TREM-2 plays a protective role in cholestasis by acting as a negative regulator of inflammation
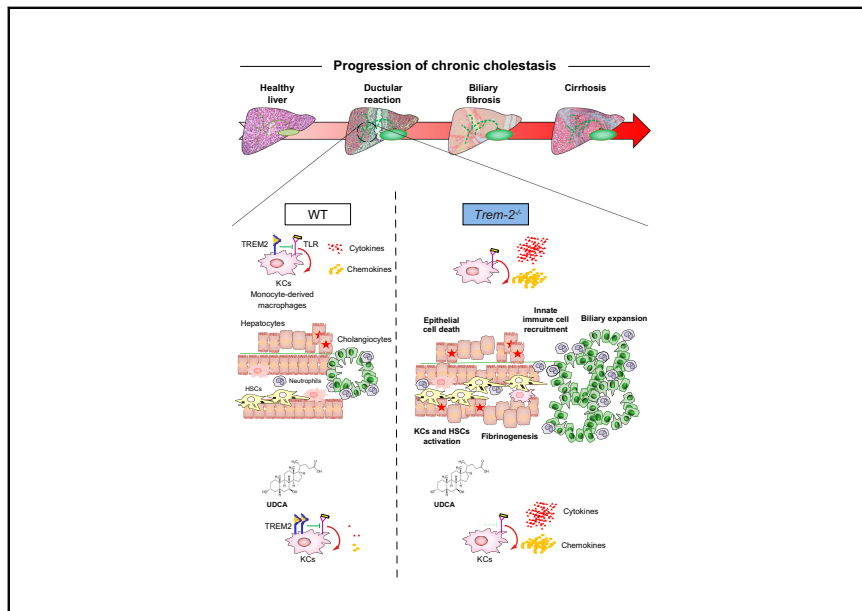
Graphical abstract

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Lay summary

Cholestasis (the reduction or cessation of bile flow) causes liver injury. This injury is exacerbated when gut-derived bacterial components interact with receptors (specifically Toll-like receptors or TLRs) on liver-resident immune cells, promoting inflammation. Herein, we show that the anti-inflammatory receptor TREM-2 dampens TLR-mediated signaling and hence protects against cholestasis-induced liver injury. Thus, TREM-2 could be a potential therapeutic target in cholestasis.

Highlights

- TREM-2 expression is upregulated in the livers of patients with PBC and PSC, and mice with cholestatic liver injury.

- *Trem-2$^{-/-}$* mice show an exacerbated inflammatory response to experimental cholestasis.

- UDCA mediates anti-inflammatory effects in KCs via TREM-2.

- TREM-2 arises as a novel therapeutic target for patients with cholestasis.

TREM-2 plays a protective role in cholestasis by acting as a negative regulator of inflammation

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Background & Aims: Inflammation, particularly that mediated by bacterial components translocating from the gut to the liver and binding to toll-like receptors (TLRs), is central to cholestatic liver injury. The triggering receptor expressed on myeloid cells-2 (TREM-2) inhibits TLR-mediated signaling and exerts a protective role in hepatocellular injury and carcinogenesis. This study aims to evaluate the role of TREM-2 in cholestasis.
Methods: TREM-2 expression was analyzed in the livers of patients with primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC), and in mouse models of cholestasis. Wild-type (WT) and Trem-2 deficient (Trem-2$^{-/-}$) mice were subjected to experimental cholestasis and gut sterilization. Primary cultured Kupffer cells were incubated with lipopolysaccharide and/or ursodeoxycholic acid (UDCA) and inflammatory responses were analyzed.

Results: TREM-2 expression was upregulated in the livers of patients with PBC or PSC, and in murine models of cholestasis. Compared to WT, the response to bile duct ligation (BDL)-induced obstructive cholestasis or alpha-naphthylisothiocyanate (ANIT)-induced cholestasis was exacerbated in Trem-2$^{-/-}$ mice. This was characterized by enhanced necroptotic cell death, inflammatory responses and biliary expansion. Antibiotic treatment partially abrogated the effects observed in Trem-2$^{-/-}$ mice after BDL. Experimental overexpression of TREM-2 in the liver of WT mice downregulated ANIT-induced IL-33 expression and neutrophil recruitment. UDCA regulated Trem-1 and Trem-2 expression in primary cultured mouse Kupffer cells and dampened inflammatory gene transcription via a TREM-2-dependent mechanism.
Conclusions: TREM-2 acts as a negative regulator of inflammation during cholestasis, representing a novel potential therapeutic target.
Lay summary: Cholestasis (the reduction or cessation of bile flow) causes liver injury. This injury is exacerbated when gut-derived bacterial components interact with receptors (specifically Toll-like receptors or TLRs) on liver-resident immune cells, promoting inflammation. Herein, we show that the anti-inflammatory receptor TREM-2 dampens TLR-mediated signaling and hence protects against cholestasis-induced liver injury. Thus, TREM-2 could be a potential therapeutic target in cholestasis.

Introduction

Cholestasis is a multifactorial pathologic condition characterized by impaired bile flow and subsequent accumulation of bile acids and other toxic substances within the liver, which trigger liver damage.[1] Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are the most common chronic cholestatic diseases in adults, characterized by progressive hepatobiliary injury that may evolve to biliary fibrosis, cirrhosis, portal hypertension, ductopenia and eventually liver failure and/or development of liver malignancies.[2]

The endogenous, choleretic and hepatoprotective bile acid (BA) ursodeoxycholic acid (UDCA) is the main therapeutic option for cholestatic diseases.[2] UDCA represents the first-line treatment for patients with PBC, yet around 40% of patients have an incomplete response, which is associated with a higher risk of progressing to end-stage liver disease and the need for liver transplantation.[3] In patients with PSC, the benefits of UDCA are still controversial.[4] Novel therapeutic strategies to treat patients with cholestatic liver injury are currently under development. Among them, nuclear receptor agonists, such as the farnesoid X receptor (FXR) agonist obeticholic acid (OCA) and the dual peroxisome proliferator-activated receptors (PPAR)/pregnane X receptor (PXR) agonist bezafibrate, are currently showing promising results in clinical trials.[5] Interestingly, the activation of FXR with OCA in cholestatic rats prevented gut barrier dysfunction and consequent bacterial translocation,[6] indicating a potential role for OCA in the gut-liver axis. Still, cholestatic diseases represent an unmet clinical challenge and further investigations are needed in order to offer affected patients effective therapeutic opportunities.

Inflammation plays a crucial role in the progression of cholestatic diseases, directing cellular cross-talk and orchestrating the perpetuation of the maladaptive regenerative mechanisms.[7,8] Recent studies have highlighted the particular importance of the gut-liver axis during cholestasis.[9] In the presence of persistent hepatobiliary damage, the gut epithelial barrier is disrupted and dysbiosis occurs, leading to the translocation of bacteria and/or bacterial products from the gut to the liver.[9] In line with this, patients with PBC or PSC are characterized by bacterial overgrowth and decreased biodiversity of the gut microbiota.[10,11] In the liver, bacterial products bind to toll-like receptors (TLRs) – which are mainly expressed in resident Kupffer cells (KCs), monocyte-derived macrophages, and hepatic stellate cells (HSCs) – thereby exacerbating inflammatory and fibrogenic responses.[12]

The family of triggering receptor expressed on myeloid cells (TREM) receptors were described as modulators of TLR-mediated signaling.[13] Overall, TREM-2 negatively regulates TLR-mediated inflammatory responses in different tissues and contexts, while TREM-1 acts as a pro-inflammatory receptor, thereby amplifying TLR-induced inflammatory gene transcription.[14] Both receptors signal through the ITAM (immuno-receptor tyrosine-based activation motif) of the adaptor protein DAP-12 (DNAX adaptor protein 12).[14] The specific ligand(s) for TREM-2 are still unknown although diverse endogenous and exogenous factors including anionic molecules, phospholipids, proteoglycans, apolipoproteins and heat shock proteins have been postulated to activate TREM-2-mediated signaling.[15] In the context of hepatocellular diseases, we previously described that TREM-2 expression is induced in the liver of patients and mice with diverse forms of hepatocellular injuries and hepatocellular carcinoma (HCC).[16,17] Additionally, TREM-2 exerts multiple protective effects in the liver, dampening inflammatory gene transcription upon TLR activation in KCs and HSCs, and thus protecting the liver from acute and chronic hepatocellular damage and hepatocellular carcinogenesis.[16,17] Given the crucial role for innate immunity and the involvement of the gut-liver axis in cholestatic diseases, the present study aims to investigate the role of TREM-2 in the development and progression of these disorders, as well as in the further understanding of the molecular mechanisms triggering the therapeutic effects of UDCA.

Material and methods

Human samples

Liver tissue samples from patients with PBC (n = 25; 10 with F3-F4 and 15 with F1-F2 fibrosis score), PSC (n = 10; 6 with fibrosis scores F3-F4) and cirrhosis of different aetiologies (n = 44), as well as healthy control liver tissues (n = 26) were used. Patient characteristics and clinical parameters are presented in Table S1 and S2.

