Research Article
Cholestasis and Autoimmune Diseases

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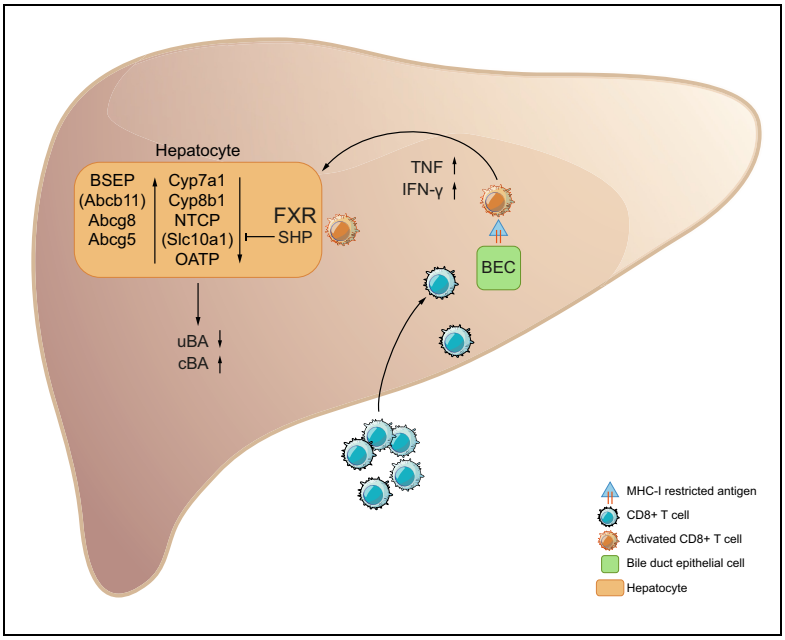
Liver infiltrating T cells regulate bile acid metabolism in experimental cholangitis

Graphical abstract



Highlights

- Antigen-specific CD8+ T cells can control bile acid metabolism in a murine model of cholangitis.
- The effect of T cells on bile acid metabolism partly depends on TNF and IFN-γ, and on T cell contact with hepatocytes.
- Understanding the effect of lymphocytes on bile acid metabolism may help in the design of combined treatment strategies.

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Lay summary

Dysregulation of bile acid metabolism and T cells can contribute to the development of cholangiopathies. Before targeting T cells for the treatment of cholangiopathies, it should be determined whether they exert protective effects on bile acid metabolism. Herein, we demonstrate that T cell-induced cholangitis resulted in decreased levels of harmful unconjugated bile acids. T cells were able to directly control synthesis and metabolism of bile acids, a process which was dependent on the proinflammatory cytokines TNF and IFN-γ. Understanding the effect of lymphocytes on bile acid metabolism will help in the design of combined treatment strategies for cholestatic liver diseases.

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Liver infiltrating T cells regulate bile acid metabolism in experimental cholangitis

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See Editorial, pages 657–659

Background & Aims: T cells are central mediators of liver inflammation and represent potential treatment targets in cholestatic liver disease. Whereas emerging evidence shows that bile acids (BAs) affect T cell function, the role of T cells for the regulation of BA metabolism is unknown. In order to understand this interplay, we investigated the influence of T cells on BA metabolism in a novel mouse model of cholangitis.

Methods: *Mdr2*$^{-/-}$ mice were crossed with transgenic K14-OVAp mice, which express an MHC class I restricted ovalbumin peptide on biliary epithelial cells (*Mdr2*$^{-/-}$xK14-OVAp). T cell-mediated cholangitis was induced by the adoptive transfer of antigen-specific CD8+ T cells. BA levels were quantified using a targeted liquid chromatography-mass spectrometry-based approach.

Results: T cell-induced cholangitis resulted in reduced levels of unconjugated BAs in the liver and significantly increased serum and hepatic levels of conjugated BAs. Genes responsible for BA synthesis and uptake were downregulated and expression of the bile salt export pump was increased. The transferred antigen-specific CD8+ T cells alone were able to induce these changes, as demonstrated using *Mdr2*$^{-/-}$xK14-OVAp recipient mice on the *Rag1*$^{-/-}$ background. Mechanistically, we showed by depletion experiments that alterations in BA metabolism were partly mediated by the proinflammatory cytokines TNF and IFN-γ in an FXR-dependent manner, a process that *in vitro* required cell contact between T cells and hepatocytes.

Conclusion: Whereas it is known that BA metabolism is dysregulated in sepsis and related conditions, we have shown that T cells are able to control the synthesis and metabolism of BAs, a process which depends on TNF and IFN-γ. Understanding the effect of lymphocytes on BA metabolism will help in the design of combined treatment strategies for cholestatic liver diseases.

Lay summary: Dysregulation of bile acid metabolism and T cells can contribute to the development of cholangiopathies. Before targeting T cells for the treatment of cholangiopathies, it should be determined whether they exert protective effects on bile acid metabolism. Herein, we demonstrate that T cell-induced cholangitis resulted in decreased levels of harmful unconjugated bile acids. T cells were able to directly control synthesis and metabolism of bile acids, a process which was dependent on the proinflammatory cytokines TNF and IFN-γ. Understanding the effect of lymphocytes on bile acid metabolism will help in the design of combined treatment strategies for cholestatic liver diseases.

Introduction

In primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), T cells are likely involved in targeting biliary epithelial cells.[1] Chronic bile duct inflammation leads to cholestasis, biliary fibrosis and potential malignant transformation in the case of PSC.[2] Adaptive immune responses are considered to contribute to early as well as late stages of disease.[3] Numerous genetic risk loci for PBC and PSC were identified that encode for genes involved in adaptive immune responses[4–6] and portal infiltrates that were predominantly composed of CD4+ and CD8+ T cells have been described in the livers of patients with PBC and PSC.[7–9] Moreover, autoantigens within the pyruvate dehydrogenase complex (PDC-E2) are targeted by lymphocytes in PBC.[10] In PSC, impaired T cell homeostasis including an imbalance of IL-17 producing T cells and regulatory T cells has been described.[11–16] Since current treatment options for these diseases are limited, T cells are an interesting target for novel combination therapies.

Keywords: TNF; IFN-γ; Interferon; Bile acid regulation; Cholangitis; T cells.
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In cholestasis, bile acids (BAs) accumulate in serum and liver and may fuel disease progression via nuclear or membrane bound BA receptors or via direct cytotoxic effects such as the induction of apoptosis or oxidative stress.[17–19] The synthesis of BAs takes place in hepatocytes by several enzymatic steps using 2 different routes. The classical BA synthesis pathway is controlled by the enzyme cholesterol 7-alpha-hydroxylase (CYP7A1), whereas the alternative BA synthesis pathway is initiated by sterol 27-hydroxylase (CYP27A1) followed by the action of 25-hydroxycholesterol 7-alpha-hydroxylase (CYP7B1). In principle, both pathways produce the same BA species that are conjugated with glycine or taurine, and secreted via the bile. The transcription factors liver X receptor (LXR) and farnesoid X receptor (FXR) control BA synthesis and homeostasis. FXR is activated by excess intestinal or hepatic BAs, leading to down-regulation of CYP7A1 and hepatic basolateral BA transporter expression, and induction of bile salt export pump (BSEP) expression.[20,21]

It is becoming increasingly clear that lipid metabolites, including BAs, influence the differentiation and function of immune cells.[22] The mainly suppressive action of BAs on immune cells could serve to reduce cholestatic liver damage. Although severe states of inflammation such as sepsis are known to profoundly alter BA metabolism via the action of cytokines such as tumor necrosis factor (TNF),[23] it is currently unknown whether T cells infiltrating the liver are able to modulate BA metabolism. In order to develop effective combination therapies for immune mediated cholangiopathies, which target both BA- and T cell-induced liver injury, it is essential to understand the role of T cells in the modulation of BA metabolism.

