The role of interleukin 17 in acute and chronic cholangitis in mice

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Für meinen kleinen Kieselstein

"And love is love is love is love is love is love is love is love;
cannot be killed or swept aside."

– Lin-Manuel Miranda –

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Abstract

Interleukin 17 is a cytokine with manifold effects and functions. It plays an important role in defending mucosal barriers from fungi and bacteria, but it is also involved as a pro-inflammatory and pathogenic trigger in chronic inflammatory and autoimmune diseases. Increased frequencies of IL-17-producing cells were found localised around the bile ducts in patients suffering from cholangiopathies - diseases that are characterised by damaged cholangiocytes. To date, both disease aetiology and pathogenesis of cholangiopathies are poorly understood and liver cirrhosis as well as end-stage liver disease may develop, since no curative treatment has been found so far.

To clarify the role of IL-17 in the pathogenesis and progression of cholangiopathies, we analysed the impact of IL-17 in two different mouse models of experimental cholangitis. To evaluate the importance of IL-17 in the onset and development of acute cholangitis, we made use of K14-OVAp mice that express an OVA peptide on cholangiocytes and develop T cell-mediated cholangitis after adoptive transfer of OVA-specific OT-1 $CD8^+$ T cells. After transferring OT-1 $CD8^+$ T cells deficient in IL-17A/F expression, we observed the development of more severe cholangitis, attributed to highly proliferative and cytotoxic $CD8^+$ T cells. We identified IL-17 to be of major importance in the early activation of cholangiocytes and their T cell-regulating abilities. IL-17-dependent upregulation of PD-L1 on cholangiocytes protected from liver inflammation through inhibition of T cell expansion and cytotoxicity.
The impact of IL-17 deficiency in the long term progression of chronic cholangitis, fibrosis and tumourigenesis was investigated in the well established $Mdr2^{-/-}$ mouse model. These mice develop sclerosing cholangitis due to the accumulation of toxic bile acids around bile ducts and harbour the risk of developing hepatocellular tumours. We did not observe altered development of chronic cholangitis or altered manifestation of liver fibrosis in $Mdr2^{-/-}$/IL-$17A/F^{-/-}$ mice, but IL-17 deficiency resulted in decreased tumour burden in mice. Although the underlying mechanisms are not clarified, the pro-tumourigenic effect of IL-17 might result from increased levels of IL-6 and PD-L1 or the altered recruitment and activation of $\gamma\delta$ T cells and neutrophils.

Overall, IL-17 expression was found to have a protective role in acute cholangitis, but tumourigenic effects in chronic cholangitis, highlighting the context dependent heterogeneity of IL-17 signalling in the setting of cholangitis. Further research therefore is necessary before targeting IL-17 can be considered for the treatment of autoimmune cholangiopathies.

Zusammenfassung

Interleukin 17 ist ein Zytokin mit vielseitiger Funktion und Wirkung. Insbesondere in der Abwehr von Pilzen und Bakterien spielt es eine zentrale Rolle. Als entzündungsfördernder Faktor ist IL-17 jedoch auch mit der Entstehung von Entzündungen und autoimmunen Erkrankungen assoziiert. In Patienten, die an Cholangiopathien leiden - Krankheiten, die mit einer Schädigung von Cholangiozyten einhergehen - wurden vermehrt IL-17-produzierende Zellen im Bereich der Gallengänge gefunden. Bis heute sind sowohl Krankheitsursache als auch Pathogenese von Cholangiopathien unzureichend erforscht. Leberzirrhose und Leberversagen können schwerwiegende Folgen sein, da es bisher keine heilende Therapie gibt.

Um die Rolle von IL-17 in der Entstehung und im Verlauf von Cholangiopathien besser zu verstehen, haben wir zwei verschiedene Mausmodelle der experimentellen Cholangitis in Hinblick auf IL-17 untersucht. Zur Klärung der Rolle von IL-17 für den Ausbruch und den Verlauf einer akuten Cholangitis wurde das K14-OVAp Mausmodell verwendet. Die Mäuse exprimieren ein OVA-Peptid auf Cholangiozyten und entwickeln eine akute T Zell-vermittelte Entzündung nach dem Transfer von OVA-spezifischen OT-1 $CD8^+$ T Zellen. Nach dem Transfer von IL-17A/F-defizienten OT-1 Zellen konnten wir eine verstärkte Cholangitis beobachten, welche auf stark proliferierende und zytotoxische $CD8^+$ T Zellen zurückzuführen war. In der frühen Phase der Entzündung wurde IL-17 als zentraler Faktor in der Aktivierung von Cholangiozyten identifiziert. In Abhängigkeit von IL-17 konnten Cholangiozyten durch die Expression von PD-L1 die T Zell-Aktivierung und -Expansion regulieren und somit vor verstärkter Entzündung schützen.
Den Einfluss von IL-17-Defizienz in der Langzeitentwicklung von chronischer Cholangitis, Leberfibrose und Lebertumoren haben wir im etablierten $Mdr2^{-/-}$ Mausmodell untersucht. Aufgrund der Anhäufung von toxischen Gallensäuren um die Gallengänge entwickeln $Mdr2^{-/-}$ Mäuse mit dem Alter eine sklerosierende Cholangitis. Wir konnten keinen veränderten Verlauf der Entzündung und der Fibroseentstehung bei $Mdr2^{-/-}$/IL-17A/$F^{-/-}$ Mäusen feststellen, jedoch eine verminderte Entstehung von Lebertumoren. Der Tumor-begünstigende Effekt von IL-17 könnte dabei auf erhöhte Spiegel von IL-6 und PD-L1 oder auf die veränderte Rekrutierung von $\gamma\delta$ T Zellen und Neutrophilen zurückzuführen sein.

Insgesamt konnten wir für IL-17 eine protektive Rolle in der akuten Cholangitis, jedoch auch einen pro-tumorigenen Effekte in der chronischen Cholangitis identifizieren, was die Diversität von IL-17 im Zusammenhang mit Entzündungen hervorhebt. Weitere Untersuchungen werden jedoch benötigt, um ein besseres Verständnis der Cholangiopathien zu gewährleisten und neue Therapieansätze zu entwickeln.

Publication List

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Congress presentations

European Network of Immunology Institutes (ENII) - Summer school 2017:
Poster presentation *"The role of interleukin 17 in acute cholangitis in K14-OVAp mice"* Stein S., Schwinge D., Lohse A. W., Schramm C.

German Association of the Study of the Liver (GASL) 2018:
Poster presentation *"The suppressive effect of IL-17-expression in antigen specific CD8+ T cells in acute experimental cholangitis in mice"* Stein S., Schwinge D., Krech T., Lohse A. W., Herkel J., Schramm C.

European Association for the Study of the Liver(EASL) 2018:
Poster presentation *"The suppressive effect of IL-17-expression in antigen specific CD8+ T cells in acute experimental cholangitis in mice"* Stein S., Schwinge D., Krech T., Lohse A. W., Herkel J., Schramm C.

German Society For Immunology (DGFI) 2019:
Poster presentation *"IL-17 mediates immune regulatory function of cholangiocytes in a mouse model of acute, T cell mediated cholangitis"* Stein S., Schwinge D., Weidemann S., Lohse A. W., Herkel J., Schramm C.

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1 Introduction

1.1 The liver as an immunological organ

The liver is not only the largest solid organ in the human body and of great importance for the human metabolism, it also appears to have unique immunological functions. Each minute 1.5 l of blood are delivered to the liver through the portal vein. A huge variety of metabolites and antigens deriving from nutrition and intestinal microbiota is collected via blood from stomach, pancreas, spleen, small and large intestine, before it drains through the liver into the central vein. Beside blood detoxification, glucose storage and bile production, the liver's main function of metabolising proteins, carbohydrates, lipids and vitamins is responsible for the generation of diverse neo-antigens that have not been recognised by the immune system before. Thus, the liver has to precisely balance between immune responsiveness and tolerance at any time, to protect the human body from excessive immune activation [1–3]. Highly specialised mechanisms of antigen presentation and cellular crosstalk orchestrate this complicated immunoregulation and classify the liver as an immunological organ [2, 4].

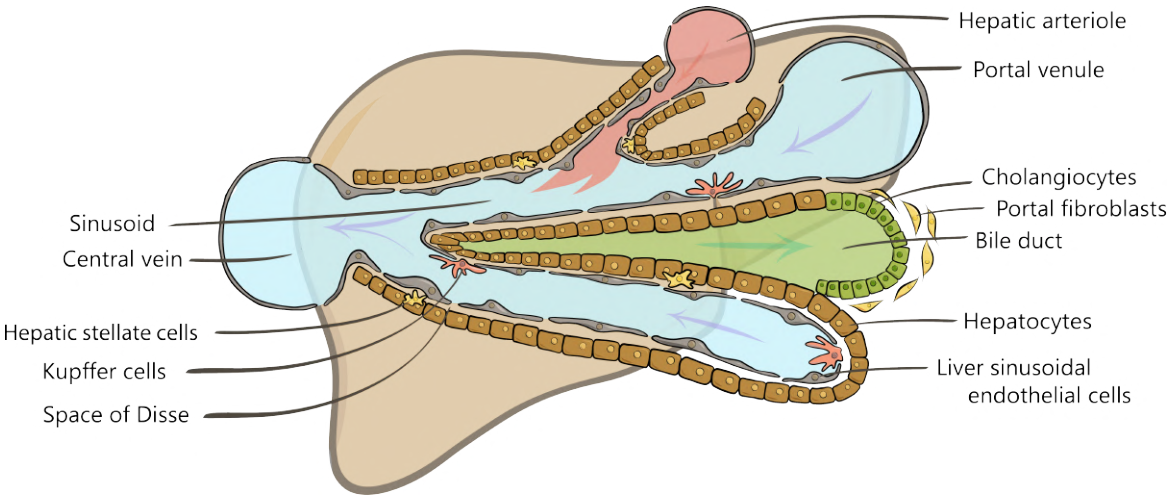

Figure 1.1: Microscopic liver anatomy: Antigen rich blood drains through the liver from hepatic arterioles and portal venues through sinusoids into the central vein. The small sinusoid diameter enables quick communication between cholangiocytes, hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and circulating immune cells.

Nearly 80 % of cells in the liver are hepatocytes that form one-cell-thick layers [5]. These hepatocyte plates are separated from the bloodstream by liver sinusoidal endothelial cells (LSECs) that form a fenestrated capillary, called sinusoid [6]. LSECs lack a basal membrane that enables the quick exchange of molecules between hepatocytes and blood [7, 8]. A small gap between LSECs and hepatocytes, the space of Disse, facilitates a niche for hepatic stellate cells (HSCs). Notably, the liver also contains the largest population of macrophages in the human body, tissue-resident Kupffer cells (KCs) [9].

1.1.1 Liver immunology

Although the liver is primarily a digestive organ, a variety of cells within the liver can exert immunological functions. Favoured by the anatomic position and vascularity, the liver is able to ensure systemic and local immune tolerance to self and foreign antigens.

Blood flowing through the sinusoids brings high quantities of microbial-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) that are recognised by pattern recognition receptors (PRRs) expressed on KCs and hepatocytes [10, 11]. PRR signalling usually induces inflammation, but in the liver, DAMPs and MAMPs are phagocytosed and degraded. Lymphocytes and other immune cells can easily cross talk to non-parenchymal liver cells, especially LSECs and HSCs, due to the small sinusoid diameter [12].
Nearly all cells in the liver can serve as antigen-presenting cells (APCs). LSECs, KCs and liver-resident dendritic cells (DCs) are well accepted and described as efficient and professional APCs, expressing major histocompatibility complex (MHC) class II and diverse co-stimulatory molecules. HSCs are still under discussion, but were also shown to express MHC class II, co-stimulatory molecules and diverse cytokines under inflammatory conditions [13]. However, also hepatic parenchymal liver cells, namely hepatocytes and cholangiocytes, can influence immunological reactions in the liver by secreting chemokines, cytokines, acute phase proteins or acting as APC [14].

1.1.2 Cholangiocytes

Cholangiocytes are the epithelial cells that line extrahepatic and intrahepatic biliary tree and therefore also named 'biliary epithelial cells'. Under physiologic conditions, the main function of cholangiocytes is the modification and transport of bile acids from the liver into the gallbladder and finally the duodenum [15]. The epithelial cells can be divided into small and large cholangiocytes. Small cholangiocytes line the intrahepatic, small bile ducts, have a cuboid shape and a high nucleus/cytoplasm ratio. On the contrary, large cholangiocytes

have a rather columnar shape, a small ratio of nucleus/cytoplasm, are highly specialised and line the bigger and extrahepatic bile ducts. Not only morphological, but also functional heterogeneity was shown, as large cholangiocytes exhibit a different expression profile of receptors and bile acid transporters [16, 17]. These differences can be explained by their different morphogenesis. While small cholangiocytes together with hepatocytes derive from liver precursor cells, epithelial cells lining the extrahepatic bile ducts directly develop from endoderm [18]. Still, all cholangiocytes share their functional structure and cellular polarity. They are densely packed to adjacent cells by tight junctions. Additional gap junctions between cholangiocytes enable rapid exchange of small molecules [19, 20]. To coordinate the absorption, secretion and modification of bile, a complex network of receptors and bile acid transporters are distributed either on the apical/luminal or the basolateral membrane. The transport is organised by the excretion of ions and organic solutes into the canalicular space, followed by osmotic entry of water. Well-studied for example is the binding of secretin to its specific receptor on the basolateral membrane of cholangiocytes. Activation of the secretin receptor induces the release of Cl into the bile duct lumen and subsequent activation of the apical Cl/HCO_3 anion exchanger 2. Thus, bicarbonate is excreted into the bile duct together with passive influx of water [19, 21].

Interestingly, cholangiocytes also express various Toll-like receptors (TLRs) and can recognise different pathogen-associated molecular patterns (PAMPs) and bacterial products. They are capable of presenting antigens, secreting cytokines and chemokines and expression of co-stimulatory molecules that enables cholangiocytes to contribute to inflammatory signalling and immune cell recruitment [22–25].

In a healthy liver, cholangiocytes are proliferative dormant and only express low levels of MHC class I and II or co-stimulatory molecules. In an inflammatory environment, after cellular stress or injury, increased proliferation and activation was observed in human and also murine cholangiocytes [15, 26]. Without co-stimulatory CD28 ligands, cholangiocytes cannot induce effective T cell activation which results in T cell tolerance. However, under conditions of cellular stress or during inflammation, expression of CD28 ligands, namely CD80 and CD86, was observed on cholangiocytes [27, 28].

Among others, TLR activation induces secretion of neutrophil attracting IL-8 and CCL2, IL-6 and dendritic cell attracting Mip-3a [23, 25, 29]. Pro-inflammatory cytokines, such as IFNγ, are potent cholangiocyte activators, leading to upregulated expression of MHC class II, ICAM-1, VCAM-1, CCL2 and CCL20 [30–32]. Many more pro-inflammatory cytokines are shown to activate cholangiocytes, particularly IL-1β, IL-6 ad IL-17 [25, 33]. *In vitro*, cytokine stimulation and direct T cell contact were shown to also induce inhibitory characteristics of cholangiocytes through secretion of prostaglandin E2 and the upregulated expression

of programmed cell death protein 1 (PD-1) ligands PD-L1 and PD-L2 [34–37]. Thus, by interacting with other immune cells cholangiocytes can critically contribute to inflammatory processes [38].

1.2 Cholangiopathies

At any time, the liver has to finely balance between tolerance and immunity which makes this organ prone to immune-mediated diseases. Chronic liver diseases that directly target the intrahepatic or extrahepatic cholangiocytes are called cholangiopathies. The most common cholangiopathies are primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), cystic fibrosis, biliary atresia, polycystic liver disease and cholangiocarcinoma.

Infections, genetic predispositions and dysregulated adaptive and/or innate immune responses are discussed as the main disease aetiologies, but disease triggers and pathogeneses are heterogeneous, complex and elusive. However, all cholangiopathies share typical characteristics of bile flow obstruction, inflammatory immune response and cholangiocyte activation and proliferation [38]. Most common consequences of the progressive cholestasis are damaged liver parenchyma and bile ducts, development of biliary fibrosis and finally end-stage liver disease. To date, there is no effective medical treatment to prevent disease progression which makes cholangiopathies a major indication for liver transplantation and a significant economic burden [39].

Although classified as rare diseases, PBC and PSC are considered to be the most frequent cholangiopathies with prevalences ranging from 1.91 to 40.2 per 100,000 inhabitants/year for PBC and 0.2 - 16.2 per 100,000 inhabitants/year for PSC, depending on the region and study parameters [40–43]. Over the last decades, prevalences of both manifestations have been increasing. Though, it is not clear whether this observation is owing to rising disease incidence or improved diagnostic procedures and awareness [41].

The genetic contribution varies widely between different cholangiopathies, but PBC and PSC are often associated with polymorphisms either connected with a disrupted barrier function and defect polarity of cholangiocytes (claudin, Notch pathway and CFTR mutations)[44–47] or with autoimmune-susceptibility loci (mutations in *HLA*, *TNFRSF14* and *IL2RA* loci) [48, 49]. In line with this, PBC and PSC are classified as presumably autoimmune or at least immune-mediated cholangiopathies [38].

1.2.1 Primary biliary cholangitis

PBC is the most frequent cholangiopathy and presents a strong female predominance. Women around the age of 55-60 are more prone to develop PBC; only 10 % of PBC patients are male [38, 50, 51]. Typical symptoms are pruritus, fatigue and jaundice. The disease process is characterised by anti-mitochondrial antibodies (AMAs) and autoreactive T cells directed against a pyruvate dehydrogenase complex (PDC-E2), resulting in specifically damaged cholangiocytes. As a consequence, especially small, intrahepatic bile ducts become inflamed, fibrotic and cholestatic [38, 52]. PBC pathogenesis is accompanied by elevated serum levels of liver aspartate and alanine aminotransferase (ASAT/ALAT), as well as alkaline phosphatase (AP), which are signs of hepatocyte and/or cholangiocyte injury [38]. Beside serum levels of immunoglobulins (IgM and IgG), both serum AP and AMAs are commonly used to ensure PBC diagnosis [53, 54].

Since autoreactive $CD4^+$ and $CD8^+$ T cells are found surrounding the injured bile ducts, the adaptive immune system is widely discussed as the most potential trigger of PBC. Although innate immune cells are also described to exhibit an altered phenotype and increased susceptibility to over-activation, T cells are the cells primarily found in inflamed areas in PBC livers [55–57]. Especially autoreactive $CD8^+$ T cells are identified as the main pathogenic effector cells through high secretion of granzyme B and interferon (IFN) γ in the early stages of PBC [58, 59]. However, advanced PBC is associated with strong type 1 T helper (Th1) response and high levels of tumor necrosis factor (TNF)α, IFNγ and interleukin (IL)-2, highlighting the importance of T cells in the disease pathogenesis [55, 60]. Furthermore, reduced frequencies and dysfunctionality of regulatory T cells (Tregs) are described in PBC patients [61, 62]. The imbalanced Th17/Treg regulation is discussed to further promote disease progression, but the role of pro-inflammatory Th17 cells as disease trigger is yet unclear.

Although patients exhibit features that are typical for autoimmune disorders, such as high levels of serum autoantibodies and autoreactive T cells, treatment with conventional immunosuppressive drugs does not improve the disease outcome [50, 63]. Except liver transplantation, the only approved therapies to counter PBC progression are treatment with ursodeoxycholic acid (UDCA) and obeticholic acid (OCA) [54]. Even though its specific mechanism is still unclear, it has been shown that administration of the naturally existing bile acid UDCA can improve markers of cholestasis and serum biochemistries, such as serum AP. Additionally, cirrhotic progression and the need for liver transplantation is delayed [63, 64]. Still, estimated 30 % of PBC patients do not respond to UDCA administration and there is no proven therapeutic effect in other cholangiopathies [65]. Nevertheless, both responders and non-responders benefit from additional treatment with obeticholic acid and bezafibrate that reduce bile acid synthesis via farnesoid X receptors [54, 63].

1.2.2 Primary sclerosing cholangitis

Men and women of all age groups can develop PSC, but in contrast to PBC more than 60 % of patients are men with a disease onset around 35 years of age, with common symptoms of itch, abdominal pain and fatigue [41, 66]. While PBC is usually restricted to small, intrahepatic bile ducts, PSC can affect both the intrahepatic and extrahepatic biliary tree. The disease is diagnosed by elevation of cholestatic serum AP and gamma-glutamyl transferase (GGT), as well as magnetic resonance cholangiopancreatography (MRCP) [48, 67].

Several mechanisms are considered to contribute to the multifactorial pathogenesis, including autoimmunity and dysregulated adaptive immune responses [48, 68]. Liver infiltrating immune cells mainly consist of $CD4^+$ and $CD8^+$ T cells and both Th1 and Th2 driven immune responses are discussed as central trigger of PSC [69–71]. Similar to observations in PBC patients, impaired Tregs and enhanced Th17 response are found in PSC livers [72, 73]. Together with increased frequencies of $\gamma\delta$-T cells and reduced infiltration of mucosal-associated invariant T (MAIT) cells, these data underline the notion of dysregulated adaptive immune cells in the disease pathogenesis [70, 74, 75].

Importantly, PSC shows an extremely high association with inflammatory bowel diseases (IBDs). More than 70 % of PSC patients have additional ulcerative colitis (UC) or, less frequently, Crohn's disease [76, 77]. This strong association suggests a related pathogenesis, since leakage of gut microbial components or pro-inflammatory antigens into the portal circulation are discussed to induce immune response in the liver accompanied by the increased recruitment of activated T cells from the gut [48, 78]. But also innate immune cells are discussed to play a central role in the induction of IBD-associated PSC, since macrophages, DCs and natural killer (NK) cells can be activated by the excessive presentation of PAMPs and MAMPs through the leaky gut [48, 68].

In PSC patients, isolated cholangiocytes exhibit enlarged size and decreased expression of ZO1 that is necessary for intact epithelial tight junctions [79]. As a result, bile acids can leak into the portal fields and trigger fibrosis. Furthermore, characteristics of cellular senescence, which is associated with excessive cytokine and chemokine secretion, are observed in PSC cholangiocytes compared to PBC and healthy bile duct cells [79, 80]. As a consequence, cholangiocytes can exhibit impaired transport function, further enhancing bile duct injuries by bile acid toxicity [50, 81].
Leakage of bile acids into the portal fields is hypothesised as trigger of immune cells recruitment leading to an activation of HSCs and portal myofibroblasts, which are the main producer of extracellular matrix (ECM) and responsible for the formation of peribiliary fibrosis [48, 82, 83]. Subsequent progression of liver fibrosis to cirrhosis, characterised by the

accumulation of connective scar tissue, also goes along with a loss of functional liver tissue and increased risk of tumour development [84, 85]. Whereas PBC progression is associated with the development of hepatocellular carcinoma (HCC), PSC patients more frequently suffer from cholangiocarcinoma (CCA) and benign or malignant gallbladder lesions [48, 86–88].

