Bile acids initiate cholestatic liver injury by triggering a hepatic specific inflammatory response

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Supplementary Results

Table S1, Hepatic mRNA expression of genes involved in inflammation and bile acids metabolism. WT=wild-type, KO=knockout, BDL=bile duct ligation. (mean±SD, [a]*p*<0.05 to WT-sham, [b]*p*<0.05 to WT-BDL)

Gene	WT-sham (n=5)	Ccl2KO-sham (n=5)	WT-BDL (n=6)	Ccl2KO-BDL (n=7)
Cxcl1	1.46±1.29	2.65±0.61	12.2±8.0[a]	11.6±4.8
Cxcl2	1.07±0.5	1.14±0.43	39.9±13.1[a]	18.8±9.4[b]
Tnfα	1.14±0.62	0.52±0.18	6.30±3.85[a]	3.13±0.96
Il-1β	1.03±0.28	1.40±0.41	2.64±0.90[a]	3.60±1.76
Egr1	1.03±0.25	1.65±0.36	3.44±1.60[a]	4.51±1.30
Bsep	1.01±0.16	0.99±0.25	0.98±0.32	0.85±0.28
Ntcp	1.01±0.13	1.06±0.36	0.68±0.18[a]	0.26±0.10[b]
Cyp7a1	1.59±1.44	2.26±0.57	1.82±0.62	0.88±0.31[b]
Mrp4	1.02±0.21	0.68±0.28	1.83±0.37[a]	1.52±0.64
Ck19	1.04±0.31	1.18±0.59	26.2±7.50[a]	13.5±3.49[b]
Col1a1	1.09±0.58	0.86±0.47	42.1±18.1[a]	28.9±12.3

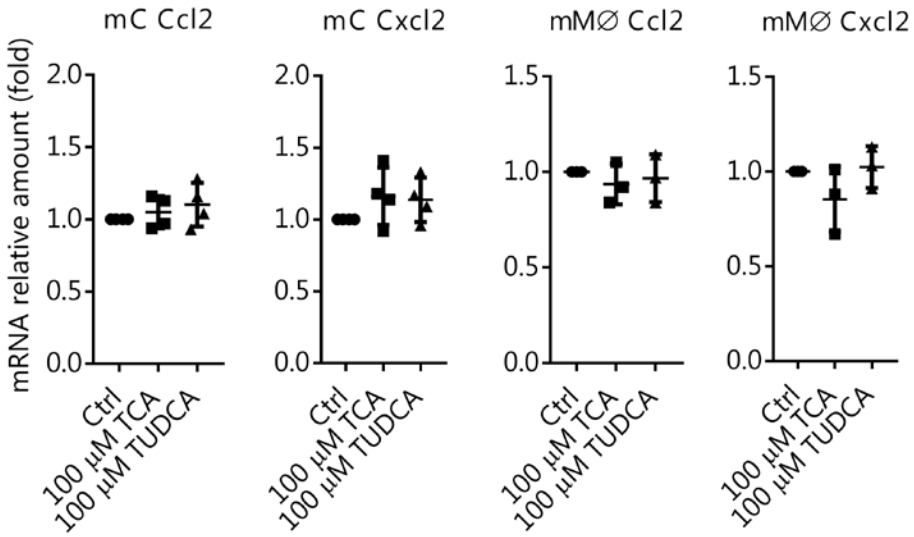


Figure S1. Taurocholic acid (TCA) did not alter Ccl2 and Cxcl2 mRNA expression in mouse cholangiocyte (mC) and macrophage (mMø) cultures. Presented above cholangiocytes were treated for 24 h. Cholangiocytes did not respond to 100 µM TCA treatment at shorter time points (3 h, 6 h, data not shown). Presented above macrophages were treated for 6 h. There was no difference at 3 h and 24 h time points either in these macrophages (data not shown). TUDCA, tauroursodeoxycholic acid. mean±SD, n≥3.