Animal models of cholestasis

Experiments with animals were performed in age-matched male WT and *Trem-2*^{-/-} mice on a C57BL/6 genetic background, that were generated as previously described.[18] Thereafter, mice were bred at the animal facility of the Biodonostia Health Research Institute (BHRI). All experiments were performed under the approval of the Animal Experimentation Ethics Committee of BHRI (CEEA15/001, CEEA15/020, CEEA18/21, CEEA19/002, CEEA19/004).

WT and *Trem-2*^{-/-} mice were subjected to bile duct ligation (BDL)-induced obstructive cholestasis in the presence and absence of an antibiotic cocktail containing ampicillin (1 g/L), neomycin (1 g/L), metronidazole (1 g/L) and vancomycin (500 mg/L).[19] Additionally, a model of chemically induced cholestasis based on ANIT administration was used. Gain-of-function experiments were performed by overexpressing TREM-2 in WT mice via intravenous tail vein injection of control or *Trem-2*-overexpressing adeno-associated viruses (AAVs), which was then followed by ANIT administration to induce cholestasis. A detailed description of the mouse models used is included in the supplementary information.

Primary cell isolation and experimental conditions

Hepatocytes, cholangiocytes, KCs and HSCs were isolated as previously reported[20,21] and gene expression analyses were performed in baseline conditions and also after incubation with lipopolysaccharide (LPS) and UDCA in KCs, as indicated in the supplementary information.

Statistical analysis

GraphPad Prism 6.00 (GraphPad Software) was used to perform the statistical analysis, after assessing normality of the data set, appropriate parametric or non-parametric tests were employed. A detailed description of the statistical analysis is included in the supplementary information.

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

Results

TREM-2 expression is upregulated in the livers of patients with PBC and PSC, positively correlating with markers of disease progression

TREM-2 mRNA levels were found increased in the livers of patients with PBC or PSC from study cohort 1, compared with cirrhotic livers (with different etiologic causes) or normal control liver tissues (Fig. 1A). Moreover, TREM-2 expression positively correlated with markers of cholestasis and macrophages when samples of patients with PBC and PSC were grouped together. Herein, TREM-2 expression correlated with the pro-inflammatory cytokines interleukin (IL)6, 8 (IL8) and 33 (IL33), the macrophage markers CD68 and CD9, as well as the marker of fibrosis collagen type 1 A 1 (COL1A1) (Fig. 1B).

Further sub-analysis including only livers from patients with PBC or PSC confirmed the correlations of TREM-2 levels with IL8 and COL1A1 in both diseases when analyzed separately (Fig. S1A,B). Additionally, positive correlations of hepatic TREM-2 expression with serum levels of markers of liver injury (i.e., alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) and the marker of cholestasis bilirubin were particularly observed in patients with PBC. Accordingly, a positive correlation between hepatic TREM-2 expression and the MELD (model for end-stage liver disease) score was also observed in this subset of patients (Fig. S1A). Moreover, a positive correlation between
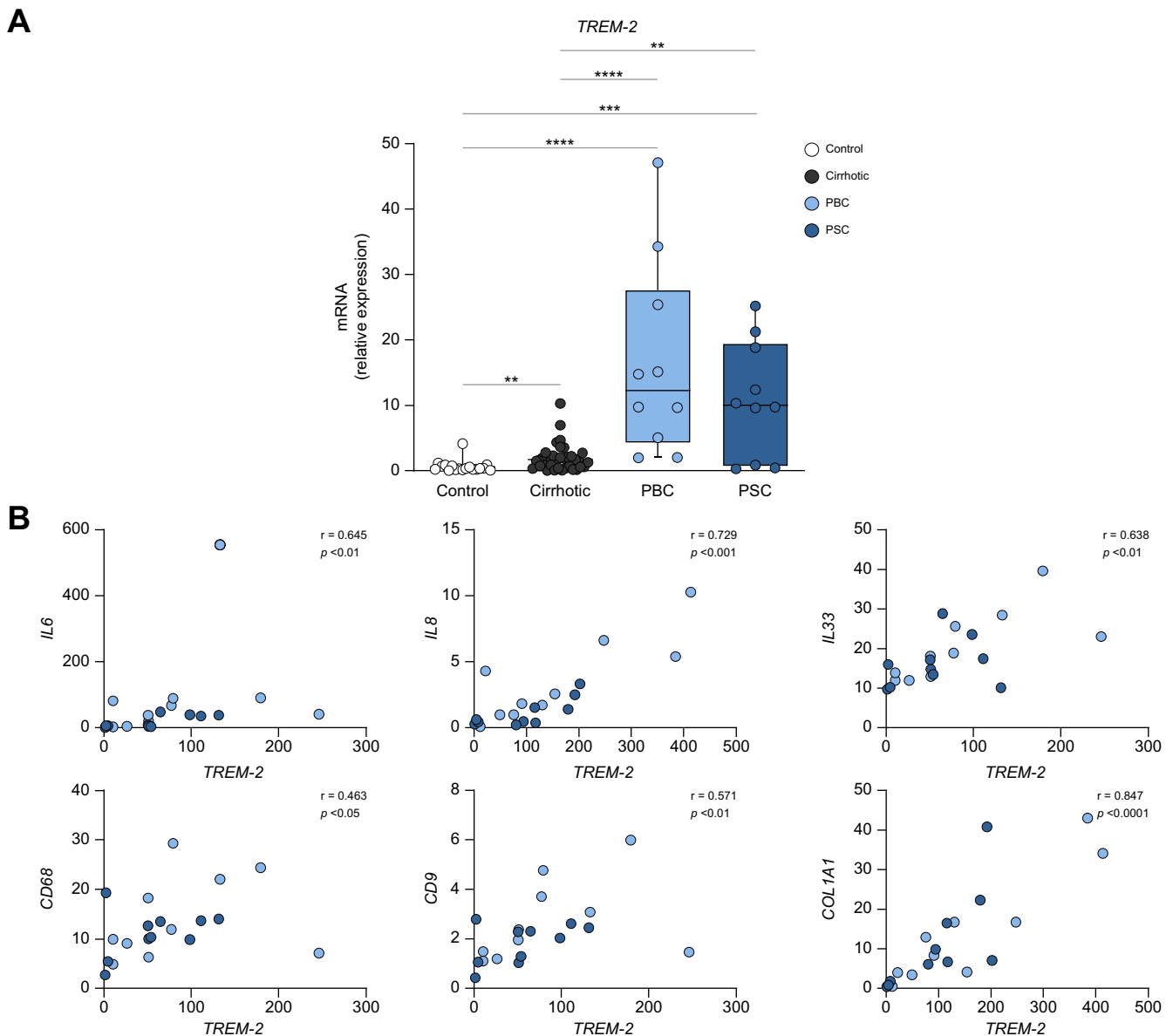

Fig. 1. TREM-2 mRNA expression in the livers of patients with cholestasis from study cohort 1. (A) TREM-2 mRNA expression in the livers of control individuals (n = 18), and patients with cirrhosis (n = 35), PBC (n = 10) or PSC (n = 10). (B) Correlation of expression (mRNA) between TREM-2 and the inflammatory markers IL6, IL8 and IL33, the macrophage markers CD68 and CD9, and the fibrosis marker COL1A1 in the liver of patients with PBC and PSC. **p <0.01; ***p <0.001 and ****p <0.0001 (Mann-Whitney test and Spearman correlation test). PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

TREM-2 expression and the levels of *IL6*, chemokine (C-X-C motif) ligand 1 (*CXCL1*), *CD9* and the marker of fibrosis *COL3A1* was confirmed in a publicly available data repository[22] that includes a gene expression array of liver tissue of patients with PBC (Fig. S2A).

Next, a comparative analysis of *TREM-2* expression was performed according to the liver fibrosis stage (*i.e.*, early (F2) *vs.* advanced (F3-F4) fibrosis) in patients with PBC and PSC. Notably, *TREM-2* was similarly upregulated in the livers of patients with F2 or F3/F4 fibrosis, when compared with normal liver tissues

(Fig. S2B). Likewise, marked *TREM-2* upregulation was found in the liver of an additional cohort of patients with early-stage PBC (F1-F2) in comparison to healthy liver tissues and patients with alcohol-related cirrhosis (Fig. S2C).