Materials and methods

Mice

C57Bl6/J, *Mdr2*$^{-/-}$, *Rag1*$^{-/-}$ and OT-1 mice were from Jackson Laboratory, Maine, USA. K14-OVAp mice were kindly provided by Kirsten Hogquist (Minnesota, USA). For the experiments *Mdr2*$^{-/-}$x*Rag1*$^{-/-}$, K14-OVApx*Rag1*$^{-/-}$, *Mdr2*$^{-/-}$xK14-OVAp and *Mdr2*$^{-/-}$xK14-OVApx*Rag1*$^{-/-}$ were generated by crossbreeding. All mice were bred and housed under specific pathogen-free conditions with 12 h light/dark cycles at the animal care facility of the University Medical Center Hamburg-Eppendorf with access to water and standard chow diet (1318 rodent diet, Altromin, Lage, Germany) ad libitum. *Mdr2*$^{-/-}$ and K14-OVAp models of cholangitis were described previously.[24,25] Animal experiments comply with the ARRIVE guidelines[26] and were approved by the review board of the State of Hamburg, Germany (G23/15, G36/16 and ORG846).

Cell isolation and adoptive transfer experiments

For induction of cholangitis, 4×10^6 splenic derived congenic OT-1 CD8+ T cells from female donors (n = 30) recognizing the ovalbumin peptide expressed on biliary epithelial cells of recipients were injected intraperitoneally into female K14-OVAp (n = 10), female K14-OVApx*Rag1*$^{-/-}$ (n = 10), female and male *Mdr2*$^{-/-}$xK14-OVAp (n = 40) and female *Mdr2*$^{-/-}$xK14-OVApx*Rag1*$^{-/-}$ (n = 5) mice. Mice were sacrificed on day 5–8 after T cell transfer. Female K14-OVAp (n = 8), female K14-OVApx*Rag1*$^{-/-}$ (n = 8), female and male *Mdr2*$^{-/-}$xK14-OVAp (n = 30) and female *Mdr2*$^{-/-}$xK14-OVApx*Rag1*$^{-/-}$ (n = 5) mice without cell transfer were used as controls. OT-1 CD8+ T cells were freshly isolated with anti-CD8-FITC antibody (Biolegend, Fell, Germany) and anti-FITC immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. For interferon gamma (IFN-γ) and TNF neutralization, anti-TNF and anti-IFN-γ (each 250 μg per mouse) or IgG1 isotype control (BioXcell, West Lebanon, USA) diluted in PBS were injected intravenously 4 h before OT-1 T cell transfer. Antibodies or isotype control were applied for a second time intraperitoneally on day 3 following cholangitis induction.

Histology

Hematoxylin Eosin (H&E) (Roth, Karlsruhe, Germany) and Sirius Red staining were performed on formalin-fixed liver sections. CK19 (Troma III) and CD45.1 (Biolegend, Fell Germany) staining was performed on cryo-frozen tissue. All pictures were taken with Biorevo (BZ-9000, Keyence, Japan). Histologies were scored by a pathologist in a blinded fashion. Liver inflammation was assessed using the modified hepatitis activity index.[27]

Serum liver enzymes

Mouse alanine aminotransferase serum levels (ALT) were measured at the Institute of Experimental Immunology and Hepatology, University Medical Center Hamburg-Eppendorf, using a COBAS Mira System (Roche Diagnostic, Mannheim, Germany).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) scans of female *Mdr2*$^{-/-}$-xK14-OVAp mice (n = 3) were performed before and 8 days after injection of 4×10^6 OT-1 CD8+ T cells with a small animal 7T MRI system (ClinScan, Bruker BioSpin GmbH, Ettlingen, Germany) according to previously described investigations.[28] For representation of morphological hepatobiliary changes maximum intensity projections (MIP) were calculated with ImageJ (National Institutes of Health, Bethesda, MD, USA) based on 3 slices.

Cell restimulation and cytokine measurements

Spleen cells and liver lymphocytes were isolated as described previously.[25] 5×10^5 cells per well were re-stimulated for 24 h with 2 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (BD Biosciences, Heidelberg, Germany) antibodies. Supernatants were analyzed for IFN-γ and TNF levels by ELISA using DuoSet® mouse ELISA kits (R&D Systems, Wiesbaden, Germany).

Primary hepatocyte and T cell co-culture

A total of 2×10^5 hepatocytes were isolated from C57Bl6/J female mice (n = 2) as previously described[29] and cultured overnight. CD3+ T cells from female C57Bl6/J mice (n = 6) were freshly isolated with anti-CD3-FITC antibody (Biolegend, Fell, Germany) and anti-FITC immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. 1×10^6/well CD3+ T cells were added directly to the hepatocytes or into TC inserts in transwell experiments (Sarstedt, Germany) and re-stimulated for 24 h with 2 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (BD Biosciences, Heidelberg, Germany) antibodies. Hepatocytes were harvested for RNA isolation and quantitative PCR analysis.

Flow cytometry

Immunofluorescence staining of cells was performed with antibodies to CD3, CD4, CD8, CD45.1 and IFN-gamma (BioLegend, Fell, Germany). Dead cells were stained with Pacific Orange-NHS (Life technologies, Darmstadt, Germany). Flow cytometry

data were analyzed with FACS Diva Software (BD Biosciences, Heidelberg, Germany).

Real-time quantitative PCR

Total RNA was extracted from liver tissue using Nucleospin Kit (Macherey-Nagel, Düren, Germany). cDNA was reverse-transcribed from total RNA (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Darmstadt, Germany). Gene expression was measured using Taqman™ primer (Table S2 and supplementary CTAT Table) and Taqman™ Universal PCR master mix. Taqman™ gene expression kits (Applied Biosystems, Darmstadt, Germany) were used for amplification and target gene expression was normalized to TATA-box binding protein Tbp or Hprt housekeeper levels by employing the ΔΔCt method.

Bile acid measurements

Targeted BA analysis was performed by HPLC-ESI-MS/MS as described previously.[30] To summarize, BA measurement comprised a quick methanol liquid-liquid extraction of liver or plasma samples after adding several internal standards. Quantitative measurement of BAs was performed using a HPLC-ESI-QqQ system in multiple reaction monitoring (MRM) (HPLC: 1200 Infinity Quaternary LC System (Agilent); Column: Accucore™ Polar Premium (2.6 µm, 150 mm × 2.1 mm i.d., Thermo Fischer Scientific Inc.); QqQ: API 4000 Q trap (ABSCIEX)). Quantification was achieved by comparison of retention times and mass transitions to respective standards.

