Research Article
Cholestasis and Autoimmune Diseases

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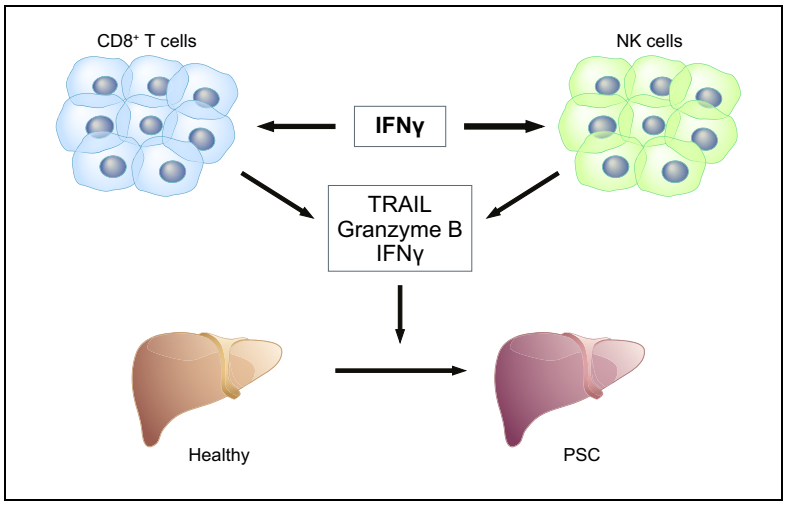
Graphical abstract



Highlights

- Patients with PSC showed increased IFNγ serum concentrations and elevated frequencies of hepatic CD56^{bright} NK cells.
- Less hepatic NK cells and CD8^{+} T cells expressing cytotoxic effector molecules after deletion of IFNγ in *Mdr2*$^{-/-}$ mice.
- Less inflammatory macrophages and more restorative macrophages after genetic deletion of IFNγ.
- Genetic deletion and blockage of IFNγ in *Mdr2*$^{-/-}$ mice attenuated liver fibrosis.

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Lay summary

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by biliary inflammation and fibrosis, whose current medical treatment is hardly effective. We observed an increased interferon (IFN)-γ response in patients with PSC and in a mouse model of sclerosing cholangitis. IFNγ changed the phenotype of hepatic CD8^{+} T lymphocytes and NK cells towards increased cytotoxicity, and its absence decreased liver cell death, reduced frequencies of inflammatory macrophages in the liver and attenuated liver fibrosis. Therefore, IFNγ-dependent immune responses may disclose checkpoints for future therapeutic intervention strategies in sclerosing cholangitis.

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Interferon-γ-dependent immune responses contribute to the pathogenesis of sclerosing cholangitis in mice

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Background and Aims: Primary sclerosing cholangitis (PSC) is an idiopathic, chronic cholestatic liver disorder characterized by biliary inflammation and fibrosis. Increased numbers of intrahepatic interferon-γ- (IFNγ) producing lymphocytes have been documented in patients with PSC, yet their functional role remains to be determined.

Methods: Liver tissue samples were collected from patients with PSC. The contribution of lymphocytes to liver pathology was assessed in *Mdr2*$^{-/-}$ *x Rag1*$^{-/-}$ mice, which lack T and B cells, and following depletion of CD90.2^{+} or natural killer (NK) p46^{+} cells in *Mdr2*$^{-/-}$ mice. Liver pathology was also determined in *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice and following anti-IFNγ antibody treatment of *Mdr2*$^{-/-}$ mice. Immune cell composition was analysed by multi-colour flow cytometry. Liver injury and fibrosis were determined by standard assays.

Results: Patients with PSC showed increased IFNγ serum levels and elevated numbers of hepatic CD56^{bright} NK cells. In *Mdr2*$^{-/-}$ mice, hepatic CD8^{+} T cells and NK cells were the primary source of IFNγ. Depletion of CD90.2^{+} cells reduced hepatic *Ifng* expression, NK cell cytotoxicity and liver injury similar to *Mdr2*$^{-/-}$ *x Rag1*$^{-/-}$ mice. Depletion of NK cells resulted in reduced CD8^{+} T cell cytotoxicity and liver fibrosis. The complete absence of IFNγ in *Mdr2*$^{-/-}$*x Ifng*$^{-/-}$ mice reduced NK cell and CD8^{+} T cell frequencies expressing the cytotoxic effector molecules granzyme B and TRAIL and prevented liver fibrosis. The antifibrotic effect of IFNγ was also observed upon antibody-dependent neutralisation in *Mdr2*$^{-/-}$ mice.

Conclusion: IFNγ changed the phenotype of hepatic CD8^{+} T cells and NK cells towards increased cytotoxicity and its absence attenuated liver fibrosis in chronic sclerosing cholangitis. Therefore, unravelling the immunopathogenesis of PSC with a particular focus on IFNγ might help to develop novel treatment options.

Lay summary: Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by biliary inflammation and fibrosis, whose current medical treatment is hardly effective. We observed an increased interferon (IFN)-γ response in patients with PSC and in a mouse model of sclerosing cholangitis. IFNγ changed the phenotype of hepatic CD8^{+} T lymphocytes and NK cells towards increased cytotoxicity, and its absence decreased liver cell death, reduced frequencies of inflammatory macrophages in the liver and attenuated liver fibrosis. Therefore, IFNγ-dependent immune responses may disclose checkpoints for future therapeutic intervention strategies in sclerosing cholangitis.

Introduction

Primary sclerosing cholangitis (PSC) is a poorly understood chronic progressive biliary disease of unknown aetiology, characterized by biliary inflammation and fibrosis, development of cholestasis, end-stage liver disease and a high risk of malignancy. Approximately 60% of patients with PSC are male and 70 to 80% have inflammatory bowel disease. Both the incidence and prevalence of PSC are increasing, indicating that current medical treatment is poorly effective.[1]

PSC has been recognized as an immune-mediated biliary disease. Genetic data provided insight into immunological loci belonging to a general pool of predisposing autoimmune risk factors, most strikingly human leukocyte antigen (HLA) associations that resemble prototypical autoimmune disorders.[2] This suggests that T cell-dependent, adaptive immune responses contribute to the immunopathogenesis of PSC. Indeed, dysregulation of apoptosis of activated CD4^{+} T cells[3] and dysfunction of regulatory T cells[4] have been observed in patients with PSC. Additionally, studies in patients with PSC showed increased T lymphocyte infiltration with a bias towards Th1 cells that

Keywords: *Mdr2* knockout; Lymphocyte cytotoxicity; NK cells; Natural killer; TRAIL; PSC; Primary sclerosing cholangitis; Fibrosis.
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localize around the bile ducts and portal tracts.[5,6] Moreover, elevated serum levels of the IFNγ-inducible chemokines CXCL9 and CXCL10 have been determined in patients with PSC,[7] and biliary epithelial cells from patients with PSC express CXCL9, CXCL10 and the IFNγ-specific transcription factors STAT1 and IRF1.[8]

Mdr2$^{-/-}$ mice, bearing a targeted disruption of the multidrug resistance gene (*Mdr2*, *Abcb4*), have been recognized as a suitable model to study inflammatory biliary disease.[9] In fact, reflecting central morphological features observed in livers of patients with PSC, biliary inflammation, ductular proliferation and onion skin type periductual fibrosis are typical signatures of liver histopathology in *Mdr2*$^{-/-}$ mice.[10] Bile duct injury upon deletion of the phospholipid flippase Mdr2 develops as a consequence of defective biliary phospholipid secretion, which results in a subsequent increase of free non-micellar and therefore potentially toxic bile acid concentrations in bile.[9,10] The murine *Mdr2* gene represents the human orthologue *MDR-3*[9,10] and mutations in *MDR-3* have been associated with biliary injury.[11] Interestingly, T effector cells seem to be involved in the pathogenesis of experimental sclerosing cholangitis in *Mdr2*$^{-/-}$ mice, since expansion of endogenous regulatory T cells have been demonstrated to diminish biliary injury and fibrosis in these animals.[12]

IFNγ has been implicated in autoimmunity[13] and a Th1 response has been associated with PSC.[5,6] However, the functional relevance has never been investigated in humans or in the *Mdr2*$^{-/-}$ mouse model so far. Thus, we aimed to study its cellular source as well as its role in liver inflammation and fibrosis in this mouse model of PSC. Our results demonstrated increased IFNγ serum levels in patients with PSC. In *Mdr2*$^{-/-}$ mice, hepatic CD8^{+} T cells and natural killer (NK) cells were the main producers of IFNγ and hepatic expression of IFNγ-inducible chemokines was enhanced. Using *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice, we observed that IFNγ changed the phenotype of hepatic CD8^{+} T cells and NK cells towards increased cytotoxicity, induced a pro-inflammatory phenotype in macrophages and aggravated liver fibrosis. The profibrotic effect of IFNγ in *Mdr2*$^{-/-}$ mice was confirmed by treatment of these animals with a neutralizing anti-IFNγ antibody. In patients with PSC, we observed enhanced frequencies of hepatic CD56^{bright} NK cells and a bias towards CD56^{bright} NK cells expressing the cytotoxic molecule TNF-related apoptosis-inducing ligand (TRAIL).