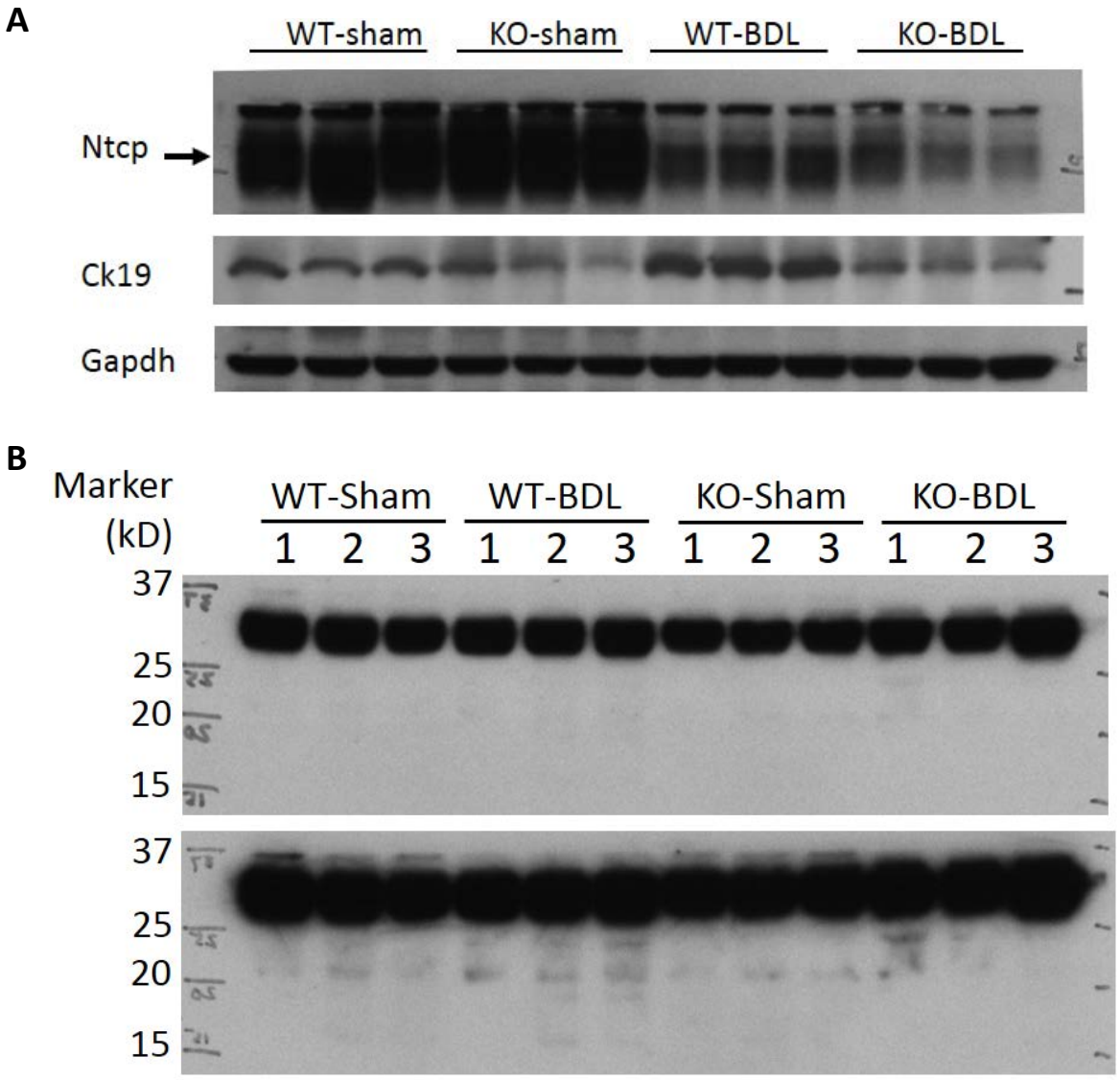


Figure S2, Western blot detection of protein expression in wild-type (WT) and Ccl2 knockout (KO) livers after BDL or sham operation. (A) reduced liver Ck19 and Ntcp protein expression in Ccl2 KO BDL mice than in WT and mice; (B) however, no caspase 3 cleavage was detected (Top, a regular exposure showing no differences among different groups; Bottom, an over-exposure

of the same blot to demonstrate that cleavage of a caspase 3 (19kD) band was not detected). Three representative animals from each group were shown.