To date, disease progression and end-stage liver disease are unavoidable for most patients, as there is no effective medical therapy available [64]. Administration of UDCA showed improvement of serum biochemistries and amelioration of disease symptoms, but no or even worsening effects in patients' survival [39, 89], so liver transplantation remains the only therapeutic option despite high recurrence rates [64, 90].

1.3 Interleukin 17

Interleukins (ILs) are a group of cytokines formerly described as immune-modulating molecules expressed by leukocytes. It is widely accepted that a variety of parenchymal and non-parenchymal cells are able to secrete and interact via interleukins. In contrast to chemokines, which induce chemotaxis of immune cells, ILs can act as activators or repressors of immune cell differentiation, proliferation and activation [91]. In humans, more than 50 ILs were found so far [92]. A prominent group is the IL-17 family, consisting of 6 members with similar protein structure: IL-17A - IL-17F. Whereas IL-17A, IL-17B, IL-17C and IL-17D are classified as pro-inflammatory cytokines, IL-17E (also known as IL-25) is discussed as an anti-inflammatory cytokine. The specific functions and effects of these four members however are poorly understood and still under investigation [93–95].

More prominent cytokines of this cytokine family are IL-17A and IL-17F that share around 60 % homology and are secreted as homodimers or heterodimers with similar sources and functions [96]. IL-17A is 10 to 30 times more potent than IL-17F and shows multiple effects in immunological processes [97]. IL-17A is indispensable for the clearance of infections with fungi or extracellular bacteria at mucosal barriers, but also induces pro-inflammatory processes and is strongly associated with inflammatory and autoimmune disorders [98–100]. IL-17 signalling is directed by five different receptor subunits: IL-17RA to IL-17RE. Both IL-17A and IL-17F bind to the dimeric IL-17RA/IL-17RC complex, which is constantly expressed by all cells [101]. The activated receptor leads to signalling via ACT1, nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK) signalling pathways and finally transcription of pro-inflammatory genes, such as *CCL2*, *CCL20*, *IL6*, *IL8* or *TNFa* [102, 103]. Particularly at mucosal barriers, pathogen-induced secretion of IL-17A increases the expression of IL-8 and granulocyte-colony stimulating factor (G-CSF), which leads to

recruitment of neutrophils to the site of infection and pathogen clearance [104]. Thus, IL-17A-deficient patients and mice show higher susceptibility to fungal or bacterial infections and aggravation of intestinal inflammation [105–108].

Many immune cells are able to secrete IL-17A, especially in response to pathogens or cellular stress, including innate lymphoid cells (ILCs), $CD4^+$ and $CD8^+$ T cells, $\gamma\delta$ T cells, MAIT cells, NK cells, natural killer T (NKT) cells, DCs, monocytes, neutrophils and mast cells [109, 110]. However, IL-17A-producing $CD4^+$ T cells (Th17 cells) and $CD8^+$ T cells (Tc17 cells) are the main cell populations associated with autoimmune disorders, such as psoriasis, IBDs, rheumatoid arthritis, multiple sclerosis and autoimmune liver diseases [109, 111–113]. $CD4^+$ T cells were the first cells described to secrete IL-17A. Today, they are named Th17 cells according to their signature cytokine. For the differentiation of naïve T cells into Th17 cells, transforming growth factor (TGF) β and IL-6 are needed in combination to induce the transcription of retinoic acid receptor-related orphan receptor γ t (RORγt) and IL-1β leads to the active expression of IL-17A or IL-17F [114]. Interestingly, without IL-6, TGFβ alone induces the transcription of forkhead box protein P3 (FoxP3) and differentiation of naïve T cells into Tregs that are in close regulatory balance to Th17 cells and responsible for immune homeostasis [115].
Similar to Th17 cells, differentiation of Tc17 cells from naïve $CD8^+$ T cells also depends on IL-1β, IL-6 and TGFβ [116]. In contrast, antigen exposure without additional cytokines results in the differentiation into conventional cytotoxic $CD8^+$ T lymphocytes (CTLs). On a functional level, both CTL and Tc17 cells secrete large amounts of IFNγ and TNFα. However, Tc17 cells express less granzyme B, which is the hallmark serum protease expressed by CTLs. Thus, Tc17 cells can be seen as rather pro-inflammatory, but less cytotoxic T cells [116, 117].

IL-17-producing cells are found accumulating around bile ducts in both PBC and PSC livers [30, 61, 73]. Cholangiocytes themselves can recognise IL-17, leading to further recruitment of Th17 and Tc17 cells to the inflamed bile ducts by secretion of CCL20 [30, 33]. Furthermore, activated cholangiocytes are able to promote the differentiation of naïve T cells into IL-17-producing Th17 or Tc17 cells by providing IL-1β, IL-6 and IL-23.
Additionally, an impaired balance of Tregs and Th17 cells, as well as elevated IL-17 serum levels are described in both cholangiopathy manifestations [72, 73, 118]. Though, the effects of increased IL-17 levels seem to be restricted to the liver. Therefore, a direct involvement of IL-17 in the pathogenesis of cholangiopathies is considered.
Moreover, IL-17 is described as strictly pro-fibrogenic through induction of KCs, HSCs and fibroblasts [119–121]. Following IL-17 signalling, KCs start secretion of pro-inflammatory and pro-fibrogenic IL-1β, IL-6, TNFα and TGFβ. Furthermore, IL-17-activated HSCs and myofibroblasts promote ECM deposition and progression of liver fibrosis [119].

The role of IL-17 in general tumourigenesis is discussed controversially, but in the liver, increased levels of IL-17 are associated with poor survival and tumour progression in both CCA and HCC patients [122–124].

Its prominent involvement in liver inflammation, fibrosis and tumourigenesis makes IL-17 a promising target in the treatment of autoimmune liver diseases and cholangiopathies. Other autoimmune conditions are already treated by blocking the IL-17/IL-17R axis, improving disease outcome. Ixekizumab, for example, is an already approved IL-17-inhibitor to treat plaque psoriasis [125]. It also shows promising effects in patients with rheumatoid arthritis, comparable to the αIL-17R antibody brodalumab [126, 127]. However, secukinumab, an αIL-17A antibody, appears to be completely ineffective in treating patients suffering from Crohn's disease [128]. Therapeutical inhibition of IL-17 signalling in liver diseases is discussed controversially, since contraindications and side effects in regard to bacterial and fungal infections are unclear. Thus, targeting IL-17 should be considered carefully, especially in diseases that are associated with defective mucosal barrier function.

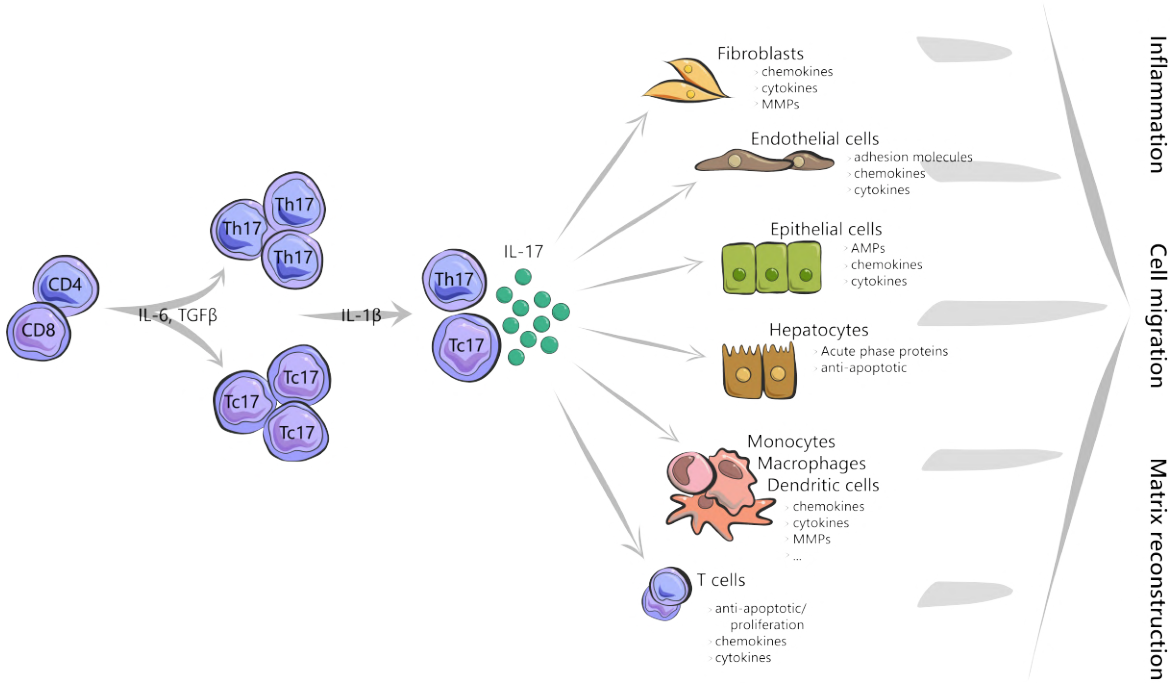

Figure 1.2: Differentiation of conventional IL-17-secreting T cells and effects of IL-17 on liver cells: Conventional T cells are the main source of IL-17A. Both Th17 and Tc17 differentiate from naïve $CD4^+$ or $CD8^+$ T cells, respectively, after activation with IL-6 and TGFβ. IL-1β induces the active expression of IL-17A, which can be recognised by a variety of cells within the liver. IL-17 induces pro-inflammatory, pro-chemotactic and pro-fibrogenic signalling in parenchymal and non-parenchymal liver cells.

1.4 Cholangiopathies in mice

Cholangiopathies arise in many different manifestations and pathologies. Thus, there is no animal model that portrays the complexity of all different cholangiopathies. Most variety in disease characteristics and experimental opportunities can be found in studies with rodents, especially mice. Disease development can be based on genetic deficiencies/knockouts, chemical induction, biliary obstruction, antigen-driven biliary injury, infections and many more.

To name only a few, the commonly used dnTGFβRII mouse spontaneously develops PBC-like periportal inflammation with production of AMAs, progressive bile duct destruction and development of fibrosis [129, 130]. Similar features are present in IL-2R$\alpha^{-/-}$ mice, which histologically resemble the PBC liver phenotype and are found to be AMA positive [131, 132]. Interestingly, both mouse models are accompanied by spontaneous development of colitis that usually is not found in the progression of PBC.
To specifically study cholestasis and biliary obstruction, bile duct ligation (BDL) is the most widely and longest used technique that can be performed in mice with any transgenic background. Disease course after BDL usually includes strong cholangiocyte proliferation and apoptosis, portal inflammation and rapid biliary fibrosis [133, 134]. Advantage, as well as disadvantage is the fast progression which is challenging to investigate in detail. Slow development of cholestasis, sclerosing cholangitis, biliary fibrosis and bile duct proliferation can be found in mice fed with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), depending on the administered concentration and the murine background [135].
The connection of pathologies in colitis and cholangitis are studied in a mouse model of chemical induced colitis by the administration of dextran sulfate sodium (DSS) that also leads to associated cholangitis and elevation of serum bile acids in CD-1 mice, although they are completely lacking a phenotype of fibrosis [136].

However, most mouse models cannot completely reflect the human immune system and the development of human cholangiopathies. To have a closer look into basic, IL-17 dependent immunological processes occurring in the context of cholangitis, we made use of two different mouse models - an antigen-driven and T cell-mediated, acute cholangitis model and a toxicity-driven, chronic cholangitis model.

1.4.1 K14-OVAp mouse model

The K14-OVAp mouse model is described as an inducible mouse model of antigen-dependent cholangitis [137]. K14-OVAp mice express the chicken ovalbumin peptide (OVAp) amino acid

sequence SIINFEKL (257-264) under control of the keratin 14 (K14) promoter. The promoter is expressed in murine epithelial cells in oesophagus, liver, skin, thymus and tongue [138]. K14-OVAp mice present the SIINFEKL peptide via MHC class I, which can be recognised by cytotoxic $CD8^+$ T cells. OT-1 mice have designed inserts for Tcra-V2 and Tcrb-V5 genes, resulting in a transgenic T cell receptor (TCR) that recognises the SIINFEKL peptide.

Adoptive transfer of OT-1 $CD8^+$ T cells into K14-OVAp mice results in lymphocytic infiltrations around the bile ducts and portal fields, development of AMAs and elevated levels of liver transaminases, but no lobular inflammation. The model shows a liver-specific female predominance. A comparable gender bias is seen in human PBC and autoimmune hepatitis (AIH) and was not shown before in a mouse model of acute cholangitis [41, 50]. The increased cholangitis severity is associated with elevated frequencies of IL-17-producing T cells. Both disease severity and IL-17 production are directly affected and reduced by the presence of testosterone in this model of acute cholangitis [139].

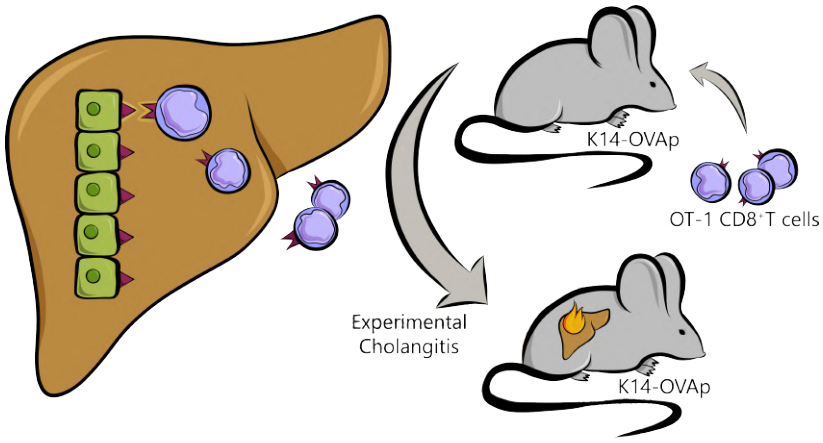

Figure 1.3: The K14-OVAp mouse model: OVA-specific OT-1 $CD8^+$ T cells are adoptively transferred into K14-OVAp recipient mice, where they recognise the OVA-peptide SIINFEKL expressed on epithelial cells, including cholangiocytes in the liver. The activation of antigen-specific OT-1 T cells leads to the development of acute cholangitis in K14-OVAp mice.

Initially, the K14-OVAp mouse model was developed to study the mechanisms of autoimmunity. K14-OVAp/OT-1 double transgenic mice were used to study positive and negative selection of autoreactive T cells in the thymus. In this model, central tolerance was broken, so mice developed lethal CD8-mediated autoimmune disease within few weeks of age, accompanied by inflammation in the portal liver fields, oesophagus and skin [138, 140]. By adoptive transfer of lymph node-derived OT-1 $CD8^+$ T cells into K14-OVAp recipient mice, expansion of antigen-specific T cells, their migration to different tissues that present the SIINFEKL antigen and manifestation of chronic skin inflammation was analysed [139].

Schwinge et al. could only detect mild inflammation of skin and oesophagus after adoptive transfer of spleen-derived OT-1 $CD8^+$ T cells into K14-OVAp mice at the time point of analysis [137].

1.4.2 Mdr2$^{-/-}$ mouse model

The most prominent mouse model of chronic cholangitis is the Mdr2$^{-/-}$ mouse model. In the liver, the phospholipid flippase "multidrug resistance protein (Mdr) 2" is expressed exclusively at the canalicular hepatocyte membrane [141, 142] and required for the excretion of basolateral absorbed phosphatidylcholine (PC) into the bile fluid [143, 144]. The PC translocation itself is necessary for the formation of micelles in the bile, leading to solubilisation of cholesterol and, on the other hand, the migration of highly detergent bile salts into the biliary tree.

In humans, defects in MDR3 flippases are associated with different cholestatic syndromes, drug-related and pregnancy-related cholestasis as well as biliary cirrhosis [44, 143, 145, 146]. Since many different mutations in the MDR3 gene were found to increase the severity of cholestatic diseases like PBC and PSC [143, 146], mice with a disruption of the Mdr2 gene were generated to study the clinical relevance of the PC transporter for disease development and treatment options.

The phospholipid flippase Mdr2 sequence in mice is more than 90 % identical and exhibits the same tissue distribution with the highly conserved MDR3 in humans [142]. The complete lack of the Mdr2 flippase results in a lack of PC and the accumulation of non-micellar, toxic bile acids in the intrahepatic biliary system. As a consequence, cholangiocytes become injured by cell membrane damage and the disruption of cell junctions [143, 147, 148].

In general, the accumulation of toxic bile acids around bile ducts leads to increased permeability of epithelial tight junctions, leakage of bile into the portal fields and subsequent portal liver inflammation already in the first weeks of age [141, 148]. Over time, Mdr2$^{-/-}$ mice develop pericholangitis, concentric periductal fibrosis, ductular proliferation and eventually, sclerosing cholangitis comparable to human PSC histology [148–150]. Additionally, chronic inflammation and fibrosis strongly favours the development of HCC, which can be observed in Mdr2$^{-/-}$ within 12-16 months of age [141, 151, 152]. Furthermore, the overall bile flow is elevated in mice with a homozygous knockout of the Mdr2 gene. The same effect can already be observed in heterozygous Mdr2$^{+/-}$ mice that have an increased bile flow rate by 60 %. On the other hand, decreased cholesterol and glutathione secretion were only detected in mice with homozygous Mdr2 disruption [147, 153].

However, the specific time course and manifestation of disease depends on the murine genetic background. Mice bred on the Friend virus B-type/N (FVB) background exhibit increased chronic hepatitis and earlier development of liver tumours compared to the murine C57 black 6 (C57Bl/6) background that favours gender-specific differences with female predominance in inflammation and tumourigenesis [154, 155].

Although the pathogenesis of chronic cholangitis seen in Mdr2$^{-/-}$ mice is caused by bile acid toxicity and varies widely from immune driven PSC and other cholangiopathies, both human and mouse disease manifestations show similarities in the histological outcome and cholangiocyte damage. Due to the continuous development of bile duct injury, periportal fibrosis and HCC, the Mdr2$^{-/-}$ mouse model is widely used to investigate disease progression and treatment options.

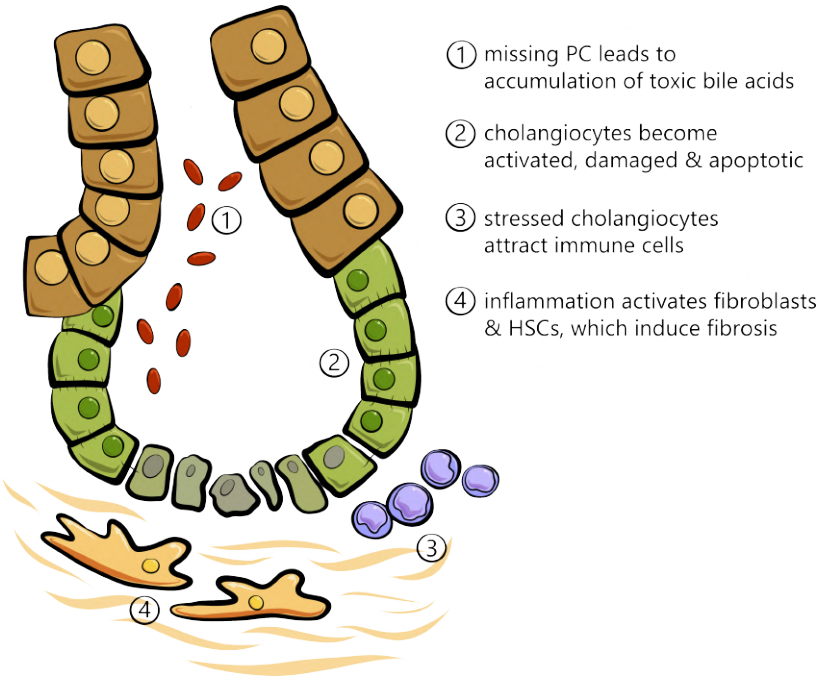

Figure 1.4: The Mdr2$^{-/-}$ mouse model: Hepatocytes in Mdr2$^{-/-}$ exhibit defective secretion of phosphatidylcholine (PC) into the bile, leading to the accumulation of toxic bile acids in the biliary tract. Consequently, cholangiocytes become activated, instable and apoptotic resulting in the recruitment of immune cells and portal inflammation. Chronic inflammation further induces activation of hepatic stellate cells (HSCs) and myofibroblasts, leading to the establishment of sclerosing cholangitis.

1.5 Aim of the study

Cholangiopathies, such as PBC and PSC, are rare and progressive liver diseases arising from cholangiocyte inflammation and bile duct injury. Neither disease aetiology nor pathogenesis are fully understood. Thus, no effective, curative treatment beside liver transplantation is available yet. Recent data have shown that a dysregulated immune response promotes the development of cholangiopathies.
Interleukin-17 was already associated with the pathogenesis of various autoimmune disorders, such as psoriasis, rheumatoid arthritis, multiple sclerosis and IBD. For some of the diseases, blocking IL-17 signalling was promising in different therapeutical approaches. In several cholangiopathies, serum levels of IL-17 were shown to be elevated and IL-17-producing cells were found to accumulate around bile ducts in these patients, indicating a contributing role of IL-17 in the pathogenesis of cholangiopathies. Still, it is not clear, whether IL-17 has a protective or harmful function.

Findings of this doctoral thesis might contribute to the understanding of the complex immunological processes in the context of cholangitis. Therefore, aim of this work was the functional analysis of IL-17 in the development and course of acute and chronic cholangitis:

(1) K14-OVAp mice develop an acute, T cell-mediated cholangitis after adoptive transfer of antigen-specific OT-1 $CD8^+$ T cells, which specifically attack the antigen-presenting cholangiocytes. To evaluate the functional role of IL-17 in the development of cholangitis, we generated donor $CD8^+$ T cells that lack the expression of IL-17A and IL-17F. First, OT-1/IL-$17^{-/-}$ $CD8^+$ T cells should be analysed concerning their general cytokine secretion, activation, proliferation and cytotoxicity. Second, the induction and course of acute cholangitis should be studied comparing the adoptive transfer of IL-17-competent OT-1 $CD8^+$ or OT-1/IL-$17^{-/-}$ $CD8^+$ into K14-OVAp mice.

(2) To investigate the effect of IL-17 deficiency in the long term disease progression, $Mdr2^{-/-}$ mice that develop a chronic cholangitis based on the accumulation of toxic bile around the bile ducts should be analysed. Beside portal inflammation, $Mdr2^{-/-}$ mice also exhibit periportal fibrosis, sclerosing cholangitis and spontaneous development of hepatocellular carcinoma with age. To analyse the role of IL-17 both in an early phase of inflammation, but also in late stage liver disease, fibrosis and tumourigenesis, $Mdr2^{-/-}$/IL-$17^{-/-}$ mice were generated and the course of disease will be investigated at different ages.

