Original Paper

Farnesoid X Receptor in Mice Prevents Severe Liver Immunopathology During Lymphocytic Choriomeningitis Virus Infection

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Key Words

NR1H4 • FXR • IFN-I • Bile acids • LCMV • Monocytes

Abstract

Background: Bile acids (BAs) are steroid molecules that are synthesized in the liver. In addition to their important role as a surfactant in solubilizing lipids and promoting the absorption of lipids in the gastrointestinal tract, they act as inflammagens. The role of BAs and their receptor farnesoid X receptor (FXR) during viral infection has not been studied in detail. **Methods:** By using FXR-deficient mice, we investigated the role of bile acid receptor FXR during infection with lymphocytic choriomeningitis virus (LCMV). The importance of FXR in inducing IFN-I and monocytes proliferation were investigated and viral titers and T cell exhaustion were analyzed at different time points. **Results:** This study shows that controlled levels of BAs activate FXR in hepatocytes and FXR in response upregulates the production of type I interferon. In turn, FXR maintains BAs within a balanced range to inhibit their toxic effects. The absence of FXR results in high levels of BAs, which inhibit the proliferation of monocytes and result in a defect in viral elimination, consequently leading to T cell exhaustion. **Conclusion:** We found that FXR contributes to IFN-I production in hepatocytes and balances BA levels to inhibit their toxic effects on monocytes.

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Introduction

The liver is a central organ that is responsible for vital functions, including the detoxification and metabolism of many drugs, the production of enzymes, cytokines, and the uptake of viruses through Kupffer cells. Additionally, the liver is important for the synthesis of bile acids (BAs). In recent years, many studies have shown that BAs not only promote the absorption of lipids because of their amphipathic nature but are also considered to be signaling molecules in hepatocytes that stimulate the production of proinflammatory cytokines [1]. However, BAs can also increase the replication of viruses such as hepatitis C [2] and hepatitis B viruses [3, 4]. Therefore, their influence on the immune system is still controversial.

Two BA receptors have been described. The first, TGR5 (also known as GPAR1, M-BAR, or BG37), is a surface receptor that belongs to the family of G protein-coupled receptors (GPCRs). TGR5 is expressed in various tissues, such as ileum and colon, sinusoidal endothelial cells, and macrophages [5-9]. The second receptor is an intracellular BA-activated nuclear receptor called farnesoid X receptor (FXR; also known as nuclear receptor subfamily 1, group H, member 4; NR1H4). FXR belongs to the nuclear hormone receptor (NHR) superfamily, which is composed of 49 functional genes [10]. Many studies have shown that both TGR5 and FXR receptors may contribute to immune function [8, 11-16]. The importance of FXR emerged from its ability to regulate many genes involved in liver cancer [17, 18], hepatic inflammation [19, 20], hepatic fibrosis [21, 22], triglycerides, cholesterol [23], glucose [24, 25], and BA homeostasis [26, 27]. FXR is mainly expressed in tissues that have contact with BAs, such as liver, intestine, and kidney [28-30]. In the liver, FXR is mainly expressed in hepatocytes (parenchymal cells), at levels as much as 20-fold higher than those in non-parenchymal cells [31, 32].

BAs are endogenous ligands for FXR. The hydrophobic BA chenodeoxycholic acid (CDCA) is the strongest activator of FXR [33, 34]. In turn, FXR induces the small heterodimer partner (SHP), which downregulates cholesterol 7 α -hydroxylase (Cyp7a1) and consequently reduces the synthesis of BAs [35]. Recently, a few reports have described the roles of FXR as a player at the immunological level [11-14, 19, 20, 36]. The change in its expression can influence the production of some cytokines, but its exact role in controlling viral infection has not yet been determined.

In our study, using FXR knockout (FXR^{-/-}) mice, we found that infection with lymphocytic choriomeningitis virus (LCMV) leads to the release of BAs; this release signals the liver, through FXR, to produce type I interferon (IFN-I) in hepatocytes. Additionally, the feedback mechanism of BAs through FXR guarantees low levels of BAs, thereby avoiding their negative role in monocyte expansion. This feedback mechanism allows efficient proliferation and infiltration of immune cells to the site of infection, consequently leading to rapid elimination of the pathogen and saving the adaptive immune cells from exhaustion due to viral overload.

Material and Methods

Mice

All experiments were performed with animals housed in single ventilated cages, under the authorization of the Veterinäramt Nordrhein Westfalen (Düsseldorf, Germany) and in accordance with the German law for animal protection. All mice were sex matched and they were used 10-14 weeks old. FXR^{-/-} and CD8^{-/-} mice were maintained on a C57BL/6 background. P14/CD45.1 mice expressing a T cell receptor (TCR) transgene specific for LCMV glycoprotein aminoacid 33 to 41 (LCMV-GP33-41) were also maintained on a C57BL/6 background and were used for adoptive transfer experiments [37]. All the experimental protocols were approved by the Nordrhein Westfalen Landesamt für Natur, Umwelt und Verbraucherschutz (Recklinghausen, Germany).

Virus, Beads

LCMV strain WE was originally obtained from Prof. Dr. F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated in L929 cells. Mice were infected intravenously with LCMV-WE at the indicated doses. Latex beads (Fluoresbrite, 4.55×10^{10} particles per mL) were obtained from Polysciences (Warrington, PA, USA) and had a diameter of 1 μ m.

