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1 Title: The protective role of liver X receptor (LXR) during fumonisin B1-induced
2 hepatotoxicity

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15 **Abbreviations:**

17 **Keywords**

18 Fumonisin, Ceramide, Liver, LXR, Hepatotoxicity

1 **ABSTRACT**

2 Fumonisin B1 (FB1), a congener of fumonisins produced by *Fusarium* species, is the most
3 abundant and most toxicologically active fumonisin. FB1 causes severe mycotoxicosis in
4 animals, including nephrotoxicity, hepatotoxicity, and disruption of the intestinal barrier.
5 However, mechanisms associated with FB1 toxicity are still unclear. Preliminary studies have
6 highlighted the role of liver X receptors (LXRs) during FB1 exposure. LXRs belong to the
7 nuclear receptor family and control the expression of genes involved in cholesterol and lipid
8 homeostasis. In this context, the toxicity of FB1 was compared in female wild-type (LXR^{+/+})
9 and LXR_{α,β} double knockout (LXR^{-/-}) mice in the absence or presence of FB1 (10 mg/kg body
10 weight/day) for 28 days. Exposure to FB1 supplemented in the mice's drinking water resulted
11 in more pronounced hepatotoxicity in LXR^{-/-} mice compared to LXR^{+/+} mice, as indicated by
12 hepatic transaminase levels (ALT, AST) and hepatic inflammatory and fibrotic lesions. Next,
13 the effect of FB1 exposure on the liver transcriptome was investigated. FB1 exposure led to a
14 specific transcriptional response in LXR^{-/-} mice that included altered cholesterol and bile acid
15 homeostasis. ELISA showed that these effects were associated with an elevated FB1
16 concentration in the plasma of LXR^{-/-} mice, suggesting that LXRs participate in intestinal
17 absorption and/or clearance of the toxin. In summary, this study demonstrates an important role
18 of LXRs in protecting the liver against FB1-induced toxicity, suggesting an alternative
19 mechanism not related to the inhibition of sphingolipid synthesis for mycotoxin toxicity.

1 INTRODUCTION

2 Fumonisin B1 (FB1) is the most abundant and most documented mycotoxin of the fumonisin
3 family, which contains more than 30 species (Wan Norhas et al. 2009). FB1 is produced mainly
4 by *Fusarium verticillioides* and *Fusarium proliferatum* and mainly contaminates corn. As FB1
5 is nephrotoxic and hepatotoxic (Voss et al. 1995; Bondy et al. 1997; Humphreys et al. 2001)
6 and exhibits deleterious effects on human and animal health, contamination levels are strictly
7 regulated in food and feed ((Ec) No 1126/2007 Commission Regulation 2007; (Ec) No
8 576/2006 Commission Recommendation 2006). Other specific related clinical diseases include
9 leukoencephalomalacia, pulmonary edema, cardiac dysfunction (Haschek et al. 2001),
10 carcinogenesis (FAO/WHO 2001), neural tube defects (Marasas et al. 2004), and disruption of
11 the intestines and immune system (Devriendt et al. 2009; Grenier et al. 2012).

12 Due to its structural similarity to sphingoid species, such as sphinganine (Sa) and sphingosine
13 (So), FB1 represents an important competitive substrate for all ceramide synthases (Cers)
14 involved in the formation of long-chain and very long-chain ceramides. Though FB1 decreases
15 the quantity of ceramide and sphingomyelin species in tissues, it also increases the levels of Sa
16 and sphingolipid terminal products, such as Sa 1-phosphate. Thus, the Sa/So ratio is used as a
17 biomarker of exposure to FB1 in animals (Wang et al. 1991; Loiseau et al. 2007, 2015).

18 Several studies have reported a link between sphingolipids and the metabolism of other lipids,
19 such as sterols and fatty acids. In the white adipose tissue of rats, *Cers4* has been identified as
20 a potent target of modulators of endogenous lipid metabolism, such as leptin (Bonzón-
21 Kulichenko et al. 2009), insulin, or changes in phospholipid transfer protein activity (PLTP α)
22 (Rosenthal et al. 2011; Stratford et al. 2001). Moreover, fatty acid elongase 1 (ELOVL1)
23 activity, which is particularly involved in saturated and monounsaturated C20- and C22-CoA
24 synthesis, is regulated by CerS2, which is essential for the production of C24-sphingolipids
25 (Ohno et al. 2010). Sphingomyelins have also been shown to be involved in the post-

1 translational regulation of master regulators of fatty acid and cholesterol metabolism, called
2 sterol regulatory element-binding proteins (SREBPs), through inhibition of SREBP cleavage-
3 activating protein (SCAP) (Scheek et al. 1997).

4 We recently reported that the kinome and transcriptome profiles of piglets exposed to toxic
5 levels of FB1 revealed that most of the effects of the mycotoxin were mediated by the regulation
6 of ceramide levels, which in turn influence protein phosphatase 2 (PP2A) and the
7 phosphoinositide 3-kinase (PI3K)/AKT signaling pathways (Régnier et al. 2017). This
8 disturbance induces inhibition of integrin-mediated cell-matrix adhesion, an inflammatory
9 response, and alters the expression of genes involved in cholesterol and fatty acid homeostasis.

10 We identified at least four modulated genes (*Abcg8*, *Scd1*, *Ldlr*, and *Fasn*) that are prototypical
11 target genes regulated by the LXR nuclear receptors.

12 LXRs belong to the nuclear receptor superfamily, which comprises 48 members in humans and
13 49 in rodents (Gronemeyer et al. 2004). Two isoforms of LXR have been described: LXR α
14 (NR1H3) and LXR β (NR1H2) (Teboul et al. 1995). These nuclear receptors are involved in
15 cholesterol and fatty acid metabolism (Ducheix et al. 2013). LXR α ^{-/-}, LXR β ^{-/-}, or LXR α/β ^{-/-} mice
16 have shown an accumulation of cholesterol esters in the liver related to a defect in cholesterol
17 excretion (Peet et al. 1998). In the intestine, LXRs limit cholesterol absorption by inducing the
18 expression of ABC transporters G5 and G8 (Yu et al. 2005; Liqing Yu et al. 2003) and facilitates
19 cholesterol excretion in high density lipoproteins (HDLs) by stimulating *Abca1*. In the liver,
20 LXRs stimulate *Abcg5* and *Abcg8*, which is necessary for the excretion of cholesterol in the
21 bile duct, and *Cyp7a1*, which is responsible for the degradation of cholesterol into biliary acids.
22 These findings suggest that LXRs play an important role in the detoxification of FB1, but this
23 has never been demonstrated. The aim of the present study was to compare the toxicity of FB1
24 in wild-type (LXR^{+/+}) and LXR α,β -deficient (LXR^{-/-}) mice. Our data indicate that LXRs control
25 the plasmatic levels of plasmatic FB1 and, therefore, its toxicity in the liver.

1 MATERIALS AND METHODS

2 Animals

3 Female LXR α,β ^{-/-} double-deficient (LXR^{-/-}) and LXR α,β ^{+/+} mice (LXR^{+/+}) with a mixed
4 C57BL6J/129SVJ genetic background were bred at INRA's transgenic rodent facility at 22±
5 2°C. Animals were 8 weeks old at the beginning of the experiment and given free access to
6 water and a basal diet (Harlan). Based on the literature, female mice were used in this study
7 because of their higher sensitivity to FB1 compared to male mice (Bondy et al. 1997; Howard
8 et al. 2001; Johnson and Sharma 2001). The experiments were carried out in accordance with
9 the European Guidelines for the Care and Use of Animals for Research Purposes (accreditation
10 number APAFIS#5917-2016070116429578).

11 Treatment with fumonisin B1

12 LXR^{-/-} and LXR^{+/+} mice were randomly divided into two groups of eight mice each. For the
13 FB1 treated group, the drinking water was supplemented with fumonisin B1 (10 mg FB1/kg
14 bw/day) for 4 weeks. Body weight and water intake were recorded weekly to adjust the quantity
15 of FB1 in the water for changes in body weight and water consumption. At the end of the
16 experiment, the mice were euthanized to collect blood and tissue samples. The liver was
17 removed, dissected, snap-frozen in liquid nitrogen, and stored at -80°C until use.

18 Gene expression studies

19 Total RNA was extracted from the liver using Trizol® reagent (Amresco, USA). Gene
20 expression profiles of six liver samples per group, randomly chosen, were obtained at the
21 GeTTRiX facility (GénoToul, Génopole Toulouse Midi-Pyrénées) using Agilent Sureprint G3
22 Mouse GE v2 microarrays (8x60K, design 074809) following the manufacturer's instructions.
23 For each sample, cyanine-3 (Cy3) labeled cRNA was prepared from 200 ng of total RNA using
24 the One-Color Quick Amp Labeling kit (Agilent Technologies, California) according to the
25 manufacturer's instructions, followed by Agencourt RNAClean XP (Agencourt Bioscience

1 Corporation, Massachusetts). Dye incorporation and cRNA yield were checked using a
2 Dropsense™ 96 UV/VIS droplet reader (Trinean, Belgium). A total of 600 ng of Cy3-labeled
3 cRNA were hybridized onto the microarray slides following the manufacturer's instructions.
4 Immediately after washing, the slides were scanned on an Agilent G2505C Microarray Scanner
5 using Agilent Scan Control A.8.5.1 software and the fluorescence signal extracted using Agilent
6 Feature Extraction software v10.10.1.1 with default parameters. Microarray data and
7 experimental details are available in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and
8 are accessible through GEO Series accession number GSE118072
9 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118072>).