2 Materials and Methods

2.1 Materials

All experiments were performed with standard laboratory equipment unless otherwise stated.

Table 2.1: Reagents

Reagent	Manufacturer	Country
general reaction and centrifuge tubes	Sarstedt	Germany
general cell culture dishes and plates	Sarstedt	Germany
general serological pipettes and tips	Sarstedt	Germany
ABTS^{TM}	Sigma-Aldrich	Germany
Acetone	Th. Geyer	Germany
Adenine	Sigma-Aldrich	Germany
Albumin Fraction V	Carl Roth	Germany
Annexin V-FITC	BD Biosciences	USA
Annexin V Binding Buffer	BD Biosciences	USA
αFITC MicroBeads	Miltenyi Biotec	Germany
Antibody Diluent	Agilent	USA
Aqua	B. Braun	Germany
β-Mercaptoethanol	Sigma-Aldrich	Germany
CD8 MicroBeads, mouse	Miltenyi Biotec	Germany
CD11c MicroBeads, mouse	Miltenyi Biotec	Germany
CellTrace^{TM} Violet Cell Proliferation Kit	Invitrogen^{TM}	USA
Cholera Toxin	Sigma-Aldrich	Germany
Collagen R solution (0.2 %)	SERVA	Germany
Collagenase NB 4G Proved Grade	Nordmark	Germany
DAB Chromogen Solution	Agilent	USA
DAB Substrate Buffer	Agilent	USA
Dexamethasone	Sigma-Aldrich	Germany

Reagent	Manufacturer	Country
DMEM, high glucose, GlutaMAX™	Gibco™	USA
EDTA (UltraPure™, 0.5 M)	Invitrogen™	USA
EGF, recombinant, human	PeproTech	USA
Entellan®	Sigma-Aldrich	Germany
Eosin Y solution (0.5 %)	Carl Roth	Germany
Epinephrine	Sigma-Aldrich	Germany
Ethanol, absolute	Th. Geyer	Germany
Ethanol, denatured	Carl Roth	Germany
Fetal calf serum (FCS)	PAA Laboratories	USA
Fluorescence Mounting Medium	Agilent	USA
GolgiPlug™ (cont. Brefeldin A)	BD Biosciences	USA
Ham's F-12 Nutrient Mix	Gibco™	USA
Hemalum solution acid acc. to Mayer	Carl Roth	Germany
HEPES (1 M)	Gibco™	USA
HGF, recombinant, human	PeproTech	USA
Hoechst 33258, Pentahydrate	Invitrogen™	USA
Human Serum	tcs bioscience	UK
Hydrocortisone	Sigma-Aldrich	Germany
Hydrogen peroxide (30 %)	Merck	Germany
IFNγ, recombinant, mouse	PeproTech	USA
IL-1β, human	Miltenyi Biotec	Germany
IL-6, human	Miltenyi Biotec	Germany
IL-17A, recombinant, mouse	PeproTech	USA
Insulin solution human	Sigma-Aldrich	Germany
Insulin-Transferrin-Selenium (ITS) (100X)	Gibco™	USA
Ionomycin calcium salt	Sigma-Aldrich	Germany
Ketamidor® (100 mg/ml)	WDT	Germany
Labelled Polymer - Dako REAL EnVision-HRP, Rabbit-Mouse	Agilent	USA
L-Glutamine (200 mM)	Gibco™	USA
MEM Vitamin Solution (100X)	Gibco™	USA
Methanol	J.T.Baker	Germany
Negative Control Compensation Beads	BD Biosciences	USA
Normal Goat Serum	Agilent	USA
OneComp eBeads™ Compensation Beads	Invitrogen™	USA

Reagent	Manufacturer	Country
OptiPrep™	STEMCELL Technologies	Canada
OVA (257-264) SIINFEKL	AnaSpec	USA
Pacific Orange™ Succinimidyl Ester	Invitrogen™	USA
Panserin 401, Serum-free, w: L-Glutamine	PAN Biotech	Germany
Paraformaldehyde solution (4 % in PBS)	Morphisto	Germany
Penicillin-Streptomycin (10'000 U/ml)	Gibco™	USA
Percoll density gradient media	GE Healthcare	UK
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma-Aldrich	Germany
Proleukin® S (IL-2)	Novartis Pharma	Switzerland
Protein Block, Serum-Free	Agilent	USA
Proteinase K, recombinant	Roche Diagnostics	Switzerland
RNase Inhibitor (20 U/μl)	Applied Biosystems	USA
Roti®-Histofix (4 %)	Carl Roth	Germany
Sirius Red	Sigma-Aldrich	Germany
Soybean Trypsin Inhibitor (STI)	Gibco™	USA
StemPro™ Accutase™ Cell Dissociation Reagent	Gibco™	USA
TaqMan™ Fast Advanced Master Mix	Applied Biosystems	USA
Tissue-Tek® O.C.T.™ Compound	Sakura	Netherlands
3,3',5-Triiodo-L-thyronine	Sigma-Aldrich	Germany
Triton® X 100	Carl Roth	Germany
Trypan blue solution (0.4 %)	Gibco™	USA
Trypsin-EDTA (0.05 %)	Gibco™	USA
Tween® 20	J.T.Baker	Germany
Water, nuclease-free	Thermo Fisher Scientific	USA
Xylazin (20 mg/ml)	WDT	Germany
Xylene	Th. Geyer	Germany

Table 2.1: αmouse-antibodies used for histology and flow cytometry

Antigen	Conjugate	Clone	Manufacturer	Country
Annexin V	FITC		BD Biosciences	USA
CD3	145-2C11		BioLegend	USA
CD3	Alexa Fluor®700	17A2	BioLegend	USA

Antigen	Conjugate	Clone	Manufacturer	Country
CD3	APC/Fire™750	17A2	BioLegend	USA
CD3	FITC	17A2	BioLegend	USA
CD3	PE/Cy7	17A2	BD Biosciences	USA
CD3	PerCP/Cy5.5	145-2C11	Invitrogen	USA
CD4	PE/Cy7	GK1.5	BioLegend	USA
CD4	PE/Dazzle™594	RM4-5	BioLegend	USA
CD8a	Alexa Fluor®700	53-6.7	BioLegend	USA
CD8a	APC/Fire™750	53-6.7	BioLegend	USA
CD8a	FITC	53-6.7	BioLegend	USA
CD8a	PerCP	53-6.7	BioLegend	USA
CD8a	V450	53-6.7	BD Biosciences	USA
CD11b	Alexa Fluor®488	M1/70	BioLegend	USA
CD11b	FITC	M1/70	BD Biosciences	USA
CD11c	PE/Dazzle™594	N418	BioLegend	USA
CD25	APC	PC61	BioLegend	USA
CD25	Brilliant Violet 421™	PC61	BioLegend	USA
CD28	37.51		BioLegend	USA
CD45.1	Alexa Fluor®647	A20	BioLegend	USA
CD45.1	Alexa Fluor®700	A20	BioLegend	USA
CD45.1	APC	A20	BioLegend	USA
CD45.1	FITC	A20	BioLegend	USA
CD45.2	APC	104	BioLegend	USA
CD45.2	FITC	104	BioLegend	USA
CD45.2	PE	104	BioLegend	USA
CD45.2	PE/Cy7	104	BioLegend	USA
CD45R/B220	PE/Cy7	RA3-6B2	BioLegend	USA
CD62L	Alexa Fluor®700	MEL-14	BD Biosciences	USA
CD62L	PE	MEL-14	BD Biosciences	USA
CD69	Brilliant Violet 421™	H1.2F3	BioLegend	USA
CD69	FITC	H1.2F3	BD Biosciences	USA
CD152/CTLA-4	PE	UC10-4F10-11	BD Biosciences	USA
CD223/LAG-3	APC	C9B7W	BioLegend	USA
CD274/PD-L1	Brilliant Violet 421™	10F.9G2	BioLegend	USA
CD274/PD-L1	PE	MIH5	BD Biosciences	USA
CD279/PD-1	Brilliant Violet 421™	29F.1A12	BioLegend	USA

Antigen	Conjugate	Clone	Manufacturer	Country
CD279/PD-1	FITC	29F.1A12	BioLegend	USA
CD326/EpCAM	APC	caa7-9G8	Miltenyi Biotec	USA
CD326/EpCAM	FITC	G8.8	BioLegend	USA
CD366/Tim-3	PE/Cy7	RMT3-23	BioLegend	USA
Donkey αRat IgG	Alexa Fluor®488		Invitrogen	USA
Goat αRat IgG	Cy5		Invitrogen	USA
Granzyme B	FITC	GB11	BioLegend	USA
IFNγ	Alexa Fluor®700	XMG1.2	BD Biosciences	USA
IL-17	APC	eBio17B7	Invitrogen	USA
IL-17	PE	TC11-18H10.1	BioLegend	USA
IL-2	PE	JES6-5H4	BD Biosciences	USA
IL-6	APC	MP5-20F3	BioLegend	USA
Ki67	Brilliant Violet 421™	16A8	BioLegend	USA
Krt-19/TROMA-III			DSHB	USA
Ly-6C	PerCP/Cy5.5	HK1.4	BioLegend	USA
Ly-6G	Brilliant Violet 421™	1A8	BioLegend	USA
NK1.1	PE	PK136	BioLegend	USA
TCR γ/δ	APC	GL3	BioLegend	USA
TCR γ/δ	FITC	UC7-13D5	BioLegend	USA
TIGIT	Brilliant Violet 421™	1G9	BD Biosciences	USA
TNFα	PE/Cy7	MP6-XT22	BioLegend	USA

Table 2.1: Buffers and Media

Buffer/Medium	Conc.	Composition	Conc.	Composition
ACK-Buffer		a. dest	10 mM	$KHCO_3$
	100 mM	EDTA	150 mM	NH_4Cl
MACS-Buffer		PBS		
	0.5 %	BSA	2.5 mM	EDTA
PBS, pH 7,4		a. dest		
	1.5 mM	KH_2PO_4	2.7 mM	KCl
	6.5 mM	Na_2HPO_4	137 mM	NaCl
Saponin-Buffer		PBS		
	2 %	BSA	0.5 %	Saponin
Tris-EDTA		a. dest	10 mM	EDTA
	10 mM	Tris	0.05 %	Tween®20
H69-medium	1:3	DMEM/Ham's F-12	26 μg/ml	Adenine
	10 ng/ml	EGF	1 μg/ml	Epinephrine
	10 %	Human Serum	400 ng/ml	Hydrocortisone
	1 X	ITS	2 mM	L-Glutamine
	100 U/ml	Pen/Strep	2 nM	Triiodo-L-thyronine
Cholangiocyte	1:1	DMEM/Ham's F-12	4 ng/ml	Dexamethasone
-medium	25 ng/ml	EGF	10 %	FCS
	1 X	ITS	2 mM	L-Glutamine
	1 X	MEM	100 U/ml	Pen/Strep
	50 μg/ml	STI	3,4 μg/ml	Triiodo-L-thyronine
Lymphocyte	1:1	Panserin		
-medium	5 %	FCS	100 U/ml	Pen/Strep

Table 2.2: Kits

Kit	Manufacturer	Country
High capacity cDNA reverse transcription kit	Applied Biosystems	USA
In Situ Cell Death Detection Kit, TMR red	Roche Diagnostics	Switzerland
mouse CCL2/MCP-1 DuoSet ELISA	R&D Systems	USA
mouse CCL20 DuoSet ELISA	R&D Systems	USA
mouse IFNγ DuoSet ELISA	R&D Systems	USA
mouse IL-2 ELISA MAX^{TM} Standard Set	BioLegend	
mouse IL-6 DuoSet ELISA	R&D Systems	USA
mouse IL-10 DuoSet ELISA	R&D Systems	USA
mouse IL-17 DuoSet ELISA	R&D Systems	USA
mouse Granzyme B Uncoated ELISA Kit	Invitrogen^{TM}	USA
mouse TNFα DuoSet ELISA	R&D Systems	USA
NucleoSpin® RNA	MACHEREY-NAGEL	Germany
Pierce^{TM} LDH Cytotoxicity Assay Kit	Thermo Fisher Scientific	USA
RNAscope® Fluorescent Multiplex Assay Kit	Advanced Cell Diagnostics	USA
RNeasy Micro Kit	Qiagen	Netherlands

Table 2.3: TaqMan® Gene Expression Assay Probes

Gene	Protein	Assay ID
Ccl2	Chemokine ligand 2	Mm00441242_m1
Ccl20	Chemokine ligand 20	Mm01268754_m1
Cd274	Programmed death-ligand 1	Mm03048248_m1
Col1a1	Collagen, type I, α 1	Mm00801666_g1
Col3a1	Collagen, type III, α 1	Mm01254476_m1
Cxcl9	Chemokine ligand 9	Mm00434946_m1
Cxcl10	Chemokine ligand 10	Mm00445235_m1
Foxp3	Forkhead box P3	Mm00475162_m1
Gzmb	Granzyme B	Mm00442837_m1
Hprt	Hypoxanthine-guanine phosphoribosyltransferase	Mm03024075_m1
Ifng	Interferonγ	Mm01168134_m1
Il1b	Interleukin-1β	Mm00434228_m1
Il6	Interleukin-6	Mm00446190_m1
Il10	Interleukin-10	Mm00439614_m1
Il17a	Interleukin-17A	Mm00439618_m1
Krt19	Keratin 19	Mm00492980_m1
Pdcd1	Programmed cell death protein 1	Mm01285676_m1
Rorc	RAR-related orphan receptor γ	Mm01261022_m1
Tgfb1	Transforming growth factor β 1	Mm01178820_m1
Tnf	Tumor Necrosis Factor α	Mm00443258_m1
Vcam1	Vascular cell adhesion protein 1	Mm01320970_m1

Table 2.4: Devices and Software

Device/Software	Developer	Country
BD™ LSR II	BD Biosciences	USA
BD FACSFlow Supply System	BD Biosciences	USA
BD FACSAria™ III	BD Biosciences	USA
BD FACSDIVA™ Software (V8.0)	BD Biosciences	USA
Biorevo BZ-9000 Fluorescence Microscope	Keyence	Japan
ClinScan, 7 T animal MRI system	Bruker BioSpin	Germany
COBAS Integra 400 plus	Roche Diagnostics	Switzerland
CUT 5062 microtome	SLEE medical	Germany
FlowJo (V10.5.3)	FlowJo LLC	USA
GraphPad Prism® (V6.01)	GraphPad Software	USA
ImageJ/FIJI (V1.52)	Wayne Rasband	USA
Infinite F50 absorbance reader	Tecan	Switzerland
Microm HM550 cyryostat microtome	Thermo Fisher Scientific	USA
NanoDrop™ 2000	Thermo Fisher Scientific	USA
NanoDrop 2000/2000c Software (V1.6.198)	Thermo Fisher Scientific	USA
PeqSTAR 2X Universal Gradient thermocycler	Peqlab (VWR)	Germany
Tecan i-control (V1.10.4.0)	Tecan	Switzerland
ViiA™ 7 Real-Time PCR System	Applied Biosystems	USA
ViiA™ 7 Software (v1.2.4)	Applied Biosystems	USA

2.2 Methods

2.2.1 Mice

To investigate the role of IL-17 in acute, but also chronic experimental cholangitis, different mouse models were used. All strains are listed in Table 2.5.

All experiments comply with the ARRIVE guidelines [156] and were approved by the review board of the State of Hamburg, Germany (G36/16 and ORG846). Mice were received from Jackson Laboratory (USA) or bred in-house at the animal facility of the University Medical Center Hamburg-Eppendorf. K14-OVAp mice were kindly provided by Kirstin Hogquist from the Center for Immunology, University of Minnesota, IL-17A/F$^{-/-}$ mice by Immo Prinz from the Institute of Immunology, Hannover Medical School and OT-1/PD-1$^{-/-}$ mice were kindly provided by Hans W. Mittrücker from the Institute of Immunology, University Medical Center Hamburg-Eppendorf. Mdr2$^{-/-}$ and K14-OVAp mice were bred homozygous and their genotype was checked regularly by PCR. K14-OVAp/IL-17A/F$^{-/-}$, OT-1/IL-17A/F$^{-/-}$ and Mdr2$^{-/-}$/IL-17A/F$^{-/-}$ mice were generated by in-house cross-breeding. All animals were housed under specific pathogen free conditions with 12 h light/dark cycles and standard chow diet (Altromin, Germany) and water available ad libitum.

Table 2.5: Transgenic mice

Mouse strains	Background	Characteristics
K14-OVAp	C57Bl/6	Expression of SIINFEKL peptide on epithelial cells
K14-OVAp/IL-17A/F$^{-/-}$	C57Bl/6	Expression of SIINFEKL peptide on epithelial cells; knockout of IL-17A and IL-17F
OT-1	C57Bl/6	Specific TCR against SIINFEKL peptide
OT-1/IL-17A/F$^{-/-}$	C57Bl/6	Specific TCR against SIINFEKL peptide; knockout of IL-17A and IL-17F
OT-1/PD-1$^{-/-}$	C57Bl/6	Specific TCR against SIINFEKL peptide; knockout of PD-1
Mdr2$^{-/-}$	C57Bl/6	Chronic cholangitis induced by toxic bile acid accumulation around bile ducts
Mdr2$^{-/-}$/IL-17A/F$^{-/-}$	C57Bl/6	Chronic cholangitis induced by toxic bile acid accumulation around bile ducts; knockout of IL-17A and IL-17F

2.2.2 Induction of acute cholangitis in mice

For the analysis of acute experimental cholangitis, only female K14-OVAp mice at an age of 7-14 weeks were used, since female mice present a stronger phenotype in this model as described before by Schwinge et al. [137]. $CD8^+$ T cells from spleens of female OT-1, OT-1/IL-17A/$F^{-/-}$ or OT-1/PD-$1^{-/-}$ donor mice were isolated with αCD8-FITC and αFITC immunomagnetic beads by magnetic cell separation (MACS) according to the manufacturer's instructions. To induce acute cholangitis, freshly isolated donor $CD8^+$ T cells were injected intravenously (i.v.) into female K14-OVAp recipient mice at a concentration of $2*10^5$ donor T cells/200 μl/recipient mouse. Onset of cholangitis was observed 4 - 5 days after adoptive cell transfer.

2.2.3 Serum liver enzymes

Serum levels of liver enzymes were measured diluted 1:4 in a. dest with a Cobas Integra 400 plus in the Institute of Experimental Immunology and Hepatology, University Medical Center Hamburg-Eppendorf.

2.2.4 Cell isolation

To isolate lymphocytes from spleen or liver draining lymph nodes, tissue was ground mechanically through 100 μm strainers with PBS. Cells were collected by centrifugation at 400 g. To reduce the numbers of erythrocytes in spleen samples, erythrocyte lysis was performed with 1x Ammonium-Chloride-Potassium (ACK) lysing buffer.

For the isolation of liver non-parenchymal cells, mouse livers were perfused with PBS and dissected mechanically. Hepatocytes and debris were sedimented twice at 40 g, and non-parenchymal cells were recovered by centrifugation over a 35 % Optiprep gradient at 400 g. To reduce the numbers of erythrocytes, lysis was performed with 1x ACK lysing buffer.

To isolate cholangiocytes, mouse livers were perfused with 0.5 mg/ml collagenase in PBS for 5 min and dissected mechanically. Tissue was digested with 2.5 mg/ml collagenase I in PBS for 20 min shaking at 37 °C and filtered with 40 μm cell strainer. Remaining tissue was digested and filtered with 5 mg/ml collagenase as before. Finally, tissue was degraded and filtered using trypsin/EDTA for 10 min shaking at 37 °C. Hepatocytes were sedimented twice at 40 g, and debris was separated using a 35 % percoll gradient at 900 g for 10 min. Purification of epithelial cells was achieved by staining with αCD326/EpCAM-APC and αCD45.2-PE antibodies for 20 min and subsequent sorting of PE-negative/APC-positive

cells using the BD FACSAria^{TM} III (FACS Sorting Core Unit, University Medical Center Hamburg-Eppendorf).

2.2.5 Cell cultivation

All media used for cell cultivation are listed in Table 2.1.

The cholangiocyte cell line H69 was cultivated in H69-medium in T25 and T75 flasks until grown confluent. Cells were splitted regularly 1:10 - 1:20 and used until passage 10.

Isolated lymphocytes from mouse spleen or liver were cultivated in flat bottom 96 well plates with mouse lymphocyte-medium for up to 48 h.

Primary mouse cholangiocytes were first grown in 48 well plates coated with collagen in mouse cholangiocyte-medium, later cultivation was performed in uncoated T25 and T75 flasks.

2.2.6 Stimulation of T-cells

To specifically stimulate T cells via TCR, whole mouse splenocytes and liver infiltrating lymphocytes were seeded at a minimum of $2*10^5$ cells per 96-well (optimum of $5*10^5$ cells/well) and restimulated for up to 48 h with 2 μg/ml coated αCD3 and 2 μg/ml soluble αCD28.

Antigen-specific stimulation of $CD8^+$ T cells was induced using APCs and the SIINFEKL peptide. Therefore, $CD11c^+$ cells (APCs) and $CD8^+$ T cells were freshly isolated from spleens of transgenic OT-1 or OT-1/IL-17A/$F^{-/-}$ donor mice using CD11c and CD8 immunomagnetic beads, respectively, and MACS according to the manufacturer's instruction. APCs were seeded at $5*10^4$ cells/96-well together with $5*10^5$ $CD8^+$ T cells/well and 0.5 μg/ml SIINFEKL peptide for up to 48 h.

To activate $CD8^+$ T cells additionally with cytokines, OT-1 or OT-1/IL-17A/$F^{-/-}$ $CD8^+$ T cells were isolated as described before, seeded at $5*10^5$ cells per 96-well together with $5*10^4$ APCs/96-well, 0.5 μg/ml SIINFEKL peptide and 50 ng/ml IL-6 and/or 10 ng/ml TGFβ for 24 h.

Supernatants of restimulated cells were analysed for levels of IFNγ, IL-17A, TNFα and granzyme B by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturers' protocols. Depending on the absolute cytokine levels, supernatants were used undiluted or diluted up to 1:200 in adequate dilution buffer. Cells were either stained for flow cytometry or lysed in lysis buffer for mRNA expression analysis.

2.2.7 Stimulation of cholangiocytes

H69 cells and primary isolated mouse cholangiocytes were seeded in 24 or 48 -well plates in corresponding medium. When grown 80 % confluent, medium was changed to fresh medium containing 10 ng/ml IFNγ and/or 10 ng/ml IL-17 for 24 h. Culture supernatants were then analysed for the absolute levels of CCL2, CCL20 and IL-6 by ELISAs according to the manufacturers' protocols in adequate dilutions. Cells were lysed in lysis buffer for mRNA expression analysis or stained for immunofluorescent analysis.