Viral uptake

Mice were intravenously infected with 2×10^6 PFU LCMV-WE. After 1, 10, and 60 min, LCMV viral titer was measured in the blood by plaque assay.

Measurement of bile acids, ALT, AST, bilirubin, and LDH

Biochemical analyses were performed by the Central Laboratory, Clinic of the University Duisburg-Essen, Essen, Germany or MVZ Laboratory Dr. Limbach, Heidelberg, Germany.

Histology

Histological analyses were performed on snap-frozen tissues and LCMV was visualized with a monoclonal antibody against LCMV nucleoprotein (VL4; made in-house). Liver macrophages (Kupffer cells) were stained with anti-F4/80 (BM8; eBioscience, San Diego, CA, USA).

Plaque assay

LCMV titers were measured with a plaque-forming assay using MC57 cells, as previously described [38].

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR

RNA was isolated from liver tissue with the RNA Mini Kit (Qiagen, Hilden, Germany) or from FACS-sorted cells with Trizol (Thermo Fisher Scientific, Waltham, MA, USA). Quantitation of RNA was performed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The RNA was reverse-transcribed to cDNA with the Quantitect Reverse Transcription Kit (Qiagen). Gene expression analysis was performed with assays from Qiagen (*GAPDH*, *Ifnar*, *Oas1*, *Mx1*, *Ifna4*, and *Ifnb1*) or Eurofins (*NP* and *GP*). For analysis, the expression levels of all target genes were normalized against glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Δ cycle threshold [Ct]). Gene expression values were then calculated by the delta delta Ct ($\Delta\Delta$ Ct) method, with the mean of the control group as the calibrator to which all other samples were compared. Relative quantities (RQs) were determined with the equation $RQ = 2^{-\Delta\Delta Ct}$.

Depletion of macrophages

Macrophages were depleted by intravenous injection of 200 μ L (50 mg) clodronate-encapsulated liposomes in phosphate-buffered saline (PBS) on day -1 of infection [39].

Sorting of F4/80⁺CD11b⁺ macrophages

Liver was digested with Liberase DNase (Roche, Basel, Switzerland) and stained with anti-F4/80 (eBioscience) and anti-CD11b (eBioscience). After incubation for 30 min, F4/80⁺CD11b⁺ cells were washed and sorted by FACS (BD FACS Aria III, BD Bioscience, Franklin Lakes, NJ, USA).

Flow cytometry

Lymphocytes were stained with anti-CD8 (53-6.7; BD Bioscience). For measurement of intracellular IFN- γ , cells were restimulated with glycoprotein 33 (GP33; Strasbourg, France) for 6 h, fixed with 2% formaldehyde (Sigma-Aldrich; Steinheim, Germany) for 10 min, permeabilized with saponin, and stained with anti-IFN- γ antibody (eBioscience).

Tetramers were provided by the National Institutes of Health (NIH) Tetramer Facility (Emory University, Atlanta, GA, USA). Staining was performed as previously described [40]. Briefly, blood and cells were stained with allophycocyanin (APC)-labeled GP33 MHC class I tetramers (GP33/H-2Db) for 15 minutes at 37°C. After incubation, the samples were stained with anti-CD8 peridinin-chlorophyll-protein complex (PerCP; BD Biosciences) for 30 min at 4°C. Erythrocytes were then lysed with 1 ml BD lysing solution (BD Biosciences), washed once, and analyzed with a flow cytometer LSR Fortessa (BD Biosciences). Absolute

numbers of GP33-specific CD8⁺ T cells were calculated by fluorescence-activated cell sorting (FACS) analysis using fluorescent beads (BD Biosciences).

Lymphocyte transfer

Lymphocyte transfer was done as previously described [41]. Briefly, 10⁷ splenocytes from P14 mice expressing CD45.1 were labelled with CFSE (1 μM, Invitrogen, Carlsbad, CA, USA) and were injected intravenously into FXR^{-/-} or C57BL/6 WT mice on day 12 after infection with 2×10⁶ PFU LCMV-WE. Two days later (on day 14 after LCMV infection), the proliferation of P14 T cells was assessed in the spleen by CFSE dilution and flow cytometry.

Bone marrow chimeras

To generate bone marrow chimeras, we irradiated recipient mice with 9.5 Gy (320 kV X-rays, 3 Gy/min, 0.35 mm copper+1.5 mm aluminium filter; Pantak-Seifert, Ahrensburg, Germany) on day -1. On the next day, 10⁷ bone marrow cells were transferred. After 15 days, clodronate liposomes were administered to ensure macrophage exchange in WT > WT, FXR^{-/-} > WT, WT > FXR^{-/-}, and FXR^{-/-} > FXR^{-/-} chimeras. LCMV infection was performed after 30 days.

Isolation of primary hepatocytes

Primary hepatocytes were isolated from murine livers by collagenase perfusion, as described previously [42]. The hepatocytes were cultured in Williams' medium E (Biochrom, Berlin, Germany) containing 10% fetal bovine serum (FBS; Biochrom), and L-glutamine-penicillin-streptomycin (Sigma-Aldrich, Steinheim, Germany).