Materials and methods

Patient samples

Peripheral blood samples for BioPlex cytokine analysis were obtained from patients suffering from PSC (n = 9), primary biliary cholangitis (PBC; n = 9) and autoimmune hepatitis (AIH; n = 9) recruited at the University Medical Center Hamburg-Eppendorf. The healthy donors were enrolled in the Hamburger Gesundkohorte (n = 8). A summary of the clinical parameters of the patients is shown in Table S1. Liver tissue samples were collected from 6 patients with PSC undergoing liver transplantation at the Department of Hepatobiliary and Transplant Surgery of the UKE. A summary of the clinical parameters of these patients is shown in Table S2. As controls, liver samples from patients undergoing liver resection due to tumour metastases were used (n = 6–8; Department of General and Visceral Surgery at the Asklepios Clinic Hamburg-Barmbek; c.f. Table S2, cholestasis in the control group was defined by elevated serum levels of gamma-glutamyl transferase (GGT) and/or alkaline phosphatase (ALP); all control patients had normal serum concentrations of bilirubin (≤1.1 mg/dl)). All patients provided informed written consent according to study protocols approved by the Ärztekammer Hamburg (PV4898, PV4081, PV4780).

Mice

Mdr2$^{-/-}$ mice (C57BL/6.129P2-Abcb4^{tm1Bor}) were kindly provided by Daniel Goldenberg (Goldyne Savad Institute of Gene Therapy, Hadassah-Hebrew University Medical Centre, Jerusalem, Israel) and *Ifng*$^{-/-}$ mice (C57BL/6.129S7-(Ifng)^{tm1Ts}/J) were kindly provided by Professor Mittrücker (Hamburg, Germany). *Mdr2*$^{-/-}$ x *Rag1*$^{-/-}$ mice (C57BL/6.129S7-*Rag1*^{tm1Mom}/129P2-Abcb4^{tm1Bor}/J/J) and *Mdr2*$^{-/-}$ x *Ifng*$^{-/-}$ mice were generated by crossbreeding of homozygous specimen of the single knockouts. Successful knockout was confirmed via PCR analysis of DNA isolated from tail biopsies. All mice received human care according to the guidelines of the National Institutes of Health and to the legal requirements in Germany. Mouse experiments were conducted according to the German animal protection law and approved by the institutional review board (Behörde für Gesundheit und Verbraucherschutz, Hamburg, Germany; G93/16) and conformed to the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines). Mice were housed in IVC cages under controlled conditions (22 °C, 55% humidity, and 12 h day-night rhythm) and fed a standard laboratory chow (LASvendi, Altromin, Germany).

Animal treatment

Depletion experiments were performed in 10-week-old male and female *Mdr2*$^{-/-}$ mice by injecting the respective antibodies (Ab) or isotype controls intraperitoneally. *Mdr2*$^{-/-}$ mice were treated with an anti-IFNγ Ab (R4-6A2)/ *InVivo*Mab rat IgG1 (HRPN; both BioXCell, Köln, Germany; both 0.5 mg/mouse) or anti-Thy1.2 Ab (30H12)/*InVivo*Mab rat IgG2b (LTF-2; both BioXCell; both 0.25 mg/mouse) or anti-asialo GM1-Ab/rabbit serum (both Wako Chemicals GmbH, Neuss, Germany; 25 µl/mouse) twice a week for 2 weeks. At the age of 12 weeks, mice were sacrificed and analysed.

Determination of liver injury

Liver/biliary injury was quantified by determination of plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALP, and GGT as well as by plasma bilirubin concentrations as previously described.[14]

Immunohistochemistry

Hematoxylin & eosin and Sirius red staining were performed as described previously.[14,15] Inflammatory activity was scored according to the modified hepatitis activity index (mHAI score;[16]).

Hydroxyproline assay

The content of hydroxyproline, a component of collagen, was measured by a spectrophotometric assay as previously described.[17]

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (*In Situ* Cell Death Detection Kit, TMR Red, Sigma-Aldrich) was performed on paraformaldehyde-fixed liver sections according to manufacturer's protocol.

Processing of human liver tissue

Liver tissue of PSC and control patients was cut into small pieces and homogenized using a gentleMACS Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently, homogenized liver tissue was filtered through different filters (500 μm – 40 μm, Greiner Bio-One, Frickenhausen, Germany) to get single cell suspensions. The lymphocytes were finally isolated by Optiprep (Sigma-Aldrich) density gradient centrifugation. Cells were stored in 90% FBS + 10% DMSO freezing medium at −196 °C.

Isolation and re-stimulation of murine hepatic non-parenchymal cells

Hepatic non-parenchymal cells (NPCs) were isolated by Percoll density gradient centrifugation. Single cell suspensions (100,000 cells/well) were re-stimulated with phorbol-12-myristate-13-acetate (50 ng/ml) and ionomycin (1 μg/ml) for 4–5 h at 37 °C. Culture supernatants of re-stimulated NPCs were collected and stored at –20 °C. For analysis of intracellular cytokine expression, brefeldin A (50 ng/ml) and monensin (1 μg/ml) were added after 30 min. For evaluation of NK cell cytotoxicity, an anti-CD107a Ab (ID4B; FITC; Biolegend, San Diego, CA) was added to the re-stimulation medium.

Determination of cytokine levels

Cytokine levels in culture supernatants of re-stimulated murine NPCs were quantified using LEGENDplex (BioLegend, San Diego, CA) according to manufacturer's instruction. IFNγ concentrations in plasma samples of patients and healthy controls were measured using a Bio-Plex Pro Human Cytokine Kit, a Bio-Plex 200 analyser, and Bio-Plex Data Analysis Software (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instruction. Values were defined as zero if the IFNγ concentration was below the detection limit (15 pg/ml) of the Bio-Plex assay.

Flow cytometry

Cells were incubated with anti-CD16/32 Ab (clone 93; BioLegend) prior to antibody staining in order to prevent unspecific binding. LIVE/DEAD Fixable Staining Kit (ThermoFisher Scientific, Waltham, MA) was used to exclude dead cells. For cell surface analysis, cells were stained with antibodies listed in Table S3. For intracellular staining, cells were fixed using the Transcription Factor Staining Buffer Set (ThermoFisher Scientific) and incubated in permeabilization buffer with antibodies listed in Table S4.

Quantitative real-time PCR analysis

Total RNA was isolated from shock-frozen liver tissue using the NucleoSpin RNA Kit (Machery-Nagel, Duren, Germany) according to the manufacturer's instruction. Primers were obtained from Metabion (Martinsried, Germany). Sequences of the primers are listed in Table S5.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 software (GraphPad software, San Diego, CA). All data are presented as mean ± SEM. For comparisons between 2 groups, a non-parametric Mann-Whitney *U* test and for more than 2 groups, a one-way ANOVA with Tukey's *post hoc* test were used. A p value of less than 0.05 was considered statistically significant with the following ranges $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

Results

Quantification of IFNγ-producing T cells and NK cells in *Mdr2*$^{-/-}$ mice

Mdr2$^{-/-}$ mice on the C57Bl/6 background[18] develop chronic biliary inflammation and fibrosis within 12 weeks.[14,19] Compared to age-matched C57Bl/6 control mice, *Mdr2*$^{-/-}$ mice showed significantly elevated plasma ALT, AST, and ALP levels whereas bilirubin was not altered. Moreover, liver inflammation, assessed by the mHAI score, and markers of fibrosis including hydroxyproline liver tissue concentrations, hepatic *Col3a1* (collagen type III alpha 1 chain) gene expression, and degree of Sirius red staining were strongly increased in *Mdr2*$^{-/-}$ mice (Fig. S1).

Since the major IFNγ-producing cells are T lymphocytes and NK cells, we analysed IFNγ-production by liver lymphocyte populations isolated from 12-week-old *Mdr2*$^{-/-}$ and C57Bl/6 control mice using flow cytometry. As shown in Fig. 1A and B, the main producers of IFNγ were CD8^{+} T cells and NK cells. Significantly increased amounts of IFNγ production by lymphocytes from *Mdr2*$^{-/-}$ mice compared to WT mice were particularly observed in CD8^{+} T cells (Fig. 1B). We further performed quantitative RT-PCR analysis of liver tissue gene expression levels of the IFNγ-inducible chemokines *Cxcl9* and *Cxcl10*, which were significantly increased in *Mdr2*$^{-/-}$ mice compared to C57Bl/6 control animals (Fig. 1C). Interestingly, analysis of IFNγ serum levels showed increased serum concentrations of IFNγ in patients with PSC compared to healthy controls (Fig. 1D). We further demonstrated elevated serum IFNγ levels in patients with other chronic liver diseases such as PBC and AIH (Fig. S2).