RNA sequencing analysis

In total 59 frozen liver tissue samples obtained via liver biopsy and taken from our department's prospective biobank were sequenced. The Ethics Committee of Hamburg (PV4081 liver.net) approved the study and all participants gave their written consent. For total RNA isolation and enrichment we used the mirVana miRNA Isolation Kit (Thermo Fischer) according to the manufacturer's protocol. Library preparation and sequencing, paired-end 2 × 75bp was performed on an Illumina MiSeq. After quality control with FastQC (v0.11.5), we used Trimmomatic (v0.36) to perform adapter (TruSeq2-PE) and low quality read trimming (4 bp sliding window, min. 15 phred score, 36 bp min read length). The trimmed FASTQ files (mean 39.5 million reads) were mapped to the human genome (GRCh38 release 86 and Gencode v25 annotation). For Genome mapping and gene counting, STAR[2] (v2.5.2a) was used with default parameters. Only uniquely mapped reads (mean 92.2%) were counted. All further analyses were performed in R. The gene count table was normalized and rlog transformed using DESeq2 (v1.18.1). After removing one outlier sample identified by

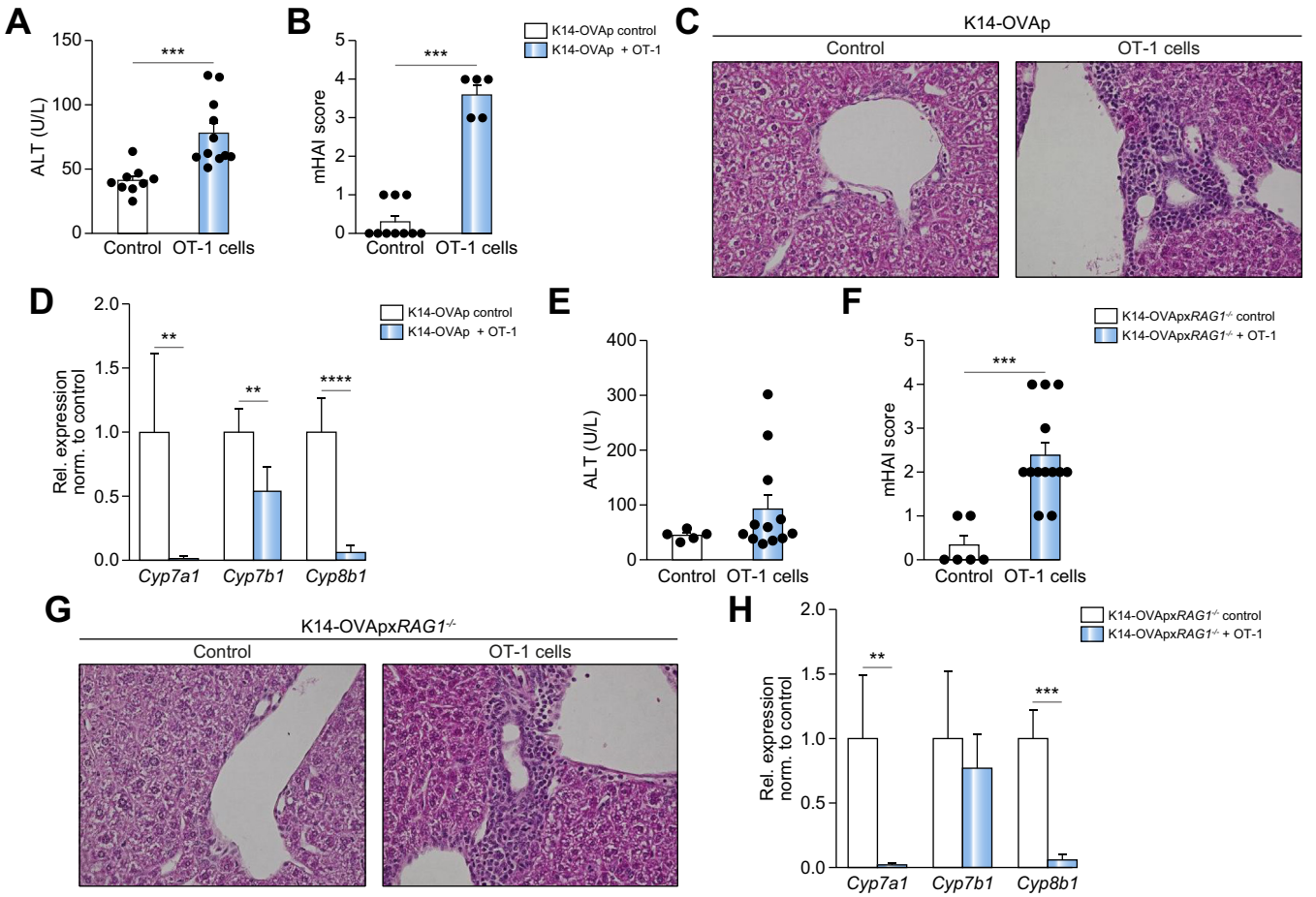

Fig. 1. Antigen-specific T cells modulate the expression of genes relevant for bile acid synthesis. (A) ALT levels (B) mHAI grading of liver inflammation, (C) representative H&E stainings and (D) mRNA expression of BA synthesis genes in whole liver tissue from female K14-OVAp animals (n = 10) on day 5 after transfer of OT-1 CD8+ T cells compared to female controls (n = 8). (E) ALT levels, (F) mHAI grading of liver inflammation, (G) representative H&E stainings and (H) mRNA expression of BA synthesis genes in whole liver tissue from female K14-OVApx*Rag1*$^{-/-}$ animals (n = 10) on day 5 after transfer of OT-1 CD8+ T cells compared to female controls (n = 8). Data were analyzed using the Mann-Whitney *U* test. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. ALT, alanine aminotransferase; BA, bile acid; mHAI, modified hepatitis activity index.

inspecting a PCA plot, 58 samples (AIH = 22, PBC = 16, PSC = 20) remained. Pearson's correlation and differential correlation were calculated with the DGCA (v1.0.1) package using the ddcorAll function. Permutation testing was used to adjust for multiple testing and a *p* value cut-off of 0.05 was used to determine differential correlation.

Statistical analysis

Differences between 2 experimental groups were assessed for statistical significance using the Mann-Whitney *U* Test. Differences between more groups were assessed using the one-way ANOVA (Analysis of Variance) test and Tukey's *post hoc* test (*p* values <0.05 were considered significant and are indicated as, **p* <0.05; ***p* <0.01; ****p* <0.001; *****p* <0.0001).

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

Suppression of bile acid synthesis in acute T cell-mediated cholangitis

To address whether T cell-induced acute cholangitis could affect BA metabolism we performed adoptive T cell transfer experiments in an inducible mouse model of antigen-dependent cholangitis.[25] Adoptive transfer of antigen-specific OT-1 CD8+ T cells in K14-OVAp recipient animals which express an MHC class I restricted ovalbumin peptide on biliary epithelial cells resulted in acute cholangitis as demonstrated by increased serum liver enzymes and histopathological periportal and portal inflammation (Fig. 1A-C). The expression of genes encoding rate-limiting enzymes of the classical and alternative BA synthesis pathways were significantly downregulated in K14-OVAp animals after OT-1 cell transfer (Fig. 1D). To determine whether T cells were responsible for modulating BA metabolism in this setting, we crossed the established K14-OVAp mouse model with *Rag1*$^{-/-}$ mice, which lack mature T and B cells. Adoptive transfer of antigen-specific OT-1 CD8+ T cells in K14-OVApx*Rag1*$^{-/-}$ mice induced portal and periportal inflammation (Fig. 1E-G). Expression of genes critical for the classical BA synthesis pathway Cyp7a1 and Cyp8b1 were found to be significantly downregulated (Fig. 1H) in recipient mice lacking mature T and B cells, suggesting the involvement of transferred T cells in suppression of BA synthesis.