Treatment of cells

For *in vitro* infections, primary hepatocytes from C57BL/6 WT mice were seeded at 3×10⁵ cells per well in a 6-well plate and were infected with LCMV-WE at a multiplicity of infection (MOI) of 1 or left uninfected. Cells were additionally treated with the FXR agonist GW4064 (1 μM; Sigma-Aldrich) or left untreated. After 6 and 24 h, hepatocytes were washed twice with PBS.

Primary hepatocytes from WT and FXR^{-/-} mice were seeded and infected as described above. Cells were additionally treated with CDCA (50 μM; Sigma-Aldrich) or left untreated. After 24 h, hepatocytes were washed twice with PBS, and RNA was isolated as described above.

Bone marrow cells were isolated from femurs and tibias of WT and FXR^{-/-} mice. After elimination of erythrocytes, bone marrow cells were cultured in very low endotoxin Dulbecco's Modified Eagle's Medium (VLE-DMEM) supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) and 0.1% 2-mercaptoethanol (β-ME; Sigma-Aldrich, Steinheim, Germany) in the presence or absence of GM-CSF. After 15 min, 2×10⁶ bone marrow cells were additionally treated with CDCA (50 μM; Sigma-Aldrich) or left untreated for additional 15 min. Cells were lysed with sodium dodecyl sulfate (SDS) buffer for immunoblotting. For analysis of Ly6C⁺ cell numbers, bone marrow cells were treated with or without CDCA (100 μM; Sigma-Aldrich) and with GM-CSF for 5 days; cells were stained for Ly6C and measured with FACS.

Immunoblotting

Bone marrow cells from WT and FXR^{-/-} mice were lysed with boiling SDS buffer (Sigma-Aldrich; 1.1% SDS, 11% glycerol, 0.1M Tris; pH 6.8) with 10% 2-mercaptoethanol (β-ME; Sigma-Aldrich). Total cell extracts were examined by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto Whatman nitrocellulose membranes (GE Healthcare, Freiburg, Germany) by standard techniques. Membranes were blocked for 1 h in 5% bovine serum albumin (BSA; PAA Laboratories, Pasching, Austria) / 5% nonfat dried milk powder (AppliChem, Darmstadt, Germany) in Tris-buffered saline (TBS, Calbiochem, San Diego, CA, USA) supplemented with 1% Tween-20 and incubated with the following antibodies: anti-phospho-p44/42 (p-Erk1/2; Cell Signaling Technologies, Danvers, MA, USA) or anti-GAPDH (Meridian Life Science, Memphis, TN, USA). Antibody binding was detected by horse radish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) antibodies (BIO RAD, Munich, Germany). Signals were detected with the BIO RAD ChemiDoc imaging system and analyzed with the manufacturer's software. Images have been cropped for purposes of presentation.

Antibody treatment

LCMV-WE-infected mice were intravenously treated with RB6-8C5 antibody (Bioxcell, West Lebanon, NH, USA; 200 µg per mouse) on day 3 after LCMV infection to deplete Gr-1⁺ myeloid cells and to analyse viral load (by plaque assay and histological analysis), or were treated additionally on days 7 and 11 after infection for T cell measurement.

Statistical analysis

Data are expressed as mean ± S.E.M. Student's *t*-test was used to detect statistically significant differences between groups. Significant differences between several groups were detected by two-way analysis of variance (ANOVA) with Bonferroni or Dunnett post hoc tests. The level of statistical significance was set at *P* < 0.05. Each data reflect results from one experiment unless it is mentioned in the figure legend that they are pooled from several experiments.

Ethics Statement

Animal experiments were carried out with approval of the "Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen" (LANUV), Germany (approval number: 84-02.04.2011.A282 and 84-02.04.2011.A246) in accordance with the German laws for animal protection. Animal care and documentation was supervised by the central animal laboratory of the University Hospital Essen, Essen, Germany.

Results

The role of bile acids and their receptor FXR during LCMV infection

To determine whether the secretion of BAs is upregulated during LCMV infection, we measured the BAs in the blood serum of C57BL/6 wild-type (WT) mice after they had been infected with the acute strain of LCMV (LCMV-WE). We found that the secretion of BAs was increased 10 days after LCMV infection (Fig. 1A). This increase is directly balanced by a feedback mechanism through FXR, because the absence of FXR led to persistently high serum levels of BAs (Fig. 1A). In line with this finding, we noticed that, in contrast to C57BL/6 WT mice, FXR^{-/-} mice exhibited high levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The lactate dehydrogenase (LDH), an indicator of parenchymal injury, appeared similar in WT and FXR^{-/-} mice at day 10 after infection, but was significantly different at day 15 and 20 indicating that WT mice recovered earlier from liver cell damage. More severe liver cell damage in FXR^{-/-} mice is also indicated by impaired bilirubin metabolism (Fig. 1B). To check whether LCMV infection leads to elevated levels of BAs directly or is arise from CD8⁺ T cell-dependent liver immunopathology, we measured BAs in C57BL/6 WT and CD8^{-/-} mice after LCMV infection. We found that CD8⁺ T cells are responsible for the destruction of hepatocytes and release of BAs (Fig. 1C). Taken together, as a consequence of infection liver cell damage is more severe in FXR^{-/-} mice than in WT mice and liver inflammation sustains only in FXR^{-/-} mice. We conclude that FXR and their BA ligands play an important role during LCMV infection.