On the other hand, the expression levels of *TREM-1* were not altered in the livers of patients with PBC or PSC, when compared with healthy controls or patients with cirrhosis, although a positive correlation with markers of inflammation and fibrosis was evident (Fig. S3A,B). Together, these findings suggest an involvement of TREM-2 in human cholestatic disease.
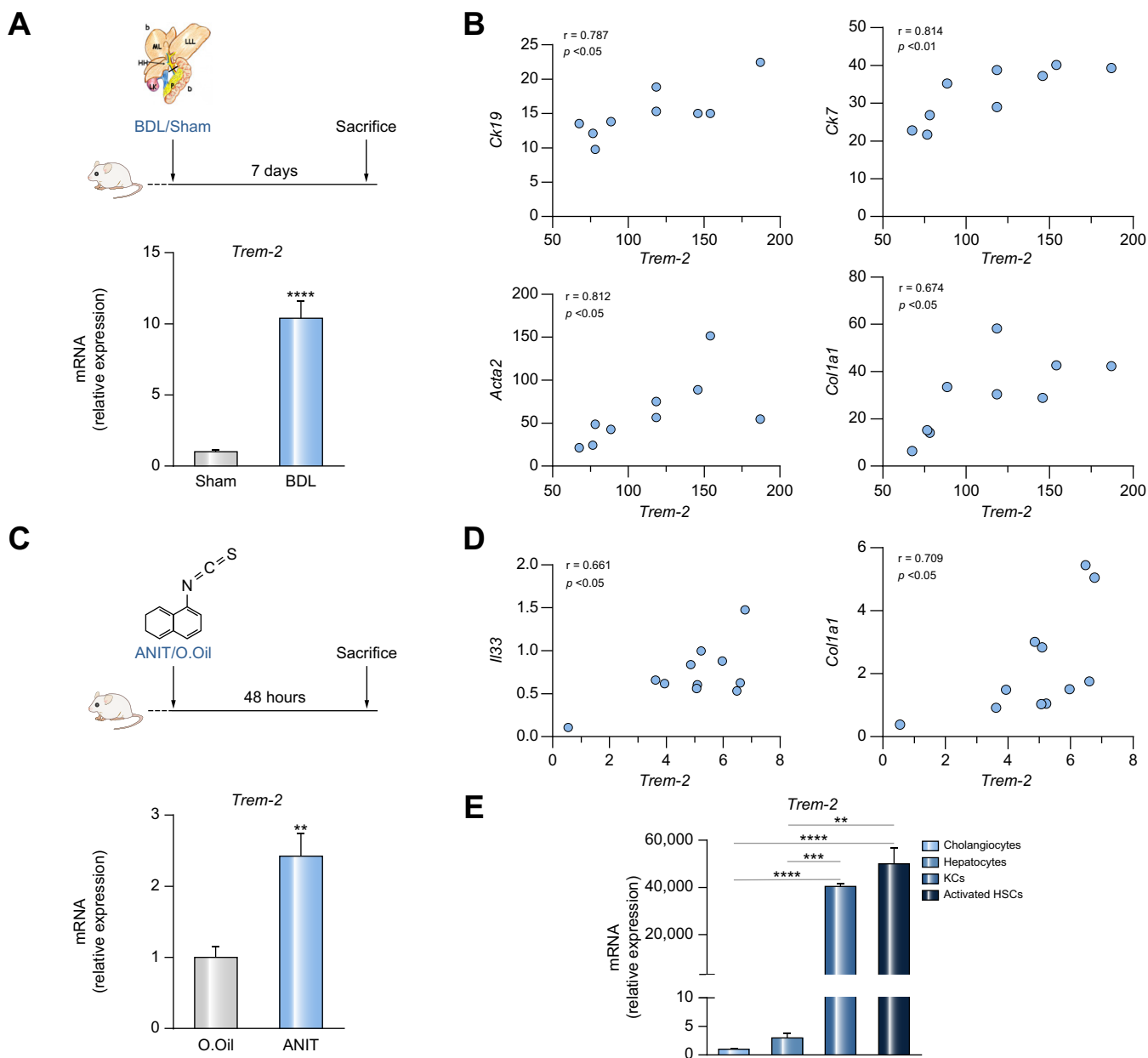

Fig. 2. *Trem*-2 mRNA expression in cholestatic mouse models and in different liver cell types. (A) Hepatic *Trem*-2 mRNA expression in mice subjected to sham or BDL operations (n = 6-9). (B) Correlation of expression (mRNA) between *Trem*-2 and *Ck19, Ck7, Acta2* or *Col1a1* in the liver of mice subjected to BDL (n = 9). (C) Hepatic *Trem*-2 mRNA expression in mice administered with the vehicle olive oil or ANIT (n = 8-9). (D) Correlation of expression (mRNA) between *Trem*-2 and *Il33* or *Col1a1* in the livers of mice exposed to ANIT (n = 9). (E) *Trem*-2 mRNA expression in WT primary mouse cholangiocytes (n = 6), hepatocytes (n = 8), KCs (n = 6) and activated HSCs (n = 5). **p <0.01; ***p <0.001 and ****p <0.0001 (Mann-Whitney-, Spearman's-, Pearson's- and Student's *t* test). ANIT, alpha-naphtylisothiocyanate; BDL, bile duct ligation; HSC, hepatic stellate cell; KC, Kupffer cell; O.Oil, olive oil.
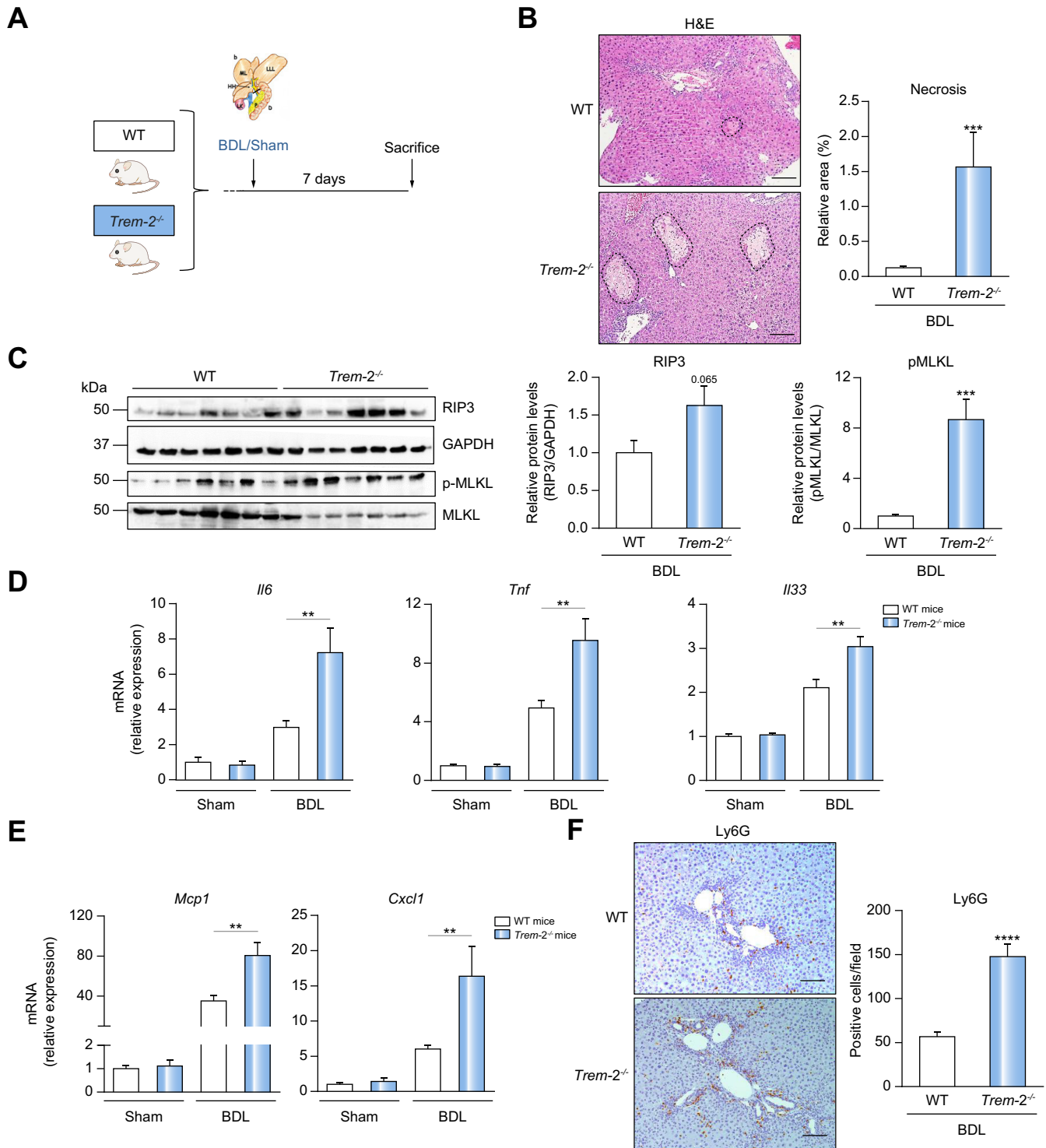
Fig. 3. Hepatic cell death and inflammation in response to BDL in WT and *Trem-2*$^{-/-}$ mice. (A) Mice were subjected to BDL (WT n = 9; *Trem-2*$^{-/-}$ n = 8) or control sham operation (WT n = 6; *Trem-2*$^{-/-}$ n = 7) and sacrificed 7 days after surgery. (B) Representative H&E images and quantification of necrotic areas. (C) Representative immunoblot and quantification of RIP3 and phospho-MLKL. (D) mRNA expression of hepatic inflammatory cytokines *Il6*, *Tnf* and *Il33*, and (E) chemokines *Mcp1* and *Cxcl1*. (F) Representative IHC images and quantification of Ly6G. Scale bars (B,F): 100 μm. **p <0.01; ***p <0.001 and ****p <0.0001 (Student's *t* test and Mann-Whitney test). BDL, bile duct ligation; IHC, immunohistochemistry; WT, wild-type.

