB3 and levels of FAS and SREBP-1c were assessed (Figure 5E). There was no significant correlation between FAS and CVB3 levels, but there was a linear correlation between SREBP-1c and viral load (Figure 5F). Furthermore, CVB3 viral levels were significantly reduced in primary cardiomyocytes when both FAS and SREBP-1c have been inhibited but not when the individual factors were inhibited alone (Figure 5G). Finally, CVB3 infection of cardiomyocytes resulted in the activation of host machinery as expression of the pattern recognition receptors, Melanoma Differentiation-Associated protein 5 (MDA5) and Toll-like receptor 3 (TLR3), were up-regulated (Figure 5H). Pretreatment of cells with either the FAS inhibitor C75 or SREBP-1c inhibitor 25-HC was able to completely blunt this antiviral response (Figure 5H and see Supplementary material online, Figure S3B).

4. Discussion

In view of the anti-inflammatory and lipid-modulating properties of LXR agonism, we examined the use of LXR agonism as a potential new line of therapy in the treatment of VM. Using two different experimental designs, we assessed the effect of LXR agonism on viral replication and cardiac inflammation during VM. In contrast to the previously reported anti-inflammatory properties of LXR agonists, we show that treatment with the agonist T0901317 prior to viral infection strongly increased CVB3-mediated mortality (albeit due to extra cardiac damage), myocardial damage, and enhanced viral presence in the heart. Further exploration showed that the presence of the viral genome was positively associated with the induction of lipogenic factors FAS and SREBP-1c, and lipid droplets were detectable in infected cardiac tissue, in particular where inflammation and cardiomyocyte damage were present. Finally, we demonstrated that targeting the dominant factor SREBP-1c, which is induced by LXR agonism, blunts the effect on viral replication in vitro (Figure 6). This study, to the best of our knowledge, is the first to suggest a causal link between CVB3 viral replication and lipid availability in vivo.

We hypothesized that LXR agonists would be beneficial during VM due to their anti-inflammatory properties in macrophages, yet no such benefit was observed. The absence of an effect of LXR agonism on reducing leucocyte infiltration was unexpected, as previous studies have demonstrated reduced inflammation not only during atherosclerosis but also during pulmonary inflammation.35,36,27,28,29 Experimental autoimmune encephalitis,29 and most recently during experimental autoimmune uveitis.30 However, the cellular and molecular players elicited during pathogen-induced myocarditis are different from those induced during sterile inflammation or during autoimmune diseases. For example, CVB3 infection activates the NFκB pathway,31 the target regulated by LXRα agonists, but the exact molecular mechanism still remains unclear and may be divergent to those involved during atherosclerosis.32 In addition, in contrast to these other pathologies, the host response to cardiac viral infection activates both innate and adaptive immunity to clear infected cardiomyocytes. Depleting a leucocyte subset alone does not always have a beneficial outcome during myocarditis as demonstrated by studies were either CD4 or CD8 T-cells, B-cells, or NK cells were targeted.25–28,30 Our study, however, did not explore the long-term outcome of LXR agonism during VM but used two different experimental models, focused on the phases when viral replication and acute leucocyte infiltration take place. Therefore, the effect of LXR agonism on the reparative phases of VM or whether there is a protective effect on cardiac function during viral infection still remains unknown. Owing to the route of drug administration of this study, we could not monitor directly the amount of LXR agonist received in the individual mice. Furthermore, we cannot exclude that there may be a dose-dependent factor at play, and that possibly at a different dose there would be a beneficial effect on cardiac inflammation. Finally, a role for LXRs during VM cannot be excluded completely without assessing disease severity and outcome in mice genetically lacking LXRs.

Despite the above-mentioned limitations, we found an undesirable effect of LXR agonism in vivo if administration is during the viraemic phase and not post viraemia. Though viral infection provoked the activation of FAS and SREBP-1c in both experimental set-ups used in this study, it was only when LXR agonism was pre-administered that the effect of viral replication and mortality was observed. This was most likely due to the effect of LXR activation occurring at the peak of when CVB3 infection exerts lipid metabolic demands.

The findings of our study suggest that caution is needed when developing LXR agonists for long-term use, such as during pathological hypertrophy,18,35 atherosclerosis, or arthritis. These agents undoubtedly
could benefit patients through their pleiotropic mechanisms, yet we suggest that they may leave patients vulnerable to various viral infections. It seems therefore necessary to strike a timely balance of these two facets of LXR agonism by limiting treatment duration in order to harness the benefits on inflammation and/or cholesterol homeostasis, yet minimize life-threatening viral infections.

Unlike other sterile inflammatory diseases, damage to the heart during VM is caused both by the virus itself and the consequent immune response. Therefore, the optimal therapeutic approach requires on the one hand blunting viral replication and on the other limiting the immune infiltration into the heart. The effect of LXR agonism on CVB3 viral replication has been unexplored up until now. Here we demonstrated, in vivo and in vitro, that CVB3 infection of cardiomyocytes is associated with increases in SREBP-1c (and to a lesser extent FAS), which is enhanced by LXR agonism. Previous studies have suggested that targeting FAS with the inhibitor C75 could have a beneficial effect on CVB3 replication. We confirmed that in vitro targeting of FAS reduces CVB3-mediated cellular damage in epithelial cells and demonstrated that targeting SREBP-1c is more effective for the protection of cardiac cells. Our data show that inhibiting lipid metabolism during viral infection of primary cardiomyocytes has a beneficial outcome on the viral response genes and consequently the viral load. Though this indicates a possible new therapeutic avenue, this needs to be supported by in vivo studies using selective FAS/SREBP-1c inhibitors.

Figure 6  Lipid availability facilitates CVB3 viral replication and is enhanced by LXR agonism. Scheme representing the proposed mechanism by which the CVB3 virus utilizes the lipogenic factors FAS and SREBP-1c to enter, replicate, and leave the cardiomyocytes. LXR agonist T09 stimulates the production of both factors, enhancing their use by the CVB3 to replicate.
Based on the in vitro findings, we demonstrate that interfering with lipid biosynthesis pathways might prove an antiviral strategy, though caution needs to be taken as we have only proved antiviral effectiveness against CVB3 and in vitro. In light of the striking similarities in the replicative cycle of several single-stranded positive RNA viruses (e.g. Enteroviruses, Poliovirus, Echovirus, Hepatitis C, etc.), it is possible that FAS and/or SREBP inhibitor may be effective against other viruses of this class, but more research is needed to corroborate this. Finally, what is becoming more apparent is that lipids as a whole are critical components of how viruses interact with the host cell, and that targeting these interactions, such as SREBP-1c, may provide new antiviral strategies for VM.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

The authors acknowledge Yoonne Sensen, Sarah Vander Peer, Kevin Custers, and Rick van Leeuwen for their contributions to the manuscript. W.H. gratefully acknowledges the Fund for Clinical Cardiovascular Research of Prof Frans Van de Werf (KU Leuven, Belgium).

Conflict of interest: none declared.

Funding

This study was supported by a research grant from the Research Foundation Flanders, Belgium (FWO-Vlaanderen-G.0800.14N to S.H. and A.-P.P.; FWO-Vlaanderen-1183211N and -1183213 to W.H.); the Netherlands Organization for Scientific Research (Vidi Grant 91796338), the Netherlands Heart Foundation (20080011 to S.H. and 2007T046 to R.A.D.B.), and the European Union (Academy Pathways and Partnerships—CARDIO-MIR, FP7-HEALTH-2010-MEDIA, FP7-HEALTH-2011-EU-Mascara, and FP7-HEALTH-2012-HOMAGE) to S.H. and A.-P.P.

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