T cell depletion reduced IFNγ production as well as liver injury in *Mdr2*$^{-/-}$ mice

Since liver CD8^{+} T cells produced increased amounts of IFNγ and expression levels of chemokines necessary for T cell and NK cell recruitment were significantly increased in livers of *Mdr2*$^{-/-}$ mice, we measured their accumulation in liver tissue of *Mdr2*$^{-/-}$ *vs.* control animals. As shown in Fig. 2A, numbers of TCRβ^{+} T cells, CD8^{+} T cells, NKT cells, and NK cells (gating strategies: Fig. S3A) were significantly enhanced in livers of *Mdr2*$^{-/-}$ mice compared to C57Bl/6 control animals. In a first attempt, we aimed at analysing the contribution of all IFNγ-producing lymphocytes, *i.e.* T cells and NK cells, to liver pathophysiology in *Mdr2*$^{-/-}$ mice by *in vivo* depletion of these cells. In C57Bl/6 mice, most lymphocytes express CD90.2 on their surface. Treatment of *Mdr2*$^{-/-}$ mice with a CD90.2 depletion antibody (anti-Thy1.2) twice a week for 2 weeks, resulted in almost complete depletion of CD4^{+} T cells, CD8^{+} T cells and NKT cells in the liver (Fig. 2B). In contrast, hepatic γδ T cells, which produced less IFNγ in *Mdr2*$^{-/-}$ compared to control mice (Fig. 1B) and which represented only a minority of liver T cells in *Mdr2*$^{-/-}$ mice (Fig. 2A), and NK cells were hardly affected by the depletion antibody (Fig. 2B). T cell depletion resulted in a significant reduction of *Ifng* mRNA expression in liver tissue (Fig. 2C). Moreover, IFNγ secretion by *ex vivo* re-stimulated residual liver leucocytes, including monocytes/macrophages, neutrophils and NK cells, was attenuated (Fig. 2D). Besides attenuation of IFNγ secretion, these cells produced significantly reduced amounts of the pro-inflammatory cytokines TNFα, IL-17 and IL-2 (Fig. S3B). Interestingly, liver NK cells still produced IFNγ but seemed to be less cytotoxic in the absence of T cells, as determined by significantly decreased numbers of NKp46^{+} NK cells

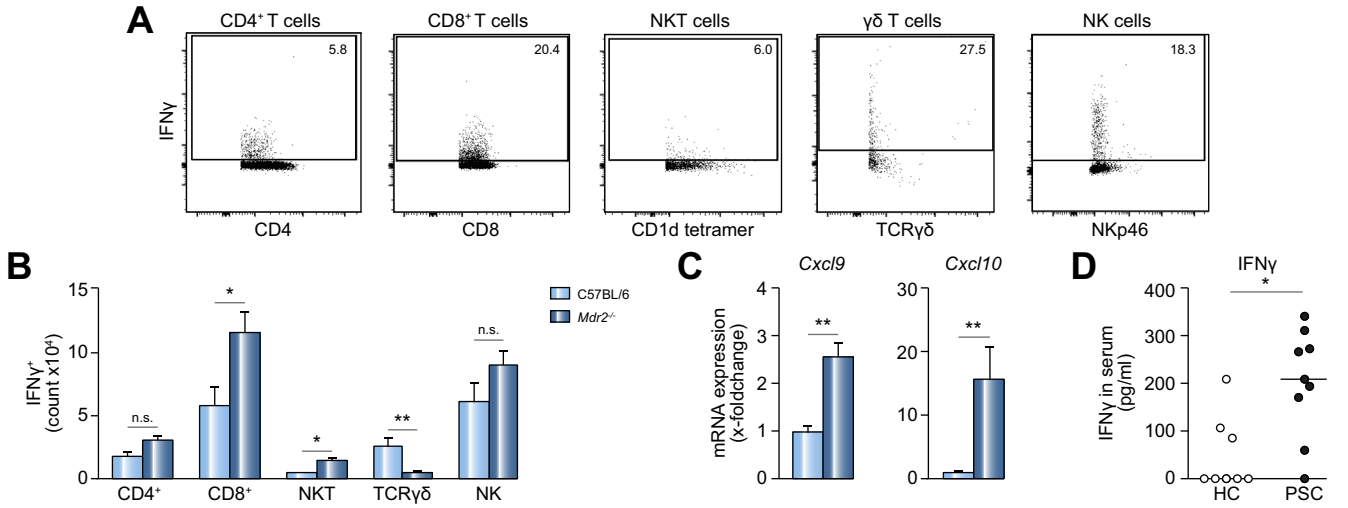

Fig. 1. IFNγ production of murine liver lymphocytes and IFNγ serum concentrations in patients with PSC. (A,B) NPCs of 12-week-old *Mdr2*$^{-/-}$ mice and C57Bl/6 controls were re-stimulated with PMA/ionomycin and expression of IFNγ was analysed by flow cytometry. (C) Hepatic gene expression levels of the IFNγ-inducible chemokines *Cxcl9* and *Cxcl10* in *Mdr2*$^{-/-}$ mice were analysed by RT-PCR and normalized to mRNA expression of C57Bl/6 mice. (D) IFNγ serum concentrations in patients with PSC and HCs were measured by a Bio-Plex Pro Human Cytokine Kit. Data: mean values ± SEM, n = 5 (mice). For statistical analysis, the non-parametric Mann-Whitney *U* test was used; n.s.: not significant, **p* ≤0.05, ***p* ≤0.01. HCs, healthy controls; NPC, non-parenchymal cells; PSC, primary sclerosing cholangitis: RT-PCR, quantitative real-time PCR.

expressing the degranulation marker CD107a (Fig. 2E). Serum ALT activities were also significantly decreased following T cell depletion (Fig. 2F) while we did not observe differences in ALP and bilirubin levels (data not shown). Liver inflammation, assessed by the mHAI score, tended to be reduced (Fig. 2G). However, development of fibrosis remained largely unaffected (Fig. 2H-J). Accordingly, serum ALT activities were significantly reduced in *Mdr2*$^{-/-}$ *x Rag1*$^{-/-}$ mice, which lack T and B cells as a result of the *Rag1* deletion, compared to *Mdr2*$^{-/-}$ mice, while the appearance of fibrosis remained unaffected (Fig. 2K and L).

Frequencies of hepatic NK cells and T cells in patients with PSC

Since we determined elevated frequencies of hepatic NK cells and CD8$^+$ T cells in *Mdr2*$^{-/-}$ mice that might play a role in the pathogenesis of PSC, we further analysed T cell and NK cell frequencies in livers of patients with PSC compared to control patients undergoing liver resection due to tumour metastases. As shown in Fig. 3, while frequencies of CD3$^+$, CD4$^+$ and CD8$^+$ T cells were unaltered (Fig. 3A), frequencies of hepatic NK cells, in particular those of CD56^{bright} NK cells, which are supposed to represent a liver-resident NK cell population,[20] were significantly increased in livers of patients with PSC. Moreover, we observed a bias towards increased frequencies of CD56^{bright} NK cells expressing the pro-apoptotic molecule TRAIL in livers of patients with PSC (Fig. 6B; gating strategy: Fig. S4). In the control group, 3 of 6 patients had cholestasis assessed by elevated levels of ALP and GGT (Table S2). However, the frequencies of NK cell populations did not correlate with cholestasis in these patients (data not shown).

Depletion of NK cells in *Mdr2*$^{-/-}$ mice reduced cytotoxicity of CD8$^+$ T cells and exerted an antifibrotic effect

Since both, NK cells and CD8$^+$ T cells were enhanced in livers of *Mdr2*$^{-/-}$ mice and depletion of CD8$^+$ lymphocytes in juvenile *Mdr2*$^{-/-}$ mice has recently been reported to exert hepatoprotective effects,[12] we depleted NK cells in 10-week-old *Mdr2*$^{-/-}$ mice using the NKp46-specific anti-asialo GM1 antibody and analysed disease pathology of the animals at an age of 12 weeks. As shown in Fig. 4A, NK cell depletion was efficient and spared the T cell subpopulations. NK cell depletion resulted in significantly reduced IFNγ-production and cytotoxicity of CD8$^+$ T cells, assessed by expression of the cytotoxic effector molecule granzyme B (GzmB) and TRAIL (Fig. 4B). Lack of NK cells did not affect liver injury and inflammation (Fig. 4C, D) but interestingly, impaired development of fibrosis (Fig. 4E-G).

Genetic ablation of *Ifng* in *Mdr2*$^{-/-}$ mice reduced cytotoxicity of CD8$^+$ T cells and NK cells and exerted an antifibrotic effect

In order to determine the functional role of IFNγ for liver injury in *Mdr2*$^{-/-}$ mice, we generated *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ double knockout mice. As expected, IFNγ was not detectable in T cells and NK cells isolated from livers of *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice (Fig. 5A). Hepatic gene expression of the IFNγ-inducible chemokines *Cxcl9* and *Cxcl10* was significantly decreased in *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice compared to *Mdr2*$^{-/-}$ mice (Fig. 5B). Accordingly, recruitment of T cells and NK cells to the liver was significantly reduced in *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice (Fig. 5C). Interestingly, IFNγ affected the phenotype of hepatic CD8$^+$ T cells and NK cells in *Mdr2*$^{-/-}$ mice. We observed increased frequencies of CD8$^+$ T cells expressing GzmB in *Mdr2*$^{-/-}$ mice compared to C57Bl/6 controls (Fig. 5D). The frequencies of both GzmB$^+$ CD8$^+$ T cells and GzmB$^+$ NK cells were significantly reduced in *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice. Moreover, frequencies of CD8$^+$ T cells and NK cells expressing TRAIL were significantly increased in *Mdr2*$^{-/-}$ mice compared to C57Bl/6 controls and TRAIL$^+$ CD8$^+$ T cells and TRAIL$^+$ NK cells were significantly reduced in *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice compared to *Mdr2*$^{-/-}$ mice (Fig. 5D).