Trem-2 is upregulated in the liver of murine models of cholestasis and is expressed in non-parenchymal KCs and HSCs

To elucidate whether TREM-2 upregulation is a characteristic and conserved event in cholestasis, its expression was assessed in different murine models of cholestasis. Compared with sham-operated animals, hepatic *Trem-2* expression was upregulated in animals with obstructive cholestasis (BDL) (Fig. 2A). Herein, *Trem-2* positively correlated with different markers of cholestatic liver injury, including the biliary markers cytokeratin (*Ck)19* and *Ck7*, as well as the markers of liver fibrosis actin alpha 2 *(Acta2)* and *Col1a1* (Fig. 2B). Furthermore, in a model of chemically induced cholestasis based on ANIT administration, *Trem-2* expression was upregulated when compared to mice receiving vehicle (olive oil) (Fig. 2C), positively correlating with *Il33* and the fibrosis marker *Col1a1* (Fig. 2D).

Confirming previous data reported by our group,[16,17] *Trem-2* expression (mRNA) was very low in hepatocytes, but markedly higher in non-parenchymal liver cells, including KCs and activated HSCs (Fig. 2E); of note, isolated and cultured mouse cholangiocytes showed almost undetectable *Trem-2* expression.

TREM-2 protects the liver against obstructive cholestasis in mice

To elucidate the role of TREM-2 in cholestasis, WT and *Trem-2* deficient mice (*Trem-2*⁻/⁻) were subjected to BDL or control sham operation, and sacrificed 7 days after the surgical procedure (Fig. 3A). No changes in serum levels of alkaline phosphatase (ALP), bilirubin, ALT and AST were observed between genotypes after BDL (Fig. S4). However, H&E staining revealed increased hepatocellular necrotic areas in the liver of *Trem-2*⁻/⁻ compared to WT mice subjected to BDL (Fig. 3B), which was also confirmed histologically through independent blinded assessment by an experienced pathologist (Fig. S5A). Low levels of apoptosis were observed under BDL in both genotypes, as measured by immunohistochemistry (IHC) and immunoblotting for the marker of apoptosis, cleaved-caspase 3 (Fig. S5B,C). However, compared to control animals, post-BDL *Trem-2*⁻/⁻ mice showed a marked tendency towards increased expression of the main marker of necroptosis, RIP3 (receptor-interacting protein kinase 3), and significantly higher levels of the phosphorylated form of the main necroptotic effector, MLKL (mixed lineage kinase domain like pseudokinase) (Fig. 3C).[23]

Therefore, the inflammatory response was analyzed in WT and *Trem-2*⁻/⁻ mice upon BDL. H&E staining analysis revealed an exacerbated inflammatory infiltrate in livers of *Trem-2*⁻/⁻ mice compared to WT mice (Fig. S6A). Additionally, when compared with controls, post-BDL *Trem-2*⁻/⁻ mice exhibited augmented hepatic transcript levels of *Il6*, tumor necrosis factor *(Tnf)* and *Il33* (Fig. 3D). Furthermore, BDL in *Trem-2*-deficient mice was associated with enhanced expression of the pro-inflammatory chemokines monocyte chemoattractant protein 1 *(Mcp1)* and *Cxcl1* (Fig. 3E), which are involved in hepatic monocyte and neutrophil recruitment, respectively. Augmented hepatic MCP1 and CXCL1 levels in *Trem-2*⁻/⁻ mice were confirmed at the protein level by Bio-Plex assay (Fig. S6B). Of note, IHC for the neutrophil marker Ly6G depicted that increased hepatic *Cxcl1* expression in *Trem-2*⁻/⁻ mice after BDL was accompanied by augmented neutrophil recruitment (Fig. 3F). This effect was associated with increased levels (mRNA) of the oxidative stress markers heme oxygenase (*Hmox*) and nitric oxide synthase 2 (*Nos2*) (Fig. S6C). No differences were observed between WT and *Trem-2*⁻/⁻ mice in the hepatic content of macrophages and T lymphocytes (Fig. S6D,E).

Since cholestasis is characterized by intrahepatic accumulation of BAs, we next assessed total BA concentration and quantified the concentration of distinct BA species after BDL in both mouse genotypes. Under baseline conditions (*i.e.*, control sham operated), the liver of *Trem-2*⁻/⁻ mice exhibited lower content of total, primary and secondary BAs when compared with WT. As anticipated, BDL led to an accumulation of total and individual molecular species of hepatic BAs, which reached the same levels in both genotypes (Fig. 4A, Fig. S7). Consequently, this increased total and primary BA concentration (BDL *vs.* sham) was more prominent in *Trem-2*⁻/⁻ compared to WT mice (Fig. S8A). In line with this, the baseline expression levels of the rate limiting enzyme in BA biosynthesis cytochrome P450 family 7 a1 (*Cyp7a1*) showed an almost statistically significant down-regulation in *Trem-2*⁻/⁻ livers (Fig. 4B), which became significant when analyzed in a larger control (non-operated) group (Fig. S8B), thus potentially explaining the baseline decrease in the liver BA concentration observed in sham-operated *Trem-2*⁻/⁻ *vs.* WT mice.

In the progression of cholestasis, ductular reaction is triggered as an adaptive event to restore the damaged and lost tissue and preserve an optimal biliary function.[7,8] In line with this process, *Trem-2*⁻/⁻ mice exhibited increased scores of biliary expansion after BDL (Fig. S9A), which was accompanied by enhanced expression (mRNA) of the biliary markers *Ck19* and *Ck7* in total liver, and by more CK19⁺ cells on IHC (Fig. 4C,D). In addition, increased levels (mRNA) of the main marker of HSC activation *Acta2* and of the main fiber accumulating in fibrosis *Col1a1*, as well as more peribiliary cells positive for αSMA (protein encoded by *Acta2*), were found in the livers of *Trem-2*⁻/⁻ mice compared to WT mice after BDL (Fig. 4E,F). Nevertheless, the increased *Col1a1* mRNA expression was not accompanied by overall changes in collagen deposition between WT and *Trem-2*⁻/⁻ mice (Fig. S9B).

Antibiotic administration abrogates some of the differences observed between WT and *Trem-2*⁻/⁻ mice subjected to obstructive cholestasis