10 For real-time quantitative polymerase chain reaction (qPCR), total RNA samples (2 µg) were
11 reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied
12 Biosystems, California). Primers for Sybr Green assays are presented in Online Resource 1.
13 Amplification reactions were performed on an ABI Prism 7300 Real Time PCR System
14 (Applied Biosystem). The qPCR data were normalized to proteasome subunit beta type-6
15 (psmb6) mRNA levels and analyzed by LinRegPCR software.

16 **Plasma analysis**

17 Plasma levels of aspartate transaminase (AST), alanine transaminase (ALT), bilirubin, alkaline
18 phosphatase, total cholesterol, LDL-cholesterol, and HDL-cholesterol were determined on a
19 biochemical analyzer COBAS-MIRA+. Plasma FB1 concentrations were assayed using the
20 Fumonisin ELISA Kit (Novakits, Nantes, France) following the manufacturer's instructions.
21 The fumonisin ELISA (LOD = 2 µg/mL in plasma) uses antibodies raised in mouse against
22 protein conjugated FB1. Plasmas were diluted by two in dilution buffer before ELISA assay.
23 The optical density was measured at 450nm and 590nm using ELISA 96-well plate reader
24 (TECAN). The concentration of FB1 in the plasma samples (pg/ml) corresponding to the
25 maximal absorbance of each extract was read from the calibration curve ($R^2 = 0.9998$).

1 **Biochemical assays**

2 *Total lipid extraction*

3 Liver samples were homogenized in 1 ml of methanol/5 mM EGTA (2:1 v/v) with FastPrep-
4 24™ (MP-Biomedicals, USA). Lipids corresponding to an equivalent of 2 mg of tissue were
5 extracted according to Bligh and Dyer method (Bligh and Dyer 1959) in
6 chloroform/methanol/water (2.5:2.5:2, v/v/v) in the presence of the internal standards. The
7 solution was centrifuged at 1500 rpm for 3 min. The organic phase was collected and dried
8 under nitrogen and then dissolved in an adequate eluent.

9 *Neutral lipids*

10 Neutral lipids were extracted as described above in the presence of including internal standards:
11 16 µg glyceryltrinonadecanoate, 6 µg stigmaterol, and 6 µg cholesteryl heptadecanoate
12 (Sigma, Saint-Quentin-Fallavier, France). Total lipids were suspended in 160 µl of ethyl acetate
13 and triglycerides, free cholesterol, and cholesterol esters analyzed by gas-liquid
14 chromatography on a focus Thermo Electron system using a Zebron-1 Phenomenex fused-silica
15 capillary column (5 m, 0.32 mm i.d., 0.50 mm film thickness). The oven temperature was
16 programmed from 200 to 350°C at a rate of 5°C/min, and the carrier gas was hydrogen (0.5
17 bar), whereas the injector and detector temperatures were set at 315°C and 345°C, respectively.
18 (Régnier et al. 2017b)

19

20 *Ceramide, sphingomyelin, sphingosine, and sphinganine*

21 Sphingolipids were extracted using the same protocol as neutral lipids using
22 dichloromethane/water/methanol (2% acetic acid) (2.5:2:2.5, v/v/v) with the addition of internal
23 standards: ceramide d18:1/15:0 (16 ng), sphingomyelin d18:1/12:0 (16 ng), sphingosine 17:0,
24 and sphinganine 17:0. Sphingolipids and internal standards were analyzed by triple quadrupole
25 mass spectrometry (QqQ MS) (Loiseau et al. 2015).

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Histology

Hematoxylin/eosin staining was performed on tissue sections (5 μm) from paraformaldehyde-fixed, paraffin-embedded liver samples. Visualization was made possible using a Leica microscope DM4000 B equipped with a LEICA DFC450 C camera (Leica microsystems). Livers were examined by light microscopy. First, the 32 liver sections (one slide per animal, 8 slides per group, and four groups) were full-blinded screened by two persons to determine the effects present for each section. Inflammatory foci (corresponding to a minimum of 5 inflammatory cells), apoptotic bodies, cell injury, and mitosis were counted in 10 distinct areas on the 200x field for each liver slice (corresponding to an average number of 600 cells) and were approved by an histopathologist (T.A.S). Values were presented as the mean of 10 fields/slice. Next, Sirius red staining was performed on tissue sections (5 μm) from formalin-fixed, paraffin-embedded liver pieces and collagen fibers stained red.

Statistical analysis

For transcriptomic, histologic, and biochemical analyses, statistical analyses were performed using Graphpad Prism for Windows (version 7.00, Graphpad software). The data were expressed as mean ± SEM. Differential effects were analyzed by two-way analyse of variance (ANOVA) followed by appropriate posthoc tests (Sidak). A p-value < 0.05 was considered significant.

Microarray data were analyzed using R (“R: a language and environment for statistical computing” 2008) and Bioconductor packages (www.bioconductor.org, v 3.0) (Gentleman et al. 2004) as described in GEO accession GSE118072. Raw data (median signal intensity) were filtered, log2 transformed, corrected for batch effects (microarray washing bath and labeling serials), and normalized using the quantile method (Bolstad et al. 2003) A model was fitted using the limma lmFit function (Smyth 2004). Pair-wise comparisons between biological

1 conditions were applied using specific contrasts to extract simple genotype and treatment
2 effects, but also interaction terms genotype:treatment. A correction for multiple testing was
3 applied using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) to control
4 the false discovery rate (FDR). Hierarchical clustering was applied to the samples and
5 differentially expressed probes using the 1-Pearson correlation coefficient as distance and
6 Ward's criterion for agglomeration. The clustering results are illustrated as a heatmap of
7 expression signals. Enrichment of Gene Ontology (GO) Biological Processes was performed
8 using the GOC API included on the string-db.org website (Szklarczyk et al. 2015).

1 RESULTS

3 Physiological follow-up during FB1 exposure

4 Exposure to FB1 for 28 days had no effect on the body weight of the mice regardless of the
5 genotype ($LXR^{+/+}$ or $LXR^{-/-}$), whereas significant effects of genotype on body weight were
6 observed (Fig. 1A). Similarly, no change in food and water intake was observed during the
7 experiment. However, an increase in body weight and water intake was observed in $LXR^{-/-}$ mice
8 compared to $LXR^{+/+}$ mice (Fig. 1B). Though body weight remained unchanged, FB1 exposure
9 led to decreased liver weight and increased spleen weight (Fig. 1C).

11 FB1 alters sphingolipid homeostasis in $LXR^{+/+}$ and $LXR^{-/-}$ mice

12 FB1 is a known pharmacological inhibitor of ceramide synthesis. The role of LXRs in the FB1-
13 induced alteration of sphingolipid metabolism was investigated and several liver sphingolipids,
14 such as ceramides, sphingomyelins, sphinganine, and sphingosine, measured in mice.

15 In both mouse strains, the sphingoid bases Sa and So were increased during FB1 exposure.
16 Nevertheless, the increase in the Sa/So ratio was slightly greater in the $LXR^{-/-}$ mice than the
17 $LXR^{+/+}$ mice (Fig. 2A).

18 Upon FB1 exposure, ceramide levels were decreased by 59% and 71% in the livers of $LXR^{+/+}$
19 and $LXR^{-/-}$ mice, respectively (Fig. 2B). Levels were significantly lower in $LXR^{-/-}$ mice than in
20 $LXR^{+/+}$ mice, but FB1 exposure had the same effect in both $LXR^{+/+}$ and $LXR^{-/-}$ mice (no
21 significant genotype/treatment interaction). When each ceramide species was measured, a
22 decrease was observed in FB1-exposed mice regardless of genotype ($LXR^{-/-}$ or $LXR^{+/+}$) (Fig.
23 2D).

24 Similarly, FB1 exposure led to a decreased level of sphingomyelin in the liver in both $LXR^{+/+}$
25 and $LXR^{-/-}$ mice (Fig. 2B). Analyzing the effect of both genotype and the treatment on each

1 sphingomyelin species, a significant decrease in SM(d18:1/16:0), SM(d18:1/16:1), and
2 SM(d18:1/22:1) and an increase in SM(d18:1/24:0) and SM(d18:1/24:1) were observed among
3 FB1-treated mice. Moreover, a significant genotype effect was observed with a decrease in
4 SM(d18:1/22:0) and an increase in SM(d18:1/24:1) (Fig. 2E). These results clearly show that
5 FB1-associated inhibition of ceramide synthesis is not dependent on the LXR.

7 **LXRs control the plasma FB1 concentration**

8 LXRs regulate cholesterol efflux from enterocytes and their absence alter the efficiency of the
9 tight junctions, resulting in an increase in plasma levels of cholesterol. We previously showed
10 that LXR pathway is activated by FB1 (Régner et al. 2017). In order to test if LXRs also
11 participate to the modulation of plasma levels of FB1, plasma FB1 concentrations were
12 measured in treated and untreated LXR ^{+/+} and LXR ^{-/-} mice. We observed that LXR ^{-/-} mice
13 treated with FB1 contained significantly higher concentrations of toxin compared to LXR ^{+/+}
14 (Fig. 3). Even though we previously showed that sphingolipid inhibition by FB1 occurs
15 independently of LXR, this result shows that LXR-dependent activity controls the
16 bioavailability of FB1.