CD8+ T cells induced severe cholangitis in a new mouse model of sclerosing cholangitis

In order to further dissect the contribution of T cells to the suppression of BA metabolism in cholangitis and to better recreate the pathogenetic mechanisms involved in human cholangiopathies, we aimed to establish a new mouse model, which allows T cell-induced acute cholangitis on the background of BA induced sclerosing cholangitis. To this end, K14-OVAp mice were crossed with *Mdr2*$^{-/-}$ mice (*Mdr2*$^{-/-}$xK14-OVAp). Antigen-specific CD8+ T cell transfer resulted in severe portal and periportal inflammation in *Mdr2*$^{-/-}$xK14-OVAp recipient mice (Fig. 2). Serum liver enzymes were significantly elevated (ALT levels: OT-1 mean ± 1,111 U/L *vs.* control mean ± 272 U/L; $p = 0.0001$) and numbers of liver infiltrating lymphocytes were significantly increased on day 8 after OT-1 CD8+ T cell transfer (Fig. 2A). Histopathological examinations revealed periportal bile infarcts (Fig. 2B,C). Fibrosis development was not significantly affected in this short-term model (data not shown). To demonstrate periductal inflammation throughout the whole liver *in vivo*, we performed MRI scans employing 3D reconstruction (Fig. 2D and Supplementary movie). In brief, the T2-weighted imaging illustrated inflamed (bright) periportal 8 days after OT-1 T cell transfer.

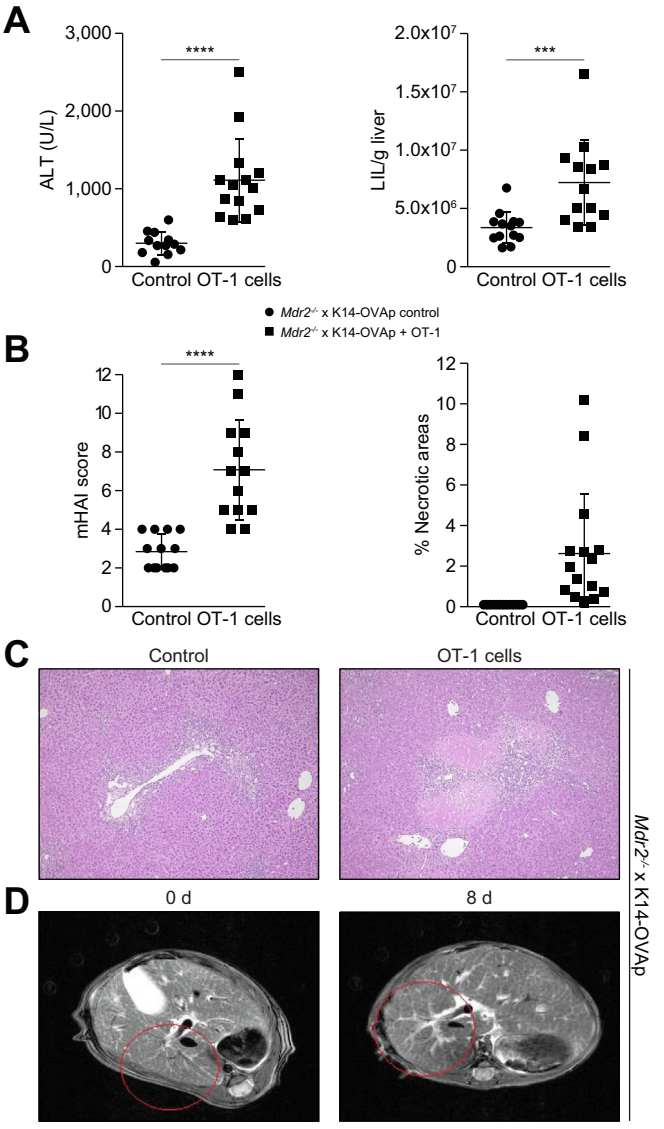

Fig. 2. Characterization of a new mouse model of CD8+ T cell-induced acute on chronic cholangitis (*Mdr2*$^{-/-}$xK14-OVAp). (A) ALT and number of LILs were determined in 7-week-old female and male *Mdr2*$^{-/-}$xK14-OVAp animals (n = 14) on day 8 after transfer of 4 × 10^6 OT-1 CD8+ T cells compared to controls (n = 11). (B) mHAI histological grading of liver sections and percentage of necrotic areas were significantly increased after cell transfer. (C) H&E staining (20×) of liver sections from *Mdr2*$^{-/-}$xK14-OVAp animals on day 8 after transfer of 4 × 10^6 OT-1 CD8+ T cells. (D) T2-weighted MR-imaging in female *Mdr2*$^{-/-}$xK14-OVAp animals (n = 3) before (0 d) and on day 8 (8 d) after cell transfer of 4 × 10^6 OT-1 CD8+ T cells. Data are expressed as the mean ± SD; n = 12–14, representing pooled data from 3 independent experiments. Data were analyzed using the Mann-Whitney *U* test. ****p* <0.0006, *****p* <0.0001. ALT, alanine aminotransferase; LILs, liver infiltrating lymphocytes; mHAI, modified hepatitis activity index.

Highly activated, antigen-specific transferred CD8+ T cells localized around bile ducts

To demonstrate that liver damage was mediated by the transferred antigen-specific T cells, we used the congenic marker CD45.1 to show that liver infiltrates contained predominantly

transferred OT-1 T cells (56% transferred OT-1 cells of total CD8 + T cells, compared to spleen: 7.7% OT-1 cells of total CD8+ T cells). Intracellular IFN-γ- and TNF-staining demonstrated that the vast majority of transferred T cells were highly activated (Fig. 3A,B). To clarify whether transferred OT-1 T cells were the main drivers of liver inflammation we performed a detailed flow cytometric analysis of liver infiltrating lymphocytes. As expected, we found alterations in different cell populations such as CD4+ T cell, monocytes and neutrophils in *Mdr2*$^{-/-}$xK14-OVAp recipient mice following OT-1 CD8+ T cell transfer (Fig. S1). However, the most striking changes occurred in the T cell population and were caused by transferred OT-1 CD8+ T cells. Re-stimulated liver infiltrating lymphocytes isolated from *Mdr2*$^{-/-}$xK14-OVAp animals on day 8 after transfer of OT-1 T cells produced large amounts of IFN-γ and TNF compared to controls. In contrast, the production of IFN-γ and TNF by splenic lymphocytes was equally low (Fig. 3C,D). Immunohistochemical staining of CD45.1 demonstrated that the transferred OT-1 cells mainly localized around CK19-positive bile ducts in areas of portal inflammation (Fig. 3E). Expression of T cell recruiting chemokines CXCL-9 and CXCL-10 as well as VCAM-1, an integrin necessary for the adhesion of leucocytes, was increased in OT-1 recipient mice (Fig. 3F).

CD8+ T cells profoundly changed bile acid metabolism in acute on chronic cholangitis

Since we had previously observed that the transfer of antigen-specific CD8+ T cells into K14-OVAp and K14OVAp*Rag1*$^{-/-}$ mice suppressed BA synthesis, we looked at whether this also takes place on the background of chronic cholangitis. The