FXR is essential for controlling viral replication in Kupffer cells

Next, we questioned whether the liver damage in FXR^{-/-} mice was due to a defect in virus control. To answer this question, we measured the viral titer in various organs after infection with LCMV. The absence of FXR led to higher viral replication in the liver, whereas FXR-deficiency had no impact on the viral titer in other investigated organs (Fig. 2A). Histological staining showed that virus is replicating in both Kupffer cells and hepatocytes in FXR-deficient mice but only in Kupffer cells in WT mice (Fig. 2B). This higher viral titer in the liver was not due to phagocytic activity, because initial viral uptake from the blood was similar in WT and FXR^{-/-} mice (Fig. 2C). This finding indicated that naive Kupffer cells have no difficulty in the phagocytosis of virus. We speculated that the Kupffer cells of FXR-deficient mice cannot control the virus; therefore, we sorted Kupffer cells from LCMV-

infected WT and FXR^{-/-} mice and measured the expression of viral genes. The expression of LCMV glycoprotein and nucleoprotein in Kupffer cells was higher in FXR^{-/-} mice than in WT mice (Fig. 2D).

To assess the phagocytic efficiency of Kupffer cells during infection, we infected WT and FXR^{-/-} mice with LCMV for 5 days. We then injected fluorescent latex beads into the mice and after 1 hour we measured the uptake of beads in the liver. Kupffer cells of WT mice are more efficient in taking up beads than Kupffer cells of FXR^{-/-} mice (Fig. 2E). From these results, we conclude that the absence of FXR leads to a defect in the ability of Kupffer cells to control virus during infection, with the consequence of higher viral replication in the liver.

FXR increases the infiltration of immune cells to the infected organs

In an earlier study, we found that Kupffer cells suppress viral replication in an IFN-I-dependent manner [42, 43]. In the current study, we hypothesized that the defect of Kupffer cells in FXR^{-/-} mice was due to a reduction in the antiviral effect. To test this theory, we measured the antiviral genes (*Oas1* and *Mx1*) in resident liver macrophages (Kupffer cells) sorted by FACS. Indeed, we found that, in the absence of FXR, the expression of antiviral genes by Kupffer cells was lower than in WT mice (Fig. 3A). Speculating that IFN-I *in situ* is reduced, we measured the gene expression of IFN-I (*Ifna4* and *Ifnb1*) in the liver under naïve conditions and after infection with LCMV. Indeed, IFN-I gene expression was significantly higher in WT mice than in FXR^{-/-} mice after infection (Fig. 3B). We concluded that the higher viral titer in Kupffer cells results from the reduction of IFN-I production in FXR^{-/-} mice.

Fig. 1. The role of bile acids and farnesoid X receptor during infection with lymphocytic choriomeningitis virus. (A) Bile acid titers in C57BL/6 wild-type (WT) and farnesoid X receptor-knockout (FXR^{-/-}) mice intravenously infected with 2 × 10⁴ PFU lymphocytic choriomeningitis virus strain WE (LCMV-WE; n = 4-5). (B) Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and bilirubin parameters were measured in the serum of C57BL/6 WT and FXR^{-/-} mice intravenously infected with 2 × 10⁴ plaque-forming units (PFU) LCMV-WE at the indicated time points (n = 4). (C) Bile acid titers measured at the indicated time points in C57BL/6 wild-type (WT) and CD8^{-/-} mice intravenously infected with 2 × 10⁶ PFU LCMV-WE (n = 3-4). The figure was adapted from diploma thesis of Caroline Krings Heinrich-Heine-University Düsseldorf. * P < 0.5; ** P < 0.01; *** P < 0.001. Statistical significance was detected by Student's *t*-test (B; LDH) or analysis of variance (ANOVA; A, B; AST, ALT, and bilirubin and C).

Fig. 2. Lymphocytic choriomeningitis virus persists in farnesoid X receptor-knockout mice. (A-B) C57BL/6 wild-type (WT) and farnesoid X receptor-knockout (FXR^{-/-}) mice were intravenously infected with 2×10^4 plaque-forming units (PFU) lymphocytic choriomeningitis virus strain WE (LCMV-WE). (A) LCMV titers in various organs of C57BL/6 WT and FXR^{-/-} mice were measured after 5 days of infection (n = 4). (B) Immunofluorescence of liver sections from C57BL/6 and FXR^{-/-} mice 5 days after LCMV-WE infection, stained for F4/80 (Kupffer cells; red) and LCMV-NP (nucleoprotein; green). Fluorescence images were captured at $20\times$ magnification (main images) or $60\times$ magnification (insets) with a Keyence BZ-9000E microscope. Scale bar = 100 μ m (main images) or 25 μ m (insets). One of

three representative images is shown (n = 3). (C) WT and FXR^{-/-} mice intravenously infected with 2×10^6 PFU LCMV. The virus uptake from blood was measured by plaque assay after 1, 10, and 60 min (n = 7 pooled from 2 independent experiments). (D) WT and FXR^{-/-} mice were intravenously infected with 2×10^4 PFU LCMV. After 5 days of infection, F4/80⁺CD11b⁺ macrophages from the liver of FXR^{-/-} and WT control mice were isolated by fluorescence-activated cell sorting (FACS). The expression of LCMV-GP and -NP was determined by quantitative real-time polymerase chain reaction (qRT-PCR; n = 5-6 pooled from 2 independent experiments). (E) WT and FXR^{-/-} mice were intravenously infected with 2×10^4 PFU LCMV-WE. After 5 days of infection, 9×10^8 fluorescent latex beads were intravenously injected into these mice. The liver's efficiency in taking up beads was measured after 1 h (n = 4). n.s. not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistical significance was detected by Student's *t*-test (A, C, D, and E).