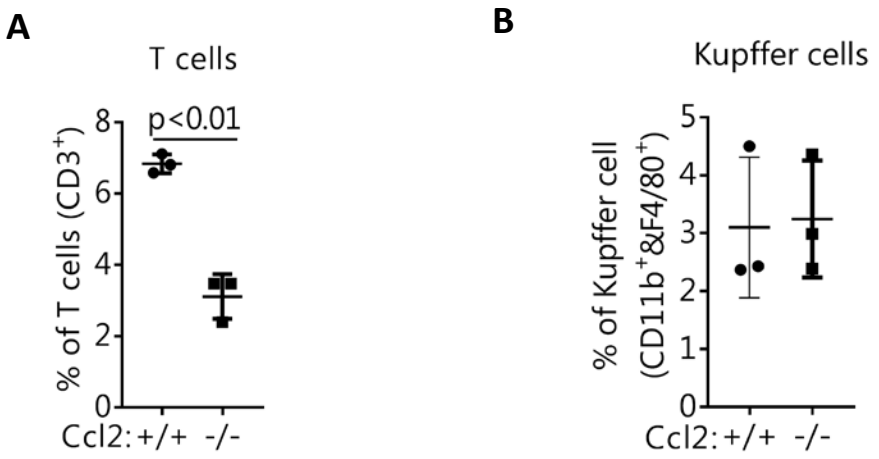


Figure S3. Quantitative assessment of T cells (CD3+) and Kupffer cells in the liver of wild-type and Ccl2 knockout liver after 7 days of bile duct ligation.

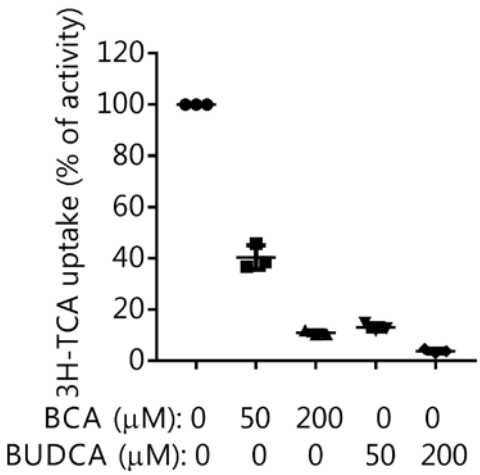
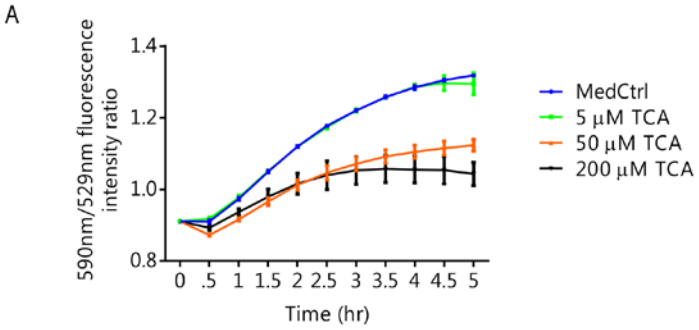


Figure S4, Biotinylated both cholic acid (BCA) and ursodeoxycholic acid (BUDCA) inhibited taurocholic acid (TCA) uptake in mouse hepatocytes. (mean±SD, n=3).



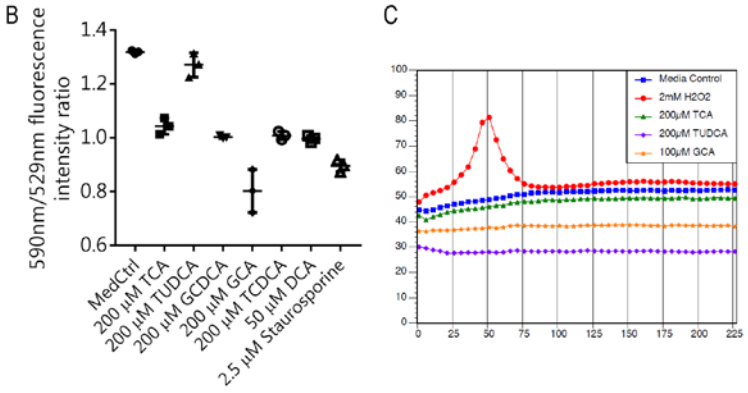


Figure S5, Bile acids reduced mitochondrial membrane potential in mouse hepatocytes. A) JC-1 assay demonstrates that taurocholic acid (TCA) altered mitochondrial membrane potential in a dose and time dependent manner. B) Effects of 5 hr treatment of a spectrum of bile acids and staurosporine on mitochondrial membrane potential. (mean±SD, n=3). C) A representative confocal microscopy measuring ROS using MitoSOX as indicatior on mouse primary hepatocytes. Confocal fluorescence of entire field at 10x. Excitation wavelength 488nm, emission wavelength 540-650nm, at least three independent experiments were performed.