In order to test the potential contribution of gut microbiota as one of the triggering factors responsible for TREM-2-mediated effects after BDL, WT and *Trem-2*⁻/⁻ mice were administered an antibiotic (Abx) cocktail for 4 weeks, and in the third week of Abx administration, BDL was conducted and mice sacrificed 7 days after the surgical procedure (Fig. 5A). Of note, Abx administration was able to abrogate the differences observed between WT and *Trem-2*⁻/⁻ mice after BDL in the hepatic expression of the pro-inflammatory cytokines *Il6, Il33* and *Tnf*, the chemokine *Mcp1*, as well as the expression of the oxidative stress markers *Hmox1* and *Nos2* (Fig. 5B-D). Interestingly, the Abx cocktail was also able to abrogate the differences detected in ductular reaction (*i.e.*, number of CK19⁺ cells) between WT and *Trem-2*⁻/⁻ mice after BDL (Fig. 5E), as well as neutrophil recruitment to the liver (Fig. S10). Together, these findings suggest pathogen-associated molecular patterns (PAMPs) derived from gut bacteria are upstream triggers for inflammation-associated ductular reaction, which are dampened by TREM-2.
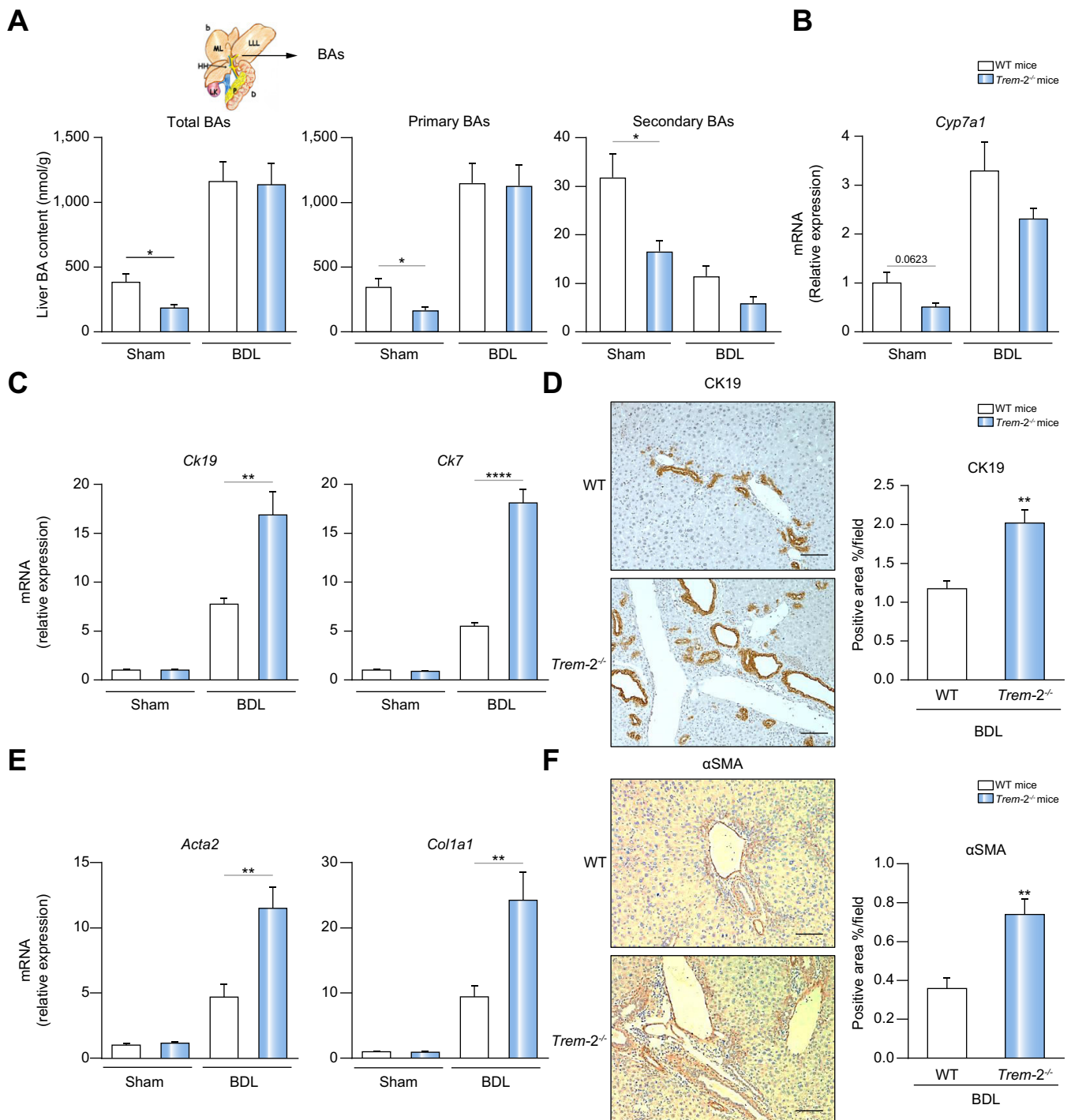
Fig. 4. Hepatic BA content and regenerative responses in WT and Trem-2⁻/⁻ mice after BDL. (A) Total, primary or secondary BA concentration (Sham: WT n = 5; $Trem2^{-/-}$ n = 6 and BDL: WT n = 7; $Trem2^{-/-}$ n = 6) in the livers of mice after BDL or sham surgery. (B) Hepatic mRNA expression of $Cyp7a1$ and (C) $Ck19$ and $Ck7$. (D) Representative IHC images and quantification of CK19. (E) Hepatic mRNA expression of $Acta2$ and $Col1a1$. (F) Representative IHC images and quantification of αSMA. (D,F) Scale bars: 100 μm. *p <0.05; **p <0.01 and ****p <0.0001 (Student's t test and Mann-Whitney test). BA, bile acid; BDL, bile duct ligation; IHC, immunohistochemistry; WT, wild-type.

TREM-2 protects the liver against ANIT-induced cholestasis

With the aim of exploring the role of TREM-2 in an alternative setting of experimental cholestasis, WT and $Trem-2^{-/-}$ mice were administered with a single oral dose of ANIT to induce chemical cholestasis or with vehicle (olive oil), and sacrificed 48 hours later (Fig. 6A). ANIT was able to trigger a higher increase in serum ALP, ALT and AST levels at 2 different doses (i.e., 50 or 75 mg/kg) in $Trem-2^{-/-}$ vs. WT mice (Fig. 6B). $Trem-2^{-/-}$ mice were also characterized by enhanced necrotic areas in the liver compared to WT mice, paralleling an augmented expression of the biliary markers $Ck7$ and $Ck19$ and a marked ductular reaction, (Fig. 6C-E). In agreement with the BDL model, ANIT-exposed
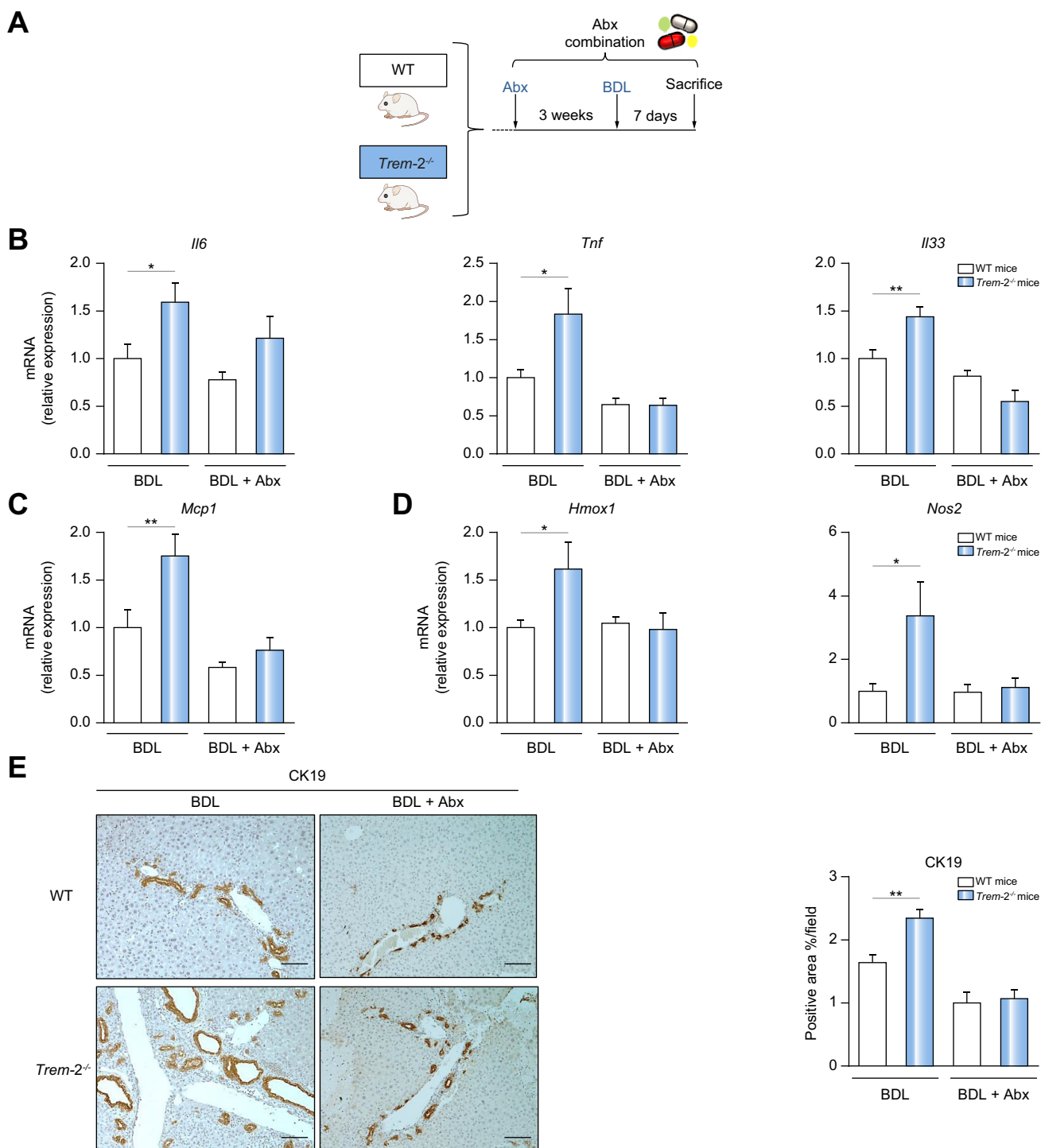
Fig. 5. Biliary expansion and inflammation after antibiotic administration and BDL in WT and *Trem-2*-/- mice. (A) Schematic representation of the experimental model (WT n = 13; *Trem-2*-/- n = 8). The groups subjected to BDL only (WT n = 9; *Trem-2*-/- n = 8) are included in the graphs for comparison. (B) Hepatic mRNA expression of *Il6*, *Tnf*, *Il33* and (C) *Mcp1*. (D) Hepatic mRNA expression of *Hmox1* and *Nos2*. (E) Representative IHC images and quantification of CK19. Scale bars (E): 100 μm. *p <0.05 and **p <0.01 in comparison to WT mice of the same experimental condition. (Student's *t* test and Mann-Whitney test). Abx, antibiotics; BDL, bile duct ligation; IHC, immunohistochemistry; WT, wild-type.

Trem-2-/- mice also exhibited increased expression (mRNA) of the pro-inflammatory cytokines *Il1β, Il6, Tnf* and *Il33* (Fig. 7A), the chemokine *Mcp1*, the oxidative stress markers *Hmox1* and *Nos2*, as well as the HSC activation marker *Acta2* (Fig. S11A-C). This was also associated with increased neutrophil recruitment and HSC

activation in these mice (Fig. 7B and Fig. S11D) while no differences regarding macrophage and lymphocyte recruitment were observed (Fig. S11E,F).