18 **Effect of FB1 exposure on the hepatic gene expression profile**

19 In order to investigate the changes in gene expression associated with the increase in plasma
20 levels of FB1 combined with the absence of the LXR nuclear receptor, a microarray analysis
21 was performed on the livers of LXR ^{+/+} and LXR ^{-/-} mice in the presence and absence of FB1
22 exposure. The statistical analysis identified thousands of genes regulated in response to FB1 in
23 each mouse strain or differentially regulated between strains. The heatmap illustrated in Fig.
24 4A is generated by the selection of the top 2000 most significantly differentially expressed

1 probes for each contrast (based on adjusted p-value; $FDR \leq 5\%$) corresponding to 4825 probes
2 overall.

3 Four main clusters showing specific gene expression profiles according to experimental
4 conditions have been identified from the hierarchical clustering of probes. Two of them
5 (clusters 2 and 4) illustrate a genotype effect and the last two (clusters 1 and 3) reflect an effect
6 of FB1 exposure.

7 Clusters 2 and 4 represent genes induced and repressed, respectively, in the absence of LXR,
8 but not dependent on FB1 exposure (Fig. 4A). The biological function analysis of cluster 2
9 revealed that LXR deficiency induces cytokine receptor interaction and catalytic activities, such
10 as the characteristic gene *Cyp2b10*. In line with the role of LXR in lipid metabolism, biological
11 function analyses of cluster 4 revealed that LXR deficiency influences the PPAR signaling
12 pathway, pyruvate, and fatty acid metabolism. A representative LXR target gene is sterol
13 regulatory element-binding transcription factor 1-c (*Srebp1-c*). Its expression is significantly
14 decreased in the absence of LXR, regardless of treatment (Fig. 4B).

15 Clusters 1 and 3 represent a panel of genes whose expression is sensitive to FB1 (Fig. 4A).
16 Biological function analysis of cluster 1 revealed that the main FB1-activated pathways are
17 focal adhesion, PI3K-AKT signaling pathways, proteoglycans in cancers, ECM-receptor
18 interaction, and chronic myeloid leukemia (Fig. 4B and Online Resource 2). Using qPCR, we
19 measured the expression of a fibrosis reference gene, collagen, type I, alpha 1 (*Coll1a1*), a
20 representative gene of cluster 1. *Coll1a1* was induced only in $LXR^{-/-}$ mice treated with FB1 (Fig.
21 4B). In contrast, FB1 reduced the expression of genes included in cluster 3 and involved in
22 metabolic pathways, amino acid degradation, amino acid metabolism, drug metabolism, and
23 bile secretion (Fig. 4B and Online Resource 3). For example, expression of UDP
24 glucuronosyltransferase 2 family, polypeptide B1 (*Ugt2b1*), involved in drug metabolism and
25 bile secretion is significantly decreased in $LXR^{-/-}$ mice treated with FB1.

LXR deficiency influences the hepatic gene expression profile in response to FB1

A Venn diagram was used to highlight LXR-dependent changes during FB1 treatment (Fig. 5A). Among the significant DEGs related to FB1, 40 were specific to LXR^{+/+} mice (34 up-regulated genes and 6 down-regulated genes) and 6056 were specific to LXR^{-/-} mice (3026 up-regulated and 3020 down-regulated). Thus, LXR deficiency reveals a broad range of FB1-sensitive genes (Fig. 5A). The 40 genes with the highest changes in hepatic gene expression in the livers of LXR^{-/-} mice (blue bars Fig. 5B and 5C) differed from those observed LXR^{+/+} mice (red bars Fig. 5B and 5C). Most of these genes are involved in inflammation, fibrogenesis, and carcinogenesis, including *Cd63* antigen, *Colla1*, *Col3a1*, chemokine (C-X-C motif) ligand 14 (*Cxcl14*), and Ras-related protein RAB7 (*Rab7b*) (Fig. 5B). Conversely, the 40 genes reduced by FB1 in LXR^{-/-} mice were largely involved in bile secretion and drug metabolism (Fig. 5C). Measuring the expression of some genes by qPCR revealed that *Colla1*, *Elovl7* (involved in the elongation of very long-chain fatty acids protein 7), and *Smpd3* (neutral sphingomyelinase) are largely up-regulated in response to FB1 in the absence of LXR, whereas, steroid 7-alpha-hydroxylase (*Cyp7b1*), transmembrane 7 superfamily member 2 (*Tm7sf2*), and ATP binding cassette subfamily G member 8 (*Abcg8*), involved in lipid and bile acid metabolism were down-regulated by FB1 (Fig. 5D).

LXR deficiency alters liver biochemistry in response to FB1

As many genes are modulated by FB1 exposure in LXR^{-/-} mice, plasma biochemistry analysis was performed to investigate the metabolic consequences of the toxin. As shown in Fig. 6A, exposure to FB1 was associated with a greater increase in plasma alanine amino transferase (ALT), aspartate amino transferase (AST), and alkaline phosphatase (ALP) in LXR^{-/-} mice than LXR^{+/+} mice (4-, 3-, and 2-fold, respectively). In addition, FB1 treatment increased plasma bilirubin levels 3- and 2-fold in LXR^{+/+} and LXR^{-/-} mice, respectively.

1 FB1 exposure also induced a significant increase in plasma levels of HDL, LDL, and total
2 cholesterol in LXR^{-/-} mice, but did not modulate the plasma level of these lipoproteins in LXR^{+/+}
3 mice (Fig. 6B). Because the accumulation of cholesterol can be hepatotoxic, we investigated
4 the effect of FB1 on liver cholesterol. As expected, an accumulation of hepatic cholesterol and
5 cholesterol esters was observed in LXR-deficient mice compared to wild-type mice (Fig. 6C);
6 however, exposure to FB1 did not have significant effects on the levels of these metabolites.
7 Interestingly, hepatic triglyceride levels were reduced in LXR^{-/-} mice and further decreased by
8 FB1 (Fig. 6C).

10 **LXR deficiency enhances liver injury in response to FB1**

11 The severity of liver lesions associated with altered plasma biochemistry were assessed on
12 hematoxylin and eosin and Sirius red stained tissues. Livers from LXR^{+/+} mice exposed to FB1
13 exhibited a small increase in histological lesions, particularly those associated with apoptosis,
14 inflammatory foci, and mitosis. Nevertheless, differences from the unexposed LXR^{+/+} mice
15 were not significant. In comparison, livers from unexposed LXR^{-/-} mice exhibited macrophage
16 infiltration and tissue fibrosis (Fig. 7A). The histopathological changes in the liver of LXR^{-/-}
17 treated mice with FB1, in comparison with control group, are characterized by obvious increase
18 in degenerative changes and apoptotic cells in comparison with control group.

19 **DISCUSSION**

20 The aim of this study was to question the role of the nuclear LXRs in the toxicity of mycotoxin
21 FB1. Modulation of the LXR pathway by FB1 in the liver of piglets was described in a previous
22 study using untargeted (transcriptomic and phospho-proteomic) approaches (Régner et al.
23 2017). In the current study, the role of LXRs in FB1 toxicity was assessed using LXR^{+/+} and
24 LXR^{-/-} mice. Though we observed no effect of LXRs on the inhibition of ceramide synthesis,
25 we demonstrated that LXRs protect against FB1-induced liver damage.

1 First, we demonstrated that exposure to the toxin does not affect body weight or food and water
2 consumption by the mice (Fig. 1), unlike what has been observed in other animal species, such
3 as pigs (Marin et al. 2006; Régnier et al. 2017). Interestingly, though the body weights of mice
4 remained unchanged, FB1 exposure decreased liver weight while increasing the spleen weight.
5 These physiological changes are in accordance with previous results from our lab and other,
6 revealing that FB1 alters hepatic lipogenesis and immune responses (Devriendt et al. 2009;
7 Régnier et al. 2017).

8 In $LXR^{+/+}$ and $LXR^{-/-}$ mice, FB1 exposure significantly decreased total ceramide content,
9 including 10 different ceramide species. These results are consistent with the transcriptomic
10 analyses of the expression of ceramide synthase 2 (*Cers2*) and sphingomyelin
11 phosphodiesterase 1 (*Smpd1*), two enzymes involved in sphingolipid metabolism that are
12 significantly inhibited by FB1 exposure, regardless of the presence of LXRs; the ceramide-
13 inhibition rate was the same regardless of genotype. The decrease in ceramide due to the
14 inhibition of ceramide synthase resulted in an increase in sphinganine and sphingosine levels
15 as described in several other studies (Loiseau et al. 2015; Masching et al. 2016; Riedel et al.
16 2016; Régnier et al. 2017a). Notably, exposure to FB1 induced a rapid elevation in sphinganine,
17 leading to a higher Sa/So ratio (Burel et al. 2013; Loiseau et al. 2007), whereas the increase in
18 sphingosine levels only occurred at a later stage concomitantly with the alterations in the liver
19 tissue (Wang et al. 1991). In the present study, we observed that a similar degree of ceramide
20 reduction was achieved in both genotypes even though different FB1 plasma levels were
21 observed. These results implied that FB1 inhibits ceramide synthesis more potently in $LXR^{+/+}$
22 mice, unless if the effect of FB1 on ceramide metabolism is already maximal in $LXR^{+/+}$ mice
23 at the dose used in the present study. Although FB1 is poorly absorbed in the intestine and the
24 majority of FB1 is excreted into the bile, the present study shows higher levels of FB1 in the