In order to examine other possible IFNγ-induced inflammatory responses, we analysed the accumulation of pro-inflammatory macrophages in livers of *Mdr2*$^{-/-}$ mice, which is critical for liver injury and fibrosis in mouse models of sclerosing cholangitis as recently described.[21] We observed a signifi-

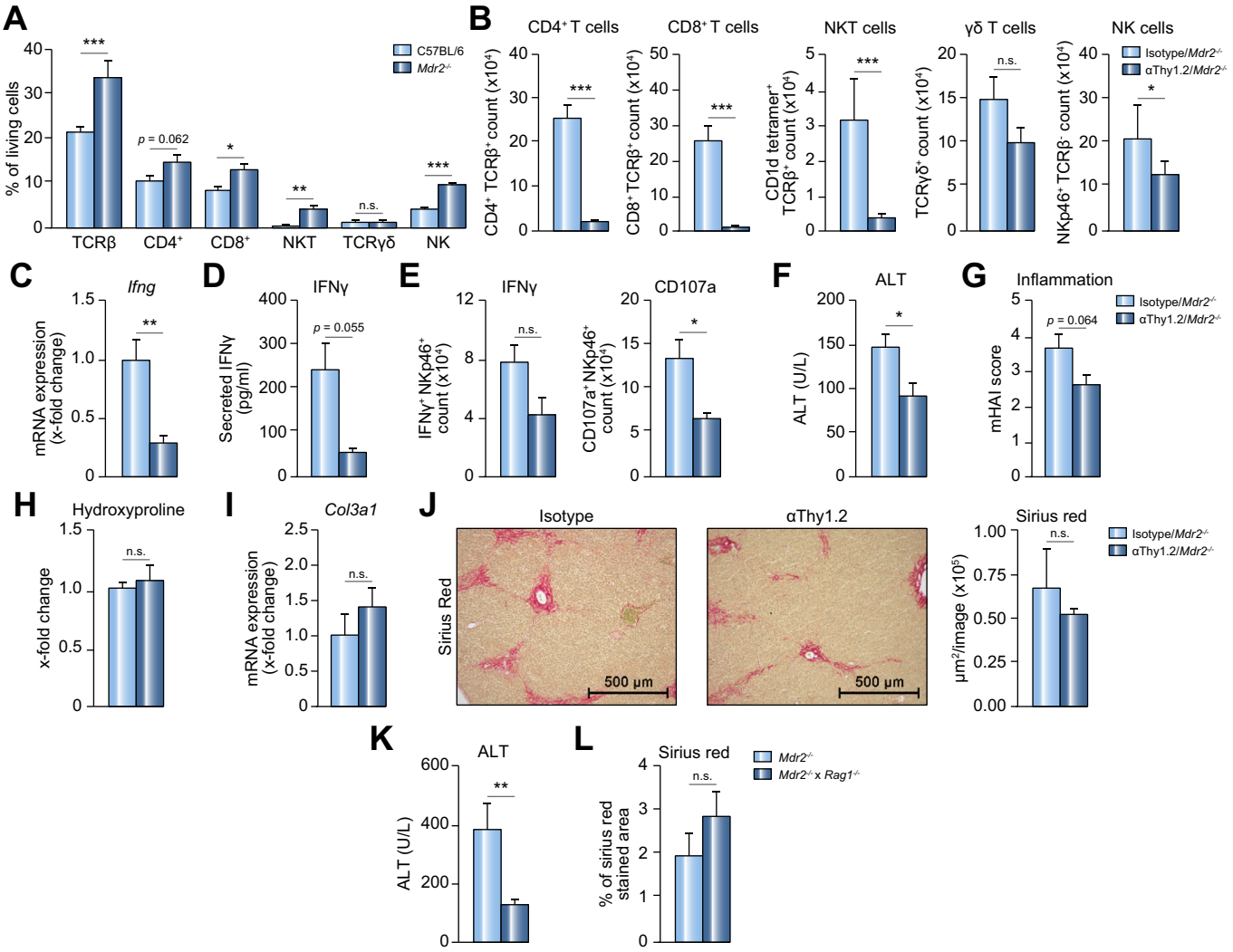

Fig. 2. T cell depletion reduced IFNγ production and liver inflammation in *Mdr2*$^{-/-}$ mice. (A) T cell and NK cell frequencies in livers of 12-week-old C57Bl/6 and *Mdr2*$^{-/-}$ mice were analysed by flow cytometry. (B) 10-week-old *Mdr2*$^{-/-}$ mice were treated with 0.25 mg anti-Thy1.2 mAb per mouse or the respective isotype control twice a week for 2 weeks. Hepatic lymphocyte depletion was analysed by flow cytometry. (C) *Ifng* mRNA expression was detected in liver tissue of antibody-treated *Mdr2*$^{-/-}$ mice and normalized to isotype-treated controls. (D) IFNγ release of re-stimulated NPCs was detected by ELISA. (E) Frequencies of IFNγ-producing and CD107a^{+} NK cells were analysed by flow cytometry. (F) ALT activities were detected in serum. (G) Liver inflammation was calculated using the mHAI score. (H) Hydroxyproline concentrations in liver tissue were determined and normalized to isotype-treated mice. (I) *Col3a1* expression was determined in liver tissue and normalized to isotype-treated mice. (J) Sirius red staining was quantified in liver slices. (K) Determination of serum ALT activities and (L) quantification of Sirius red staining was performed in *Mdr2*$^{-/-}$ *vs.* *Mdr2*$^{-/-}$ x *Rag1*$^{-/-}$ mice. Data: mean values ± SEM, n = 5 – 8. For statistical analysis, the non-parametric Mann-Whitney *U* test was used; n.s.: not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. ALT, alanine aminotransferase; mAb, monoclonal antibody; mHAI, modified hepatitis activity index; NK, natural killer; NPCs, non-parenchymal cells. (This figure appears in colour on the web.)

cant increase of infiltrating CD11b^{+}CCR2^{+} monocyte-derived macrophages co-expressing CX_3CR1[22] in *Mdr2*$^{-/-}$ mice compared to C57Bl/6 mice, and a significant decrease of restorative CD11b^{+}CX_3CR1^{+} macrophages, which do not express CCR2 (Fig. 5E, gating strategy: Fig. S5). Accordingly, hepatic expression of the CCR2^{+} cell recruiting chemokine *Ccl2* was significantly increased in *Mdr2*$^{-/-}$ *vs.* C57Bl/6 mice (Fig. 5F). Importantly, *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice showed a tendency towards lower hepatic expression levels of *Ccl2* and reduced frequencies of inflammatory CD11b^{+}CCR2^{+} and CD11b^{+}CX_3CR1^{+}CCR2^{+} monocyte-derived macrophages but significantly increased frequencies of restorative CD11b^{+}CX_3CR1^{+} macrophages compared to *Mdr2*$^{-/-}$ mice (Fig. 5E,F), indicating reduced liver inflammation in *Mdr2*$^{-/-}$ mice in the absence of IFNγ.

As we observed lower frequencies of cytotoxic CD8^{+} T and NK cells in livers of *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice compared to *Mdr2*$^{-/-}$ mice, we analysed liver cell death using TUNEL staining. As shown in Fig. 6A, TUNEL-positive hepatocytes were almost absent in *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice compared to massive cell death observed in livers of *Mdr2*$^{-/-}$ mice. However, despite the virtual absence of TUNEL^{+} liver cells, serum ALT activities were not affected in *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ mice compared to *Mdr2*$^{-/-}$ mice, although serum AST levels were significantly reduced (Fig. 6B). In addition, despite reduced frequencies of inflammatory monocyte-derived macrophages in the absence of IFNγ, liver inflammation as detected by the mHAI score (Fig. 6C) was not affected. However, we observed a significant reduction of liver fibrosis in the absence of IFNγ, as demonstrated by significantly reduced fibrosis markers, including hydroxyproline tissue concentrations (Fig. 6D), degree of Sirius red staining (Fig. 6E) and *Col3a1* gene expression (Fig. 6F). Since IFNγ was described to exert either pro-[23] or antifibrotic[24] effects, we per-

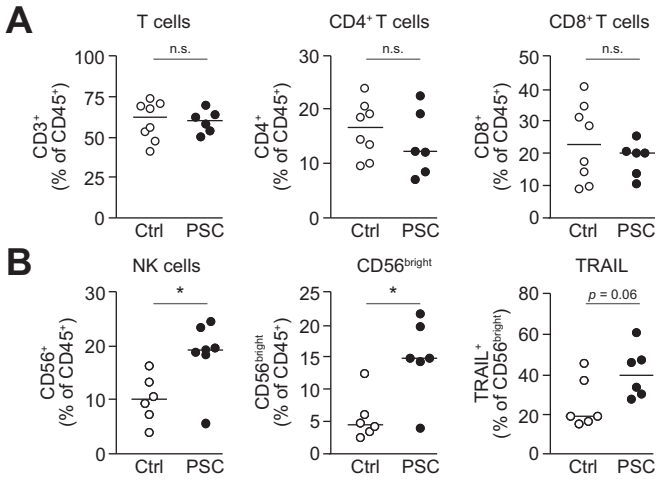

Fig. 3. T cell and NK cell frequencies in livers of patients with PSC. (A,B) Liver lymphocytes of patients with PSC undergoing liver transplantation were analysed by flow cytometry. Ctrl: control patients undergoing liver resection due to tumour metastases. Data: mean values ± SEM, n = 6–8. For statistical analysis, the non-parametric Mann-Whitney *U* test was used; n.s.: not significant, $^{*}p \leq 0.05$. NK, natural killer; PSC, primary sclerosing cholangitis.

formed additional experiments using an appropriate IFNγ-neutralizing antibody. As shown in Fig. 6G, administration of the anti-IFNγ antibody twice a week for 2 weeks to *Mdr2*$^{-/-}$ mice also decreased fibrogenesis in these animals. Hence, our data strongly indicate that IFNγ has a profibrotic effect in *Mdr2*$^{-/-}$ mice thereby driving disease pathology.