2.2.8 Co-cultivation of cholangiocytes and T cells

To co-cultivate primary mouse cholangiocytes and T cells, cholangiocytes were grown 70 % confluent in 48 well plates in mouse cholangiocyte-medium (Tab. 2.1). Freshly isolated $CD8^+$ T cells from spleens of OT-1 or OT-1/IL-17A/$F^{-/-}$ donor mice were added in a concentration of $5*10^5$ cells/well and cultivated together with cholangiocytes. Control groups of $CD8^+$ T cells without cholangiocytes were additionally stimulated with $5*10^4$ APCs/well and 0.5 μg/ml SIINFEKL peptide. After 48 h of incubation, culture supernatants were analysed for cytokine levels of CCL20, IFNγ, IL-6 and IL-17 by ELISAs according to the manufacturers' protocols. Cells were lysed in lysis buffer to analyse the gene expression by qPCR.

2.2.9 Characterisation of $CD8^+$ T cells

To characterise activation of $CD8^+$ T cells from OT-1 and OT-1/IL-17A/$F^{-/-}$ donor mice, cells were isolated and purified using CD8 immunomagnetic beads and MACS as described before.

Proliferation of $CD8^+$ T cells was assessed using the CellTrace^{TM} Violet Cell Proliferation Kit. Briefly, $CD8^+$ T cells were stained with celltrace dye according to manufacturer's protocol and seeded at $5*10^5$ cells/96-well together with $5*10^4$ APCs/well and 0.5 μg/ml SIINFEKL peptide. After 48 h incubation, proliferation was analysed by flow cytometry.

Cytotoxicity of antigen-specific-stimulated OT-1 and OT-1/IL-17A/$F^{-/-}$ $CD8^+$ T cells was analysed using the Pierce^{TM} LDH Cytotoxicity Assay Kit following the manufacturer's protocol. In brief, target cells ($CD11c^+$ cells) were seeded at $5*10^4$ cells/well together with 0.1 μg/well SIINFEKL peptide and effector cells ($CD8^+$ T cells) in ratios of 20:1, 10:1, 5:1

and 1:1 (effector:target) for 6 h. Cytotoxic-induced release of Lactate dehydrogenase (LDH) from target cells was measured in an absorbance reader. Cytotoxicity was quantified as:

$$\%\ Cytotoxicity = \frac{Ext\ _{sample} - Ext\ _{eff.\ spont.} - Ext\ _{tar.\ spont.}}{Ext\ _{tar.\ max.} - Ext\ _{tar.\ spont.}} * 100\ \%$$

2.2.10 Flow cytometry

Immunofluorescent surface staining of liver derived cells or splenocytes was performed with antibodies to CD3, CD4, CD8a, CD11b, CD11c, CD25, CD45.1, CD45.2, CD45R/B220, CD62L, CD69, CD152/CTLA-4, CD233/LAG-3, CD274/PD-L1, CD279/PD-1, CD326/EpCAM, CD366/Tim-3, Ly-6C, Ly-6G, NK1.1, TCR-$\gamma\delta$ and TIGIT conjugated with different fluorescent dyes (Tab. 2.1) for 20 min.
Dead cells were stained with Pacific Orange (PacO)-Succinimidyl Ester for 20 min and excluded in the flow cytometric analysis.
For intracellular cytokine staining, cells were restimulated with 1 μl/ml Golgi Plug^{TM} (Brefeldin A) and restimulated with 10 ng/ml PMA and 1 μg/ml ionomycin in adequate medium for 3-5 h depending on the cell type. Cells were then fixed in 4 % PFA, perforated in saponin buffer and stained for granzyme B, IFNγ, IL-2, IL-17, Ki67 and TNFα.
Cells undergoing apoptosis were stained with annexin V-FITC in Annexin V Binding Buffer according to the manufacturer's protocol.

Flow cytometry was performed using a BD LSR II cytometer and analysed with FlowJo software V10.6.0.

2.2.11 Histology

Formalin-fixed and paraffin-embedded liver tissue was cut into 3-4 μm thick sections and stained with Haematoxylin/Eosin (H&E) staining according to standard procedures to evaluate the degree of liver inflammation. All histological scorings were performed by pathologists in a blinded fashion according to the modified hepatitis activity index (mHAI) [157], consisting of four scoring criteria: **A** representing the grade of interface hepatitis, **B** the grade of confluent necrosis, **C** the amount of spotty necrosis/apoptosis and **D** the grade of portal inflammation.

Sirius Red staining was performed according to standard protocols and used to characterise the deposition of collagen fibers. The staining was analysed by quantification of the positive-stained area in liver sections with ImageJ (V1.52).

2.2.12 Immunofluorescent stainings

Freshly frozen liver tissue was cut into 6 μm thick sections and used for immunofluorescent stainings of CK-19, CD45.1, CD326, PD-L1, *Il1b* and *Il6* mRNA, cell nuclei and apoptotic DNA fragmentation.

To localise CD45.1$^+$ cells, sections were fixed 10 min in acetone and blocked with PBS containing 1 % bovine serum albumin (BSA) for 1 h. To stain bile ducts, samples were afterwards incubated with TROMA III antibody diluted 1:200 in PBS 1 h at room temperature (RT) followed by incubation with donkey-αrat-IgG-Alexa Fluor®488 (1:1000 in 1 % BSA/PBS) together with αCD45.1-FITC (1:100 in 1 %BSA/PBS) for 1 h at RT. Finally, nuclei were stained with HOECHST 33258 diluted 1:10 000 in PBS for 1 min.

To stain *Il1b* and *Il6* mRNA on fresh frozen liver samples, *in situ* hybridisation was performed using the RNAscope® Multiplex Fluorescent Assay combined with a co-staining of CK-19. Shortly, sections were fixed with cold 4 % PFA for 15 min and dehydrated in ethanol series from 50 % to 100 %. The tissue was treated with provisioned protease IV for 30 min at RT and unspecific binding was blocked using 1 % BSA and 5 % goat serum in PBS for 1 h. Afterwards, sections were stained with TROMA III and goat-αrat-IgG-Cy5 as described above. Next, samples were incubated with *Il1b* and *Il6* probes and several amplificators at 40 °C following the RNAscope® instructions. Lastly, nuclei were stained with provided DAPI for 1 min.

The surface expression of PD-L1 and CD326 in cultured cholangiocytes was stained directly in cell culture dishes. Remaining medium was removed, cells were fixed in 4 % PFA for 15 min and unspecific binding was blocked with PBS containing 1 % BSA for 1 h. Next, cells were incubated with αCD326-FITC and αPD-L1-PE for 1 h and analysed subsequently.

Apoptotic DNA fragmentation was stained with the TUNEL-method using the In Situ Cell Death Detection Kit according to the developer's protocol. Additionally, co-staining of CK-19 was performed with the TROMA III antibody as described before to identify apoptotic cholangiocytes.

All slides stained with immunofluorescent antibodies were mounted with fluorescence mounting medium and stored at 4 °C.

2.2.13 Real-time qPCR

Total RNA was isolated from liver tissue or cell cultures using the Nucleospin RNA Kit. Due to the low amounts of RNA, mRNA from sorted cholangiocytes was isolated with

the RNeasy Micro Kit. Afterwards, cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit. All kits were used according to the manufacturers' protocols. For quantitative real-time PCR analysis, expression of *Ccl2*, *Ccl20*, *Cd274*, *Col1a1*,*Col3a*,*Cxcl9*, *Cxcl10*, *Foxp3*, *Gzmb*, *Ifng*, *Il1b*, *Il6*, *Il10*, *Il17a*, *Krt19*, *Pdcd1*, *Rorc*, *Tgfb1*, *Tnf* and *Vcam1* mRNA was analysed using TaqMan^{TM} Fast Advanced Master Mix and TaqMan® Gene Expression Assays. The primer sequences are listed in Table 2.3. Target gene expression was normalised to *Hprt* mRNA expression and the fold-induction was quantified by normalisation to control groups using the ΔΔCt method shown in the following equations:

$$\begin{aligned} \Delta\, Ct &= Ct_{\,Hprt} - Ct_{\,target\ gene} \\ \Delta\Delta\, Ct &= \Delta\, Ct_{sample} - \Delta\, Ct_{control\ group} \\ x - fold\ expression &= 2^{-\Delta\Delta\, Ct} \end{aligned}$$

2.2.14 Magnetic resonance imaging

Magnetic resonance imaging (MRI) of Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17A/F$^{-/-}$ mice were performed to monitor the onset and development of liver tumours. Thus, mice were anaesthetised by inhalation of 2 % isoflurane and 98 % oxygen at a flow rate of 500 ml/min and supervised for respiration and body warming during the MRI measurement. Starting with mice at the age of 55 weeks until an age of 65/66 weeks, mice were scanned in 2 week intervals in a small animal 7 T MRI system in the Department of Diagnostic and Interventional Radiology, University Medical Center Hamburg-Eppendorf as described before [158, 159].

2.2.15 Statistical analysis

Statistical analyses were performed with GraphPad Prism (V.6.01) software. If not mentioned otherwise, data are presented as means +/− SD. Differences between 2 groups were calculated using the Mann-Whitney U test or multiple t test for comparing two time-dependent groups. Comparisons between 3 or more groups were performed using ordinary one-way-ANOVA with confidence intervals of 95 %. Significant differences were marked as follows: ****: $p < 0.0001$; ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

3 Results

IL-17 is a cytokine with various functions and effects. It is described as a pathogenic and pro-inflammatory trigger that enhances inflammation in rheumatoid arthritis, psoriasis, asthma and other autoimmune disorders [99, 100]. On the other hand, IL-17 has important anti-microbial features and is indispensable at mucosal barriers for the defence against fungal pathogens and extracellular bacteria [98, 104].
IL-17 producing cells have been associated with the pathogenesis of different cholangiopathies and were found around bile ducts in livers of PBC, PSC, AIH and alcoholic liver disease patients [30, 61, 73]. However, the distinct role of IL-17 in the pathogenesis and progression of cholangiopathies and autoimmune liver diseases remains unclear.
As the specific function of IL-17 in a complex system probably differs depending on the disease setup, we used two different mouse models to analyse the functional role of IL-17 in both acute and chronic experimental cholangitis.

3.1 IL-17 in experimental acute cholangitis

We used K14-OVAp mice as a T cell-mediated, acute model of experimental cholangitis. The K14-OVAp mice express an OVA peptide - the SIINFEKL sequence - under the promoter of keratin 14. Thus, epithelial cells including cholangiocytes, present the OVA peptide on the MHC I. The experimental cholangitis is induced by adoptive transfer of OVA-specific $CD8^+$ T cells from OT-1 mice. To evaluate the role of IL-17A and IL-17F in the induction and course of acute cholangitis *in vivo*, we generated transgenic OT-1 mice lacking the expression of IL-17A/F by cross-breeding.

3.1.1 Characterisation of OT-1 and OT-1/IL-$17^{-/-}$ $CD8^+$ T cells

First, we aimed to investigate, whether OT-1/IL-$17^{-/-}$ $CD8^+$ T cells themselves show an altered phenotype compared to OT-1 $CD8^+$ T cells due to the lack of autocrine IL-17 signalling. To that end, freshly isolated $CD8^+$ T cells from spleens of OT-1 and OT-1/IL-$17^{-/-}$ mice were stimulated via TCR with a combination of αCD3 and αCD28, or antigen-specific

with APCs and the SIINFEKL antigen for 24 h (Fig. 3.1). Both stimulations led to activation of CD8$^+$ T cells and to secretion of cytokines, namely IFNγ, TNFα and IL-17A. Antigen-specific stimulation induced stronger activation of CD8$^+$ T cells shown by increased levels of IFNγ and IL-17A compared to TCR-specific stimulated CD8$^+$ T cells. However, OT-1/IL-17$^{-/-}$ cells secreted similar levels of IFNγ and TNFα compared to IL-17-competent OT-1 CD8$^+$ T cells, but no IL-17A.

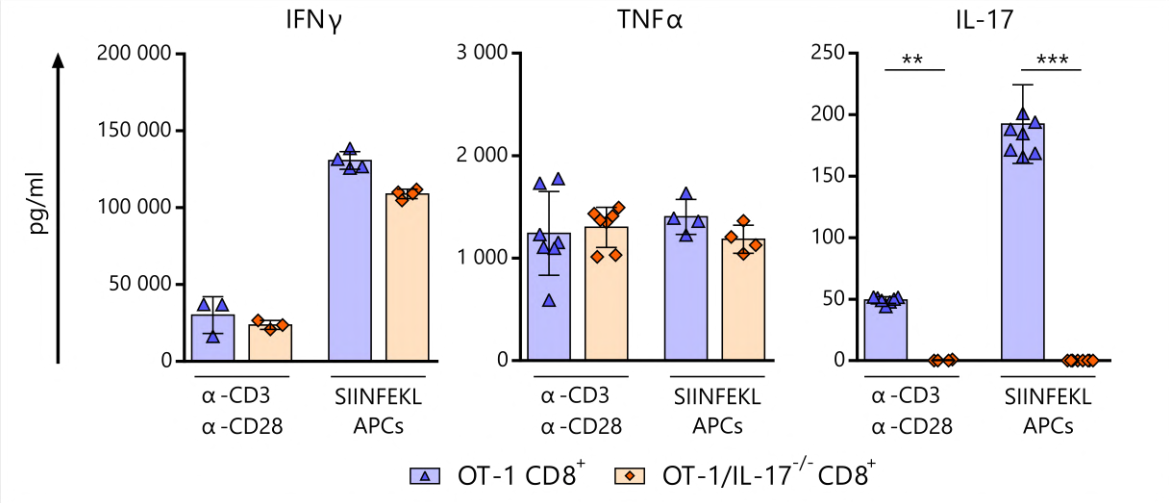

Figure 3.1: OT-1 and OT-1/IL-17$^{-/-}$ CD8$^+$ T cells have similar cytokine expression after restimulation *in vitro*: CD8$^+$ cells were isolated from spleens of OT-1 or OT-1/IL-17$^{-/-}$ donor mice and stimulated with αCD3/αCD28 or APCs/SIINFEKL peptide for 24 h. Cytokines in the supernatants were detected by ELISA.

To further exclude an altered phenotype of OT-1/IL-17$^{-/-}$ CD8$^+$ T cells *in vivo*, various lymphocyte features were analysed *in vitro* (Fig. 3.2). The proliferative capacity of OT-1 and OT-1/IL-17$^{-/-}$ CD8$^+$ T cells after antigen-specific stimulation with SIINFEKL peptide and APCs was similar (Fig. 3.2A) and repetitive stimulation with αCD3 did not lead to differences in the induction of apoptosis (data not shown). Surface expression of markers associated with T cell activation, in particular CD25, CD62L, CD69 and PD-1, were analysed by flow cytometry on freshly isolated OT-1 and OT-1/IL-17$^{-/-}$ CD8$^+$ T cells and did not show any significant differences (Fig. 3.2B). In a co-culture of freshly isolated CD8$^+$ T cells and APCs together with SIINFEKL peptide for 4 h, we analysed the cytotoxicity of antigen-activated CD8$^+$ T cells in different effector/target -cell ratios. The expression of IL-17 in OT-1 CD8$^+$ T cells did not influence their cytotoxicity, reflected by similar amounts of apoptotic APCs in this assay (Fig. 3.2C).

Taken together, the knockout of IL-17 in OT-1 CD8$^+$ T cells did not alter the activation, cytotoxicity or cytokine secretion of OT-1 CD8$^+$ T cells.

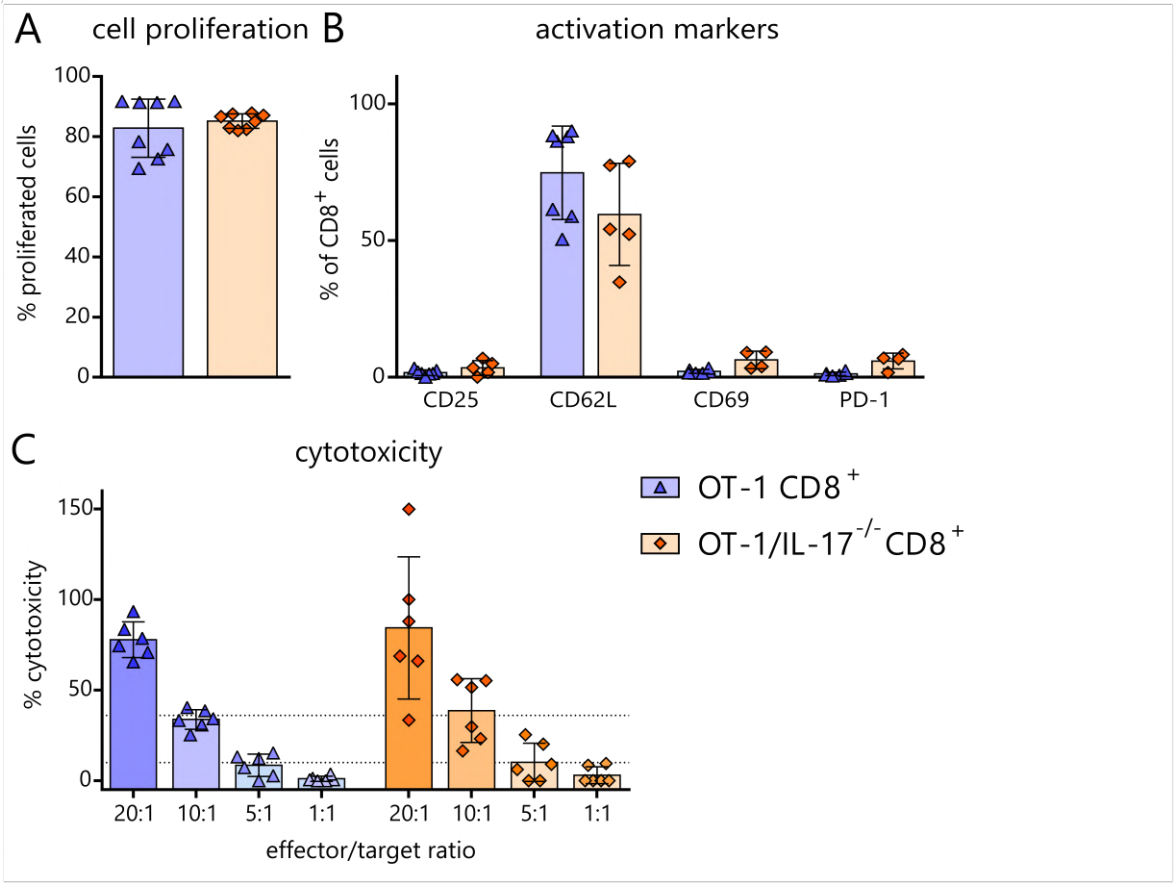

Figure 3.2: OT-1 CD8$^+$ T cells activation, proliferation and cytotoxicity is independent of IL-17 expression *in vitro*: CD8$^+$ T cells were freshly isolated from spleens of OT-1 or OT-1/IL-17$^{-/-}$ donor mice. **(A)** Lymphocytes were stained with CellTrace^{TM} Violet Cell Proliferation Kit, stimulated with APCs/SIINFEKL peptide for 48 h and analysed by flow cytometry. **(B)** Surface activation markers were stained on isolated CD8$^+$ T cells and measured by flow cytometry. **(C)** T cell cytotoxicity was analysed by the release of LDH in a coculture of CD8$^+$ T cells and APCs for 4 h in different effector/target cell ratios.

3.1.2 The role of IL-17 expression in the induction of cholangitis

To elucidate the effects of IL-17 expression on liver disease induction *in vivo*, we used the K14-OVAp mouse model of acute cholangitis. The experimental cholangitis was induced by transferring OVA-specific OT-1 CD8$^+$ T cells or OT-1/IL-17$^{-/-}$ CD8$^+$ T cells into K14-OVAp recipient mice that present the OVA peptide SIINFEKL on cholangiocytes. Within 5 days after adoptive OT-1 CD8$^+$ T cell transfer, K14-OVAp mice presented weight loss and poor body condition compared to PBS-treated control mice (Fig. 3.3A). A specific signal for liver inflammation in humans and mice is the release of liver transaminases by stressed and damaged hepatocytes, which can be measured in serum. Compared to healthy control

mice, adoptive transfer of antigen-specific OT-1 CD8$^+$ T cells resulted in increased serum levels of liver transaminases ALAT and ASAT (Fig. 3.3B). To further assess the degree of liver inflammation, histological evaluation of H&E-stained liver sections was performed by a pathologist in a blinded fashion. Lymphocytic infiltration around bile ducts in K14-OVAp livers and elevated hepatitis activity (mHAI) score were detected after OT-1 T cell transfer (Fig. 3.3C).

Of interest, we observed more severe cholangitis in K14-OVAp recipient mice after adoptive transfer of OT-1/IL-17$^{-/-}$ CD8$^+$ T cells compared to recipient mice transferred with IL-17-competent OT-1 cells. The knockout of IL-17 in transferred CD8$^+$ T cells resulted in enhanced levels of liver transaminases and increased mHAI score compared to the transfer of IL-17-competent CD8$^+$ T cells.