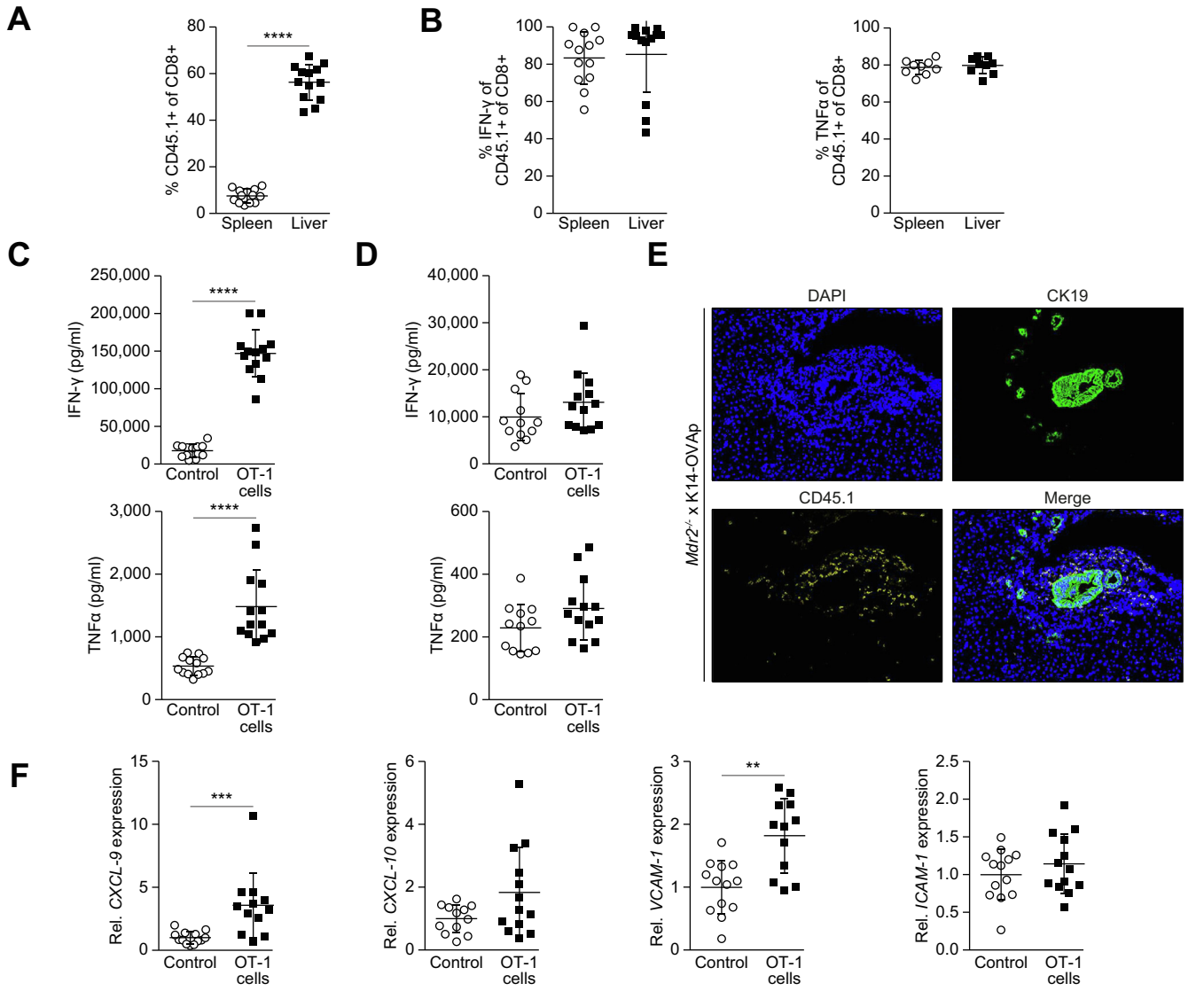

Fig. 3. Transferred antigen-specific CD8+ **T cells are highly activated and localize around bile ducts in the inflamed liver.** 4 × 10^6 OT-1 CD8+ CD45.1+ T cells were injected into 6-week-old female and male *Mdr2*$^{-/-}$xK14-OVAp mice (n = 14). On day 8 after injection mice were sacrificed and analyzed compared to control animals without cell transfer (n = 11). (A) The percentages of CD8+ CD45.1+ T cells within spleen and liver were determined by flow cytometry. (B) The activation status of transferred OT-1 CD8+ CD45.1+ T cells and endogenous CD8+ T cells in spleen and liver of *Mdr2*$^{-/-}$xK14-OVAp mice was determined by flow cytometric staining of intracellular IFN-γ and TNF. (C) Liver and (D) spleen derived lymphocytes were re-stimulated for 24 h with anti-CD3 and anti-CD28 antibodies and IFN-γ and TNF levels were measured in the supernatants using ELISA. (E) Transferred OT-1 CD8+ T cells localized around bile ducts as demonstrated by immunohistological staining of CD45.1 (yellow), CK19 (green) and DAPI (blue) 20×. (F) Relative mRNA expression of chemokine ligands in liver tissue from *Mdr2*$^{-/-}$xK14-OVAp mice on day 8 after cell transfer compared to controls. Data are expressed as mean ± SD; n = 12–14, representing pooled data from 3 independent experiments. Data were analyzed using the Mann-Whitney *U* test. $^{**}p$ <0.005, $^{***}p$ <0.0005, $^{****}p$ <0.0001.

expression of genes encoding rate-limiting enzymes of the classical (*Cyp7a1* and *Cyp8b1*) and alternative (*Cyp7b1*) BA synthesis pathways were significantly downregulated in *Mdr2*$^{-/-}$xK14-OVAp mice after antigen-specific CD8+ T cell transfer (Fig. 4A). Moreover, the expression of the gene encoding for hepatocellular BA export (*Abcb11*, encoding BSEP) was upregulated and for basolateral BA uptake (NTCP and OATP encoded by *Slc10a1* and *Slco1b2*, respectively) were downregulated. Significant changes in the expression of *Nr0b2* (encoding for small heterodimer protein [SHP]) and *Nr5a2* (encoding for LRH-1) linked the regulation of BA synthesis to an activation of the FXR-signaling pathway, a nuclear receptor critically involved in the metabolic and postprandial regulation of BA homeostasis. Next we assessed whether these expression changes reflected altered BA levels of unconjugated and conjugated BAs in serum and livers of *Mdr2*$^{-/-}$xK14-OVAp mice after transfer of antigen-specific CD8+ T cells. Corresponding to the changes observed in gene expression, T cell-induced cholangitis resulted in decreased levels of total unconjugated BAs and increased levels of conjugated BAs in serum and liver (Fig. 4B,C). We performed TUNEL staining to determine whether the changes in BA metabolism were related to parenchymal cell death/apoptosis. A significant increase of apoptotic cells was detected in the inflamed portal fields but not in liver lobul of *Mdr2*$^{-/-}$xK14-OVAp mice after transfer of antigen-specific CD8+ T cells compared to controls (Fig. S2). These results indicated that T cell-induced liver inflammation in chronic cholangitis orchestrates a transcriptional program that ultimately resulted in the inhibition of BA synthesis and uptake and an induction of biliary BA secretion independent of parenchymal cell death/apoptosis.

In time-course kinetic experiments, we observed a correlation of OT-1 T cell recruitment to the liver with increasing liver damage (Pearson r = 0.8815) and suppression of BA synthesis. From day 5 on, transferred CD8+ OT-1 T cells could be detected in the livers of *Mdr2*$^{-/-}$xK14-OVAp recipient mice, with recruited cell numbers more than doubled, resulting in increased liver enzymes on day 7 (Fig. 5A). The increase in T cell recruitment to the liver was associated with the aforementioned changes in expression of genes involved in BA metabolism (Fig. 5B), together leading to a decrease of potentially harmful unconjugated BA concentrations in the liver.