1 plasma of LXR^{-/-} mice. We postulate that LXRs contribute directly to the bioavailability of FB1
2 by inhibiting its absorption in the intestine and/or increasing bile degradation in the liver.
3 The LXR^{-/-} mouse model associated with the development of liver diseases provided an
4 opportunity to investigate the effects of FB1 exposure. Beaven et al. demonstrated that LXR^{-/-}
5 mice are susceptible to liver fibrosis in different experimental models, whereas FB1 is also
6 associated with the induction of liver fibrosis and injury (Voss et al. 1995; Howard et al. 2002).
7 In order to investigate the specific liver damage induced by FB1 in LXR^{-/-} mice, microarray
8 analysis of the hepatic transcriptome was performed. Until now, only a few transcriptional
9 pathways modulated by FB1 exposure have been characterized. Recently, we observed that
10 exposure of pigs to FB1 for 1 month activates the AKT/PTEN pathway and decreases lipid
11 metabolism. In the present study, both of these pathways were modulated by FB1, but these
12 changes occurred specifically in the LXR^{-/-} mice, suggesting that pigs are more sensitive to FB1
13 than mice and that LXR is critical in protecting mice against FB1-associated damages.
14 The modulation of the transcriptome by FB1 was more pronounced in LXR^{-/-} mice than in
15 LXR^{+/+} mice, and the induction of characteristic genes of fibrosis, such as *Colla1*, *Col3a1*, or
16 *Anxa2*, became evident (Asselah et al. 2005; Yang et al. 2017). Overall, the main regulatory
17 pathways increased by FB1 in LXR^{-/-} mice involved carcinogenesis, actin regulation, and
18 inflammation, confirming the critical role of LXRs in avoiding FB1 hepatotoxicity. Conversely,
19 bile acid degradation appears as one of the most significant pathway down-regulated by FB1
20 in LXR^{-/-} mice, suggesting that excretion of FB1 from the liver is impaired in absence of LXR.
21 To investigate hepatotoxicity in LXR^{-/-} mice exposed to FB1, biochemical parameters were
22 measured. An increase in plasma levels of AST, ALT, and alkaline phosphatase was observed
23 in response to FB1 only in LXR^{-/-} mice. These enzymes are predominantly expressed in
24 hepatocytes, and the large increase in their plasma levels is associated with hepatocyte injury
25 and necrosis (Scheig 1996; Schmidt and Schmidt 1993). These results underlined the

1 hepatotoxic effects of FB1 in absence of LXRs in mice. These findings have also been observed
2 in high-dose FB1 exposure studies (National Toxicology Program 2001). Consistent with this
3 point, histological analysis showed a specific increased in inflammation foci, necrosis, mitosis
4 and fibrosis in LXR^{-/-} mice exposed to FB1. Alongside, we showed that the absence of LXRs
5 increased plasma levels of FB1, suggesting that exacerbated toxicity in LXR knockout mice is
6 most likely attributed to the higher toxin levels in these animals..

7 In addition, FB1-treated LXR^{-/-} mice exhibited elevated levels of plasma HDL-cholesterol,
8 LDL-cholesterol, and total cholesterol characteristic of FB1-induced hepatotoxicity (Howard
9 et al. 2002; Voss et al. 1995). Thus, we postulate that the absence of LXRs disrupts cholesterol
10 metabolism and increases the bioavailability of FB1 *via* the intestine. A possible mechanism
11 that can explain the role of LXRs in modulating the effects of FB1-toxicity may involve
12 LPCAT3. LPCAT3 is a phospholipid remodeling enzyme under LXR control that modulates
13 intestinal fatty acid and cholesterol absorption (Rong et al. 2013; Wang et al. 2016).

14 Overall, this study suggests that, in the absence of LXRs, FB1 accumulates in plasma and
15 organs potentially through a defect in intestinal absorption and/or bile acid degradation.
16 Therefore, LXRs act, at least in part, as regulators of the bioavailability of FB1. Thus, in the
17 absence of LXRs, FB1 accumulates in the plasma and liver and induces deleterious effects,
18 such as fibrosis and liver injury.

19 20 **CONCLUSION**

21 Our results show that LXRs are critical to preventing toxic effects associated with FB1
22 exposure. Previous studies suggested that sphingolipids act as mediators of FB1 toxicity
23 (Benlasher et al. 2012; Merrill et al. 1996; Riley et al. 2001) because these metabolites are
24 involved in inflammatory processes and liver pathologies (Holland et al. 2011; Raichur et al.
25 2014; Turpin et al. 2014). Here, we highlight the key role of LXR in regulating FB1

1 bioavailability regardless of ceramide metabolism. LXR deficiency does not further enhance
2 the inhibition of sphingolipid synthesis (ceramide, sphinganine, sphingosine, and
3 sphingomyelin) by FB1, but has severe consequences on FB1-induced toxicity in the liver. This
4 demonstrates that FB1 hepatotoxicity is not related solely to the inhibition of sphingolipid
5 synthesis.

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15 **CONFLICT OF INTEREST**

16 The authors declare that they have no conflict of interest.

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1 **Figure legends**

2 **Fig. 1 Physiological follow-up during FB1 exposure.** LXR^{+/+} or LXR^{-/-} mice were exposed to
3 FB1 at 10 mg/kg bw/day for 28 days. (a) Body weight was followed every week over the 28
4 days. (b) Daily food and water intake. (c) Liver and spleen weight relative to body weight after
5 28 days of exposure. Data are presented as mean ± SEM (n=8/group). T, significant treatment
6 effect; G, significant genotype effect; TxG, significant interaction with p≤0.05

7
8 **Fig. 2 FB1 alters sphingolipid homeostasis in LXR^{+/+} and LXR^{-/-} mice.** LXR^{+/+} or LXR^{-/-}
9 mice were exposed to FB1 at 10 mg/kg bw/day for 28 days. (a) Sphinganine and sphingosine
10 levels in liver samples and the sphinganine (Sa)/sphingosine (So) ratio. (b) Ceramide and
11 sphingomyelin levels in liver samples. (c) Relative mRNA expression of *CerS2* and *Smpd1* in
12 liver samples quantified by qPCR. (d) Hepatic quantity of d18:1 ceramide species. (e) Hepatic
13 quantity of d18:1 sphingomyelin species. Data are presented as mean ± SEM (n=8/group). T,
14 significant treatment effect; G, significant genotype effect; TxG, significant interaction with
15 p≤0.05

16
17 **Fig. 3 LXR controls the plasma FB1 concentration.** Plasma levels of fumonisin B1 in LXR^{+/+}
18 and LXR^{-/-} mice. Data are presented as mean ± SEM (n=8/group). T, significant treatment
19 effect; G, significant genotype effect; TxG, significant interaction with p≤0.05

20
21 **Fig. 4 FB1 exposure leads to specific changes in the hepatic gene expression profile.** LXR^{+/+}
22 or LXR^{-/-} mice were exposed to FB1 at 10 mg/kg bw/day for 28 days. Gene expression profiles
23 were analyzed in liver samples from LXR^{+/+} or LXR^{-/-} mice exposed to CTRL or FB1
24 (n=6/group) using Agilent microarrays. The top 2000 most significantly regulated probes
25 (FDR≤5%), based on adjusted p-value, were selected for each contrast (5 contrasts and 4825

1 probes overall). (a) The gene expression profiles of 4825 probes are illustrated as a heatmap.
2 Red and green indicate values above and below the mean averaged centered and scaled
3 expression values (Z-score), respectively. Black indicates values close to the mean. According
4 to the probe clustering (left panel), four gene clusters exhibited specific gene expression
5 profiles, which are described on the right of the Heatmap. (b) Enriched Gene Ontology (GO)
6 functions ($p \leq 0.01$) in each cluster and relative mRNA expression by qPCR of a gene that is
7 characteristic of each cluster. qPCR data are presented as mean \pm SEM (n=8/group). T,
8 significant treatment effect; G, significant genotype effect; TxG, significant interaction with
9 $p \leq 0.05$

10
11 **Fig. 5 LXR deficiency influences the hepatic gene expression profile in response to FB1.**

12 (a) Venn diagram comparing differentially expressed genes ($FDR \leq 1\%$) in response to FB1
13 exposure in $LXR^{+/+}$ and $LXR^{-/-}$ mice. Up-regulated genes and down-regulating genes are
14 represented in bold and regular font, respectively. (b) and (c) panels are Bar plots representation
15 of the Fold Change (in Log2) of the relative gene expression in response to FB1 exposure, for
16 the top 40 most significant ($FDR \leq 1\%$) up-regulated (b) or down-regulated (c) gene expression
17 in $LXR^{-/-}$ mice (Blue bars) and in $LXR^{+/+}$ mice (Red bars). (d) Relative gene expression of
18 *Coll1a1*, *Smpd3*, *Elovl7*, *Cyp7b1*, *Tm7sf2*, and *Abcg8* in liver samples quantified by qPCR. Data
19 are presented as mean \pm SEM (n=8/group). T, significant treatment effect; G, significant
20 genotype effect; TxG, significant interaction with a $p \leq 0.05$

21
22 **Fig. 6 LXR deficiency alters liver biochemistry in response to FB1. $LXR^{+/+}$ or $LXR^{-/-}$ mice**

23 were exposed to FB1 at 10 mg/kg bw/day for 28 days. (a) Plasma ALT, AST, ALP, and bilirubin
24 levels. (b) Plasma cholesterol (HDL, LDL, and total). (c) Hepatic cholesterol, esterified
25 cholesterol (EC), and triglyceride (TG) levels. Data are presented as mean \pm SEM (n=8/group).