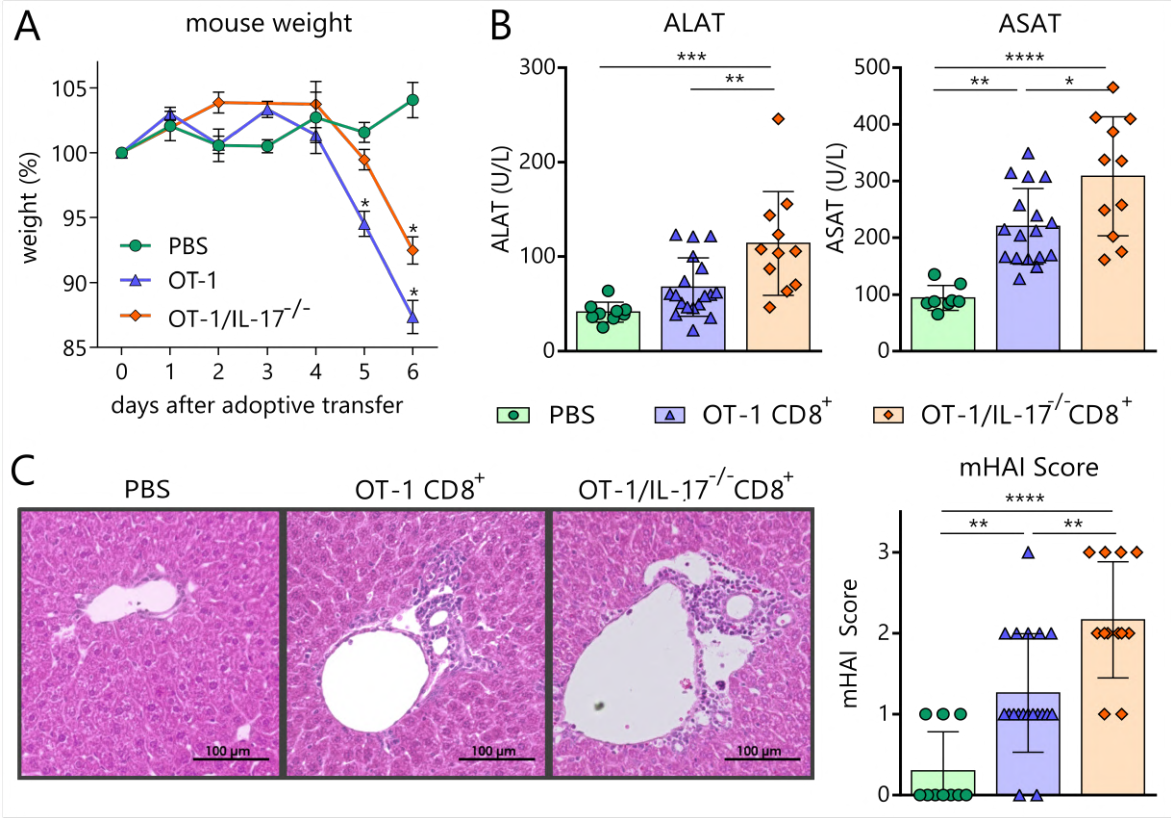

Figure 3.3: Enhanced liver inflammation in mice transferred with OT-1/IL-17$^{-/-}$ CD8$^+$ T cells: 5 days after adoptive transfer of OT-1 or OT-1/IL-17$^{-/-}$ CD8$^+$ T cells into K14-OVAp mice, cholangitis severity in recipient mice was assessed using **(A)** the weight loss relative to the initial mouse weight, **(B)** serum levels of liver transaminases and **(C)** histological activity score of H&E-stained liver sections. ((A) weights are depicted as means +/− SEM, asterisks mark significant differences compared to control group)

To investigate, whether the more severe disease phenotype observed after adoptive transfer of OT-1/IL-17$^{-/-}$ CD8^{+} T cells is associated with increased cell death in K14-OVAp recipients, TUNEL stainings were performed on liver cryosections, which labelled free DNA fragments to indicate apoptotic cell death. Independent of the IL-17 expression in transferred T cells only few apoptotic lymphocytes and rarely apoptotic cholangiocytes and hepatocytes were detected around the portal fields (Fig. 3.4).

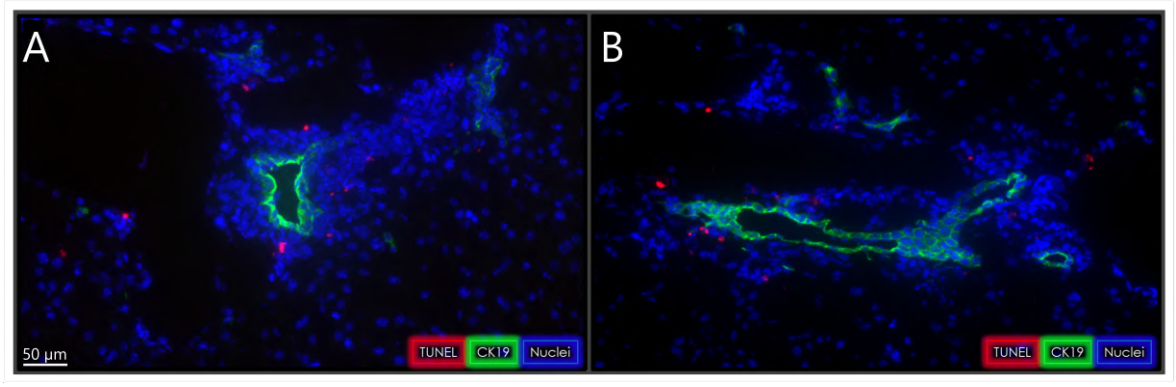

Figure 3.4: Only few cells around inflamed bile ducts are apoptotic in experimental cholangits: After transfer of antigen-specific **(A)** OT-1 or **(B)** OT-1/IL-17$^{-/-}$ CD8+ T cells, liver tissue of K14-OVAp recipient mice was frozen freshly. Cryosections were stained for CK19 (green), nuclei (blue) and free DNA fragments reflecting apoptotic cells (red).

Since differences in apoptosis induction were excluded for the observed phenotype, we performed qPCR analyses of different signature genes for inflammation, chemotaxis and immune-regulation in whole liver tissue of K14-OVAp mice on day 5 after transferring antigen-specific OT-1 or OT-1/IL-17$^{-/-}$ CD8^{+}T cells as well as PBS-treated animals (Fig. 3.5).

Compared to PBS-treated healthy mice, overall pro-inflammatory and chemotactic genes were upregulated in mice with experimental cholangitis (Fig. 3.5A). Only mRNA expression of *Krt19* was clearly downregulated in livers of recipient animals after adoptive transfer of antigen-specific OT-1 and OT-1/IL-17$^{-/-}$ cells. In the liver, Krt19 is specifically expressed by cholangiocytes. However, downregulation of the usually constitutively expressed Krt19 was not associated with apoptotic cholangiocytes, as seen before in Fig. 3.4. The highest expression of *Ccl20*, *Il1b* and *Il6* mRNA was observed in liver tissue of recipient mice after transfer of IL-17-competent OT-1 CD8^{+} T cells (Fig. 3.5B). These cytokines can be produced by cholangiocytes, the target cells of antigen-induced experimental cholangitis. Moreover, mRNA expression of pro-inflammatory *Ifng*, cytotoxic *Gzmb*, activation marker *Pdcd1* and chemotactic *Vcam1* and *Cxcl9* was elevated in mice following OT-1 CD8^{+} T cell transfer compared to PBS-treated control mice. However, the transfer of OT-1 CD8^{+} T cells

lacking IL-17 expression significantly increased the mRNA expression of these genes, which supports the aggravated inflammation seen in these mice.

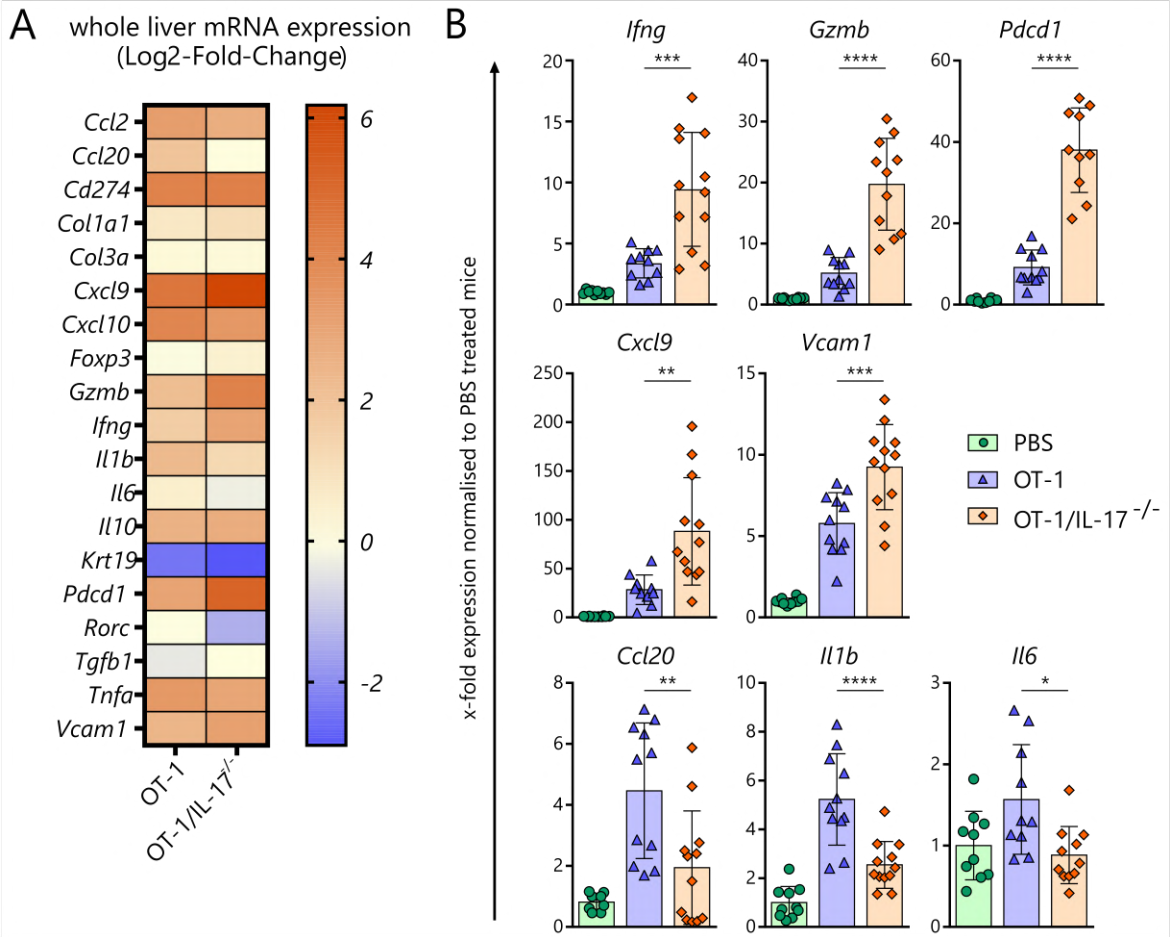

Figure 3.5: Genes relevant for inflammation and immune signalling are upregulated in livers after induction of experimental cholangitis: mRNA expression of pro-inflammatory, chemotactic and immune-regulating genes was investigated by qPCR analysis in K14-OVAp livers 5 days after transferring OT-1 or OT-1/IL-17$^{-/-}$ cells and control animals. **(A)** Log2-fold change of gene expression compared to PBS-treated control mice and **(B)** x-fold expression normalised to PBS-treated control mice showed upregulation of various pro-inflammatory genes and altered expression profile after transfer of IL-17-deficient cells.

As the mRNA expression of various chemokines and chemotactic cytokines, in particular *Ccl2*, *Ccl20*, *Cxcl9*, *Cxcl10*, *Il1b* and *Il6* were elevated after disease induction (Fig. 3.5A), we analysed which cells were infiltrating the site of inflammation and were responsible for the severe inflammation seen in K14-OVAp mice transferred with OT-1/IL-17$^{-/-}$ CD8^{+} T cells. To this end, we isolated liver infiltrating immune cells on day 5 after transfer of OT-1 or OT-1/IL-17$^{-/-}$ CD8^{+} T cells and analysed different endogenous immune cell populations using flow cytometric analysis.

All cell populations were defined after excluding cell debris, doublets and dead cells. The analysis of endogenous cell populations in K14-OVAp recipient mice after disease induction revealed no differences in the infiltration of $CD4^+$ T cells ($CD4^+$, $CD3^+$), NK cells ($NK1.1^+$, $CD3^-$) or B cells ($B220^+$, $CD3^-$), whereas populations of neutrophils ($CD45^+$, $CD11c^{low}$, $CD11b^+$, $Ly6G^+$) and inflammatory monocytes ($CD45^+$, $CD11c^{low}$, $CD11b^+$, $Ly6G^-$, $Ly6C^+$) were increased in inflamed livers after adoptive transfer (Fig. 3.6).

Moreover, after adoptive transfer of IL-17-competent OT-1 $CD8^+$ T cells, we observed specific infiltration of $\gamma\delta$-T cells ($CD3^+$, TCR-$\gamma\delta^+$), NKT cells ($NK1.1^+$, $CD3^+$) and eosinophil granulocytes ($CD45^+$, $CD11c^{low}$, $CD11b^+$, $Ly6G^-$, SSC-A^{high}) into the liver of K14-OVAp recipient mice.

Overall, different infiltration patterns of distinct immune cell populations were observed depending on the expression of IL-17 in disease-inducing, antigen-specific OT-1 T cells. However, infiltrating populations of endogenous immune cells were rather small compared to the high numbers of $CD8^+$ T cells which were recruited into the liver, especially after transfer of OT-1/IL-$17^{-/-}$ $CD8^+$ T cells.

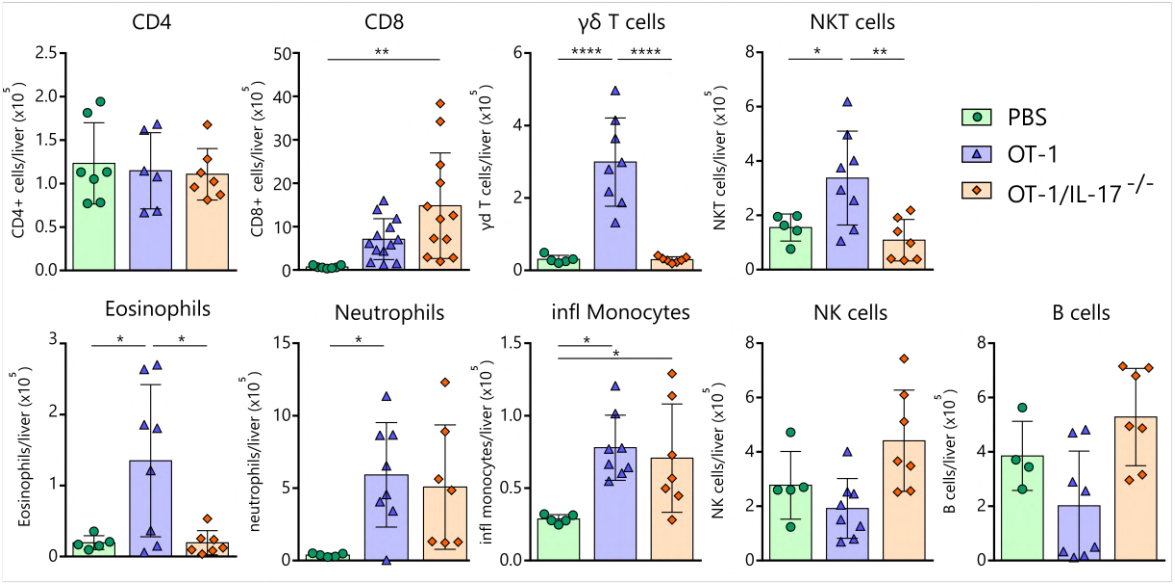

Figure 3.6: Distinct recruitment of endogenous immune cell populations into the liver after transfer of antigen-specific $CD8^+$ T cells: 5 days after adoptive transfer of OT-1 or OT-1/IL-$17^{-/-}$ $CD8^+$ T cells into K14-OVAp mice, liver infiltrating leukocytes were isolated, stained and distinguished by flow cytometric analysis.

3.1.3 The effect of IL-17$^{-/-}$ in cholangitis-inducing CD8^{+} T cells *in vivo*

To differentiate between endogenous K14-OVAp CD8^{+} T cells and transferred, antigen-specific CD8^{+} T cells *in vivo*, we made use of the congenic background of the OT-1 and OT-1/IL-17$^{-/-}$ mice, which express the CD45.1 allele in all haematopoietic cells. On the contrary, endogenous leukocytes of K14-OVAp mice express the CD45.2 variant. The different isoforms can be stained specifically and allow the determination of CD8^{+} T cell origin.

To investigate, whether the observed differences in disease severity were associated with an IL-17-dependent recruitment or localisation of transferred CD8^{+} T cells in the liver, immunofluorescent stainings of CD45.1, CK19 and nuclei were performed on cryo-frozen liver sections from K14-OVAp recipient mice 5 days following induction of experimental cholangitis. The histological analysis showed that antigen-specific CD8^{+} T cells were recruited into the liver and accumulated around the antigen-presenting cholangiocytes (Fig. 3.7). The staining also confirmed that the majority of inflammatory cells around the portal fields consisted of congenic CD45.1^{+} CD8^{+} T cells. Though, no histological differences were observed comparing the adoptive transfer of IL-17-competent and -deficient OT-1 CD8^{+} T cells in regard to the cells localisation (comparison not shown).

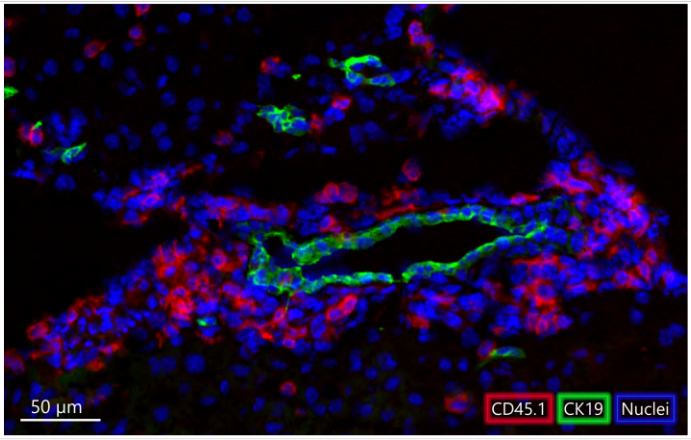

Figure 3.7: Localisation of the congenic OT-1 CD8^{+} T cells in the liver: 5 days after transfer of antigen-specific OT-1 CD8^{+} T cells, liver tissue of K14-OVAp recipient mice was frozen freshly. Cryosections were stained for CD45.1 (red), CK19 (green) and nuclei (blue).

As the cholangitis-inducing OT-1 and OT-1/IL-17$^{-/-}$ CD8^{+} T cells localise close to the cholangiocytes, we next analysed T cell activation and cytokine secretion of the congenic CD8^{+} T cells to investigate their role in cholangitis severity. To this end, lymphocytes from K14-OVAp livers were isolated on day 5 after disease induction. Endogenous K14-OVAp and transferred OT-1 CD8^{+} T cells were separated using the congenic CD45.1 marker. After PMA/ionomycin restimulation, frequencies of cytokine-positive cells were detected by flow

cytometry. Absolute levels of cytokine secretion were measured in cell culture supernatants after TCR-specific restimulation with αCD3 and αCD28 using ELISAs. The majority of cytokines were produced by transferred antigen-specific OT-1 and OT-1/IL-17$^{-/-}$ CD8$^+$ T cells and only to small amount by endogenous T cells (Fig. 3.8). High frequencies of transferred T cells secreted pro-inflammatory IFNγ and cytotoxic granzyme B. High levels of IL-17 secretion were only seen in IL-17-competent CD8$^+$ T cells. Of interest, OT-1/IL-17$^{-/-}$ cells showed increased secretion of granzyme B compared to IL-17-secreting OT-1 CD8$^+$ T cells. The absolute levels of produced cytokines confirmed the flow cytometric analyses.

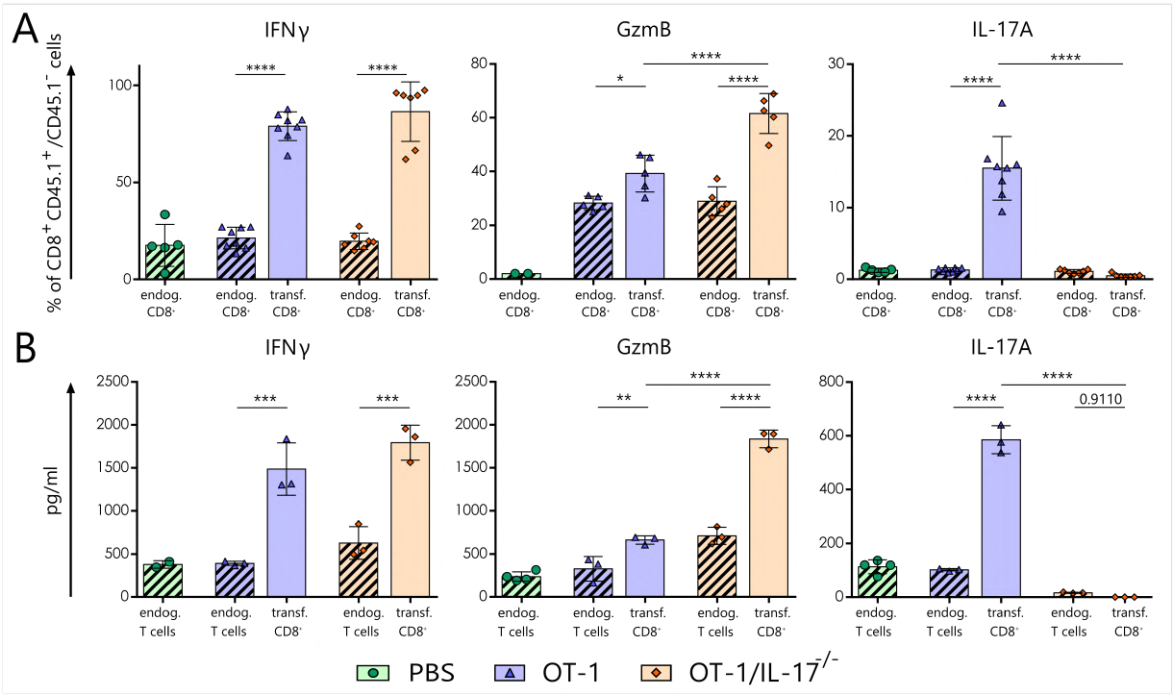

Figure 3.8: IL-17-deficient, antigen-specific OT-1 CD8$^+$ T cells exhibit an altered cytokine secretion *ex vivo*: 5 days following disease induction, lymphocytes were isolated from K14-OVAp livers and congenic cells were separated from endogenous immune cells via CD45.1 expression. Lymphocytes were restimulated **(A)** with PMA/ionomycin for 3 h and analysed by flow cytometry or **(B)** with αCD3/αCD28 for 24 h and cytokine levels in the supernatants were analysed by ELISA.

Since the transferred CD8$^+$ T cells were highly activated in the liver, we next asked whether the antigen-specific OT-1 and OT-1/IL-17$^{-/-}$ CD8$^+$ T cells were only recruited into the liver or also into lymphoid organs. Thus, isolated lymphocytes from livers, spleens and liver-draining lymph nodes from K14-OVAp recipient mice were analysed by flow cytometry following T cell transfer as described before. Overall, the congenic CD8$^+$ T cells were found in liver, spleen and lymph nodes after transfer (Fig. 3.9). Interestingly, increased frequencies of antigen-specific CD8$^+$ T cells were detected in recipient livers and spleens after transfer

of OT-1 cells lacking IL-17 expression compared to transfer of IL-17-competent $CD8^+$ T cells.