Next, we explored whether experimental TREM-2 over-expression in the liver could protect against ANIT-induced
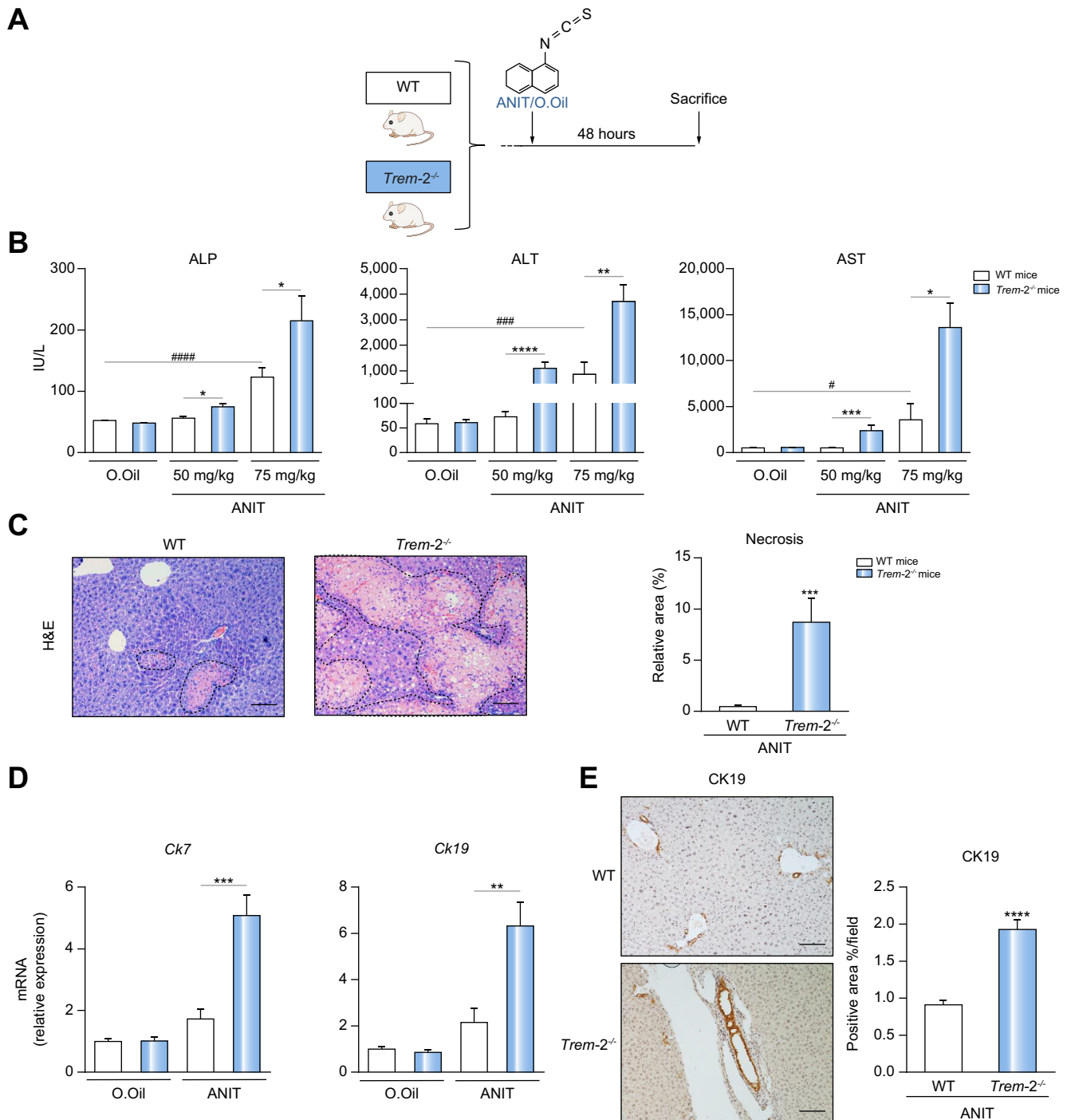
Fig. 6. Liver injury and ductular reaction in response to ANIT administration in WT and _Trem-2_^{-/-} mice. (A) Schematic representation of the experimental model (O.Oil: WT n = 8 and _Trem-2_^{-/-} n = 8; ANIT 50 mg/kg: WT n = 11 and _Trem-2_^{-/-} n = 12; ANIT 75 mg/kg: WT n = 9 and _Trem-2_^{-/-} n = 6). (B) Serum levels of liver enzymes (C) Representative H&E images and quantification of necrotic areas in mice exposed to ANIT 50 mg/kg. (D) Hepatic mRNA expression of _Ck19_ and _Ck7_. (E) Representative IHC images and quantification of CK19. Scale bars (C, E): 100 μm. *p <0.05; **p <0.01; ***p <0.001 and ****p <0.0001; #p <0.05; ###p <0.001 and ####p <0.0001 in comparison to O.Oil-administered mice of the same genotype. (Student's t test and Mann-Whitney test). ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANIT, alpha-naphtylisothiocyanate; AST, aspartate aminotransferase; IHC, immunohistochemistry; O.Oil, olive oil; WT, wild-type.

cholestasis. Thus, control or _Trem-2_ overexpressing AAVs were intravenously injected into WT mice, ANIT (75 mg/kg) was administered 72 hours later and mice were sacrificed 48 hours after ANIT administration (Fig. 7C) As expected, the injection of AAVs containing _Trem-2_ produced a marked increase of _Trem-2_ expression within the liver (Fig. 7D). Interestingly, TREM-2 overexpression

was able to selectively downregulate the expression of the pro-inflammatory cytokine _Il33_ compared to mice injected with the control AAV (Fig. 7D), whereas no differences were observed in the other aforementioned cytokines/chemokines (data not shown). Notably, a reduction in neutrophil recruitment after TREM-2 over-expression was also observed (Fig. 7E).
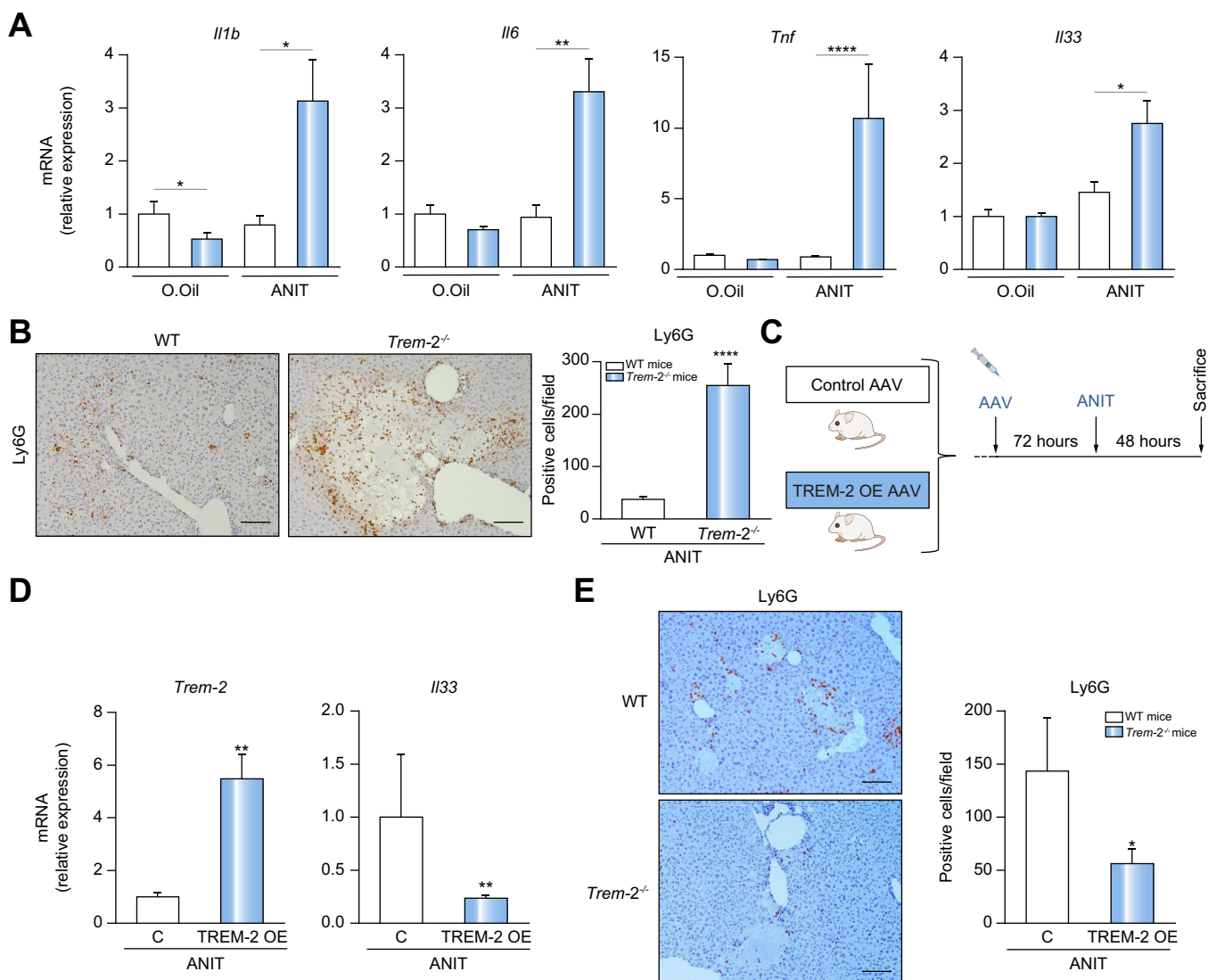
Fig. 7. Liver inflammation in WT and *Trem-2*^-/- mice, and in *Trem-2*-overexpressing WT mice, in response to ANIT administration. (A) Hepatic mRNA expression of *Il1β*, *Il6*, *Tnf* and *Il33* in ANIT-exposed mice (50 mg/kg). (B) Representative IHC images and quantification of Ly6G. (C) Schematic representation of the experimental model (control AAV n = 7; *Trem-2*-overexpressing AAV n = 9). (D) Hepatic mRNA expression of *Trem-2* and *Il33*. (E) Representative IHC images and quantification of Ly6G. Scale bars (B, E): 100 μm. *p <0.05; **p <0.01 and ****p <0.0001 ((A-D) Student's *t* test and Mann-Whitney test, (E) One-tailed Student's *t* test). AAV, adeno-associated virus; ANIT, alpha-naphtylisothiocyanate; C, control; IHC, immunohistochemistry; OE, overexpression; O.Oil, olive oil; WT, wild-type.