1 T, significant treatment effect; G, significant genotype effect; TxG, significant interaction with
2 p ≤ 0.05

3
4 **Fig. 7 LXR deficiency enhances liver injury in response to FB1.** LXR^{+/+} or LXR^{-/-} mice were
5 exposed to FB1 at 10 mg/kg bw/day for 28 days. (a) Representative hematoxylin and eosin or
6 Sirius red staining of liver sections. Scale bars, 100 μm. (b) Histological scoring of apoptosis,
7 inflammatory foci, mitosis, and necrosis in 10 distinct areas from LXR^{+/+} and LXR^{-/-} mice
8 exposed or not exposed to FB1. Field 200X. Data are presented as mean ± SEM (n=8/group).
9 T, significant treatment effect; G, significant genotype effect; TxG, significant interaction with
10 p ≤ 0.05

11
12 **Online Resource 1: Oligonucleotide sequences for real-time PCR**

13
14 **Online Resource 2: Pathways significantly up-regulated specifically in LXR^{-/-} mice treated
15 with FB1**

16
17 **Online Resource 3: Pathways significantly down-regulated specifically in LXR^{-/-} mice
18 treated with FB1**

Figure 1

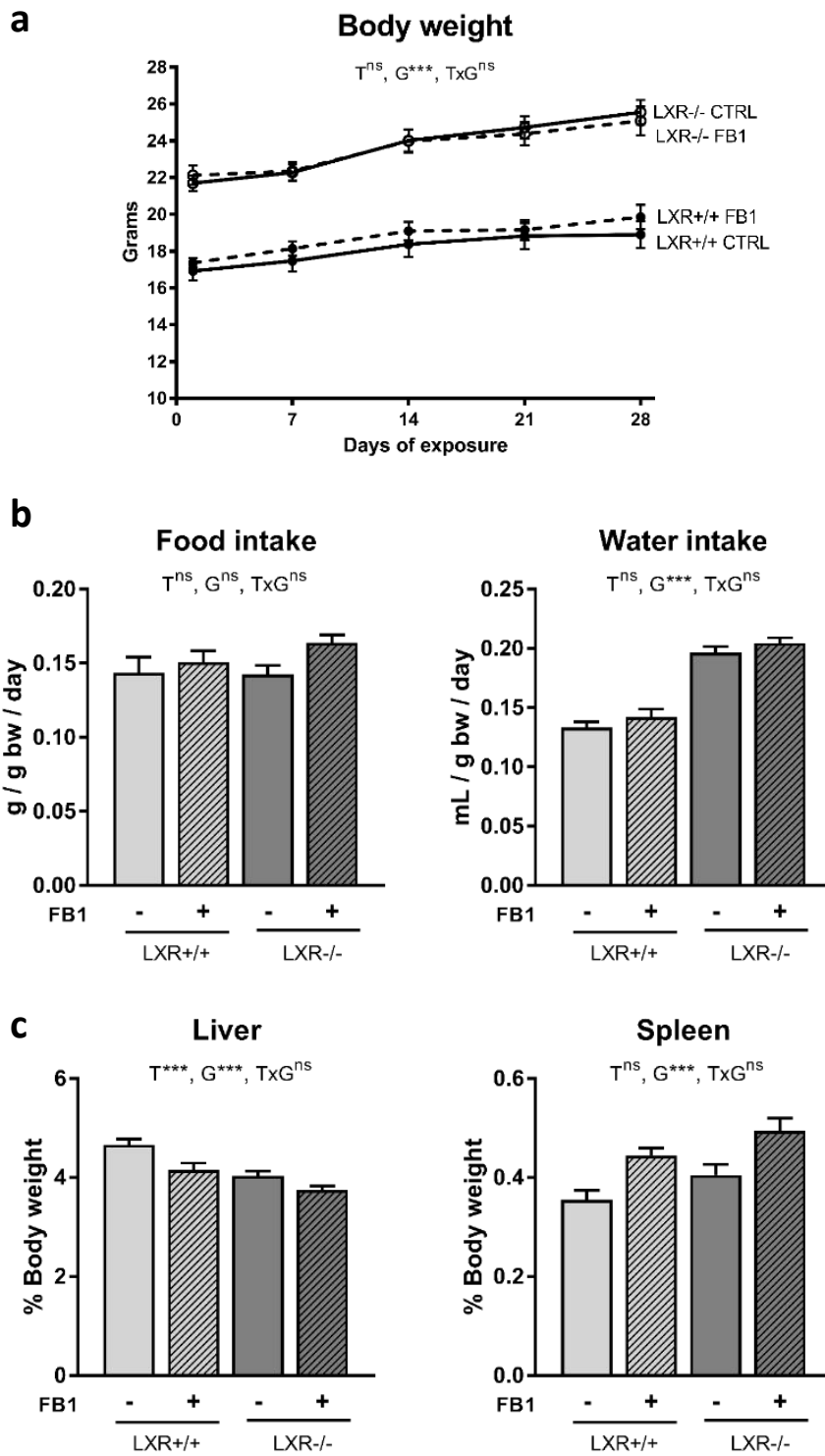
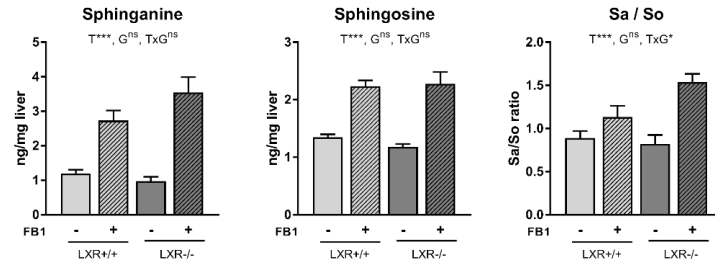
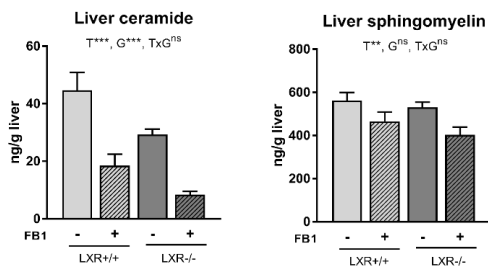


Figure 2

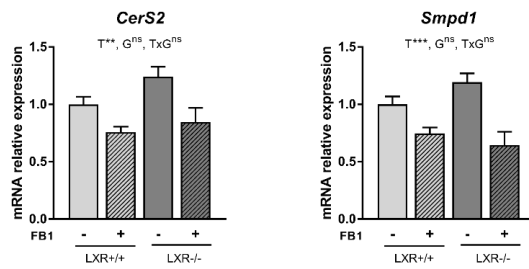
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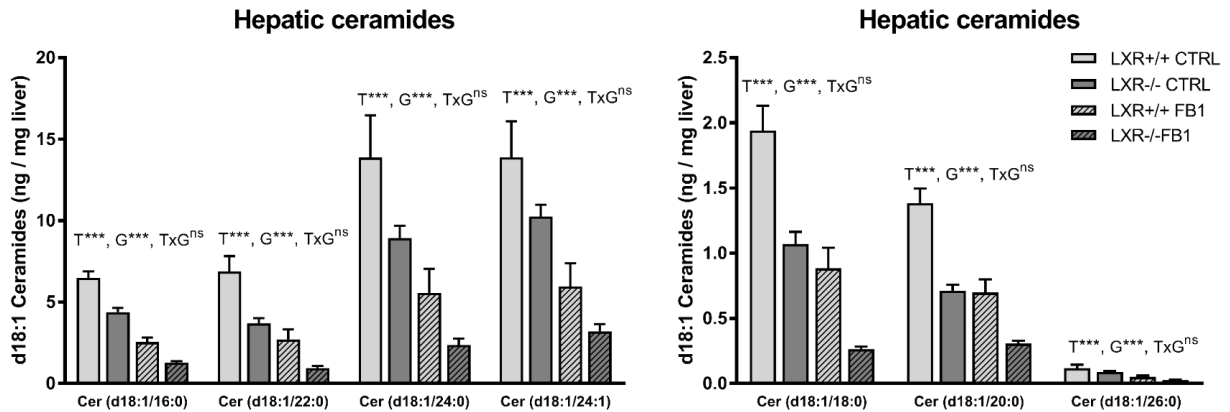
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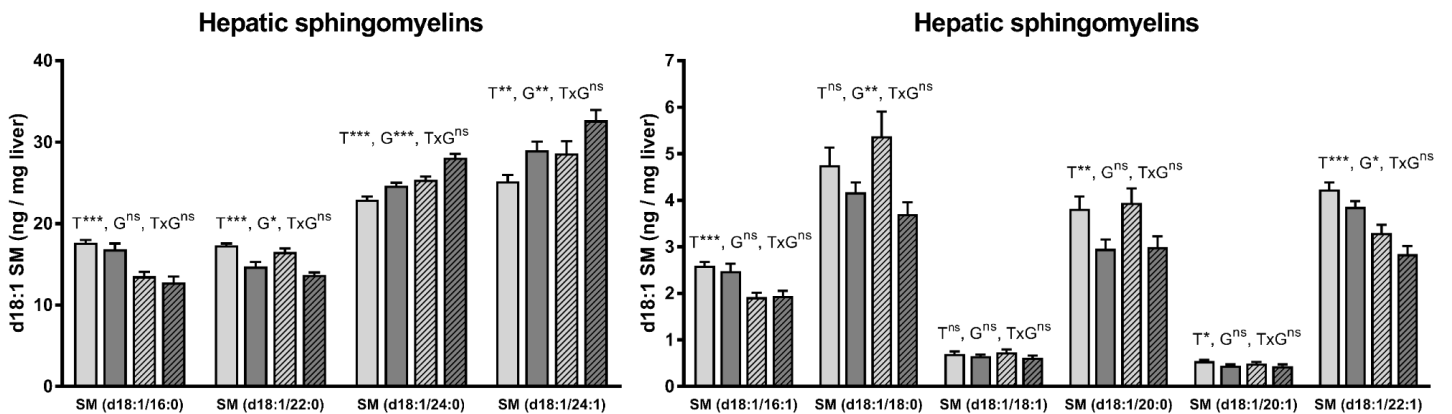
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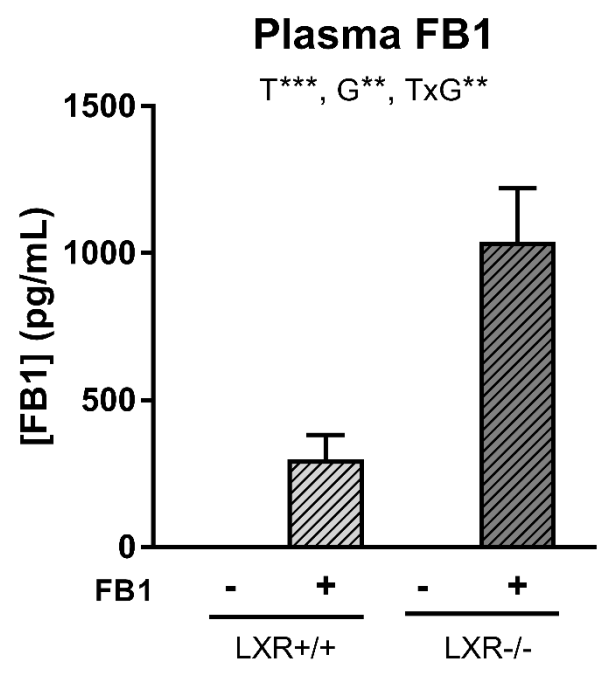