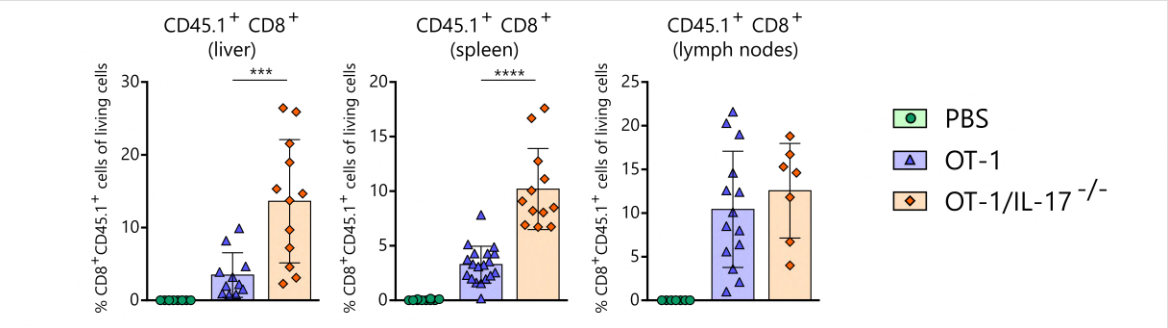

Figure 3.9: Less congenic OT-1 $CD8^+$ T cells were found in K14-OVAp livers and spleens after transfer of IL-17-competent cells: 5 days after disease induction lymphocytes were isolated from K14-OVAp livers, spleens and liver-draining lymph nodes. Congenic $CD45.1^+$ $CD8^+$ T cells were detected using flow cytometry.

To further characterise the different amount of transferred cells in K14-OVAp recipient livers, we performed time kinetic analysis and determined the absolute number of OT-1 or OT-1/IL-$17^{-/-}$ cells in the liver at different days after adoptive transfer. Only very few congenic cells were detected in K14-OVAp recipient livers 2 days after induction of experimental cholangitis (Fig. 3.10A). On day 3 following disease induction, we found similar numbers of recruited OT-1 and OT-1/IL-$17^{-/-}$ $CD8^+$ T cells. On day 5 after adoptive transfer, the absolute numbers of antigen-specific OT-1 $CD8^+$ T cells in the liver exceeded the number of initially transferred cells, namely 200,000 congenic $CD8^+$ T cells, with significantly increased numbers of OT-1/IL-$17^{-/-}$ $CD8^+$ T cells compared to OT-1 $CD8^+$ T cells.

Thus we analysed the proliferation and apoptosis of transferred OT-1 and OT-1/IL-$17^{-/-}$ $CD8^+$ T cells after induction of experimental cholangitis. Surface expression of CD25, the α-subunit of the IL-2 receptor, which is not only a marker for T cell activation but also an indicator for T cell proliferation, was upregulated on both IL-17-competent and -deficient OT-1 T cells already 3 days after adoptive transfer (Fig. 3.10B). Importantly, decreased expression of CD25 on IL-17-competent OT-1 $CD8^+$ T cells was observed 5 days after adoptive T cell transfer, whereas CD25 expression on OT-1/IL-$17^{-/-}$ $CD8^+$ T cells remained stable. Additionally, intracellular stainings of IL-2 and Ki67, marker of cell proliferation, confirmed the decreased proliferation of transferred IL-17-expressing OT-1 $CD8^+$ T cells compared to OT-1/IL-$17^{-/-}$ $CD8^+$ T cells. Furthermore, annexin V staining showed increased rate of apoptosis in IL-17 expressing OT-1 cells (Fig. 3.10C).

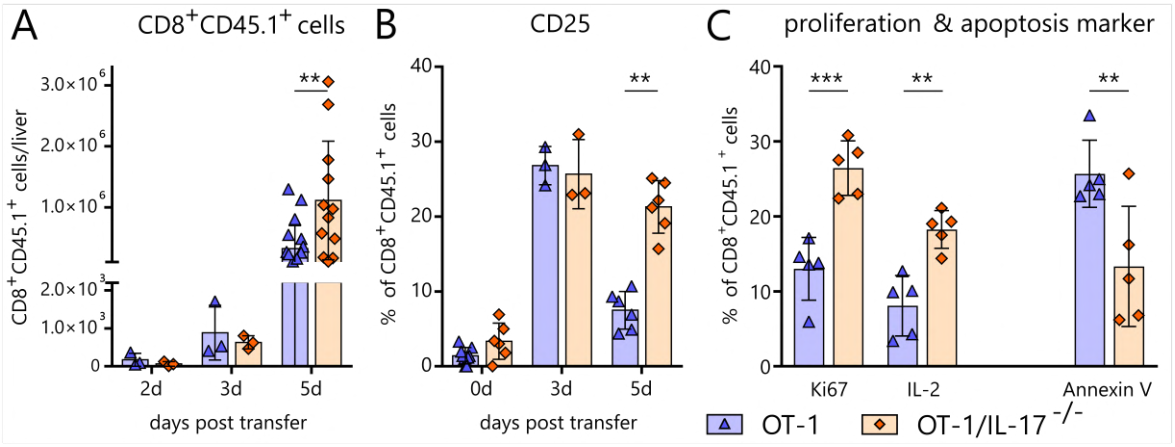

Figure 3.10: IL-17-deficient OT-1 CD8$^+$ T cells show higher proliferation in K14-OVAp livers: Lymphocytes were isolated from livers of K14-OVAp mice transferred with OT-1 or OT-1/IL-17$^{-/-}$ cells and analysed by flow cytometry. **(A)** Absolute cell numbers of congenic CD8$^+$ T cells were detected in livers 2, 3 and 5 days after transfer. **(B)** Surface expression of CD25 and **(C)** annexin V were measured immediately by flow cytometry, whereas intracellular levels of Ki67 and IL-2 were detected after 3 h restimulation with PMA/ionomycin. Frequencies of annexin V, Ki67 and IL-2 were analysed on day 5 after disease induction.

To investigate, whether the decreased proliferation and enhanced apoptosis rate seen in IL-17-competent OT-1 T cells after disease induction were mediated by other cells via co-inhibitory receptors, OT-1 and OT-1/IL-17$^{-/-}$ CD8$^+$ T cells were stained for CTLA4, LAG3, TIGIT, Tim3 and PD-1 using flow cytometric analysis on day 5 after adoptive transfer. We observed expression of all co-inhibitory receptors on the surface of transferred OT-1 CD8$^+$ T cells (Fig. 3.11) independent of their IL-17 expression. Interestingly, notably high surface expression of the co-inhibitory PD-1 was observed on both IL-17-competent and -deficient OT-1 CD8$^+$ T cells.

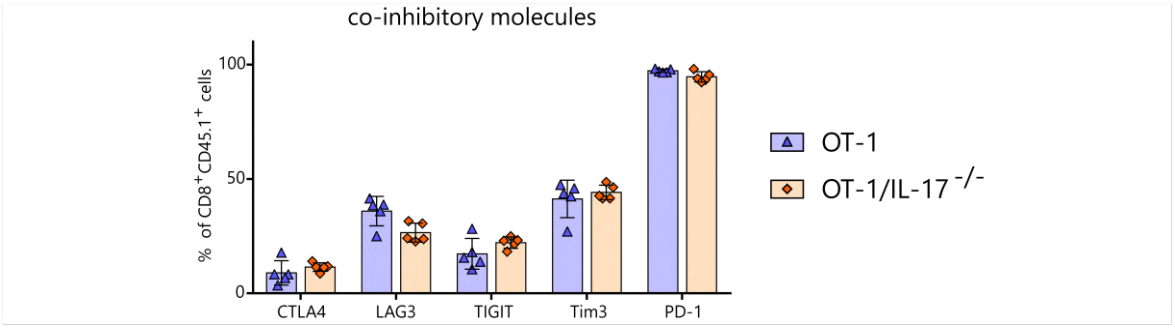

Figure 3.11: Surface expression of co-inhibitory markers on transferred OT-1 CD8$^+$ T cells *ex vivo* is independent of IL-17 expression: Congenic lymphocytes were isolated from livers of K14-OVAp mice transferred with OT-1 or OT-1/IL-17$^{-/-}$ CD8$^+$ T cells and analysed by flow cytometry for the surface expression of different co-inhibitory molecules.

3.1.4 Characterisation of cholangiocytes during inflammation

In K14-OVAp mice, cholangiocytes present the SIINFEKL antigen on their surface via MHC I. After adoptive transfer, antigen-specific OT-1 $CD8^+$ T cells accumulate around the bile ducts (Fig. 3.7). This makes cholangiocytes the main target cells in this model of experimental cholangitis. Hence, cholangiocytes are exposed to high levels of pro-inflammatory and cytotoxic cytokines secreted by disease-inducing OT-1 T cells.

Adoptive transfer of OT-1 $CD8^+$ T cells lacking IL-17 expression led to severe cholangitis and elevated liver damage, accompanied by higher proliferative capacity and stronger pro-inflammatory and cytotoxic activation of transferrred $CD8^+$ T cells (Fig. 3.8, Fig. 3.10). Many co-inhibitory receptors were upregulated on the surface of transferred antigen-specific OT-1 $CD8^+$ T cells. Thus, we next elucidated, whether activated cholangiocytes might contribute to the altered regulation of transferred T cells.

To assess the activation of cholangiocytes during cholangitis, cryo-frozen liver sections of K14-OVAp recipient mice were stained 5 days following adoptive transfer for mRNA expression of *Il1b* and *Il6*, cytokines secreted by activated cholangiocytes [30, 61], using *in situ* hybridisation techniques. After induction of experimental cholangitis, positive signals of both *Il1b* and *Il6* mRNA were observed in cholangiocytes, which were identified by the specific expression of CK19 (Fig. 3.12). Expression of the pro-inflammatory cytokines indicate an activated phenotype of cholangiocytes challenged by the transferred OT-1 $CD8^+$ T cells *in vivo*.

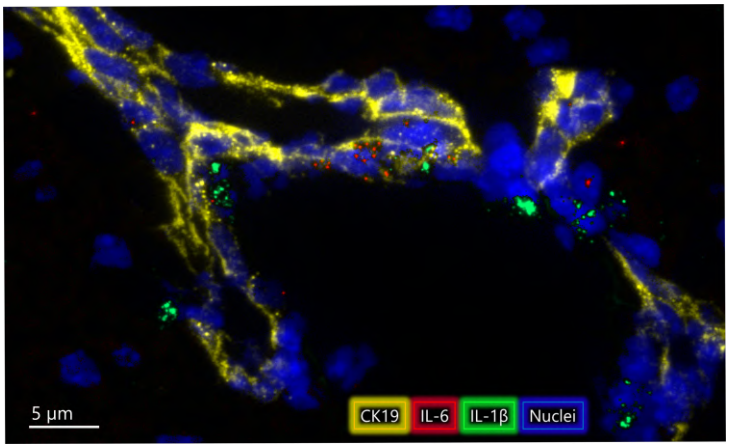

Figure 3.12: Cholangiocytes are activated during experimental cholangitis and express pro-inflammatory cytokine mRNA: After transfer of antigen-specific OT-1 $CD8^+$ T cells, cryo-frozen liver sections of K14-OVAp recipient mice were stained for CK19 (yellow), expression of *Il1b* (green) and *Il6* (red) mRNA and nuclei (blue) by *in situ* hybridisation.

To further analyse the activation of cholangiocytes and their contribution to the differences in observed cholangitis severity after transfer of IL-17-competent or -deficient OT-1 $CD8^+$ T cells, epithelial cells from K14-OVAp recipient livers were isolated 5 days after disease induction. Cholangiocytes were identified using the epithelial cell marker CD326 (EpCAM) which - in the liver - is specific for biliary epithelial cells. $CD326^+$ cells were stained and analysed using flow cytometry. To perform qPCR analysis, isolated cells were purified by flow cytometric based cell sorting of the $CD326^+/CD45^-$ population.
Compared to healthy control mice, cholangiocytes from mice with experimental cholangitis exhibited elevated surface and mRNA expression of *Cd247*, encoding for the inhibitory PD-1 ligand (PD-L1) (Fig. 3.13). However, expression of *Cd274*, as well as *Il6* mRNA was significantly higher in cholangiocytes challenged with IL-17-competent OT-1 $CD8^+$ T cells compared to cholangiocytes after transferring OT-1/IL-$17^{-/-}$ $CD8^+$ T cells . In accordance with the mRNA expression, highest frequencies of intracellular IL-6 and surface PD-L1 were observed on cholangiocytes after transfer of OT-1 $CD8^+$ T cells by flow cytometric analysis (Fig. 3.13B).

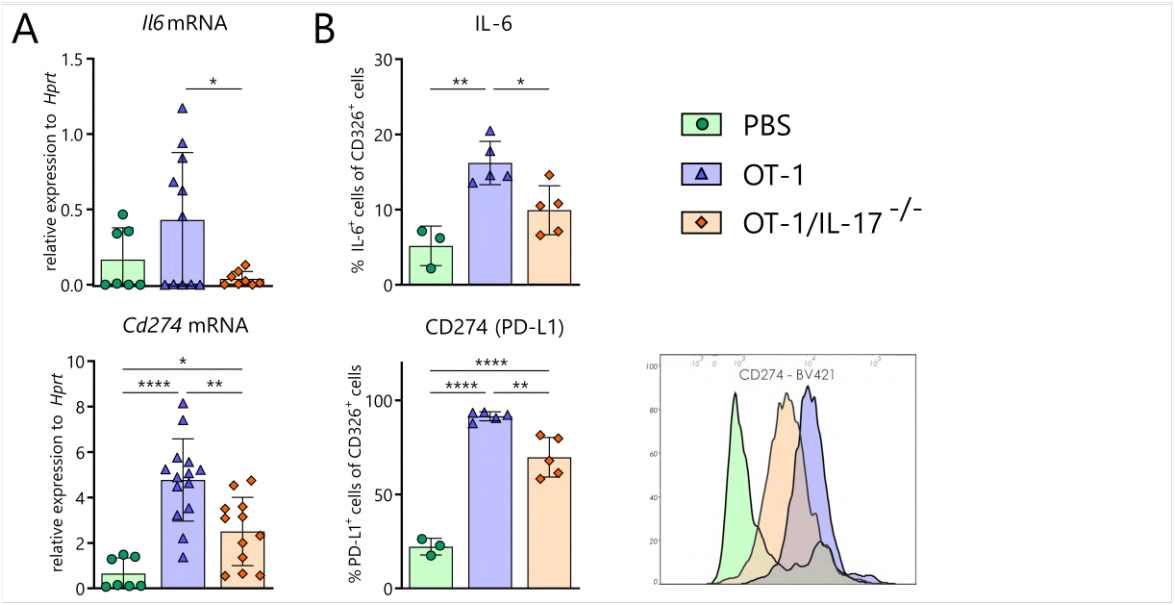

Figure 3.13: Activation of cholangiocytes during cholangitis is dependent on IL-17: Cholangiocytes were isolated from K14-OVAp livers 5 days after transfer of antigen-specific OT-1 $CD8^+$ T cells and identified by the expression of CD326. **(A)** mRNA expression of different genes was analysed in $CD326^+$ $CD45^-$ sorted cells by qPCR analysis. **(B)** Surface expression of CD274/PD-L1 was analysed immediately by flow cytometry, intracellular levels of IL-6 after 5 h PMA/ionomycin restimulation.

3.1.5 Functional role of IL-17 for cholangiocyte activation

Upon stimulation, cholangiocytes not only upregulate inhibitory surface molecules, but are also able to release cytokines and chemokines that can impact the activation of T cells. Since the activation of cholangiocytes and their upregulation of inhibitory PD-L1 *in vivo* was dependent on IL-17 expression by transferred OT-1 CD8$^+$ T cells, we further determined the functional role of IL-17 for the immune-regulating ability of cholangiocytes.
To this end, primary mouse cholangiocytes, which present the OVA peptide, were challenged with antigen-specific OT-1 or OT-1/IL-17$^{-/-}$ CD8$^+$ T cells *in vitro* and expression and secretion of different cytokines and chemokines were measured by qPCR analysis and ELISAs.

Cholangiocytes encountered with IL-17-competent OT-1 CD8$^+$ T cells secreted the highest levels of CCL20 and IL-6, indicating a stronger activation compared to cholangiocytes co-cultured with OT-1/IL-17$^{-/-}$ CD8$^+$ T cells (Fig. 3.14A). Moreover, IL-17-competent OT-1 CD8$^+$ T cells exhibited decreased expression of IFNγ than OT-1/IL-17$^{-/-}$ T cells when co-cultured with cholangiocytes, suggesting either inhibition or weaker activation of IL-17-expressing CD8$^+$ T cells. The relative expression of *Ifng*, *Il17a* and *Il6* mRNA confirmed levels of secreted cytokines.

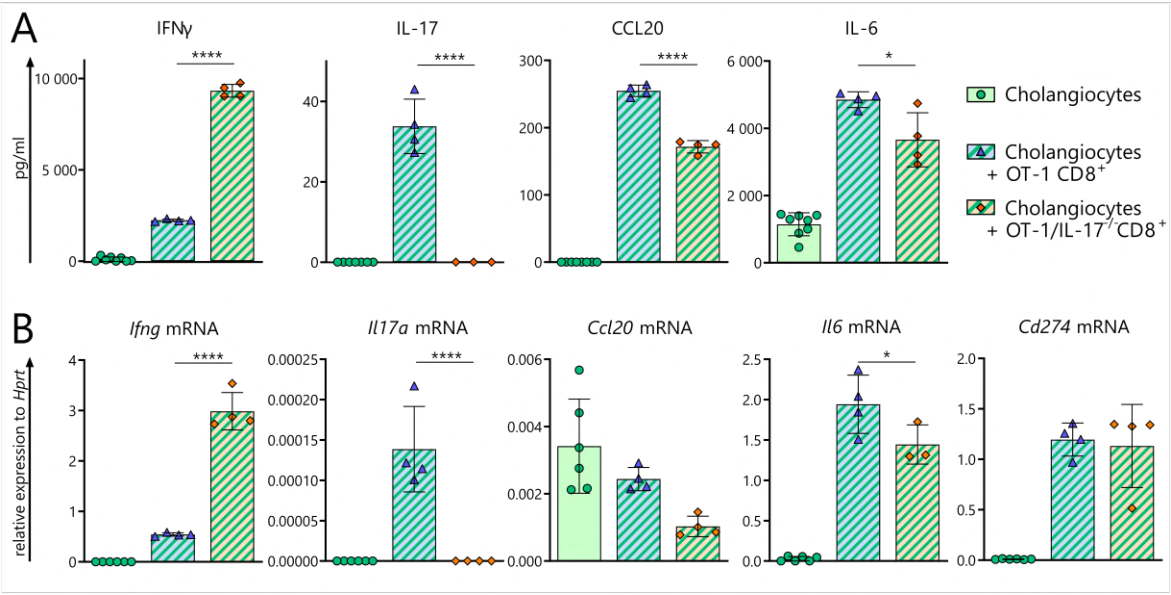

Figure 3.14: Stimulation of cholangiocytes with antigen-specific CD8$^+$ T cells *in vitro* is dependent on IL-17 expression: Cultivated mouse cholangiocytes were stimulated with freshly isolated CD8$^+$ T cells from spleens of OT-1 or OT-1/IL-17$^{-/-}$ donor mice for 48 h. **(A)** Absolute levels of cytokines in culture supernatants were detected by ELISA. Cells from co-cultures were harvested and **(B)** mRNA expression of different genes was analysed by qPCR.

To further characterise the IL-17-dependent activation of cholangiocytes observed *in vitro* (Fig. 3.14) and *in vivo* (Fig. 3.13), we investigated the expression of PD-L1 and different chemokines after stimulation of primary mouse cholangiocytes with pro-inflammatory cytokines *in vitro*. In accordance with previous findings [30], we could show that upon stimulation with pro-inflammatory IFNγ, cholangiocytes upregulated mRNA expression and secretion of chemokines and ligands, namely CCL2 and PD-L1 (Fig. 3.15). Stimulation of primary mouse cholangiocytes with IL-17A led to increased secretion of CCL20 and IL-6 (Fig. 3.15B). Notably, the expression of *Cd274* mRNA was highest, when cholangiocytes were stimulated with a combination of IFNγ and IL-17A.

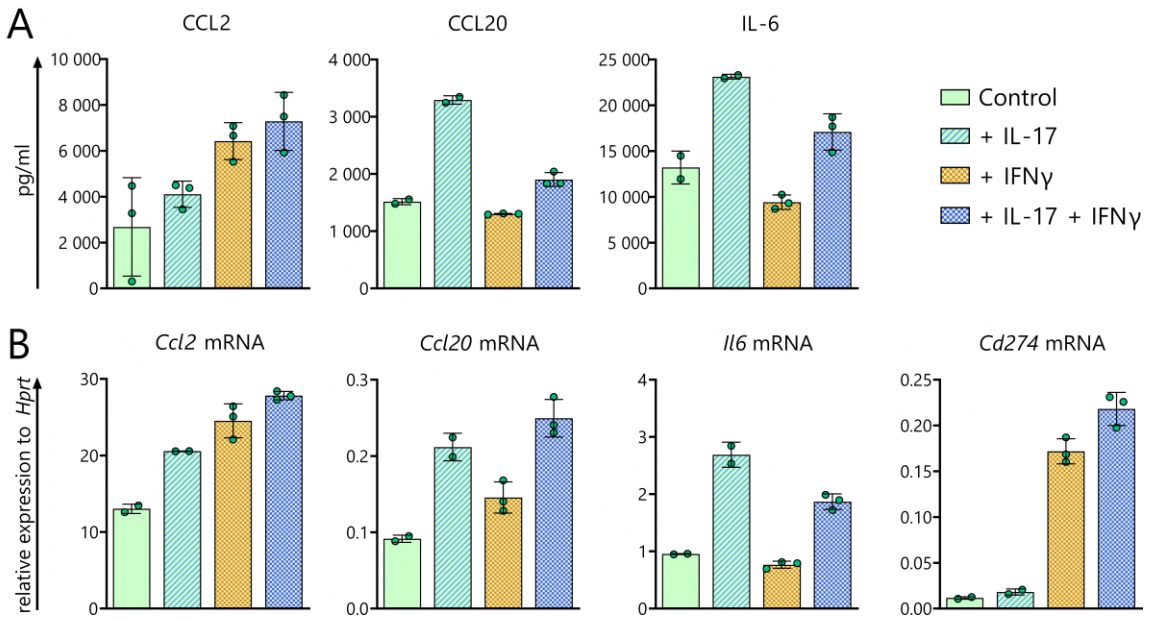

Figure 3.15: Primary cholangiocytes are sensitive for IL-17A and IFNγ: Primary mouse cholangiocytes were isolated from K14-OVAp mice. Cultivated cells were grown 80 % confluent and stimulated with IL-17A and/or IFNγ for 24 h. **(A)** Cytokine and chemokine levels in culture supernatants were detected by ELISA. **(B)** mRNA expression of *Ccl2*, *Ccl20*, *Il6* and *Cd274* was analysed by qPCR analysis.