UDCA regulates LPS-triggered inflammatory responses in KCs via a TREM-2-dependent mechanism

There is still limited knowledge regarding the molecular mechanisms of action of UDCA in the liver, particularly in non-parenchymal liver cells.[2] Therefore, we evaluated the effect of LPS, UDCA or the combination of LPS plus UDCA on *Trem-1* and *Trem-2* expression in primary KCs isolated from WT mice. Incubation with LPS upregulated the expression of the pro-inflammatory receptor *Trem-1* while it diminished the expression of the anti-inflammatory receptor *Trem-2* in KCs (Fig. 8A). Interestingly, UDCA alone exerted the opposite effect, thereby decreasing *Trem-1* and increasing *Trem-2* expression. Importantly, UDCA counteracted the effects induced by LPS on both *Trem-1* and *Trem-2* in KCs, decreasing *Trem-1* and augmenting *Trem-2* expression compared to KCs incubated with LPS alone (Fig. 8A). Importantly, this effect was specific for UDCA, since

neither cholic acid (CA) nor taurine-conjugated forms of CA (taurocholic acid, TCA) or UDCA (tauroursodeoxycholic acid, TUDCA) impacted LPS-dependent modulation of *Trem-1* and *Trem-2* (Fig. S12). Additionally, UDCA did not impact on the modulation of Trem receptors by LPS in HSCs (Fig. S13). These data indicate that UDCA might modulate inflammatory responses specifically in KCs by regulating *Trem-1* and *Trem-2* expression. To test this hypothesis, primary KCs were isolated from WT and *Trem-2*^-/- mice, and then incubated with LPS or a combination of LPS plus UDCA, before inflammatory responses in these conditions were assessed. As previously reported, LPS strongly induced the expression of the pro-inflammatory cytokines *Il6* and *Tnf*, as well as the chemokine *Cxcl1* in KCs derived from both mouse genotypes, with more prominent effects in *Trem-2*^-/- KCs. Notably, the addition of UDCA diminished the expression of these mediators in WT KCs, whereas no effect was
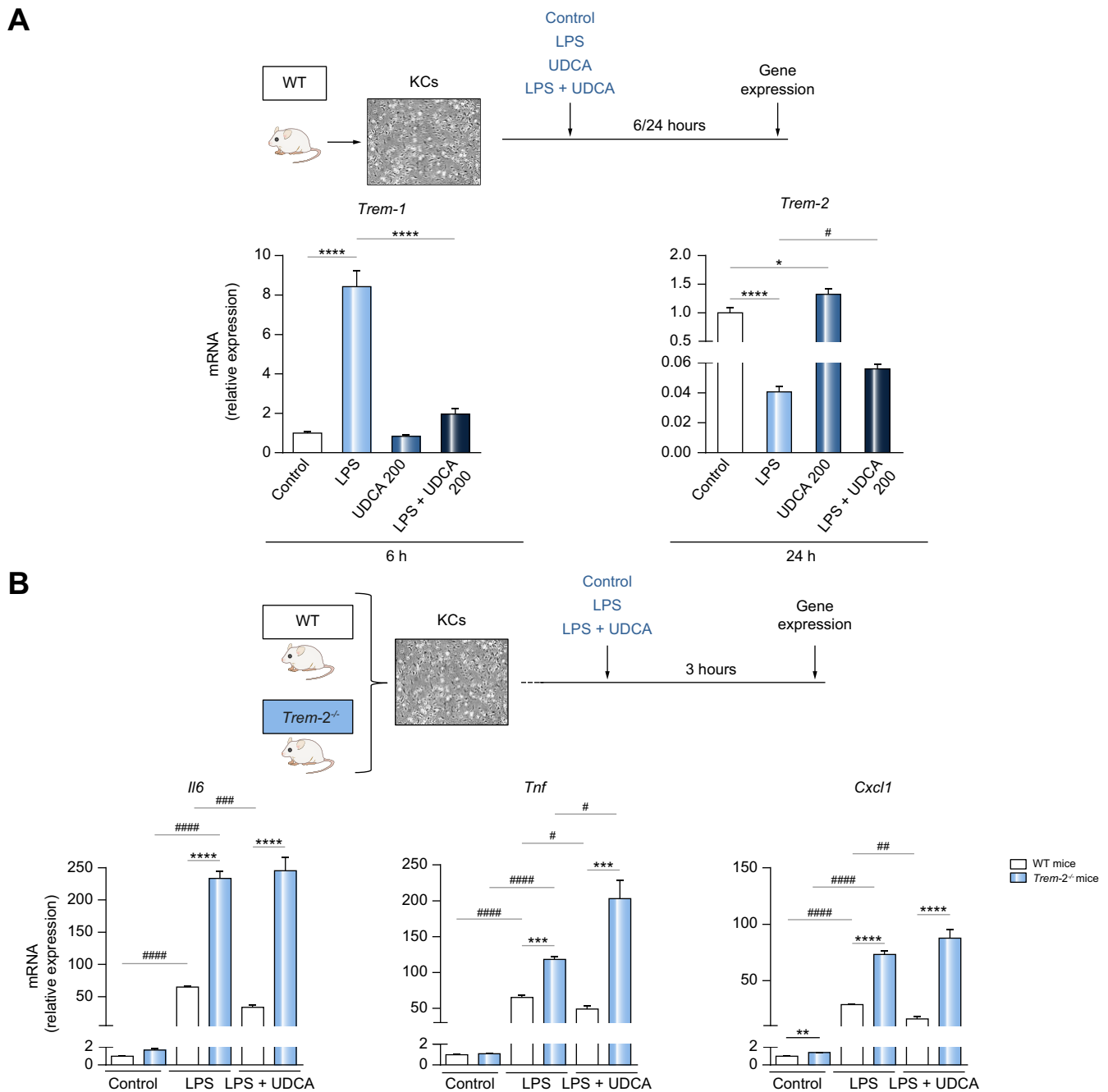
Fig. 8. Effects of UDCA alone and upon LPS incubation in primary cultured mouse KCs. (A) Schematic representation of the experimental model and *Trem-1* and *Trem*-2 mRNA levels in primary KCs (n = 4-6). (* ANOVA followed by Tukey's *post hoc* test. # When comparing LPS and LPS+UDCA groups Student's *t*-test was employed). (B) Schematic representation of the experimental model and *Il6, Tnf and Cxcl1* mRNA levels in WT and *Trem-2^-/-* primary KCs (n = 3-4). KCs were treated with LPS (100 ng/ml), UDCA (200 μM) or LPS (100 ng/ml) + UDCA (200 μM). *p <0.05; **p <0.01; ***p <0.001 and ****p <0.0001, in comparison to WT KCs following the same experimental conditions; #p <0.05; ##p <0.01; ###p <0.001 and ####p <0.0001, in comparison to control or LPS incubated KCs of the same genotype (Student's *t* test). KC, Kupffer cell; LPS, lipopolysaccharide; UDCA, ursodeoxycholic acid; WT, wild-type.

observed in *Trem-2^-/-* KCs (Fig. 8B), indicating that UDCA modulates inflammatory gene transcription in KCs via a TREM-2-dependent mechanism.

Discussion

Over the last few years, research focused on TREM-2 has unraveled its essential role in the regulation of inflammatory responses in different tissues and disease contexts.[15] In the liver, TREM-2 dampens inflammatory responses triggered by TLR activation in non-parenchymal cells, thereby exerting multifactorial protective mechanisms to defend the organ from hepatocellular injury and carcinogenesis.[16,17] Based on these observations, we hypothesized that TREM-2 may modulate inflammatory responses in cholestasis.