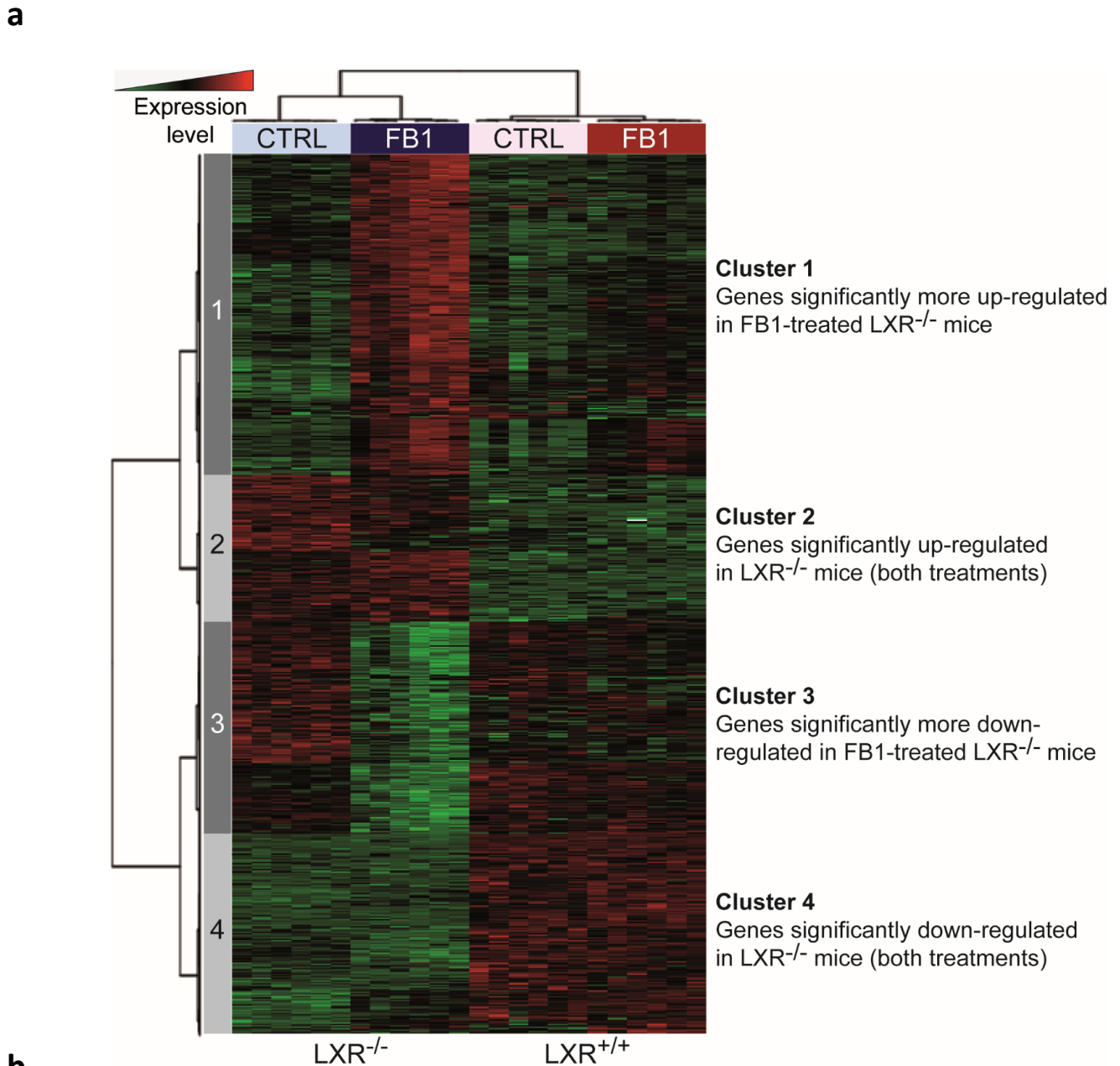
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e



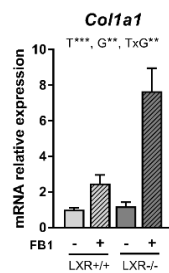




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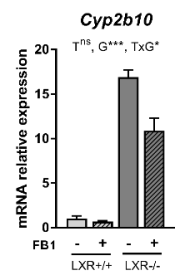
Cluster 1

- Focal adhesion
- PI3K-AKT signaling pathways
- Proteoglycans in cancers
- ECM-receptor interaction
- Chronic myeloid leukemia



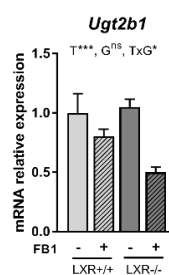
Cluster 2

- Cytokine receptors interaction
- Catalytic activity



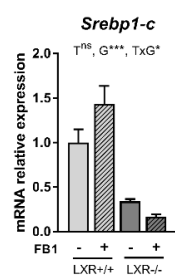
Cluster 3

- Metabolic pathways
- VAL, LEU and ILEU degradation
- ALA, ASP and GLUT metabolism
- Drug metabolism
- Bile secretion