Discussion

In this report, we analysed the role of IFNγ in chronic biliary disease. We observed increased IFNγ serum concentrations in patients with PSC in a concentration range similar to the significantly elevated IFNγ levels detected in patients with PBC and AIH, corroborating previous results demonstrating a Th1-type immune response in PBC and AIH.[7] In addition, we recently determined significantly increased serum concentrations of the IFNγ-inducible chemokines CXCL10 and CXCL11 in patients with PSC.[25] This finding underscores previous findings by others, who detected increased accumulation of IFNγ-producing T cells and IFNγ-inducible downstream molecules in livers of patients with PSC.[5,8] Since the functional role of IFNγ on the immunopathogenesis of PSC has not been investigated so far, we used the well-described *Mdr2*$^{-/-}$ mouse model of chronic biliary disease resembling PSC.[9,10] Biliary disease in these animals has previously been thought to depend on the accumulation of toxic bile acids,[10] however, emerging evidence also argues for a role of immune cells, particularly IL-17-producing T cell subsets, in mediating liver injury and fibrosis in *Mdr2*$^{-/-}$ mice.[26,27] Here we observed that CD8^{+} T cells and NKT cells produced significantly increased amounts of IFNγ in *Mdr2*$^{-/-}$ mice compared to C57Bl/6 controls, however, CD8^{+} T cells and NK cells showed the highest frequencies of IFNγ-producing cells. TCRβ^{+} T cells significantly increased in livers of *Mdr2*$^{-/-}$ mice compared to C57Bl/6 controls, and the most obvious and significant increase was again observed in the CD8^{+} T cell and NK cell subpopulations, however, CD4^{+} T cells and NKT cells also produced IFNγ. In order to investigate

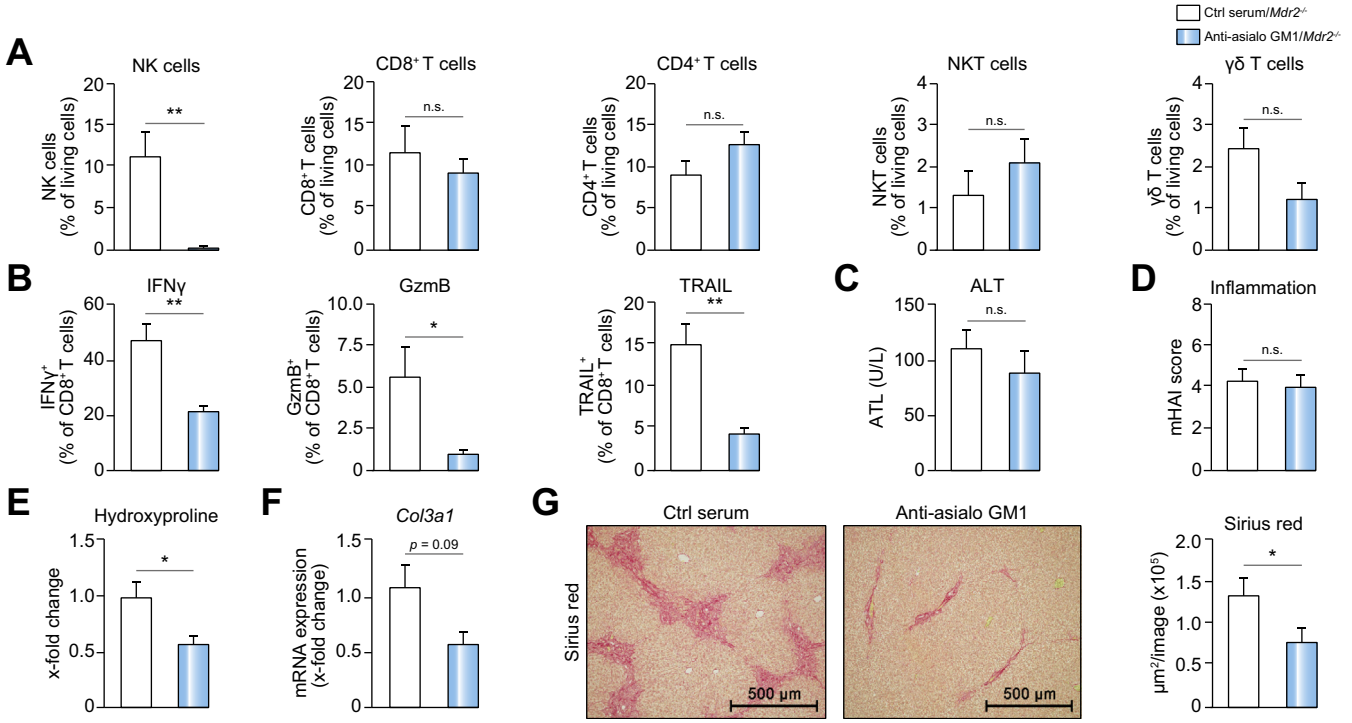

Fig. 4. NK cell depletion reduced CD8^{+} T cell cytotoxicity and provided an antifibrotic effect in *Mdr2*$^{-/-}$ mice. (A) 10-week-old *Mdr2*$^{-/-}$ mice were treated with 25 µl anti-asialo GM1 antibody per mouse or the respective control (ctrl) serum twice a week for 2 weeks. Hepatic lymphocyte depletion was analysed by flow cytometry. (B) Hepatic frequencies of IFNγ^{+}, GzmB^{+} and TRAIL^{+} CD8^{+} T cells were determined. (C) ALT activities were measured in serum. (D) Liver inflammation was calculated using the mHAI score. (E). Hydroxyproline concentrations in liver tissue were determined and normalized to ctrl serum-treated mice. (F) *Col3a1* mRNA expression was determined in liver tissue and normalized to ctrl serum-treated mice. (G) Sirius red staining was quantified in liver slices. Data: mean values ± SEM, n = 5. For statistical analysis, the non-parametric Mann-Whitney *U* test was used; n.s.: not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. ALT, alanine aminotransferase; mHAI, modified hepatitis activity index; NK, natural killer. (This figure appears in colour on the web.)