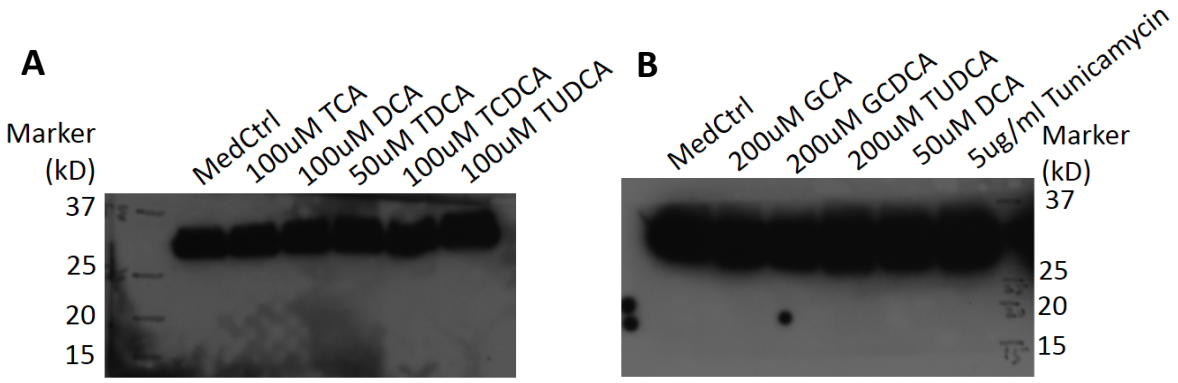


Figure S6, Western blot detection of caspase 3 in bile acids treated hepatocytes from mouse (A) and human (B). Cell were treated for 24 hr. The blots were intentionally over-exposed to determine whether a cleaved caspase 3 band around 19kD could be detected. None was seen.