To investigate the protein expression of PD-L1, primary cholangiocytes were stimulated with pro-inflammatory cytokines and analysed by immunofluorescent staining (Fig. 3.16). Although the staining only allowed qualitative, but no quantitative analysis, a clear upregulation of surface PD-L1 was detected after stimulation with IFNγ and IFNγ + IL-17A.

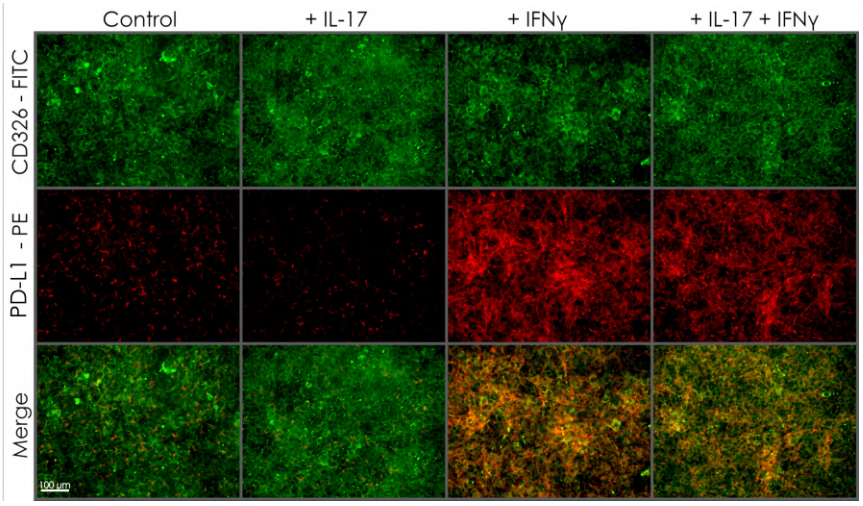

Figure 3.16: Mouse cholangiocytes upregulate surface PD-L1-dependent on IFNγ: Cultivated mouse cholangiocytes were grown 80 % confluent and stimulated with IL-17 and/or IFNγ for 24 h. Cells were stained with fluorescent antibodies against CD326 (green) and PD-L1 (red).

Activated cholangiocytes do not only secrete chemokines, they also express various cytokines that can influence the activation of antigen-specific OT-1 CD8$^+$ T cells. *In vivo*, expression of *Il1b* and *Il6* mRNA was observed in activated cholangiocytes after induction of experimental cholangitis in K14-OVAp mice (Fig. 3.12). Both IL-1β and IL-6 in combination with TGFβ were shown to promote the differentiation of naïve CD8$^+$ T cells into Tc17 cells [114, 116]. To investigate, whether cytokines secreted by stressed cholangiocytes have an impact on the differentiation of OT-1 and OT-1/IL-17$^{-/-}$ CD8$^+$ T cells and therefore influence the severity of cholangitis observed in K14-OVAp mice, we stimulated IL-17-competent and -deficient OT-1 CD8$^+$ T cells with APCs, SIINFEKL antigen and additional cytokines for 24 h *in vitro*.

The stimulation of CD8$^+$ T cells with additional TGFβ or IL-6 reduced the expression of IFNγ, but not IL-17 (Fig. 3.17). IL-17 expression was highly enriched in OT-1 CD8$^+$ T cells, when stimulated in the presence of TGFβ and IL-6, whereas the IFNγ expression was highly reduced. However, the reduced secretion of IFNγ was observed independent of IL-17 expression in OT-1 CD8$^+$ T cells.

In summary, the antigen-specific binding of OT-1 CD8$^+$ T cells to the SIINFEKL-presenting cholangiocytes was sufficient to induce T cell activation. The lack of IL-17 expression in OT-1 CD8$^+$ T cells led to reduced cytokine and chemokine secretion from primary cholangiocytes and decreased inhibitory function. Moreover, cytokines released by activated cholangiocytes affected CD8$^+$ T cell activation and their pro-inflammatory potential.

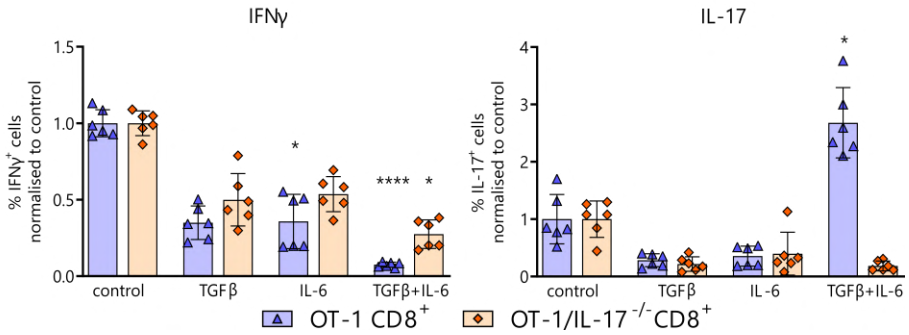

Figure 3.17: Stimulation of CD8$^+$ T cells with IL-6 and TGFβ influences T cells activation: CD8$^+$ T cells were isolated from spleens of OT-1 and OT-1/IL-17$^{-/-}$ mice and stimulated with APCs, SIINFEKL antigen and additional IL-6 and/or TGFβ for 24 h. Intracellular expression of IFNγ and IL-17A was stained after 3 h PMA/ionomycin restimulation and analysed by flow cytometry. All frequencies are normalised to CD8$^+$ T cells without cytokine stimulus (controls) and asterisks mark significant differences compared to control groups.

3.1.6 Blocking the PD-1/PD-L1 axis in experimental cholangitis

The PD-1/PD-L1 interaction seems to be crucial for the regulation of T cell activation and cholangiocyte protection. CD8$^+$ T cells upregulate the co-inhibitory receptor PD-1 after activation, which was observed independent of IL-17 expression (Fig. 3.11). On the other side, cholangiocytes are able to express the corresponding ligand PD-L1 on their surface, especially after IFNγ exposition. We could show that the upregulation of PD-L1 expression *in vivo* was dependent on IL-17 (Fig. 3.13). The impaired inhibition of T cells by cholangiocytes resulted in enhanced activation, cytotoxicity and proliferation of OT-1/IL-17$^{-/-}$ CD8$^+$ T cells *in vivo* and could explain why animals transferred with IL-17-deficient CD8$^+$ T cells exhibited more severe experimental cholangitis. To confirm this hypothesis, we next investigated the development of cholangitis after adoptive transfer of OT-1/PD-1$^{-/-}$ CD8$^+$ T cells.

Transfer of PD-1-lacking OT-1 CD8$^+$ T cells induced even more severe cholangitis compared to IL-17-deficient OT-1 CD8$^+$ T cells in recipient animals. Rapid weight loss and highly increased serum levels of liver transaminases were observed already on day 4 after adoptive transfer (Fig. 3.18A, B). In line with that, higher pathological inflammation scores were detected in H&E-stained liver sections of mice transferred with OT-1/PD-1$^{-/-}$ CD8$^+$ T cells (Fig. 3.18C).

To further analyse this phenotype, whole liver mRNA levels of key genes found in former analyses were investigated (Fig. 3.19). Expression of *Gzmb* mRNA was highly increased, while levels of *Ifng* mRNA were not altered compared to the transfer of OT-1/IL-17$^{-/-}$ CD8$^+$

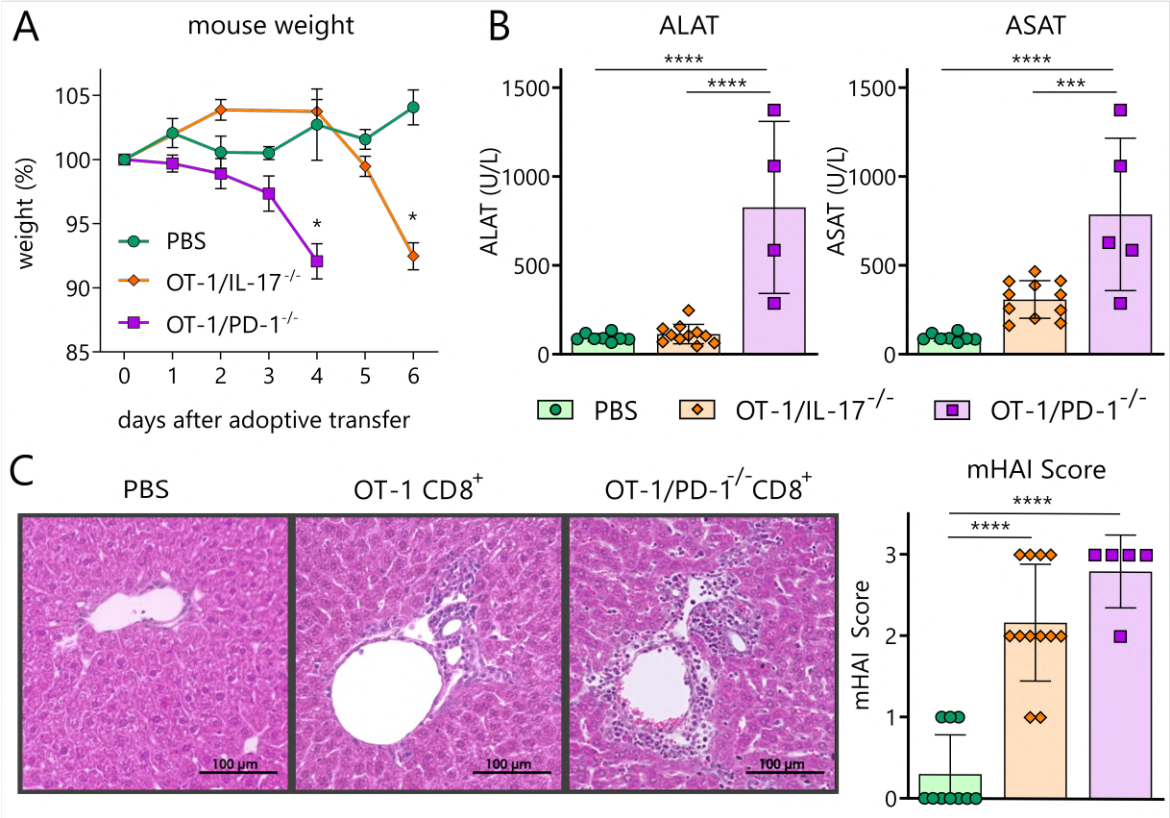

Figure 3.18: Severe liver inflammation in mice transferred with OT-1/PD-1$^{-/-}$ CD8^{+} T cells: 4 days after adoptive transfer of OT-1/PD-1$^{-/-}$ CD8^{+} T cells into K14-OVAp mice, cholangitis severity was assessed in recipient mice using **(A)** the relative weight loss, **(B)** serum levels of liver transaminases and **(C)** mHAI score of H&E-stained liver sections. ((A) weights are depicted as means +/− SEM, asterisks mark significant differences compared to control group)

T cells. Moreover, not only mRNA expression of the co-inhibitory receptor *Pdcd1*, but also its ligand *Cd274* were significantly reduced in recipient livers 4 days after disease induction with OT-1/PD-1$^{-/-}$ CD8^{+} T cells.

Concisely, our data showed that the PD-1/PD-L1 interaction between transferred OT-1 T cells and cholangiocytes is an important regulatory process and is crucial to control T cell expansion and activation in the context of antigen-specific induced experimental cholangitis.

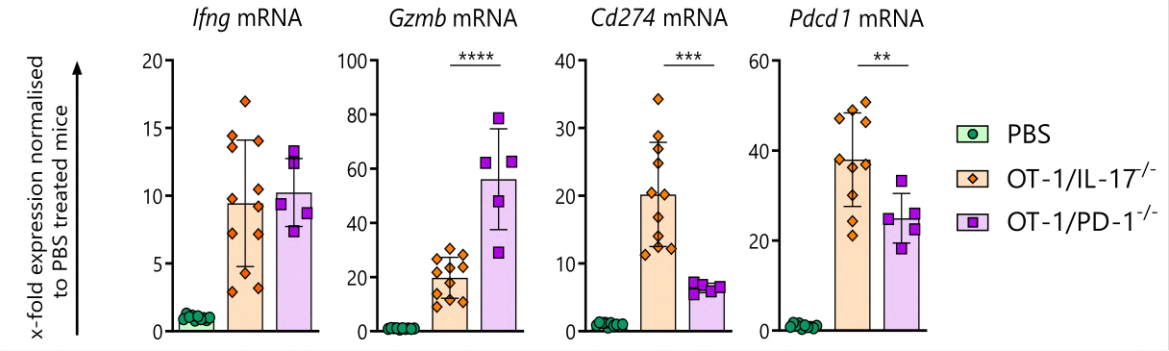

Figure 3.19: Transfer of OT-1/PD-1$^{-/-}$ CD8^{+} T cells induces cytotoxic gene expression in whole liver tissue: 4 days after induction of experimental cholangitis by transfer of OT-1/PD-1$^{-/-}$ CD8^{+} T cells, x-fold mRNA expression normalised to PBS-treated control mice was analysed in whole liver tissue using qPCR.

3.1.7 Summary - IL-17 in experimental acute cholangitis

The distinct role of IL-17 in the induction and progression of cholangitis was poorly defined so far. Taken together, we could show that the adoptive transfer of cholangitis-inducing OT-1 CD8^{+} T cells lacking IL-17 expression led to an increased severity of experimental cholangitis. This phenotype was driven by highly activated and cytotoxic OT-1/IL-17$^{-/-}$ CD8^{+} T cells and their uncontrolled proliferation.

In contrast, IL-17-competent OT-1 CD8^{+} T cells were able to induce enhanced activation of cholangiocytes. By upregulation of PD-L1 and secretion of chemokines and cytokines, cholangiocytes were able to protect from liver inflammation through inhibition of T cell expansion and cytotoxicity.

3.2 IL-17 in chronic cholangitis

IL-17 was shown to promote fibrosis as well as chronic inflammation [100, 120, 121]. To investigate the effects of IL-17 in a chronic mouse model of sclerosing cholangitis, the well-established Mdr2$^{-/-}$ mouse model was used. Knockout of the canalicular phospholipid flippase Mdr2 leads to accumulation of bile acids around the bile ducts resulting in a toxic cholangiocyte damage, chronic inflammation and the formation of periportal fibrosis. By cross-breeding, we generated Mdr2$^{-/-}$ mice that additionally lack the expression of IL-17A and IL-17F to investigate the role of IL-17 in the development and long term disease progression of chronic liver inflammation, fibrogenesis and tumour progression. Due to a weaker development of liver inflammation and fibrosis, also liver tumours arise slower in male Mdr2$^{-/-}$ mice. Hence, only female mice were analysed in this experiment. These data were generated together with Moritz Taube and data are labelled accordingly.

3.2.1 IL-17 in the development of cholangitis and biliary fibrosis

The disease development in Mdr2$^{-/-}$ mice can be divided into different stages. At very young age (1-2 months), Mdr2$^{-/-}$ mice suffer from the initial toxic liver damage and acute inflammation. Only few weeks later, the severe portal inflammation changes into a milder, but chronic cholangitis accompanied by established periductal fibrosis around 3-4 months of age. From 9-12 months of age on, mice suffer from severe sclerosing cholangitis and spontaneous development of hepatocellular carcinoma [148, 150].

To investigate the role of IL-17 in the development and maintenance of chronic inflammation and fibrosis, we analysed Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice at different stages of disease: (1) in the early phase of cholangitis (5 weeks old) featured by acute, severe inflammation, but low fibrosis; (2) in an intermediate phase (15 weeks old) with established fibrosis and (3) in a late stage of liver cirrhosis and tumour development (>65 weeks old).

First, we explored, whether the constitutive knockout of IL-17 in Mdr2$^{-/-}$ mice influences the overall body condition and development of inflammation. To this end, we monitored the body weight over time, but did not detect any differences between Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice (Fig. 3.20A). In line with that, serum levels of liver transaminases did not differ between the groups (Fig. 3.20B).

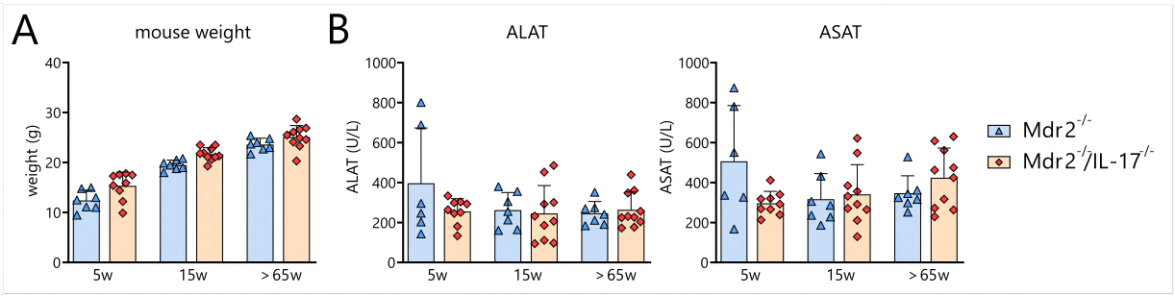

Figure 3.20: Constitutive knockout of IL-17 in Mdr2$^{-/-}$ mice does not alter severity of inflammation: Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice were monitored over months for their **(A)** body weight and **(B)** serum levels of liver transaminases.

To further characterise the liver damage over time, sections of the livers were analysed using H&E and Sirius Red stainings. Blinded scoring by a pathologist showed that the histological activity score decreased with age, as the acute inflammation changes to a chronic phenotype (Fig. 3.21B). In contrast, the deposition of collagen fibers around the portal fields, stained by the Sirius Red agent, increases over time (Fig. 3.21C). Interestingly, only in the very early phase of fibrosis and inflammation, Mdr2$^{-/-}$/IL-17$^{-/-}$ mice exhibited a milder disease phenotype compared to IL-17-competent Mdr2$^{-/-}$ mice, in particular a lower mHAI score and reduced Sirius Red-stained area were observed.

Since the inflammation in Mdr2$^{-/-}$ mice is induced by a toxic-mediated damage of cholangiocytes, different immune populations are recruited to the site of inflammation. IL-17 is known to induce the secretion of neutrophil and NK cell attracting chemokines [104, 160]. To investigate, whether the expression of IL-17 influences the recruitment in experimental chronic cholangitis, we isolated leukocytes from Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ livers at different ages and analysed various immue cell populations by flow cytometric analysis (Fig. 3.22). All cell populations were defined after excluding cell debris, doublets and dead cells. Many of the infiltrating immune cells were CD3^{+} T lymphocytes, but the recruitment was independent of IL-17 expression. Of interest, at an age of 5 weeks Mdr2$^{-/-}$/IL-17$^{-/-}$ mice showed increased numbers of $\gamma\delta$ T cells (CD3^{+}, TCR-$\gamma\delta^{+}$) , but reduced recruitment of neutrophils (CD3^{-}, Ly6G^{+}). Whilst the infiltration of neutrophils did not altered to later time points between Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice, recruitment of $\gamma\delta$ T cells into livers of IL-17-deficient mice remained increased over time. The infiltration of NK cells (CD3^{-}, NK1.1^{+}) into the liver tended to increase over time, but was only significantly elevated in young Mdr2$^{-/-}$/IL-17$^{-/-}$ mice.

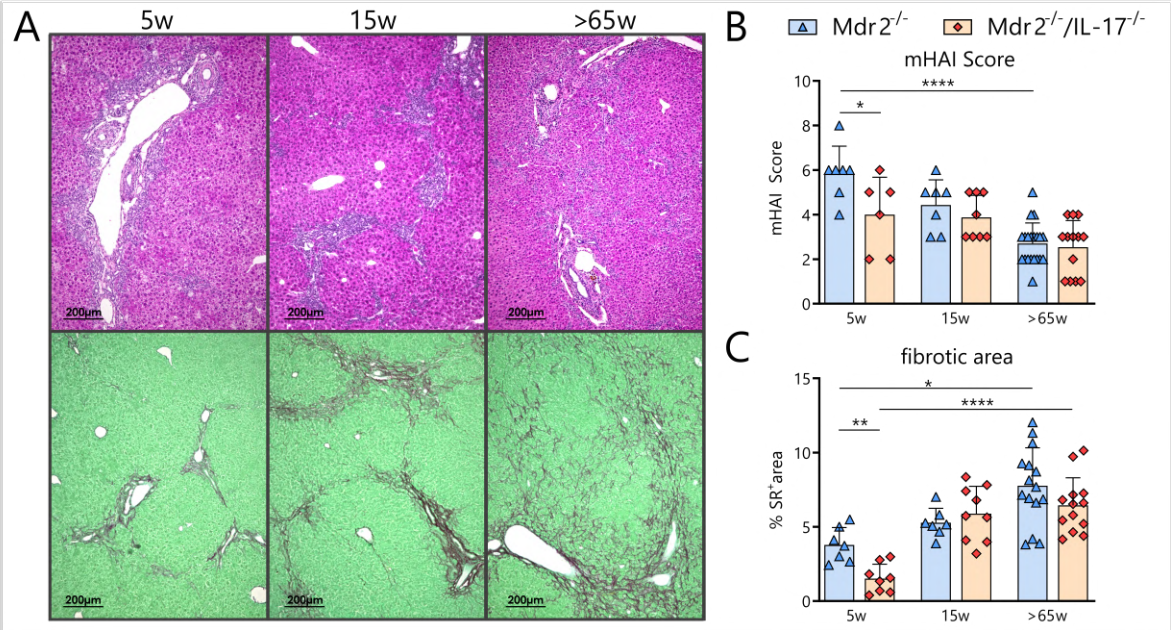

Figure 3.21: Independent of IL-17 expression, Mdr2$^{-/-}$ mice develop chronic inflammation and liver fibrosis: Liver inflammation and fibrosis were evaluated in Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice of different age. **(A)** Representative H&E- and Sirius Red-stained liver sections of Mdr2$^{-/-}$ mice. **(B)** The mHAI score of H&E-stained liver sections and **(C)** quantification of Sirius Red-stained liver sections.