Next, we measured the infiltration of immune cells in the liver of WT and FXR^{-/-} mice 5 days after LCMV infection (Fig. 3C). We observed a reduction in the number of Ly6C⁺Ly6G⁺ (granulocytes) and Ly6C⁺Ly6G⁻ cells (monocytes) in the liver (Fig. 3D) and of Ly6C⁺Ly6G⁺ cells in the blood and spleen of FXR^{-/-} mice (Fig. 3E) but there was no significant difference in the myeloid populations in the bone marrow of naive mice (Fig. 3F). This suggests that initially, FXR^{-/-} mice do not have defect in the generation of myeloid cells. We questioned whether the inhibition of monocyte expansion was due to the absence of FXR or to high levels of BAs. To answer this question, we first treated bone marrow cells from WT or FXR^{-/-} mice with CDCA or left the cells untreated as control. CDCA is considered to be the strongest FXR agonist at the half maximal effective concentration (EC₅₀) of 50 μ M [34, 44]. After this, cells were stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF). Activation

Fig. 3. Farnesoid X receptor increases the infiltration of immune cells to the infected organs (A) WT and FXR^{-/-} mice were infected with LCMV-WE. On day 5 after infection with 2×10^4 plaque-forming units (PFU) of LCMV, F4/80⁺CD11b⁺ cells in the liver were sorted by FACS. The expression of antiviral genes *Oas1* and *Mx1* was measured by qRT-PCR (n = 4). (B) WT and FXR^{-/-} mice were intravenously infected with 2×10^6 PFU LCMV-WE or left uninfected. On day 6 after infection, the expression of *Ifna4* and *Ifnb1* was measured in naïve and infected livers (n = 4-5). (C-E) WT and FXR^{-/-} mice were infected with 2×10^4 PFU LCMV-WE. On day 5 after infection, Ly6C⁺Ly6G⁺ cells were counted in the liver by FACS (C; n = 3). Ly6C⁺Ly6G⁺ and Ly6C⁺Ly6G⁻ cells were measured in the liver under naïve conditions and on day 3 and 5 after infection (D; n = 3-4). Ly6C⁺Ly6G⁺ cells were measured in the blood and spleen in naïve mice and on day 5 after infection (E; n = 3-4). (F-H) Bone marrow cells were isolated from naïve WT and FXR^{-/-} mice. Indicated cells were analyzed by FACS (F; n = 4). 2×10^6 bone marrow cells were treated with the endogenous FXR ligand chenodeoxycholic acid (CDCA, 50 μ M) for 15 minutes or left untreated before incubation with GM-CSF for additional 15 minutes. Phospho-ERK1/2 (pERK1/2) and GAPDH was measured in total cell extracts by immunoblotting. One of three representative blots is shown (G; n = 3). 2×10^6 bone marrow cells were treated with GM-CSF in the presence or absence of CDCA (100 μ M). After 5 days, Ly6C⁺ cells were analyzed by FACS (H; n = 3). (I) WT mice were intravenously infected with 2×10^4 PFU LCMV-WE. On day 3 after infection, mice were treated with RB6-8C5 antibody or left untreated. LCMV titers in liver, spleen, kidney, and lung were measured on day 5 (n = 3). (J) Immunofluorescence of liver sections stained for F4/80 (red) and LCMV-NP (nucleoprotein; green). Fluorescent images were captured at $20 \times$ magnification with a Keyence BZ-9000E microscope. Scale bars, 100 μ m (main images) or 25 μ m (insets). One of three representative images is shown (n = 3). n.s., not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistical significance was detected by Student's *t*-test (A, B, D, E, F, H and I).

Fig. 4. Farnesoid X receptor activation in hepatocytes leads to production of type I interferon. (A–B) Primary hepatocytes from wild-type (WT) mice were infected with lymphocytic choriomeningitis virus strain WE (LCMV-WE) or left uninfected. Cells were treated with the synthetic farnesoid X receptor (FXR) agonist GW4064 (1 μ M) or left untreated. After 6 and 24 h, virus glycoprotein gene expression (LCMV-GP) (A) or interferon beta 1 (*IFNβ1*) gene expression (B) was measured by quantitative real-time polymerase chain reaction (qRT-PCR) ($n = 5$ –6 pooled from 2 independent experiments). (C) WT and FXR^{-/-} primary hepatocytes were infected with LCMV-WE or left uninfected. Cells were treated with the FXR agonist chenodeoxycholic acid (CDCA; 50 μ M) or left untreated. After 24 h, *IFNβ1* expression was measured in these cells ($n = 3$). (D) Primary hepatocytes from WT mice were infected with LCMV-WE or left uninfected. Type I interferon receptor (*Ifnar*), myxovirus (influenza) resistance 1 (*Mx1*) and 2'-5'-oligoadenylate synthetase 1 (*Oas1*) gene expression was measured after 24 h by qRT-PCR ($n = 6$ pooled from 2 independent experiments). n.s., not significant; * $P < 0.5$; ** $P < 0.01$; *** $P < 0.001$. Statistical significance was detected by Student's *t*-test (A, B, C, and D).