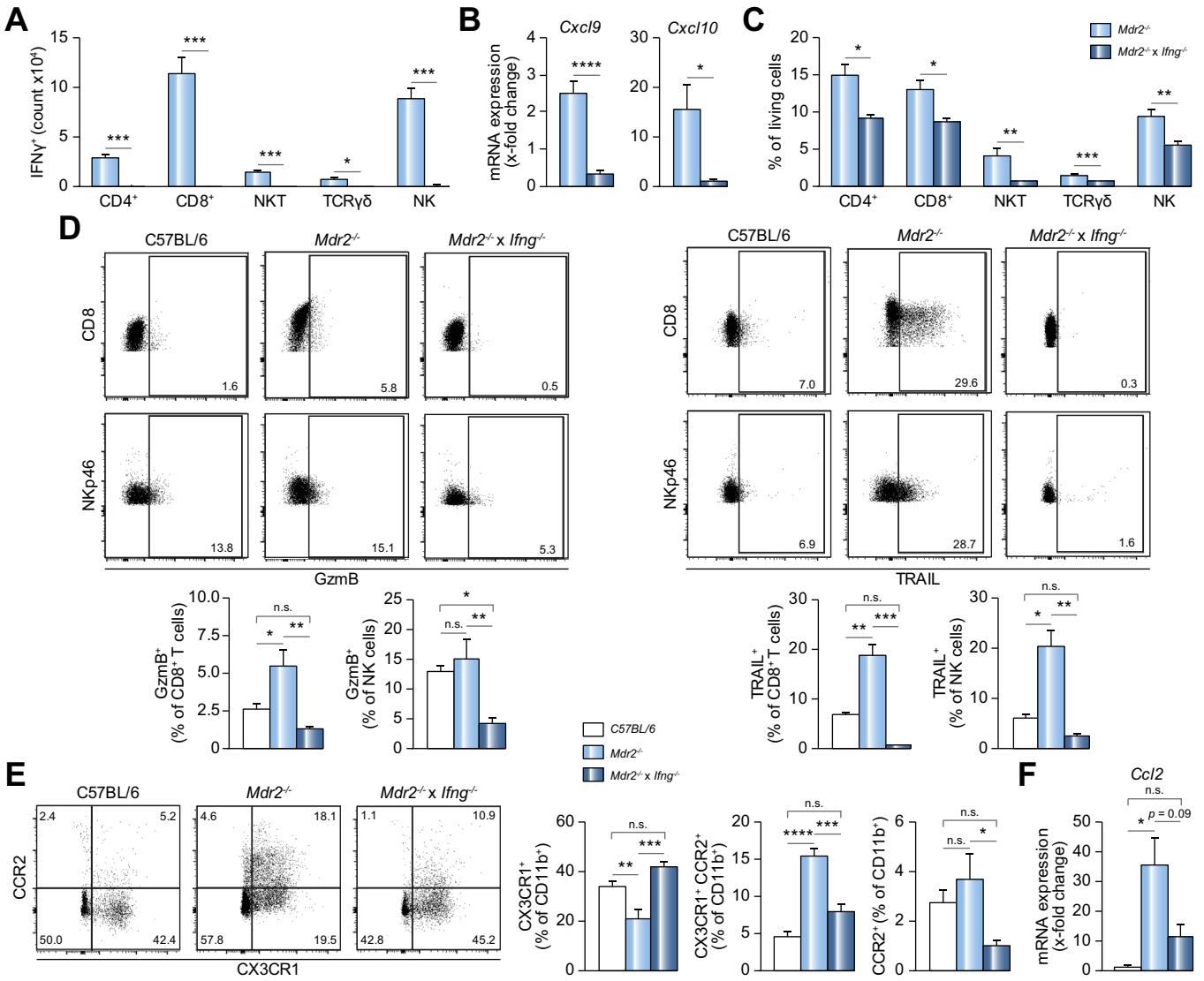

Fig. 5. Genetic ablation of IFNγ in *Mdr2*$^{-/-}$ mice reduced CD8$^+$ T cell and NK cell cytotoxicity and shifted the monocyte/macrophage phenotype towards an anti-inflammatory phenotype. (A-E) 12-week-old *Mdr2*$^{-/-}$, *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ and C57Bl/6 mice were analysed: (A) Total cell numbers of IFNγ$^+$ liver lymphocytes were analysed by flow cytometry. (B) Gene expression levels of *Cxcl9* and *Cxcl10* were measured in liver tissue and normalized to C57Bl/6 mice. (C) Frequencies of liver T cell subtypes and NK cells were determined by flow cytometry. (D) Frequencies of GzmB$^+$ and TRAIL$^+$ CD8$^+$ T cells and NKp46$^+$ NK cells, as well as (E) frequencies of CCR2$^+$ and/or CX$_3$CR1$^+$CD11b$^+$ monocyte-derived macrophages were analysed by flow cytometry. (F) Expression of *Ccl2* mRNA was analysed in liver tissue and normalized to C57Bl/6 mice. Data: mean values ± SEM, n = 5. For comparisons between 2 groups, a non-parametric Mann-Whitney *U* test and for more than 2 groups, a one-way ANOVA with Tukey's *post hoc* test was used; n.s.: not significant, $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. NK, natural killer.

whether these cells contribute to liver injury in *Mdr2*$^{-/-}$ mice, we depleted CD90.2$^+$ cells using an anti-CD90.2 antibody. CD90.2 is primarily expressed by T cells. Our results demonstrated that in contrast to γδ T cells, CD4$^+$ T cells, CD8$^+$ T cells and NKT cells were largely depleted by the antibody while frequencies of NK cells were reduced but still detectable. CD90.2$^+$ cell depletion resulted in a significant decrease of hepatic *Ifng* expression, however, the remaining NK cells still produced IFNγ. Interestingly, lower frequencies of NK cells expressing the degranulation marker CD107a were detectable, which might be a result of reduced production of IL-2 and other pro-inflammatory cytokines, which induce cytotoxicity of NK cells.[28] As a consequence, serum ALT activities were significantly reduced following CD90.2$^+$ cell depletion. The mHAI score showed only a tendency towards reduced inflammation, however, this score is less sensitive than quantitative determination of pro-inflammatory cytokines, which were significantly reduced in antibody-treated mice. In contrast, development of liver fibrosis was not affected by the absence of T cells. Similar effects were observed in *Mdr2*$^{-/-}$ *x Rag*$^{-/-}$ mice, which lack T and B cells, but not NK cells. These results indicate that T cells are the primary producers of pro-inflammatory cytokines such as IL-2, IFNγ, IL-17 and TNFα, thereby mediating liver injury in *Mdr2*$^{-/-}$ mice. However, the residual production of IFNγ by NK cells might have contributed to liver fibrosis, which was neither reduced in *Mdr2*$^{-/-}$ x *Rag1*$^{-/-}$ mice nor following CD90.2$^+$ cell depletion. Indeed, a profibrotic effect of IFNγ has previously been described in experimental steatohepatitis.[23]

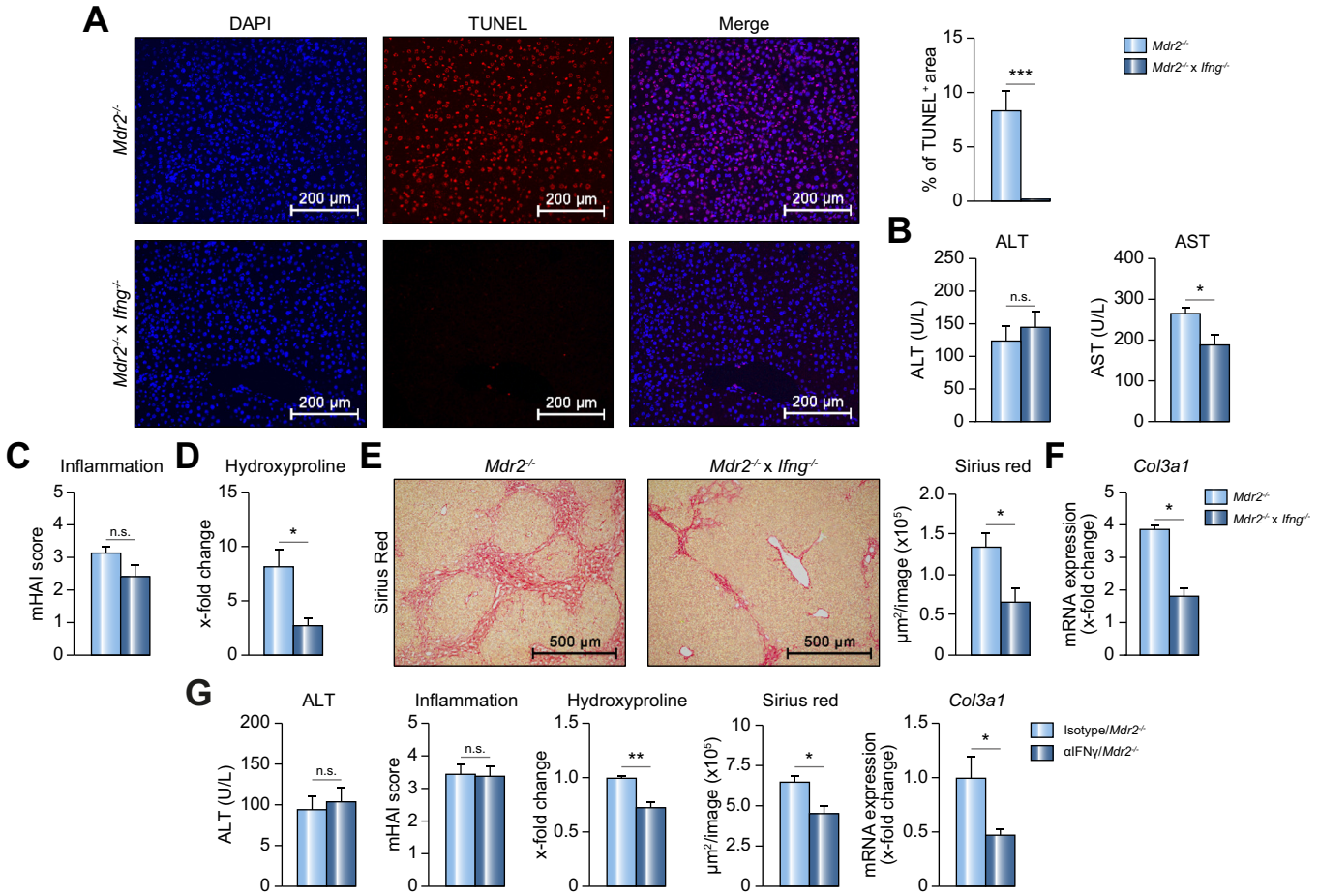

Fig. 6. Genetic ablation of IFNγ in *Mdr2*$^{-/-}$ mice exerted an antifibrotic effect. (A-G) 12-week-old *Mdr2*$^{-/-}$, *Mdr2*$^{-/-}$ *x Ifng*$^{-/-}$ and C57Bl/6 mice were analysed: Liver cell death was detected by TUNEL staining and co-staining of DAPI-positive cell nuclei. (A) TUNEL staining was quantified in liver slices. (B) ALT and AST activities were measured in serum. (C) Liver inflammation was calculated using the mHAI score. (D) Hydroxyproline concentrations in liver tissue were determined and normalized to C57Bl/6 mice. (E) Sirius red staining was quantified in liver slices. (F) *Col3a1* expression was determined in liver tissue and normalized to C57Bl/6 mice. (G) 10-week-old *Mdr2*$^{-/-}$ mice were treated with 0.5 mg anti-IFNγ mAb per mouse or the respective isotype control twice a week for 2 weeks. Data: mean values ± SEM, n = 5. For statistical analysis, the non-parametric Mann-Whitney *U* test was used; n.s.: not significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; mAb, monoclonal antibody; mHAI, modified hepatitis activity index; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling. (This figure appears in colour on the web.)