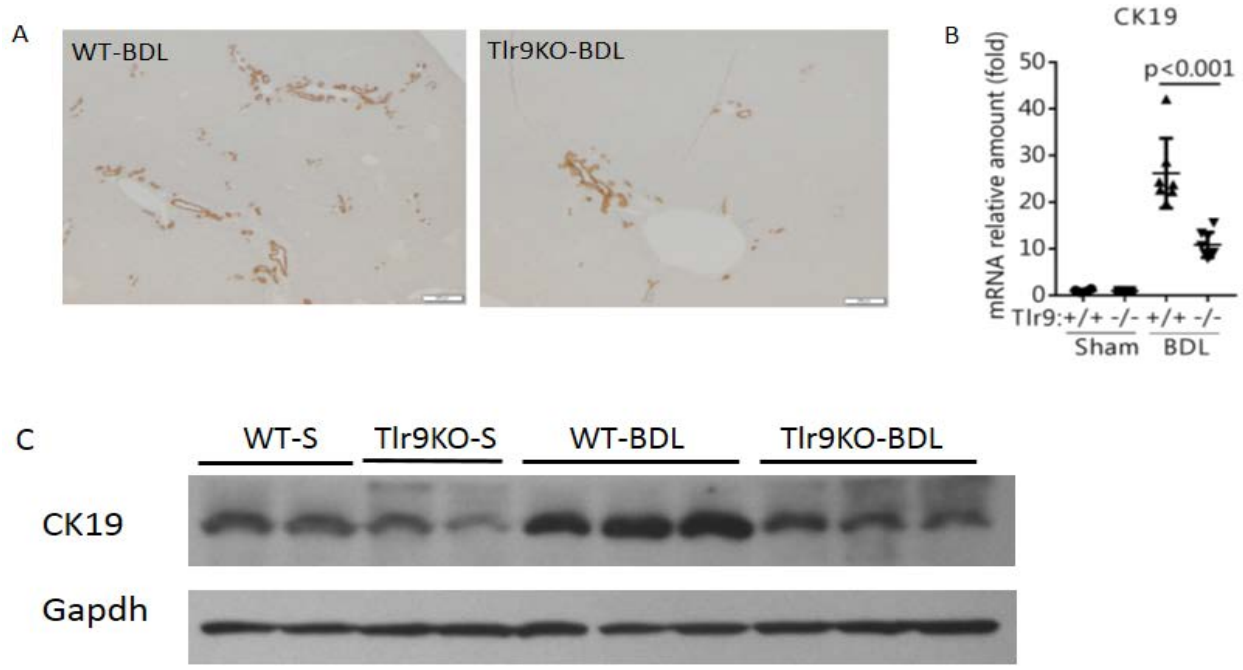


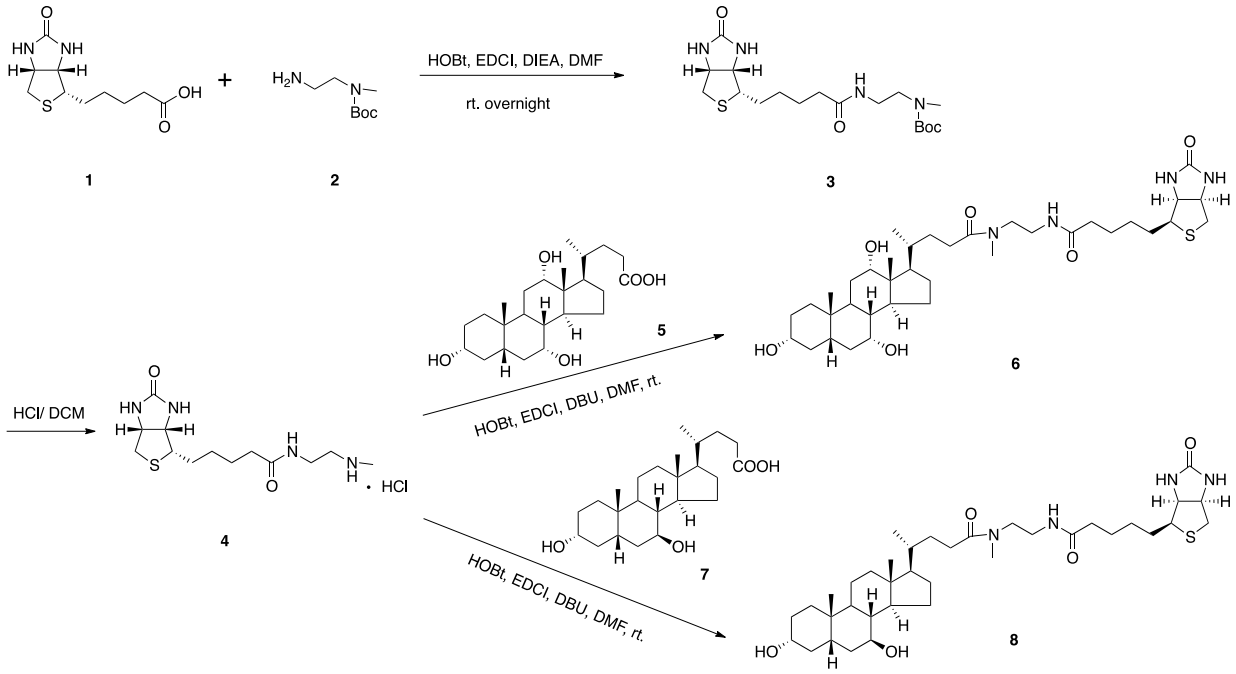
Figure S7, Marked reduction of bile duct proliferation in Tlr9 conventional knock out (KO) mouse livers after bile duct ligation (BDL) for 7 days. A) Representative immunohistochemistry labelling of cytokeratin 19 (CK19) in liver section (bar=200µm); B) CK19 mRNA expression in

wild-type (WT) and Tlr9 KO mouse livers; C) Western blot of CK19 from total liver lysate from WT and Tlr9 KO livers.

Supplementary Methods

Synthesis of biotinylated cholic acid and ursodeoxycholic acid.

These bile acid conjugates were designed and made to inhibit the uptake of bile acids. The scheme of their syntheses procedure are following.



Synthesis of *N*-Boc-N-methylethylenediamine-Biotin (**3**): In 25 mL of anhydrous DMF, Biotin (**1**, 11.68 g, 47.82 mM), 1.5 eq HOBt (9.69 g), 2.0 eq EDCI (18.34 g) were magnetically stirred for 2 hr. Then 2.0 eq DIEA (15.78 mL) and *N*-Boc-N-methylethylenediamine (**2**, 10.0g, 57.38 mM) was added, the reaction mixture was stirred overnight. The reaction was terminated by adding 500 mL of water. The reaction mixture was extracted with DCM (3×150mL) and washed with 1N HCl (3×100mL), 1N NaOH (3×100mL), and brine (2×100mL). The organic layer was dried over Mg_2SO_4, filtered, and evaporated under vacuum to give the title compound **3** (14.52 g), yield 75.82%, white solid.