First, we found that TREM-2 expression is upregulated (and correlates with markers of disease progression) in the liver of patients with cholestasis (both early- and late-stage) compared to control healthy tissue and cirrhotic livers, as well as in the livers of mice subjected to different models of cholestasis. This upregulation might be a response mechanism to dampen inflammation that is maintained under the presence of chronic injury, like previous reports in other liver disease settings.[16,17] In fact, considering that *Trem-2* expression was mainly found in mouse KCs and activated HSCs, the increased levels of *TREM-2* in the liver upon cholestasis could reflect the recruitment and/or proliferation of *TREM-2*-expressing cell populations, as well as an induced expression in KCs and monocyte-derived macrophages upon activation as a compensatory mechanism to halt the inflammatory burden.[17]

In experimental models of cholestasis, *Trem-2*^{-/-} mice exhibit increased levels of cell death, particularly necroptosis, a type of cell death previously identified as an important contributor to injury in patients with PBC and in mice subjected to BDL.[24] Underlying this effect, TNF, which is found upregulated in *Trem-2*^{-/-} mice after BDL and ANIT, was reported as an important trigger of necroptosis.[25] Consequently, we hypothesize that TREM2 dampens necroptosis, potentially by impacting on TNF expression and/or secretion.

Cell death favors inflammatory responses in the liver and necroptosis is particularly regarded as an immunogenic type of cell death, favoring inflammatory responses.[25] Indeed, *Trem-2*^{-/-} mice display upregulated *Il1β*, *Il6*, *Tnf*, *Il33*, *Mcp1* and *Cxcl1* expression upon BDL and ANIT administration. Enhanced expression of inflammatory mediators in the liver of *Trem-2*^{-/-} mice is associated with increased recruitment of neutrophils to the liver, which may potentiate the inflammatory response and sustain the induction of necroptosis, thus favoring disease worsening and progression. These inflammatory mediators found upregulated in *Trem-2*^{-/-} mice are crucial in the progression of cholestatic diseases in humans and mice.[8,26] Their expression is upregulated in patients with PBC[27] and PSC[28] and they are also present in the inflammatory microenvironment derived from ductular reactive cells.[7] Additionally, peripheral monocytes derived from patients with PBC show an exacerbated reaction to LPS, featuring increased secretion of IL1β, IL6, IL8 and TNF, among others.[29] Of note, experimental upregulation of TREM-2 in the livers of WT mice selectively reduced IL33 expression and neutrophil recruitment after ANIT administration; IL33 is a pro-inflammatory cytokine that is elevated in the serum of patients with PBC[30] and that promotes cholangiocyte proliferation in mouse models of biliary injury and repair.[31] Notably, the CXCL1-CXCR2 axis, which drives neutrophil recruitment to the liver, plays a pivotal role in cholestasis, as *Cxcr2*^{-/-} mice are protected from BDL-mediated injury.[32] On the other hand, the recruitment of macrophages was not affected in *Trem-2*^{-/-} mice after cholestatic injury. Recent studies based on single-cell RNA sequencing techniques have unraveled specific TREM-2-expressing macrophage subtypes that accumulate in different contexts, including obesity, non-alcoholic fatty liver disease and cirrhosis.[33,34] Therefore, although a similar content of the total macrophages in the liver of both genotypes of mice is observed, sophisticated techniques describing immune populations in detail may unveil differences in the recruitment of specific macrophage subtypes.

BAs accumulate during cholestasis and promote epithelial cell death and inflammatory responses.[1] Interestingly, under control conditions, *Trem-2*^{-/-} mice display lower hepatic BA concentrations compared to livers of WT mice, but reached the same BA concentration upon BDL. These results are accompanied by downregulation of *Cyp7a1* in *Trem-2*^{-/-} vs. WT mice under baseline conditions. Overall, these findings suggest that *Trem-2*^{-/-} mice may have developed an adaptive mechanism to maintain low BA concentrations in their livers to mitigate the harmful effect of toxic BAs. The dramatic increase in BAs induced by BDL may overwhelm this mechanism, resulting in similar BA levels. The molecular mechanisms linking TREM-2 and BA metabolism and their potential implications in liver disease progression are still far from clear, thus posing an interesting research opportunity.

Signals derived from bacterial products that translocate from the gut to the liver are now regarded as crucial mediators of chronic cholestasis.[9] Indeed, gut-derived bacterial products sensitize hepatocytes to BA-induced injury and are needed to establish liver damage in experimental models of cholestasis.[35] In line with this, antibiotic-based gut sterilization abrogated some of the differences observed between WT and *Trem-2*^{-/-} mice after cholestasis. This is in agreement with our previous findings in the setting of carbon tetrachloride-induced liver injury, where TREM-2-mediated effects were shown to be triggered by gut-derived PAMPs.[16] By contrast, in the context of cholestasis, some parameters were not abrogated by the administration of antibiotics, suggesting TREM-2 may also modulate TLR signaling due to the binding of ligands beyond PAMPs, including danger-associated molecular patterns released from dying epithelial cells. Alternatively, TREM-2 might also exert protective effects in cholestasis by a TLR-independent mechanism. Of note, in the setting of liver regeneration after partial hepatectomy, TLRs do not play a prominent role, as *Tlr*^{-/-} mice responded similarly to WT mice after partial hepatectomy,[36] but *Trem-2*^{-/-} mice show increased expression of inflammatory mediators and hepatocyte proliferation in this setting.[17]

In this study, we report a novel mechanism by which UDCA may impact inflammatory responses in non-parenchymal liver cells. Our results suggest that UDCA is able to modulate *Trem-1* and *Trem-2* expression exclusively on KCs and this impacts on the inflammatory responses of these cells, as the addition of UDCA upon LPS incubation downregulated *Il6*, *Tnf* and *Cxcl1* gene transcription only in WT KCs, while this effect was abrogated in *Trem-2*^{-/-} KCs. Importantly, these inflammatory mediators play a prominent role in the progression of cholestatic diseases.[8,26] These results suggest that the regulation of TREM-1 and TREM-2 expression may represent a putative mechanism of UDCA anti-inflammatory action in non-parenchymal liver cells, unraveling a novel important role for UDCA in modulating inflammatory responses. In line with this, the anti-inflammatory properties of a conjugated form of UDCA (ursodeoxycholyl lysophosphatidylethanolamide or UDCA-LPE) in RAW264.7 macrophages and murine primary KCs were recently reported,[37] thus reinforcing the immunomodulatory role of UDCA in non-parenchymal cells.

In summary, cholestatic damage triggers the wound healing response, in which epithelial and non-epithelial cells cooperate in an effort to restore the lost liver parenchyma and its functions. In this context, bacterial products derived from the intestinal

compartment bind to TLRs in the liver, mainly in KCs and HSCs, upregulating inflammatory gene transcription. Persistent inflammatory mediators act in an autocrine and paracrine fashion, also promoting cholestasis. In the absence of TREM-2, the natural break to TLR-mediated signaling disappears, therefore, TLR-mediated inflammatory gene transcription in KCs and HSCs is amplified, resulting in an exacerbated response to cholestasis. Therefore, TREM-2 arises as a novel regulator of inflammatory responses in cholestasis, and its activation could represent a promising therapeutic strategy for patients with cholestasis. Moreover, some of the therapeutic benefits of UDCA supplementation in cholestasis may be mediated by the regulation of TREM-1 and TREM-2 expression in KCs.

Abbreviations

AAV, adeno-associated virus; Abx, antibiotics; Acta 2; actin alpha 2, smooth muscle; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANIT, alpha-naphtylisothiocyanate; AST, aspartate aminotransferase; BA, bile acid; BDL, bile duct ligation; CK7, cytokeratin 7; CK19, cytokeratin 19; COL1A1, collagen type 1 alpha 1; CXCL1, chemokine (C-X-C motif) ligand 1; CYP7A1, cytochrome P450 7A1; FXR, farnesoid X receptor; HCC, hepatocellular carcinoma; HMOX, heme oxygenase; HSC, hepatic stellate cell; IL, interleukin; IHC, immunohistochemistry; KC, Kupffer cell; LPS, lipopolysaccharide; MCP1, monocyte chemoattractant protein 1; MLKL, mixed lineage kinase domain like; NOS2, nitric oxide synthase 2; P/S, penicillin-streptomycin; PAMP, pathogen-associated molecular pattern; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; RIP3, receptor-interacting protein 3; TLR, toll-like receptor; TNF, tumor necrosis factor; TREM-2, triggering receptor expressed on myeloid cells 2; UDCA, ursodeoxycholic acid.

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

IL, AA-L, AE, AE-B, OS, PA, JJGM, MV, TL, M Marzioni, DAM, PMR, MJP, JMB: study concept and design, analysis and interpretation of data, drafting of the manuscript. IL, AA-L, PO, IO, MH, AE, AE-B, EH, PM, M Milkiewicz, FR-R, MJM, MV, PMR, MJP, JMB, acquisition of data. IL, AAL, AE-B, AE, PMR, MJP, JMB: statistical analysis. LB, PMR, JMB, MJP: obtained funding.

Data availability statement

The data that support the findings of this study are available from the corresponding authors upon request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.05.044.

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Author names in bold designate shared co-first authorship

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