In order to further elucidate the role of IFNγ for NK cell cytotoxicity and liver fibrosis in sclerosing cholangitis, we generated *Mdr2*$^{-/-}$ x *Ifng*$^{-/-}$ mice. These animals displayed reduced numbers of hepatic T cells and NK cells. Most strikingly, frequencies of NK cells and CD8^{+} T cells expressing the cytotoxic effector molecules GzmB or TRAIL were significantly reduced in *Mdr2*$^{-/-}$ x *Ifng*$^{-/-}$ compared to *Mdr2*$^{-/-}$ mice. The reduction of lymphocyte cytotoxicity in the absence of IFNγ can be explained by the observation that the cytotoxic function of CD8^{+} T cells is enhanced by autocrine production of IFNγ.[29] Likewise, NK cell activation is promoted by several cytokines including IFNγ.[28]

We further showed that depletion of NK cells impaired expression of inflammatory and cytotoxic molecules by CD8^{+} T cells. This is in line with previous studies demonstrating that besides their own cytotoxic function, NK cells activate Th1 and CD8^{+} T cell responses during viral infection, which is triggered by NK cell-mediated activation of dendritic cells (DCs) driving polarisation of cytotoxic CD8^{+} T cells and Th1 cells.[30] Moreover, upon exposure to tumour cells, NK cells were shown to attract DCs, which start to express chemokines that recruit effector CD8^{+} T cells to the tumour environment. This mechanism seems to support the tumouricidal activity of NK cells.[31] In addition, NK cells have been shown to promote CD8^{+} T cell responses against cytomegalovirus (CMV). Here, NK cells control CMV early, leading to impaired activation of immunosuppressive plasmacytoid DCs, while conventional DCs were activated and accelerated antiviral CD8^{+} T cell responses.[32] Furthermore, a direct interaction of NK cells and CD8^{+} T cells has been shown in hepatitis B virus (HBV) infection. In contrast to CMV infection, CD8^{+} T cells were impaired during chronic HBV infection, which has been attributed to TRAIL-mediated killing of HBV-specific CD8^{+} T cells by NK cells, while CMV-specific CD8^{+} T cells from the same patient remained unaffected. Moreover, Epstein–Barr virus- and influenza virus-specific CD8^{+} T cells from patients with chronic HBV infection were not affected by NK cell killing.[33] Hence, it seems that NK cell-induced killing of HBV-specific CD8^{+} T cells is specific for this virus. Moreover, several other mechanisms for the impairment of the HBV-specific CD8^{+} T cell response during chronic infection have been proposed.[34] Thus, the findings discussed above point to a role of NK cells in CD8^{+} T cell activation through stimulation and recruitment of DCs, and you might speculate that by this mech-

anism, NK cells also contribute to activation of $CD8^+$ T cells in *Mdr2*$^{-/-}$ mice.

It has previously been shown that depletion of $CD8^+$ T cells between the second and fourth week of life decreased levels of ALP in juvenile *Mdr2*$^{-/-}$ mice.[12] However, in explant livers of patients with PSC undergoing liver transplantation, we observed unaltered frequencies of hepatic $CD8^+$ T cells compared to controls. In contrast, liver NK cells, in particular those that have been described to exhibit a liver-resident phenotype,[20] were significantly elevated in the explant livers of patients with PSC, showing a bias towards increased TRAIL expression. It has been shown that direct activation of NK cells by polyinosinic-polycytidylic acid or IFNγ induced killing of hepatic stellate cells and attenuated the severity of liver fibrosis in murine models.[24] However, although IFNγ was described to abrogate profibrogenic TGFβ signalling in hepatic stellate cells *in vitro*,[35] IFNγ deficiency prevented hepatic inflammation and fibrosis in a chronic steatohepatitis model, which was associated with a reduction of macrophage accumulation in the liver.[23] Indeed, it has been shown recently that $CCR2^+$ inflammatory monocyte-derived macrophages mediate fibrosis in a mouse model of sclerosing cholangitis.[21] Since we observed an inhibition of hepatic $CCR2^+$ macrophage accumulation in *Mdr2*$^{-/-}$ x *Ifng*$^{-/-}$ mice, it would be conceivable that IFNγ indirectly accelerates hepatic fibrosis in sclerosing cholangitis via recruitment of inflammatory monocyte-derived macrophages.

Macrophages are activated by damage-associated molecular patterns, which are generated in response to hepatocellular damage. We have observed a reduced cytotoxic phenotype of $CD8^+$ T cells and NK cells along with a strong inhibition of liver cell death in *Mdr2*$^{-/-}$ x *Ifng*$^{-/-}$ mice compared to *Mdr2*$^{-/-}$ mice. A critical role of NK cells for the pathogenesis of sclerosing cholangitis in the *Mdr2*$^{-/-}$ mouse model was further demonstrated by depression of the inflammatory and cytotoxic phenotype of $CD8^+$ T cells as well as by reduction of liver fibrosis upon NK cell depletion. Notably, gene variation analysis indicated a significant reduction of HLA alleles specific for inhibitory NK cell receptors in patients with PSC.[36] Moreover, genetic variations were also observed in a ligand for the activating NKG2D receptor, the major histocompatibility complex class I chain-related molecule MIC-A, which has been proposed as a candidate locus for HLA-encoded disease susceptibility to PSC.[37] Hepatic NK cells display a pronounced cytotoxic phenotype.[28] Indeed, we observed a relatively high frequency of $GzmB^+$ NK cells in C57Bl/6 mice that was not further enhanced in *Mdr2*$^{-/-}$ mice but significantly reduced in the absence of IFNγ. Also, hepatic NK cells constitutively express TRAIL,[28] which is highly upregulated by viral infection in the liver.[38] In our mouse studies, we demonstrated a strong upregulation of TRAIL in hepatic NK cells from *Mdr2*$^{-/-}$ mice, which was significantly reduced in *Mdr2*$^{-/-}$ x *Ifng*$^{-/-}$ mice. Interestingly, cholangiocytes of patients with PSC express increased amounts of the TRAIL receptor 5 arguing for TRAIL-dependent lysis of these cells by NK cells.[39] Moreover, agonistic anti-TRAIL receptor 5 antibodies induced cholangitis and cholestatic liver injury in mice showing the typical histological appearance reminiscent of human PSC.[37] Hence, IFNγ is likely to induce cholangiocyte and/or hepatocyte death by enhancing NK cell cytotoxicity, which finally results in activation of inflammatory macrophages and development of fibrosis. Taken together, our study demonstrates that IFNγ-dependent immune responses may represent checkpoints for therapeutic intervention in sclerosing cholangitis.

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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

GR, LB, SW, AEL, DS, TP, SH, BS, LUH, AEZ, KJO, CS, and MA: Acquisition, analysis and interpretation of data, statistical analysis. GT, KN, and RB: study concept and design, study supervision, and interpretation of data. GT and RB: obtained funding. GT and KN: preparation of the manuscript. All authors were involved in the critical revision of the manuscript.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.05.023.

References

Author names in bold designate shared co-first authorship

[1] Lazaridis KN, LaRusso NF. Primary sclerosing cholangitis. N Engl J Med 2016;375(25):2501–2502. https://doi.org/10.1056/NEJMc1613273.

[2] Chung BK, Hirschfield GM. Immunogenetics in primary sclerosing cholangitis. Curr Opin Gastroenterol 2017;33(2):93–98. https://doi.org/10.1097/MOG.0000000000000336.

[3] Schoknecht T, Schwinge D, Stein S, Weiler-Normann C, Sebode M, Mucha S, et al. CD4+ T cells from patients with primary sclerosing cholangitis exhibit reduced apoptosis and down-regulation of proapoptotic Bim in peripheral blood. J Leukoc Biol 2017;101(2):589–597. https://doi.org/10.1189/jlb.5A1015-469R.

[4] **Sebode M**, **Peiseler M**, Franke B, Schwinge D, Schoknecht T, Wortmann F, et al. Reduced FOXP3(+) regulatory T cells in patients with primary sclerosing cholangitis are associated with IL2RA gene polymorphisms. J Hepatol 2014;60(5):1010–1016. https://doi.org/10.1016/j.jhep.2013.12.027.

[5] Dienes HP, Lohse AW, Gerken G, Schirmacher P, Gallati H, Lohr HF, et al. Bile duct epithelia as target cells in primary biliary cirrhosis and primary sclerosing cholangitis. Virchows Arch 1997;431(2):119–124.

[6] Liaskou E, Jeffery LE, Trivedi PJ, Reynolds GM, Suresh S, Bruns T, et al. Loss of CD28 expression by liver-infiltrating T cells contributes to pathogenesis of primary sclerosing cholangitis. Gastroenterology 2014;147(1):221 e7–232 e7. https://doi.org/10.1053/j.gastro.2014.04.003.