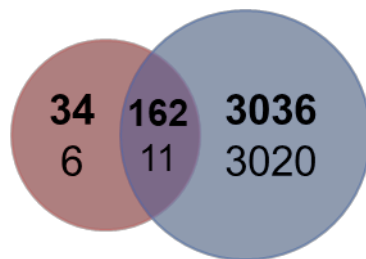
Cluster 4

- PPAR signaling pathway
- Pyruvate metabolism
- Fatty acid metabolism



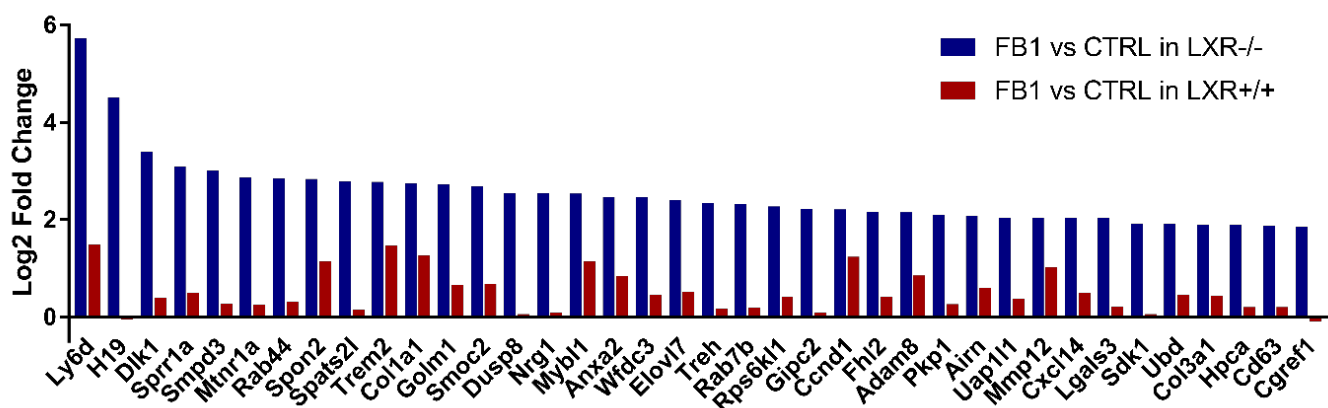
a

DEG (p<0.01) in response to FB1
Specific to LXR+/+

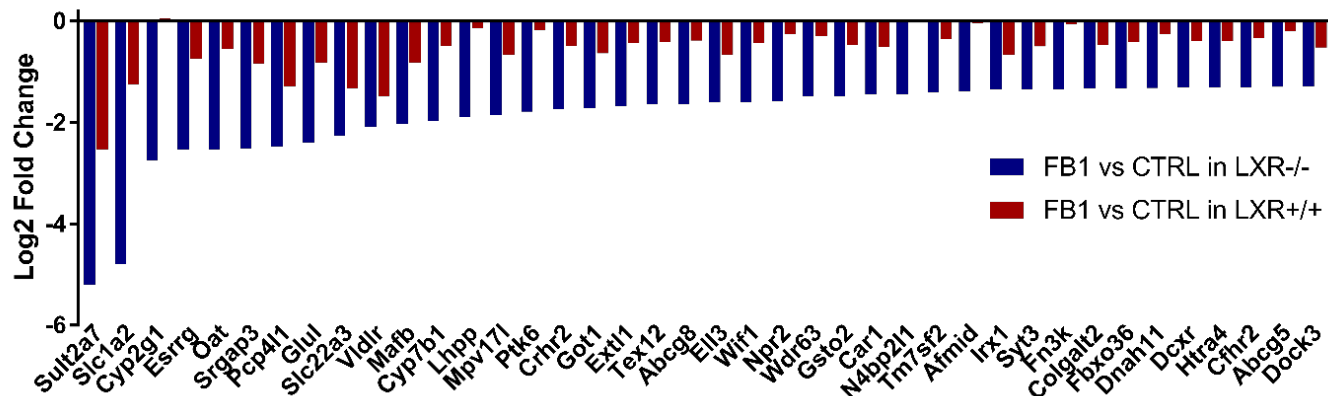


DEG (p<0.01) in response to FB1
Specific to LXR-/-

b



c



d

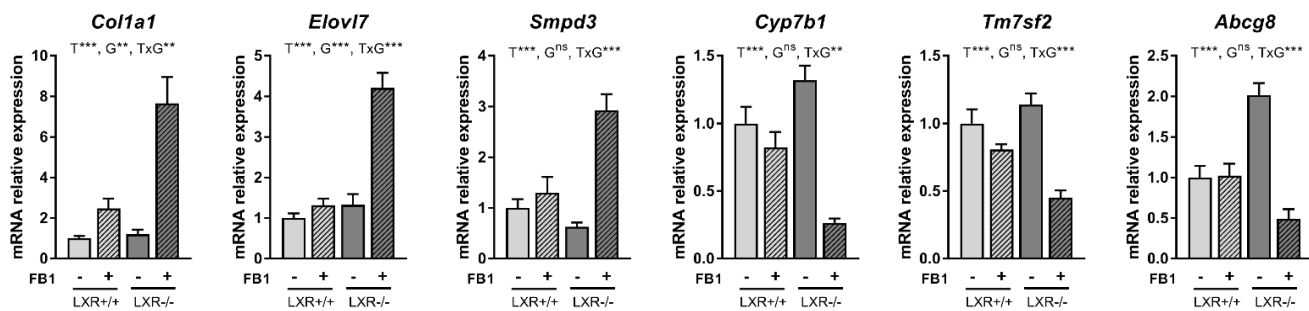
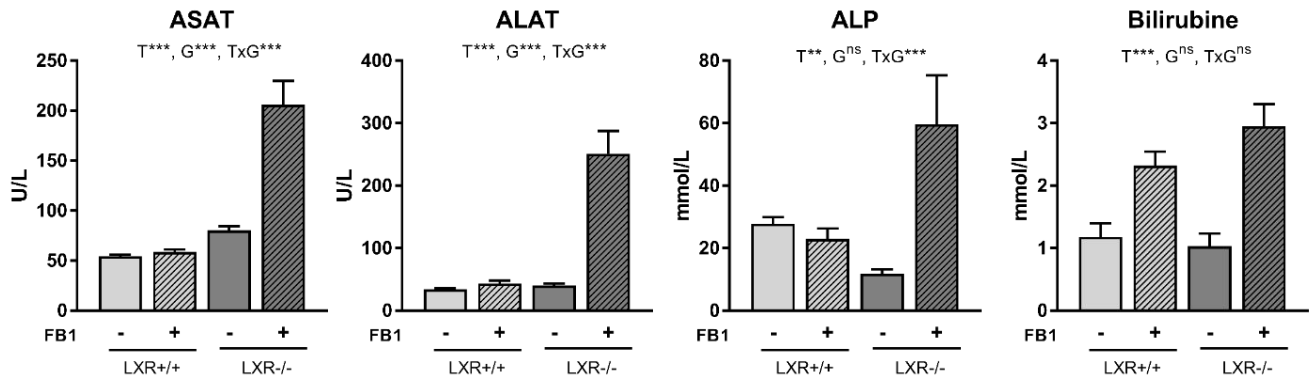
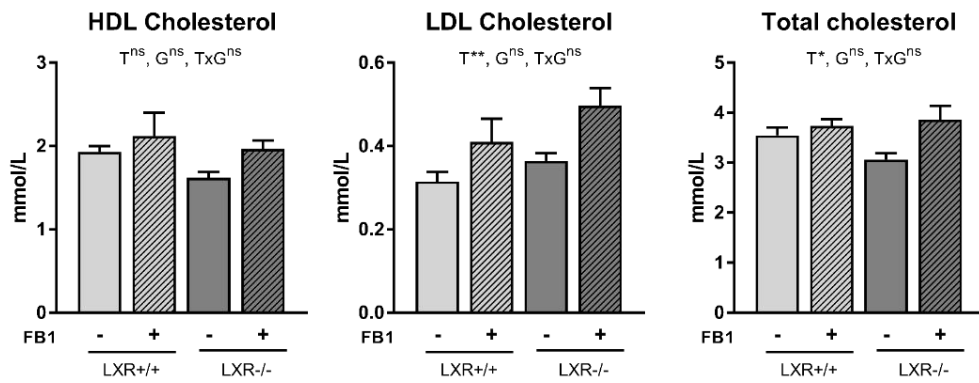