To confirm the hypothesis that transferred OT-1 CD8+ T cells themselves were able to induce these changes, we performed cell transfer experiments into *Mdr2*$^{-/-}$xK14-OVApx*Rag1*$^{-/-}$

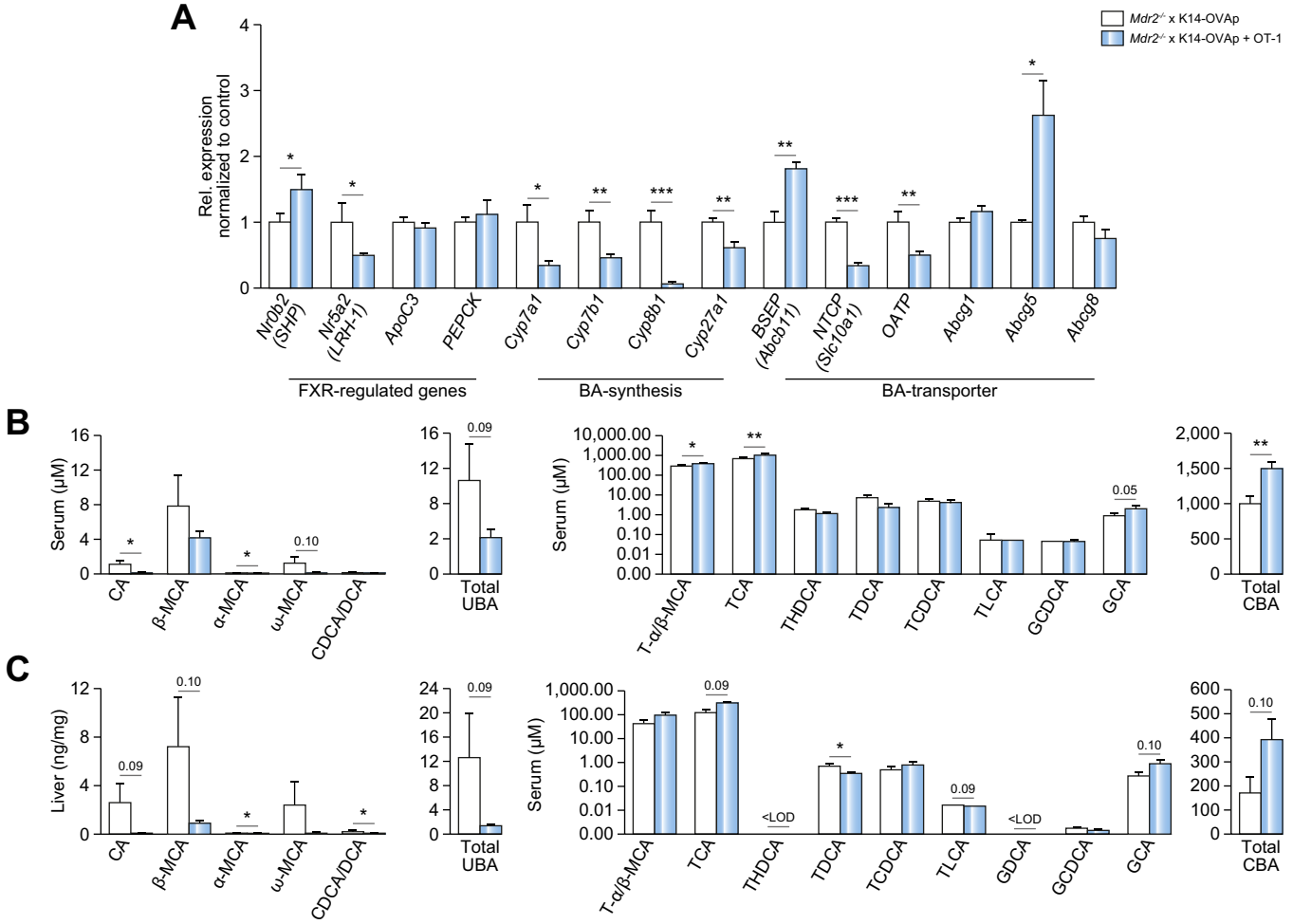

Fig. 4. T cell-induced cholangitis profoundly modulated BA metabolism. (A) mRNA expression of FXR regulated genes, BA synthesis and transporter genes in whole liver tissue from female and male *Mdr2*$^{-/-}$xK14-OVAp animals (n = 14) on day 8 after cell transfer compared to untreated controls (n = 13). Levels of uBAs and cBAs were measured in the (B) serum (µM) and (C) liver tissue (ng/mg) of male and female *Mdr2*$^{-/-}$xK14-OVAp animals on day 8 after transfer compared to untreated controls. Data were analyzed using the Mann-Whitney *U* test. **p* <0.05, ***p* <0.005, ****p* <0.0005. BA, bile acid; CA, cholic acid; cBA, conjugated BA; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; MA, muricholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; THDCA, taurodehydrocholic acid; TLCA, taurolithocholic acid; uBA, unconjugated BA.