with GM-CSF was assessed by measuring phosphorylation of ERK (Fig. 3G). In bone marrow cells of WT or FXR^{-/-} mice treated *in vitro* with CDCA phosphorylation of ERK was reduced (Fig. 3G) which means that CDCA can inhibit the effect of GM-CSF independently of FXR.

To analyze the effect of BAs on the proliferation of Ly6C⁺ cells, we treated bone marrow cells with GM-CSF in the presence or absence of CDCA. After 5 days, we noticed a reduction in the number of Ly6C⁺ cells after treatment with CDCA (Fig. 3H). This reduction was not FXR-dependent because FXR-deficient as well as WT bone marrow cells had reduced numbers of Ly6C⁺ cells in the presence of CDCA (Fig. 3H). Again, this finding means that bile acids (BAs) can inhibit GM-CSF signaling independent of FXR.

To study the role of Ly6C⁺Ly6G⁺ granulocytes and Ly6C⁺Ly6G⁻ monocytes during LCMV infection, we depleted these cell populations early after viral infection by using a monoclonal anti-Gr-1 antibody (clone RB6-8C5). This antibody recognizes both antigens Ly6G and Ly6C [45]. Like FXR-deficient mice, RB6-8C5-treated WT mice, which lacked these cell populations, exhibited an increase in viral replication in the liver and, to a lesser extent, in the lung, but exhibited no change in viral replication in the spleen and kidney (Fig. 3I). Histological staining showed more viral replication in Kupffer cells and in a few single localized hepatocytes in RB6-8C5-treated mice (Fig. 3J).

In conclusion, we found that the absence of FXR leads to high BA levels, which influence monocyte expansion by inhibiting the GM-CSF signaling pathway and consequently affects the viral control. The ability of BAs in reducing GM-CSF signaling was FXR-independent.

FXR activation in hepatocytes leads to production of IFN-I

To study the role of FXR in hepatocytes during LCMV infection independent of the effect of BAs, we infected primary hepatocytes with LCMV in the presence or absence of the synthetic FXR agonist GW4064. Interestingly, we found that activation of FXR led to the inhibition of viral replication (Fig. 4A). The antiviral effect of FXR was due to the upregulation of *IFNβ1* in hepatocytes (Fig. 4B). Even without infection, the activation of FXR with GW4064 led to significantly higher *IFNβ1* expression (Fig. 4B). Similarly, after LCMV infection of WT or FXR^{-/-} hepatocytes in the presence or absence of the FXR agonist CDCA, the expression of *IFNβ1* was highest in hepatocytes in which FXR was activated with CDCA, whereas FXR-deficient hepatocytes showed no upregulation of *IFNβ1* mRNA (Fig. 4C). To determine whether the hepatocytes can respond to IFN-I, we measured the expression of the type I interferon receptor (*Ifnar*) and antiviral genes (*Oas1*, *Mx1*) in naive and LCMV-infected hepatocytes. A significant upregulation was detected after infection with LCMV, a finding indicating that hepatocytes can respond to the antiviral cytokine (Fig. 4D). We conclude that FXR in hepatocytes can play an antiviral role during LCMV infection.

Lack of FXR potentiates CD8⁺ T cell exhaustion and viral persistence

Next we investigated whether the absence of FXR during LCMV infection can influence the adaptive immune system. LCMV-specific CD8⁺ T cells in the blood and spleen were counted in infected WT and FXR^{-/-} mice. A (normal) proliferation of CD8⁺ T cells was observed on day 8; thereafter, the proliferation of CD8⁺ T cells did not increase in FXR^{-/-} mice (Fig. 5A, B). The function of CD8⁺ T cells was also impaired in FXR^{-/-} mice, as determined by an insufficient ability of CD8⁺ T cells to produce IFN-γ after *in vitro* re-stimulation with the LCMV peptide GP33 (Fig. 5C). This finding points to an exhaustion of CD8⁺ T cells in FXR^{-/-} mice.

To determine whether high levels of BAs can influence CD8⁺ T cell priming, we transferred carboxyfluorescein succinimidyl ester (CFSE)-labeled LCMV-specific CD8⁺ T cells (P14) to WT and FXR^{-/-} mice on day 12 after LCMV infection at a time point at which BA levels are high. CD8⁺ T cells proliferated similarly in WT and FXR^{-/-} mice, a finding indicating that high levels of BAs do not alter CD8⁺ T cell priming (Fig. 5D). To determine whether the absence of monocytes and granulocytes can lead to impairment of CD8⁺ T cells, we counted CD8⁺ T cells in WT mice after treatment with the RB6-8C5 antibody. Indeed, the absence of monocytes or granulocytes was associated with a reduction in the number of LCMV-specific CD8⁺ T cells in treated mice, as compared to untreated mice (Fig. 5E). The exhaustion of CD8⁺ T cells in FXR^{-/-} mice was accompanied by viral persistence in liver, spleen, lung and kidney (Fig. 5F). From these results, we conclude that the absence of FXR leads to exhaustion of CD8⁺ T cells and to viral persistence.