Figure 6

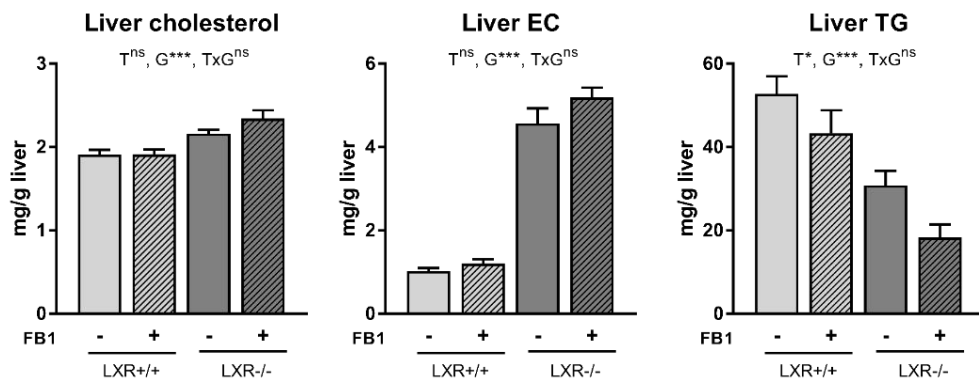
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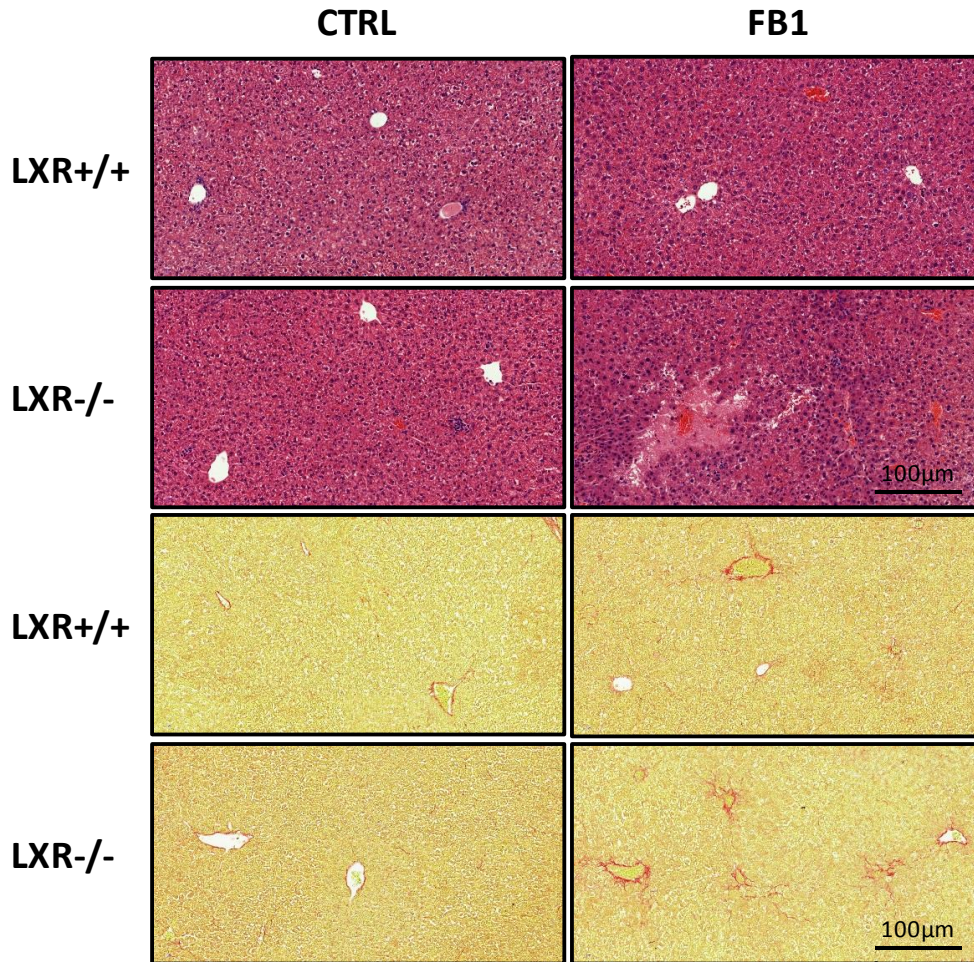
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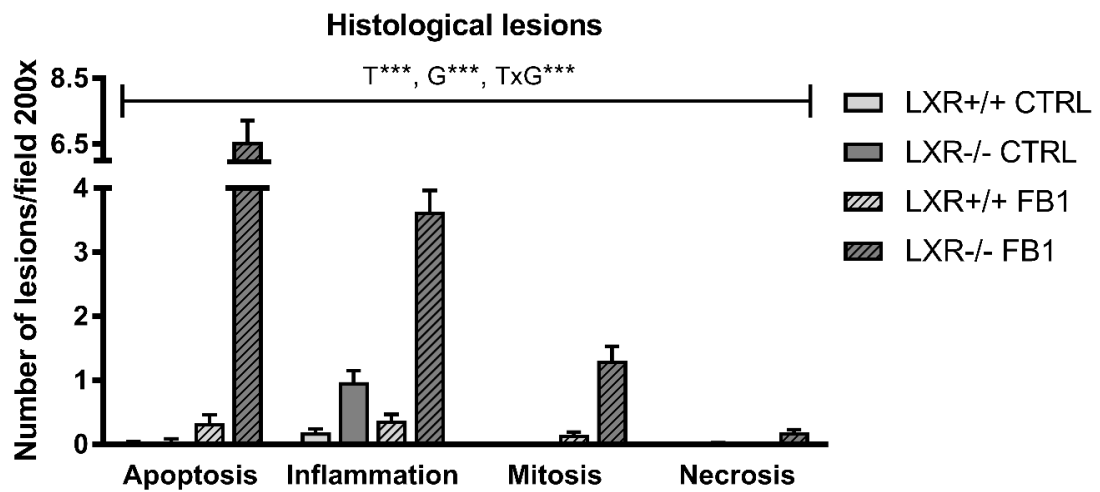
c



a



b



Online Resource 1: Oligonucleotide sequences for real-time PCR

Gene	NCBI Refseq	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Abcg8</i>	NM_026180	ATCCATTGGCCACCCTTGT	GCGTCTGTCGATGCTGGTC
<i>CerS2</i>	NM_029789	CAGCTCTGCACCGGACG	GGTTAAGTTCACAGGCAGCCAT
<i>Col1a1</i>	NM_007742	GGCTCCTGCTCCTCTTAGGG	TCGGGTTTCCACGTCTCAC
<i>Cyp2b10</i>	NM_009999	TTTCTGCCCTTCTCAACAGGAA	ATGGACGTGAAGAAAAGGAACAAC
<i>Cyp2e1</i>	NM_000773	CTGTGTTCCAGGAGTACAAGAACAAG	TCCTTCCATGTGGGTCCATT
<i>Cyp7b1</i>	NM_007825	ACATGGTGACACTTTCCTGTCTTC	GAACTTCTGAAAGCTTAATTGTTTTGG
<i>Elovl7</i>	NM_029001	TCATGGAGAACCGGAAGCC	AATGTCACATCGAAACGAGTAACCT
<i>Got1</i>	NM_010324	TCCAGATCCCCGCAAGGT	CCTTCCTCACTACTGGCAAACC
<i>Psmb6</i>	NM_008946	GGAAGTCTCCACAGGGACCA	AACCACGCCCCCATTAAACT
<i>Smpd1</i>	NM_011421	TGTTTACCAGCTGATGCC	AGGCGGAGGCCAGGG
<i>Smpd3</i>	NM_018667	CCAATGGGTGCAGCTTCG	AACAATTCTTTGGTCTGAGGTG
<i>Srebp1-c</i>	NM_011480	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGGCCAG
<i>Tm7sf2</i>	NM_028454	AAGGCCTGGAAGTGAAGGACA	ACCAGAGCCTGGAAGCCAT
<i>Ugt2b1</i>	NM_152811	GTTTTCTCTGGGATCAATGGTTAAA	TTTCTTACCATCAAATCTCCACAGAAC

Online Resource 2: Pathways significantly up-regulated specifically in LXR^{-/-} mice treated with FB1

Pathway description	Observed gene count	False discovery rate
Focal adhesion	33	5.04e-10
PI3K-Akt signaling pathway	41	9.61e-09
Proteoglycans in cancer	31	2.28e-08
ECM-receptor interaction	17	1.9e-06
Chronic myeloid leukemia	15	2.33e-06
Amoebiasis	19	4.27e-06
Osteoclast differentiation	19	6.32e-06
MicroRNAs in cancer	20	9.93e-06
Protein digestion and absorption	15	3.51e-05
Viral carcinogenesis	23	4.66e-05
Pathways in cancer	30	0.000117
HTLV-I infection	26	0.000217
Glioma	11	0.00024
Regulation of actin cytoskeleton	22	0.000402
p53 signaling pathway	11	0.000629
Cytokine-cytokine receptor interaction	23	0.00105
MAPK signaling pathway	23	0.00134
Hepatitis B	16	0.00161
Colorectal cancer	10	0.00164
Prostate cancer	12	0.00164
Chemokine signaling pathway	18	0.00173
Pancreatic cancer	10	0.00173
Endocytosis	20	0.00178
Endocytosis	8	0.00249
Melanoma	10	0.00284
Natural killer cell-mediated cytotoxicity	13	0.00369
Bacterial invasion of epithelial cells	10	0.00524
Thyroid cancer	6	0.00648
Transcriptional misregulation in cancer	15	0.00947
Thyroid hormone synthesis	9	0.0105
Platelet activation	13	0.0107
Vasopressin-regulated water reabsorption	7	0.0107
Small cell lung cancer	10	0.0116
Neurotrophin signaling pathway	12	0.0131
Dilated cardiomyopathy	10	0.0142
Ras signaling pathway	18	0.0148
Cell cycle	12	0.0152
Leukocyte transendothelial migration	12	0.0152
Bladder cancer	6	0.0152
Leishmaniasis	8	0.0192
Estrogen signaling pathway	10	0.0199
Phagosome	14	0.0216
Apoptosis	9	0.0225
Glutathione metabolism	7	0.0259
Hippo signaling pathway	13	0.027
Gap junction	9	0.0306
Thyroid hormone signaling pathway	11	0.0306
NF-kappa B signaling pathway	9	0.0389

Online Resource 3: Pathways significantly down-regulated specifically in LXR^{-/-} mice treated with FB1 (p<0.01)

Pathway description	Observed gene count	False discovery rate
Metabolic pathways	83	3.84e-17
Valine, leucine, and isoleucine degradation	10	2.13e-05
Alanine, aspartate, and glutamate metabolism	8	0.000108
Arginine and proline metabolism	9	0.000305
Phenylalanine metabolism	6	0.000305
Glyoxylate and dicarboxylate metabolism	6	0.000767
Nitrogen metabolism	5	0.00118
Propanoate metabolism	6	0.00213
Drug metabolism - other enzymes	7	0.00377
Microbial metabolism in diverse environments	13	0.00377
Biosynthesis of amino acids	8	0.00693
Tyrosine metabolism	6	0.00716
Pyrimidine metabolism	9	0.00834
Tryptophan metabolism	6	0.00834
Selenocompound metabolism	4	0.00834
Pantothenate and CoA biosynthesis	4	0.00834
Peroxisome	8	0.00834
beta-Alanine metabolism	5	0.00915
Linoleic acid metabolism	6	0.00915
2-Oxocarboxylic acid metabolism	4	0.00915
Serotonergic synapse	10	0.0105
Glycerophospholipid metabolism	8	0.013
Bile secretion	7	0.0151
Glycine, serine, and threonine metabolism	5	0.0212
Carbon metabolism	8	0.0345