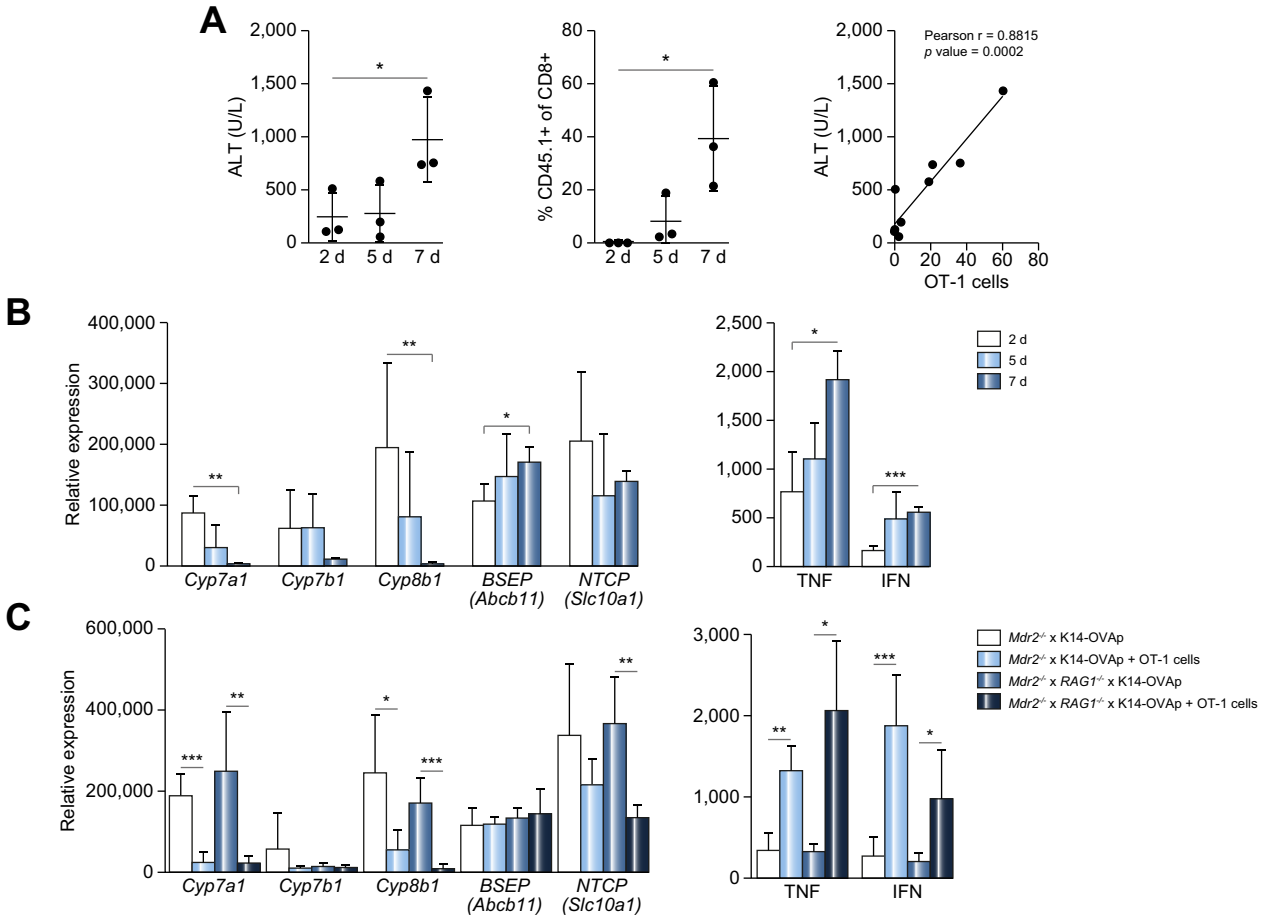

Fig. 5. CD8+ T cells targeting bile ducts were associated with changes in bile acid metabolism. 4×10^6 OT-1 CD8+ CD45.1+ T cells were injected into 6-week-old female *Mdr2*$^{-/-}$xK14-OVAp mice (n = 3). (A) ALT serum levels were measured and recruitment of transferred OT-1 CD8+ CD45.1+ T cells into the livers was determined by flow cytometry at different time points. (B) Time course of mRNA expression of bile acid synthesis and transporter genes and *Tnf* in liver tissue from *Mdr2*$^{-/-}$xK14-OVAp mice after transfer of 4×10^6 OT-1 CD8+ CD45.1+ T cells. (C) mRNA expression of bile acid synthesis and transporter genes and *Tnf* in liver tissue of female *Mdr2*$^{-/-}$xK14-OVApxRag1$^{-/-}$ (n = 10) and female *Mdr2*$^{-/-}$xK14-OVAp mice (n = 10) on day 8 after transfer of 4×10^6 OT-1 CD8+ CD45.1+ T cells compared to controls. Data were analyzed using the Mann-Whitney *U* test. **p* <0.05, ***p* <0.005, ****p* <0.0005. ALT, alanine aminotransferase.

mice, which lack endogenous mature T and B cells. Histological staining confirmed that transferred OT-1 cells were recruited to the liver and again localized around bile ducts (Fig. S3). Moreover, OT-1 T cell transfer in recipient mice on the *Rag1* knockout background led to a significant downregulation of genes responsible for BA synthesis, similar to observations in recipient mice with mature endogenous lymphocytes (Fig. 5C).

TNF and IFN-γ mediated T cell-induced changes in bile acid metabolism

To investigate the mechanism by which OT-1 CD8+ T cells could mediate changes in BA metabolism in *Mdr2*$^{-/-}$xK14-OVAp mice, the expression of proinflammatory cytokines in the liver was measured after adoptive CD8+ T cell transfer (Fig. S4). Recipient mice with mature endogenous lymphocytes showed hepatic upregulation of several proinflammatory cytokines such as IL-1β, IL-6, IL-12α and IL-12β upon cell transfer. Most of these cytokines were expressed at lower levels in recipient mice on the *Rag1* background. Since K14-OVAp mice on the *Rag1* knockout background showed suppression of BA synthesis, we searched for changes in cytokine expression also evident in *Rag1* knockout mice. The expression of both TNF and IFN-γ was not only increased after recruitment of OT-1 cells to the liver, but was also higher in *Mdr2*$^{-/-}$xK14-OVApx*Rag1*$^{-/-}$ recipients compared to RAG1-positive controls (Fig. 5C). In line, a significant negative correlation was observed between IFN-γ- and TNF-expression and the expression of BA synthesis genes *Cyp7a1* and *Cyp8b1* (Fig. S5). To determine the functional role of TNF and IFN-γ for the observed changes in gene expression, mice were treated with blocking anti-TNF and anti-IFN-γ antibodies. The inhibition of TNF and IFN-γ in combination did not affect the recruitment of transferred OT-1 CD8+ T cells into the liver or the severity of liver damage or parenchymal cell apoptosis rate (Fig. 6A-C and Fig. S6). However, we observed a significant upregulation of *Cyp7a1* and *Cyp8a1* expression (Fig. 6D) and alterations in BA transporter genes (Fig. 6E) which were related to significant changes in the expression of *Nr0b2* and *Nr5a2* (Fig. 6F) linking the upregulation of BA synthesis to the FXR-signaling pathway. Of note, *in vitro* transwell experiments showed that the modulation of BA metabolism by T cells was not only mediated by the release of cytokines but also dependent on cell-to-cell interaction between hepatocytes and T cells (Fig. 6G and Fig. S7).

Reduced expression of BA synthesis genes in human livers of patients with autoimmune, T cell driven liver diseases

To translate the findings that T cell related cytokines may also contribute to the control of BA metabolism in human disease,