[7] Landi A, Weismuller TJ, Lankisch TO, Santer DM, Tyrrell DL, Manns MP, et al. Differential serum levels of eosinophilic eotaxins in primary sclerosing cholangitis, primary biliary cirrhosis, and autoimmune hepatitis. J Interferon Cytokine Res 2014;34(3):204–214. https://doi.org/10.1089/jir.2013.0075.

[8] Mueller T, Beutler C, Pico AH, Shibolet O, Pratt DS, Pascher A, et al. Enhanced innate immune responsiveness and intolerance to intestinal endotoxins in human biliary epithelial cells contributes to chronic

cholangitis. Liver Int 2011;31(10):1574–1588. https://doi.org/10.1111/j.1478-3231.2011.02635.x.
[9] **Fickert P**, **Pollheimer MJ**, Beuers U, Lackner C, Hirschfield G, Housset C, et al. Characterization of animal models for primary sclerosing cholangitis (PSC). J Hepatol 2014;60(6):1290–1303. https://doi.org/10.1016/j.jhep.2014.02.006.
[10] Fickert P, Fuchsbichler A, Wagner M, Zollner G, Kaser A, Tilg H, et al. Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. Gastroenterology 2004;127(1):261–274.
[11] Jacquemin E, De Vree JM, Cresteil D, Sokal EM, Sturm E, Dumont M, et al. The wide spectrum of multidrug resistance 3 deficiency: from neonatal cholestasis to cirrhosis of adulthood. Gastroenterology 2001;120(6):1448–1458.
[12] Taylor AE, Carey AN, Kudira R, Lages CS, Shi T, Lam S, et al. Interleukin 2 promotes hepatic regulatory T cell responses and protects from biliary fibrosis in murine sclerosing cholangitis. Hepatology 2018;68(5):1905–1921. https://doi.org/10.1002/hep.30061.
[13] Green DS, Young HA, Valencia JC. Current prospects of type II interferon gamma signaling and autoimmunity. J Biol Chem 2017;292(34):13925–13933. https://doi.org/10.1074/jbc.R116.774745.
[14] Barikbin R, Neureiter D, Wirth J, Erhardt A, Schwinge D, Kluwe J, et al. Induction of heme oxygenase 1 prevents progression of liver fibrosis in Mdr2 knockout mice. Hepatology 2012;55(2):553–562. https://doi.org/10.1002/hep.24711.
[15] **Segnani C**, **Ippolito C**, Antonioli L, Pellegrini C, Blandizzi C, Dolfi A, et al. Histochemical detection of collagen fibers by sirius red/fast green is more sensitive than van gieson or sirius red alone in normal and inflamed rat colon. PloS One 2015;10(12). https://doi.org/10.1371/journal.pone.0144630 e0144630.
[16] Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995;22(6):696–699.
[17] Uchinami H, Seki E, Brenner DA, D'Armiento J. Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis. Hepatology 2006;44(2):420–429. https://doi.org/10.1002/hep.21268.
[18] Potikha T, Stoyanov E, Pappo O, Frolov A, Mizrahi L, Olam D, et al. Interstrain differences in chronic hepatitis and tumor development in a murine model of inflammation-mediated hepatocarcinogenesis. Hepatology 2013;58(1):192–204. https://doi.org/10.1002/hep.26335.
[19] **Barikbin R**, **Berkhout L**, Bolik J, Schmidt-Arras D, Ernst T, Ittrich H, et al. Early heme oxygenase 1 induction delays tumour initiation and enhances DNA damage repair in liver macrophages of Mdr2(/) mice. Sci Rep 2018;8(1):16238. https://doi.org/10.1038/s41598-018-33233-0.
[20] Harmon C, Robinson MW, Fahey R, Whelan S, Houlihan DD, Geoghegan J, et al. Tissue-resident Eomes(hi) T-bet(lo) CD56(bright) NK cells with reduced proinflammatory potential are enriched in the adult human liver. Eur J Immunol 2016;46(9):2111–2120. https://doi.org/10.1002/eji.201646559.
[21] **Guicciardi ME**, **Trussoni CE**, Krishnan A, Bronk SF, Lorenzo Pisarello MJ, O'Hara SP, et al. Macrophages contribute to the pathogenesis of sclerosing cholangitis in mice. J Hepatol 2018;69(3):676–686. https://doi.org/10.1016/j.jhep.2018.05.018.
[22] Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. Nat Rev Immunol 2017;17(5):306–321. https://doi.org/10.1038/nri.2017.11.
[23] Luo XY, Takahara T, Kawai K, Fujino M, Sugiyama T, Tsuneyama K, et al. IFN-gamma deficiency attenuates hepatic inflammation and fibrosis in a steatohepatitis model induced by a methionine- and choline-deficient high-fat diet. Am J Physiol Gastrointest Liver Physiol 2013;305(12):G891–G899. https://doi.org/10.1152/ajpgi.00193.2013.
[24] Radaeva S, Sun R, Jaruga B, Nguyen VT, Tian Z, Gao B. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. Gastroenterology 2006;130(2):435–452. https://doi.org/10.1053/j.gastro.2005.10.055.
[25] Langeneckert AE, Lunemann S, Martrus G, Salzberger W, Hess LU, Ziegler AE, et al. CCL21-expression and accumulation of CCR7(+) NK cells in livers of patients with primary sclerosing cholangitis. Eur J Immunol 2019. https://doi.org/10.1002/eji.201847965.
[26] **Tedesco D**, **Thapa M**, Chin CY, Ge Y, Gong M, Li J, et al. Alterations in intestinal microbiota lead to production of interleukin 17 by intrahepatic gammadelta T-cell receptor-positive cells and pathogenesis of cholestatic liver disease. Gastroenterology 2018;154(8):2178–2193. https://doi.org/10.1053/j.gastro.2018.02.019.
[27] Berkhout L, Barikbin R, Schiller B, Ravichandran G, Krech T, Neumann K, et al. Deletion of tumour necrosis factor alpha receptor 1 elicits an increased TH17 immune response in the chronically inflamed liver. Sci Rep 2019;9(1):4232. https://doi.org/10.1038/s41598-019-40324-z.
[28] Shi FD, Ljunggren HG, La Cava A, Van Kaer L. Organ-specific features of natural killer cells. Nat Rev Immunol 2011;11(10):658–671. https://doi.org/10.1038/nri3065.
[29] Bhat P, Leggatt G, Waterhouse N, Frazer IH. Interferon-gamma derived from cytotoxic lymphocytes directly enhances their motility and cytotoxicity. Cell Death Dis 2017;8(6). https://doi.org/10.1038/cddis.2017.67 e2836.
[30] Mailliard RB, Son YI, Redlinger R, Coates PT, Giermasz A, Morel PA, et al. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. J Immunol 2003;171(5):2366–2373.
[31] Wong JL, Berk E, Edwards RP, Kalinski P. IL-18-primed helper NK cells collaborate with dendritic cells to promote recruitment of effector CD8+ T cells to the tumor microenvironment. Cancer Res 2013;73(15):4653–4662. https://doi.org/10.1158/0008-5472.CAN-12-4366.
[32] Robbins SH, Bessou G, Cornillon A, Zucchini N, Rupp B, Ruzsics Z, et al. Natural killer cells promote early CD8 T cell responses against cytomegalovirus. PLoS Pathog 2007;3(8). https://doi.org/10.1371/journal.ppat.0030123 e123.
[33] Peppa D, Gill US, Reynolds G, Easom NJ, Pallett LJ, Schurich A, et al. Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. J Exp Med 2013;210(1):99–114. https://doi.org/10.1084/jem.20121172.
[34] Schuch A, Hoh A, Thimme R. The role of natural killer cells and CD8(+) T cells in hepatitis B virus infection. Front Immunol 2014;5:258. https://doi.org/10.3389/fimmu.2014.00258.
[35] Weng H, Mertens PR, Gressner AM, Dooley S. IFN-gamma abrogates profibrogenic TGF-beta signaling in liver by targeting expression of inhibitory and receptor Smads. J Hepatol 2007;46(2):295–303. https://doi.org/10.1016/j.jhep.2006.09.014.
[36] Karlsen TH, Boberg KM, Olsson M, Sun JY, Senitzer D, Bergquist A, et al. Particular genetic variants of ligands for natural killer cell receptors may contribute to the HLA associated risk of primary sclerosing cholangitis. J Hepatol 2007;46(5):899–906. https://doi.org/10.1016/j.jhep.2007.01.032.
[37] Van Steenbergen W, De Goede E, Emonds MP, Reinders J, Tilanus M, Fevery J. Primary sclerosing cholangitis in two brothers: report of a family with special emphasis on molecular HLA and MICA genotyping. Eur J Gastroenterol Hepatol 2005;17(7):767–771.
[38] Stegmann KA, Robertson F, Hansi N, Gill U, Pallant C, Christophides T, et al. CXCR6 marks a novel subset of T-bet(lo)Eomes(hi) natural killer cells residing in human liver. Sci Rep 2016;6:26157. https://doi.org/10.1038/srep26157.
[39] **Takeda K**, **Kojima Y**, Ikejima K, Harada K, Yamashina S, Okumura K, et al. Death receptor 5 mediated-apoptosis contributes to cholestatic liver disease. Proc Natl Acad Sci U S A 2008;105(31):10895–10900. https://doi.org/10.1073/pnas.0802702105.