Intrinsic expression of FXR has no impact on immune cells

To determine whether the defect in granulocyte and monocyte infiltration, the impaired virus-specific T cell number and virus control in FXR^{-/-} mice was immune cell intrinsic, we developed bone marrow chimeras by engrafting FXR^{-/-} or control WT bone marrow into FXR^{-/-} or WT mice. Mice were irradiated and reconstituted with donor bone marrow, and treated at day 15 with liposomal clodronate to exchange the resident Kupffer cells as well. 30 days after reconstitution, chimeric mice were infected with LCMV. FXR^{-/-} mice that received bone marrow from WT donor mice exhibited lower infiltration of granulocytes into the liver (Fig. 6A), whereas grafting FXR^{-/-} bone marrow into WT recipients restored the number of patrolling granulocytes in the liver to normal levels (Fig. 6A). Moreover, at later time points, only FXR^{-/-} mice exhibited a lower number of LCMV-specific CD8⁺ T cells, whereas the intrinsic absence of FXR in immune cells did not influence the number of CD8⁺ T cells in spleen and liver (Fig. 6B). Next we wanted to ensure that the absence of FXR in immune

Fig. 5. Lack of farnesoid X receptor potentiates CD8⁺ T cell exhaustion and viral persistence. (A-C) C57BL/6 wild-type (WT) and farnesoid X receptor-knock-out (FXR^{-/-}) mice were infected with 2×10^6 plaque-forming units (PFU) lymphocytic choriomeningitis virus strain WE (LCMV-WE). Fluorescence-activated cell sorting (FACS) analysis of total CD8⁺ T cells and virus-specific GP33⁺ CD8⁺ T cells measured in blood at the indicated time points (A) and spleen on day 12 (B) ($n = 6-8$ pooled from 2 independent experiments). (C) FACS analysis of interferon gamma (IFN- γ) CD8⁺ T cells measured in splenocytes on day 12 after infection without or after restimulation with GP33-peptide for 6 hours ($n = 3$). (D) WT and FXR^{-/-} mice were intravenously infected with 2×10^6 PFU LCMV-WE. After 12 days of infection, 10^7 splenocytes from P14/CD45.1 mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into infected C57BL/6 WT or FXR^{-/-} mice. Proliferation of CD45.1⁺ CD8⁺ T cells was assessed by CFSE dilution in the spleen 2 days after transfer. Blots show cells gated on CD45.1⁺ CD8⁺ T cells. One representative set of data is shown ($n = 3$). (E) C57BL/6 WT mice were intravenously infected with 2×10^4 PFU LCMV-WE. On days 3, 5, and 11 after infection, mice were treated with RB6-8C5 antibody or left untreated. FACS analysis of virus-specific GP33⁺ CD8⁺ T cells measured on day 12 after infection in the spleen of treated or untreated WT mice ($n = 5$). (F) WT and FXR^{-/-} mice were infected with 2×10^6 PFU LCMV-WE. Viral titers were analyzed in the indicated organs by plaque assay on day 12 after infection ($n = 5-6$ pooled from 2 independent experiments). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistical significance was detected by Student's *t*-test (B, C, E, and F) or analysis of variance (ANOVA) (A).

dependent experiments). (C) FACS analysis of interferon gamma (IFN- γ) CD8⁺ T cells measured in splenocytes on day 12 after infection without or after restimulation with GP33-peptide for 6 hours ($n = 3$). (D) WT and FXR^{-/-} mice were intravenously infected with 2×10^6 PFU LCMV-WE. After 12 days of infection, 10^7 splenocytes from P14/CD45.1 mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into infected C57BL/6 WT or FXR^{-/-} mice. Proliferation of CD45.1⁺ CD8⁺ T cells was assessed by CFSE dilution in the spleen 2 days after transfer. Blots show cells gated on CD45.1⁺ CD8⁺ T cells. One representative set of data is shown ($n = 3$). (E) C57BL/6 WT mice were intravenously infected with 2×10^4 PFU LCMV-WE. On days 3, 5, and 11 after infection, mice were treated with RB6-8C5 antibody or left untreated. FACS analysis of virus-specific GP33⁺ CD8⁺ T cells measured on day 12 after infection in the spleen of treated or untreated WT mice ($n = 5$). (F) WT and FXR^{-/-} mice were infected with 2×10^6 PFU LCMV-WE. Viral titers were analyzed in the indicated organs by plaque assay on day 12 after infection ($n = 5-6$ pooled from 2 independent experiments). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Statistical significance was detected by Student's *t*-test (B, C, E, and F) or analysis of variance (ANOVA) (A).

cells exerts no influence on viral control. Measurements of viral titer in various organs showed that the viral titer was independent of the presence or absence of FXR in immune cells (Fig. 6C). These results exclude the role of FXR in immune cells, a finding indicating that FXR expression in non-immune cells prevents severe liver immunopathology during LCMV infection.