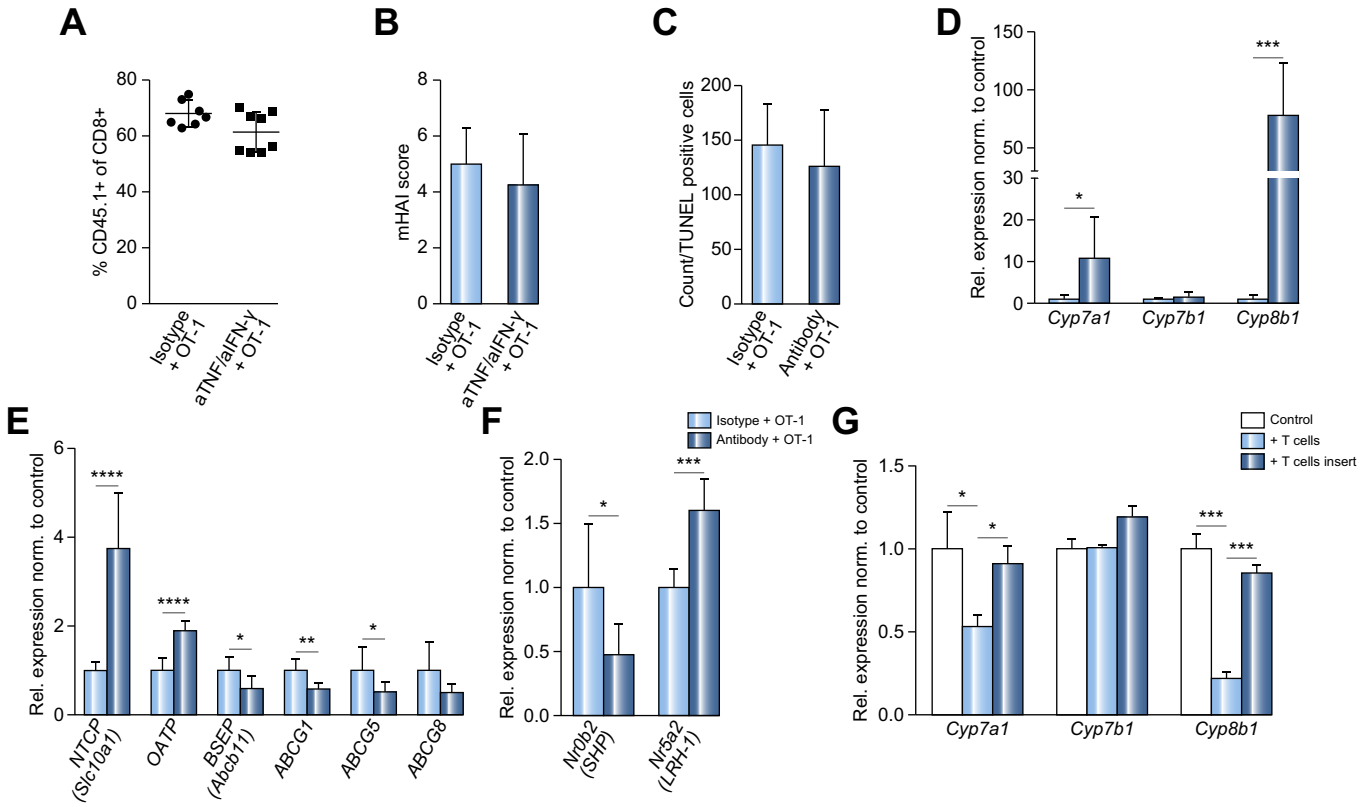

Fig. 6. TNF and IFN-γ were involved in T cell-induced changes in BA metabolism. 7-week-old female *Mdr2*$^{-/-}$xK14-OVAp animals were analyzed on day 8 after OT-1 cell transfer and administration of aTNF/aIFN-γ antibody (n = 8) or isotype control (n = 7) were applied 4 hours before and on day 3 following cholangitis induction. (A) Recruitment of transferred OT-1 CD8+ CD45.1+ T cells, (B) mHAI histological grading and (C) quantification of TUNEL positive cells of liver sections. (D) mRNA expression of BA synthesis, (E) transporter genes and (F) FXR regulated genes in whole liver tissue. (G) qPCR-analysis from primary hepatocytes isolated from female C57Bl6/J animals co-cultured for 24 h with T cell receptor stimulated CD3+ T cells in transwell or direct co-culture experiments. Data were analyzed using the Mann-Whitney *U* test. $^{*}p$ <0.05, $^{**}p$ <0.005, $^{***}p$ <0.0005, $^{****}p$ <0.0001. BA, bile acid; mHAI, modified hepatitis activity index.

we investigated gene expression in whole liver tissue from liver biopsies obtained from patients with PBC (n = 16), AIH (n = 22) and PSC (n = 20). RNA sequencing analysis showed a negative correlation between genes responsible for BA synthesis and hepatic BA uptake with the expression of a set of known TNF- and IFN-γ-regulated genes (Fig. 7).

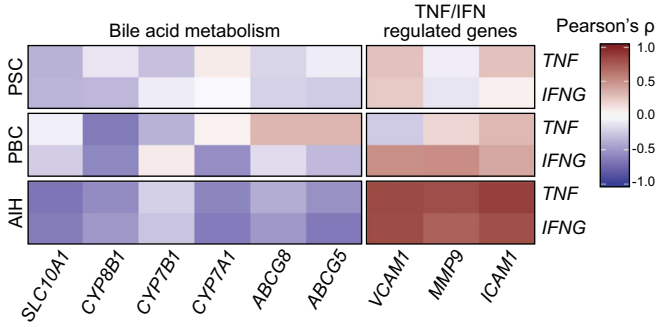

Fig. 7. RNA sequencing of liver biopsies revealed negative correlation between expression of TNF and IFN-γ and bile acid synthesis genes. Log fold-change in mRNA expression in correlation to the expression of TNF and IFN-γ expression in tissue obtained by liver biopsy from patients with AIH (n = 22), PBC (n = 16) and PSC (n = 20). AIH, autoimmune hepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

Discussion

T cells significantly contribute to the pathogenesis of PBC and PSC and represent a potential therapeutic target in autoimmune cholestatic liver diseases.[8,9,31–33] Emerging data suggest a suppressive effect of BAs on the functioning of T cells,[22] but so far it has not been investigated whether T cells in turn could affect BA metabolism. In the new mouse model described here, which combines chronic and acute T cell-induced bile duct injury, we could show for the first time that recruitment of CD8+ T cells targeting bile ducts resulted in profound changes in BA metabolism, signified by a reduction of BA uptake and synthesis, and increased BA export from hepatocytes. CD8+ T cell recruitment to the liver led to reduced intrahepatic levels of unconjugated BAs that could be explained by a profound downregulation of enzymes regulating both the classical and alternative BA synthesis pathways (Cyp7a1, Cyp8b1 and Cyp27a1, Cyp7b1). In addition, transfer of T cells reduced the expression of basolateral bile acid uptake transporters Ntcp and Oatp and increased the expression of the BSEP, which ultimately resulted in increased serum levels of conjugated BAs and reduced intrahepatic levels of toxic, unconjugated BAs. Mechanistically, the proinflammatory cytokines IFN-γ and TNF were at least in part responsible for the observed changes in BA metabolism. IFN-γ and TNF act as a key drivers of liver inflammation[34–37] and blocking TNF is emerging as a targeted treatment option for difficult to treat

AIH.[38] Inhibition of both of these cytokines in mice using an antibody-based approach restored the suppression of genes involved in BA synthesis, export and hepatic uptake *in vivo*, without changing the degree of liver inflammation. This effect on BA metabolism was also seen in *in vivo* experiments blocking both cytokines separately. However, an additional effect of blocking IFN-γ on liver inflammation cannot be excluded and should be further explored (Fig. S6). Mechanistically, IFN-γ and TNF could influence FXR-mediated signaling pathways. The nuclear receptor FXR is the master regulator of BA homeostasis.[20,21] Activated FXR negatively regulates the expression of different genes involved in BA synthesis and basolateral hepatic BA uptake, whereas it induces the expression of genes involved in hepatic BA export.[39] We here report a significant regulation of the SHP and LRH-1, which act downstream of FXR to suppress the expression of Cyp7a1. However, we cannot exclude an effect on other signaling pathways since it has been previously described that TNF can lead to a rapid downregulation of NTCP expression by FXR-independent mechanisms.[40,41]

TNF has been shown to activate the MAP/JNK pathway and to downregulate the expression of hepatocyte nuclear factor 4 (HNF-4) through MEKK1 dependent pathways resulting in an inhibition of *Cyp7a1* transcription in hepatocytes.[42,43] In addition, sepsis is known to lead to profound changes in BA metabolism, with the proinflammatory cytokines TNF and IFN-γ likely involved in the impairment of hepatocellular transporters including MRP2, OATP, BSEP and NTCP.[23,44] However, in contrast to our findings, endotoxin-induced inflammation has been characterized by the downregulation of hepatic BSEP expression in rodents and primary human hepatocytes.[41,45,46] Inflammatory cytokines such as TNF, IL-1β and IFN-γ have been reported to repress genes regulating BA transport and synthesis in several inflammatory models, including necrotizing enterocolitis, lipopolysaccharide treatment, IL-6 or turpentine treatment and *C. rodentium* infection.[47–50] However, it remained unclear which cell type mediated these effects and it was unknown whether liver infiltrating T cells could themselves modulate BA metabolism. In the current study, we showed for the first time *in vivo* that T cells were able to induce profound changes in BA metabolism independent of hepatocyte apoptosis. Interestingly, our *in vitro* data suggested that T cell contact with hepatocytes was required in addition to the release of cytokines, a mechanism that clearly requires further investigations. Traditional views regard T cells as harmful in cholestatic liver injury, but treatment targeting T cells in cholestatic liver disease has so far been disappointing.[9,51] Herein, we describe a mechanism by which the liver enables proinflammatory CD8+ T cells to regulate BA metabolism. It is tempting to speculate that this happens to protect the liver from additional cholestatic damage and that an unselective targeting of T cells in the liver may not be effective in immune-mediated cholangiopathies.

We suggest that treatment regimens targeting T cells, in addition to activation of FXR to suppress the increase in BA synthesis resulting from depletion of T cells, could be tested in preclinical models of immune-mediated cholangitis in the future.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

F.G., C.J., B.E., B.H., J.Hee, C.S., D.S. designed the study and wrote the manuscript.

F.G., C.J., B.E., B.H., S.W., J.D., S.S., N.B., C.C., F.A.S., B.W., M.P., F. J., A.C., D.S. performed and analyzed experiments. A.F., A.C., A.W. L., H.I., J.Her. critical revision of the manuscript for important intellectual content

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Supplementary data

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Author names in bold designate shared co-first authorship

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