Synthesis of N-methylethylenediamine-Biotin hydrocholoride (**4**): While stirring compound **3** in methylene chloride solution (10.01 g in 60 mL) dry hydrogen chloride gas was pumped into the solution for 2.5 h at room temperature. After stopping hydrogen chloride, the reaction mixture was stirred for another 1.5 hr at the same temperature. The resulting reaction mixture was evaporated under vacuum and then dried in vacuum to give the title compound **4** (8.34 g). Yield: 99.1%, white solid.

Synthesis of CA-N-methylethylenediamine-Biotin (**6**): In 10 mL of anhydrous DMF, cholic acid (**5**, 2.04 g, 5 mM), 1.5 eq HOBt (1.08 g), 2.0 eq EDCI (1.98 g) were magnetically stirred for 2 hr. Then 3.6 eq DBU (2.79 g) and **4** (1.52 g, 4.51 mM) were added, the reaction mixture was then stirred for overnight. The reaction was terminated by adding 100 mL of water. The reaction mixture was extracted with DCM (3×30mL) and washed with 1N HCl (3×30mL), 1N NaOH (3×30mL), and brine (2×40mL). The organic layer was dried over Mg_2SO_4, filtered, and evaporated under vacuum to give a yellow oil. The crude product was further purified by silica gel column chromatography to give the title compound **6**, (1.76 g), yield 51.2%, white solid.

Synthesis of UDCA-N-Methylenediamine-Biotin (**8**): In 10 mL of anhydrous DMF, ursodeoxycholic acid (**7**, 2.99 g, 7.61 mM), 1.5 eq HOBt (1.64 g), 2.0 eq EDCI (3.01 g) were magnetically stirred for 2 hr. Then 3.6 eq DBU (4.24 g) and **4** (2.32 g, 6.87 mM) were added and the reaction mixture stirred overnight. The reaction was terminated by adding 100 mL of water. The reaction mixture was extracted with DCM (3×30mL) and washed with 1N HCl (3× 30mL), 1N NaOH (3×30mL), and brine (2×40mL). The organic layer was dried over Mg_2SO_4, filtered, and evaporated under vacuum to give a yellow oil. The crude product was further purified by silica gel column chromatography to give the title compound **8** (2.35 g), yield 45.8%, white solid.

Mitochondrial membrane potential (JC-1 assay) and cellular reactive oxygen species (DCFDA) assays. Mouse hepatocytes were plated in collagen-coated 48-well plate (6×10^4 cells/well) and maintained in 5% CO_2 incubator for 24 hr. Cells were stained with 2 µM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyaniniodide (JC-1) from Life Technologies in Williams' E media containing 5% FBS for 30 min and washed twice with culture media. After adding designated concentrations of bile acid to the cells, fluorescent intensity was determined with a

BioTek Synergy 2 plate reader (Winooski, VT) at wavelengths of 485 nm (excitation) and 530 and 580 nm (emission) for 5 hr with 5 min interval between each read. The ratio of green and red fluorescence signal intensities was calculated and presented as a parameter for the mitochondrial membrane potential $\Delta\Psi$, independent of the mitochondrial mass. For detecting cellular reactive oxygen species, we used a DCFDA assay kit from Abcam (Cambridge, MA) by following the manufacturer's instruction.

MitoSOX assays. To determine whether bile acids treatment leads to mitochondria release of oxidative species, we used MitoSOX in two different assays, i.e. flow cytometry and confocal fluorescent microscopy. When confocal fluorescent microscopy was used, mouse hepatocytes were plated on collagen-coated Falcon Culture Slides. Twenty-four hours post plating, the culture slide was set in a heated chamber (37˚C) with 5% CO_2 on a Zeiss LSM 710 DUO microscope. Fluorescent intensity (Excitation 488nm, Emission 540-650nm) of an entire field at 10x was measured in 4 hr time period at 5 min interval. H_2O_2 (2mM) served as positive control.

Table S2, Patients with cholestatic liver disorders

Diagnosis	Number of cases
Cholestatic Hepatitis	3
Drug cholestasis	2
Cholangitis	3
Autoimmune hepatitis	1
Choledochal cyst	1
Giant Cell hepatitis	1
Vanishing bile duct syndrome	1