Fig. 6. Intrinsic expression of farnesoid X receptor has no impact on immune cells. Wild-type (WT) and farnesoid X receptor-knockout (FXR^{-/-}) mice were lethally irradiated and one day later were reconstituted with 10⁷ bone marrow cells from either FXR^{-/-} or WT control mice. On day 15, all recipient mice were intravenously treated with clodronate liposomes. Thirty days later, mice were infected with 2×10⁴ plaque-forming units (PFU; A and C) or 2×10⁶ PFU (B) lymphocytic choriomeningitis virus strain WE (LCMV-WE). (A) The number of Ly6G⁺ and Ly6C⁺ cells was measured in the liver on day 5 after LCMV infection (n = 4-5 pooled from 2 independent experiments). (B) Virus-specific GP33⁺ CD8⁺ T cells in the spleen and liver were counted by flow cytometry on day 12 after LCMV-WE infection (spleen, n = 6-7; liver, n = 4-5 pooled from two independent experiments). (C) Viral titer in various organs measured on day 5 after infection (n = 3). n.s., not significant, * *P* < 0.5; ** *P* < 0.01. Statistical significance was detected by Student's *t*-test (A, B, and C).

Fig. 7. Role of farnesoid X receptor during lymphocytic choriomeningitis virus infection. Illustrated figure of the role of farnesoid X receptor (FXR) during lymphocytic choriomeningitis virus strain WE (LCMV-WE) infection. WT, wild-type; FXR^{-/-}, FXR knockout; IFN β , interferon beta; GM-CSF, granulocyte-macrophage colony-stimulating factor.

Discussion

The results of the current study showed that FXR expression in hepatocytes is very important for sensing free BAs during LCMV infection which are released from hepatocytes in a CD8⁺ T cell-dependent manner. Activation of the nuclear receptor FXR led to upregulation of IFN-I expression. Additionally, FXR inhibits the hyperproduction of BAs, thereby avoiding their toxic effect on monocyte proliferation and infiltration into the liver. This infiltration

was essential in helping Kupffer cells to control viral infection. The absence of FXR reduces IFN-I production and the proliferation and patrolling of immune cells in the liver and leads to higher viral replication (Fig. 7) and, consequently, to severe immunopathology and CD8⁺ T cell exhaustion.

The functionality of Kupffer cells is crucial during viral infection. Their role is not limited to capturing viral particles; they also inhibit viral replication in an IFN-I-dependent manner [42, 43]. Additional study showed that prevention of liver disease during viral infection is mediated by macrophages through getting IFN-I support from plasmacytoid dendritic cells (pDCs) [46]. In this study, we found that in the liver IFN-I production is FXR-dependent. Moreover, during infection, FXR guarantees granulocyte and monocyte infiltration into the liver, where these cells assist Kupffer cells in viral control. During bacterial infection, the efficiency of Kupffer cells in the elimination of bacteria depends rather more on the interaction between these cells and granulocytes than on their own ability to phagocytize and digest bacteria [47, 48]. In this study, we found that monocytes and granulocytes that infiltrate the liver during LCMV infection can assist Kupffer cells in controlling virus. However, it remains to be determined whether the direct interaction of monocytes and granulocytes with Kupffer cells is sufficient to overcome viral infection or whether monocytes and granulocytes have additional mechanism by which they participate in antiviral control.

The fact that hepatocytes can upregulate the expression of IFNAR and antiviral genes during infection and can inhibit viral replication in an IFN-I-dependent manner illustrates their contribution to viral control. Similar results were also observed by using immortalized human hepatocytes showing that HCV can induce IFN β and the antiviral protein 2',5'-oligoadenylate synthetase 1 (*Oas1*) mRNA expression [49]. This finding raises the question: which role do BAs play during HCV infection, and consequently, is FXR involved in this process. However, other study showed that cholestasis and BAs induced during chronic viral infection seem to play a role in resistance to IFN because they can inhibit the induction of antiviral proteins [3, 50, 51]. Our results show that BAs can play a yin and yang role in viral control. On one hand, it leads to IFN-I production through FXR activation, on the other hand, it inhibits monocytes and granulocytes expansion.

As we mentioned above, FXR is not only expressed in hepatocytes but also in other organs like kidney and intestine [28-30]. Although our *in vitro* results showed that FXR and BAs have a direct effect in the upregulation of *IFN β 1* expression and inhibiting GM-CSF signaling, it should be taken in consideration that FXR regulates also the expression of the ileal bile acid transporters, such as ASBT, OST α -OST β and the cytosolic ileal bile acid binding protein IBABP (FABP6) [52, 53], which may modify gastrointestinal tract physiology and food absorption and consequently can influence the immune response.

Although some studies have found that FXR is also expressed in RAW264.7 macrophages, blood-derived macrophages, and Kupffer cells [12, 54] our study showed that the absence of FXR in immune cells does not influence LCMV control, whereas the absence of FXR in non-immune cells is crucial in reducing viral replication and maintaining the efficiency of the adaptive immune system.

In conclusion, we found that FXR expression by hepatocytes plays an important role in overcoming viral infection, inhibition of T cell exhaustion and preventing immunopathology.

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