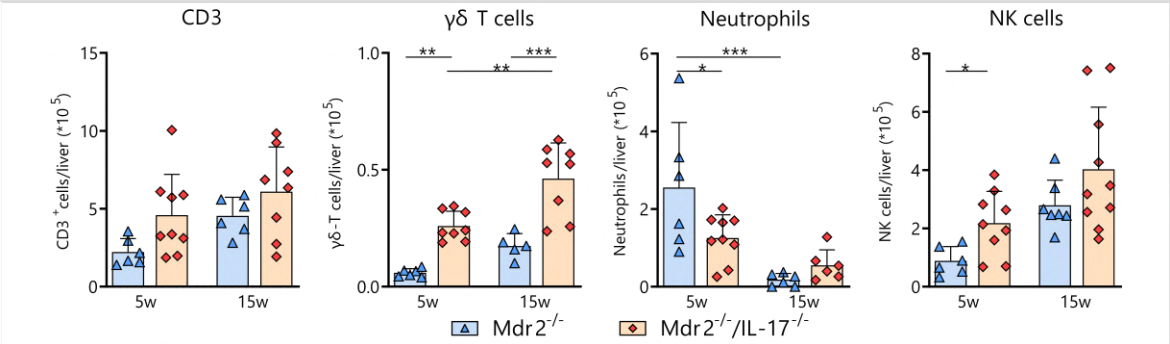

Figure 3.22: IL-17 deficiency in Mdr2$^{-/-}$ mice increases recruitment of $\gamma\delta$ T cells into inflamed livers: Infiltrating leukocytes were isolated from livers of Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice at an age of 5 weeks and 15 weeks. Immune cell populations were distinguished and analysed by flow cytometry.

Altogether, the knockout of IL-17A and IL-17F in Mdr2$^{-/-}$ mice led to slightly improved inflammation and fibrogenesis at an early age of 5 weeks. This was accompanied by reduced infiltration of neutrophils, but elevated numbers of $\gamma\delta$ T cells. With increasing age, the IL-17 expression had no effect on the severity of cholangitis or the development of liver fibrosis.

3.2.2 IL-17 in the development of liver tumours

Mdr2$^{-/-}$ mice were shown to spontaneously develop liver tumours over time [141, 151]. To monitor the tumourigenesis, regular MRI scans were performed with mice beginning at an age of 55 weeks until all Mdr2$^{-/-}$ mice showed noticeable liver structures (Fig. 3.23A) at an age of 65 weeks. Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice were sacrificed and macroscopically abnormal structures were counted on the liver surface (Fig. 3.23B). Nodules with a diameter smaller than 2 mm were categorised as "small tumours", whereas bigger structures were measured and categorised as "large tumours". Strikingly, less tumours were detected in IL-17-deficient Mdr2$^{-/-}$ mice (Fig. 3.23C). Additionally, the mean tumour size tended to be larger in IL-17-competent Mdr2$^{-/-}$ mice.

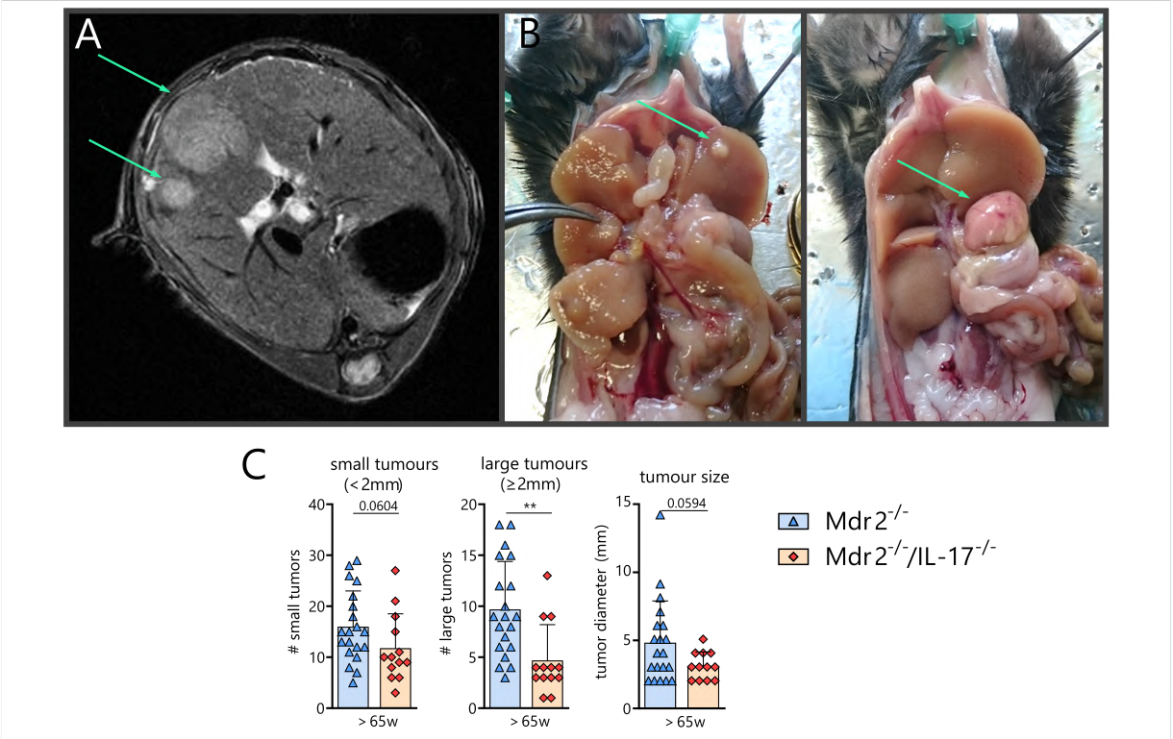

Figure 3.23: Knockout of IL-17 protects Mdr2$^{-/-}$ mice from tumour development: (A) Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice were monitored by regular MRI scans until the development of liver tumours. **(B, C)** Mice were sacrificed at an age of 65 weeks. Macroscopically tissue abnormalities were counted and measured on the liver surface. (Green arrows indicate liver tumours and surface abnormalities)

To further characterise the tumours arising in Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice, liver sections were analysed using H&E and Sirius Red stainings (Fig. 3.24A). Pathologists classified the larger, established tumours in Mdr2$^{-/-}$ mice as hepatocellular carcinomas confirming the observations described by M. Katzenellenbogen et al. [151, 152]. Different dysplastic nodule

structures were found in liver tissue of both Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice regarding size, presence of tumour capsules and the accumulation of fat vacuoles. However, only few tissue abnormalities were found in Mdr2$^{-/-}$/IL-17$^{-/-}$ mice. Hence, a direct comparison of these structures between IL-17-competent and -deficient mice was not possible.

The severity of liver inflammation and fibrosis in 65 week old mice was not altered dependent on the expression of IL-17. At young age, IL-17-competent mice showed increased inflammatory and fibrotic parameters. Thus, different pro-inflammatory and pro-tumourigenic genes were analysed in whole liver tissue of Mdr2$^{-/-}$ and Mdr2$^{-/-}$/IL-17$^{-/-}$ mice and compared to their expression at different ages (Fig. 3.24B).

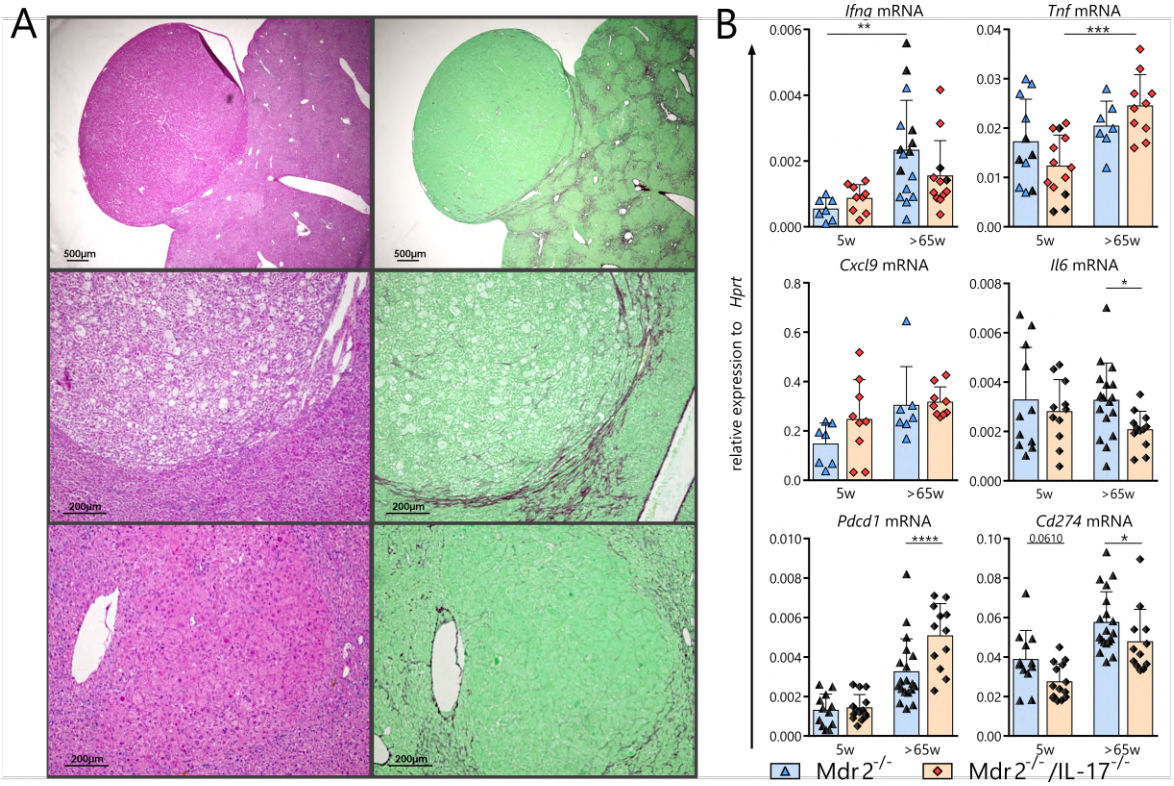

Figure 3.24: Characterisation of liver tissue including tumours: Mdr2$^{-/-}$ and Mdr2$^{-/-}$, IL-17$^{-/-}$ mice were sacrificed at an age of 65 weeks. **(A)** Liver sections were stained with Sirius Red and H&E to evaluate tissue abnormalities (representative pictures of different structures are shown). **(B)** Whole liver mRNA expression was investigated by qPCR analysis. Dark symbols indicate data generated together with M. Taube.

Although mRNA expression of pro-inflammatory *Ifng* and *Tnf* increased with the age of mice, no significant differences were observed between IL-17-expressing and IL-17-deficient Mdr2$^{-/-}$ mice. Interestingly, old Mdr2$^{-/-}$ mice with higher tumour burden also showed elevated mRNA expression of *Il6* and *Cd274*. On the other hand, decreased mRNA expression of *Pdcd1* was observed in these mice compared to Mdr2$^{-/-}$/IL-17$^{-/-}$ mice.

3.2.3 Summary - IL-17 in chronic cholangitis

Summarizing, IL-17 expression in the Mdr2$^{-/-}$ mouse model of chronic cholangitis and biliary fibrosis, did not show any relevance for the overall inflammation, as well as the establishment of liver fibrosis and cirrhosis. Only at the very early stage of inflammation, IL-17 expression might play a pathogenic role, represented by the recruitment of neutrophils into the liver, elevated fibrosis and inflammation. Of great interest, IL-17 deficiency led to a decreased tumour burden in Mdr2$^{-/-}$ mice, although it is still unclear, whether the decreased appearance is based on a delayed onset of tumour growth or a reduction of tumourigenesis itself. However, elevated mRNA expression of *Il6* and *Cd274* in IL-17-competent Mdr2$^{-/-}$ mice of older age could give a mechanistic hint for the increased tumour burden in these mice.

4 Discussion

Cholangiopathies are chronic and progressive diseases damaging the bile duct epithelial cells until the physiological bile transportation and regulation can not be fulfilled anymore. Chronic liver inflammation, cirrhosis and finally end-stage liver diseases are common consequences, as to date no curative therapy was found. Both disease aetiology and pathology are widely unclear, although many hypotheses claim dysregulated immune responses together with genetic predispositions to be trigger of cholangiopathies, like PBC and PSC [50, 52]. The cytokine IL-17 was already associated with the pathogenesis of many autoimmune, chronic or inflammatory diseases, since IL-17 signalling triggers recruitment of different immune cells and the activation of pro-inflammatory processes [91, 100]. In livers of patients suffering from cholangiopathies, IL-17-producing cells were found localised close to the bile duct epithelium. However, the impact of IL-17 in disease pathogenesis and progression is poorly understood.

In this thesis, we therefore aimed to analyse the functional role of IL-17 in different settings of cholangiocyte inflammation. We investigated the role of IL-17 in the development and maintenance of acute and chronic cholangitis as well as the importance of IL-17 signalling for cholangiocyte activation in liver inflammation. A better understanding of the complex role of different cytokines and mediators should contribute to the clarification of disease pathology and to the development of new therapeutical approaches.

4.1 IL-17 in acute cholangitis in mice

T cells significantly contribute to the pathogenesis of cholangiopathies and were found together with IL-17 around intrahepatic biliary tracts in patients suffering from PSC [161]. However, the role of T cell-secreted IL-17 for disease induction has not been investigated.

To address that question, we analysed the established K14-OVAp mouse model of inducible, acute cholangitis and observed that the lack of IL-17 in the initial phase of T cell-driven inflammation resulted in a more severe disease phenotype. In brief, we found that the missing IL-17 expression in disease-inducing OT-1 $CD8^+$ T cells increased cholangitis severity in the

K14-OVAp mice, characterised by enhanced histological mHAI score and increased liver enzymes (Fig. 3.3). Moreover, severe cholangitis was associated with highly activated and proliferative $CD8^+$ T cells. Mechanistically, we identified the IL-17-dependent induction of cholangiocytes and subsequent upregulation of the co-inhibitory ligand PD-L1 to be of major importance to control the activation of T cells (Fig. 3.13).

The ligands PD-L1 and PD-L2 have been described to specifically inhibit T cell proliferation and activation after binding the PD-1 receptor. They can be expressed by parenchymal and non-parenchymal cells in the liver, such as T cells, hepatocytes, cholangiocytes, KCs and LSECs [34, 162]. Our data now demonstrated that cholangiocytes are also able to upregulate PD-L1 expression after T cell-mediated initiation of inflammation in the K14-OVAp mouse model. Importantly, we found a significantly increased expression of PD-L1 on antigen-presenting cholangiocytes after the transfer of IL-17-secreting OT-1 T cells compared to mice transferred with OT-1/IL-$17^{-/-}$ T cells (Fig. 3.13). In general, inflammatory cytokines, such as IFNγ and TNFα have been described as potent inducers of PD-L1 expression [36, 163]. However, also IL-17 signalling could potentially influence the expression of PD-L1 via multiple pathways, supporting our findings of an IL-17-dependent regulation of PD-L1 on cholangiocytes. For instance, binding of IL-17A/F to IL-17 receptors was described to induce downstream NF-κB in epithelial cells, which can directly bind and activate PD-L1 promoter regions [164, 165]. Similarly, AKT signalling was shown to be induced by IL-17/IL-17R interactions, which further activates NF-κB expression and also enhances the translation of constitutively expressed *CD274* mRNA by blocking microRNAs in cholangiocytes [163, 166–168]. Further mechanisms include the transcription factor STAT3 that was found to co-stimulate *Cd274* expression and is induced by IL-17 signalling [122, 169–171].
Our *in vivo* and *in vitro* data now clearly demonstrated that the presence of IL-17 significantly enhanced the expression of PD-L1 on cholangiocytes and thereby directly affected the activation and expansion of T cells (Fig. 3.14, Fig. 3.15). Still, detailed analyses of involved pathways in the IL-17-dependent PD-L1 expression in cholangiocytes remain unidentified.

Binding of PD-L1 to its receptor PD-1 can inhibit T cell proliferation, but also influences the effector T cell function [172]. After induction of experimental cholangitis in the K14-OVAp mice, high expression of the receptor PD-1 on both IL-17-competent and –deficient OT-1 $CD8^+$ T cells was observed (Fig. 3.11), highlighting the importance of the PD-L1/PD-1 axis as a T cell-regulating mechanism. Increased PD-1 expression on activated $CD8^+$ T cells has been associated with reduced secretion of granzyme B [172]. Vice versa, elevated PD-L1 expression in epithelial cells was reported to protect from $CD8^+$ cytotoxicity *in vitro* [35, 37].
In line with this, we found decreased frequencies of granzyme B-secreting $CD8^+$ T cells in livers with increased PD-L1 expression on cholangiocytes after transferring IL-17-competent

OT-1 T cells (Fig. 3.8). Moreover, the lack of PD-L1/PD-1 interactions after transferring OT-1/PD-1$^{-/-}$ CD8$^+$ T cells resulted in high *Gzmb* expression in liver tissue (Fig. 3.19).
In general, stronger activation of cholangiocytes was observed after stimulation with IL-17 *in vitro* and *in vivo*, also resulting in increased expression of chemokine mRNA, in particular *Ccl2* and *Ccl20* (Fig. 3.5). Moreover, also *Il1b* and *Il6* mRNA was elevated in inflamed cholangiocytes (Fig. 3.5, Fig. 3.12). Supply of IL-1β and IL-6 by cholangiocytes was shown to favour Th17 and Tc17 differentiation [33] and CCL20-secreting cholangiocytes were described to promote recruitment of CCR6$^+$ Th17 and Tc17 cells towards the bile ducts [30]. Consistent with these findings, not only less cytotoxic CD8$^+$ T cells but also increased numbers of IL-17-secreting OT-1 CD8$^+$ T cells were found in the K14-OVAp recipient animals transferred with IL-17-competent OT-1 CD8$^+$ T cells (Fig. 3.8). Although increased frequencies of Tc17 cells were found in autoimmune and chronic liver diseases and were associated with a pathogenic role in disease development due to the high secretion of IL-17, we concluded that increased levels of IL-17 and reduced levels of granzyme B in the K14-OVAp recipient animals could be responsible for the milder inflammation observed [30, 112, 113, 173].

As described before, interactions of T cell expressed PD-1 with its ligand PD-L1 can reduce T cell proliferation and induce T cell apoptosis [34]. Thus, the lack of IL-17-dependent PD-L1 and missing PD-1/PD-L1 interactions might be responsible for uncontrolled proliferation of the transferred OT-1 T cells. In accordance with that, we observed rapid proliferation and reduced apoptosis in transferred OT-1/IL-17$^{-/-}$ CD8$^+$ T cells during experimental cholangitis, characterised by decreased annexin V staining and increased protein expression of Ki67 and IL-2 (Fig. 3.10).
It has been well described that large amounts of IL-2, which are necessary for T cell proliferation, inhibit the differentiation of naïve T cells into Th17 and Tc17 cells [116, 174–176]. Vice versa, IL-2 promotes the differentiation into Tregs and is essential for the maintenance of their suppressive capacity. Thus, mutations in the IL2R locus are associated with lower frequencies of Tregs in PSC patients and are discussed to trigger the development of cholangiopathies [72]. Altered recruitment of Tregs into livers or spleens of K14-OVAp recipient mice was not observed (data not shown). Still, high levels of IL-2 observed after transferring IL-17-deficient OT-1 CD8$^+$ T cells might reflect another mechanism inhibiting the less inflammatory Tc17 cells and favouring cytotoxic CD8$^+$ T cells, which drive the severe cholangitis seen in the K14-OVAp mice.

An imbalanced expression of PD-1 and PD-L1 was also detected in inflamed livers of PBC and AIH patients [162, 177] and was shown to be associated with a poor prognosis in many manifestations of cancer in humans [178–180]. Furthermore, checkpoint inhibitor therapy with αPD-1 treatment was reported to induce individual cases of secondary sclerosing cholangitis,

supporting the importance of the PD-L1/PD-1 axis as immune cell-regulating mechanism in the liver [181, 182]. Thus, increased frequencies of IL-17^{+} cells seen in PBC and PSC patients might reflect a self-protecting mechanism of inflamed cholangiocytes, but further studies are needed to clarifiy the significance of IL-17 signalling and PD-L1 regulation in cholangiocytes.

Inhibitors of IL-17 signalling are under discussion for the treatment of many autoimmune and inflammatory diseases. Antibodies against IL-17A or the IL-17RA - namely brodalumab (αIL-17RA), ixekizumab (αIL-17A) and secukinumab (αIL-17A) - are already used for treating plaque psoriasis [109, 183]. However, indications of this therapeutical approach for treating cholangiopathies are discussed controversially, since PSC patients are often infested with fungal or bacterial infections of the biliary tract and IL-17 blockade could lead to severe infectious consequences [85, 184–186].
Interestingly, blockade of IL-17 signalling was tested as a therapeutic approach in the IL-2R$\alpha^{-/-}$ mouse model, which develops autoimmune cholangitis and additional colitis with high frequencies of IL-17-producing cells [131, 132]. After depleting IL-17A, Yang et al. described a milder course of colitis, but more severe cholangitis in IL-2R$\alpha^{-/-}$ mice [187]. Thus, blocking the IL-17 axis in cholangiopathies should be considered cautiously.

The IL-17-dependent regulation of PD-L1 and also other T cell controlling mechanisms of cholangiocytes should clearly be studied further in detail. Especially in the context of cholangiopathies that are associated with bacterial infections of the biliary tract, IL-17 could play a crucial role by inducing defensive anti-microbial responses and additionally protecting cholangiocyte survival through T cell inhibition. Not only expression of PD-L1, but also the secretion of prostaglandins by cholangiocytes might reflect an important inhibitory mechanism in an inflammatory environment. Certainly, there are many more cytokines and chemokines secreted by activated cholangiocytes and the close interplay of bile duct cells and immune cells should be investigated in more detail, since the manifold and immune-regulating functions of liver parenchymal cells are not clarified enough so far.

4.2 IL-17 in chronic cholangitis in mice

In the K14-OVAp mouse model of acute experimental cholangitis, we identified a protective effect of IL-17 expression in the initial inflammatory phase of the disease. We next wanted to investigate the role of IL-17 in the disease progression of chronic cholangitis since a strictly pro-fibrogenic role of IL-17 has been proposed in skin, lung and liver fibrosis [119, 121]. We therefore analysed Mdr2$^{-/-}$ mice that additionally lack the expression of IL-17A and IL-17F regarding liver inflammation, fibrogenesis and tumourigenesis.

In this mouse model of chronic cholangitis, we identified a pathogenic role of IL-17 for the development of HCC. We did not only observe less tumours, but also overall reduced size of tumours in Mdr2$^{-/-}$/IL-17$^{-/-}$ mice (Fig. 3.23). However, it is not clear whether IL-17 deficiency reduced the overall tumourigenesis or only delayed the development of HCC.

In the development of tumours in general, IL-17 is discussed controversially. Anti-tumourigenic effects of IL-17 were described in the development, growth and metastasis of subcutaneously induced cancer, which could be related to enhanced levels of IFNγ secreted by NK and T cells [188]. On the contrary, in a mouse model of induced colon cancer, IL-17-deficient mice showed decreased tumour numbers and growth. In line with these findings, decreased levels of *IL17* mRNA were found in patients with colon cancer and treatment of a colon cancer cell line with IL-17F inhibited tumour angiogenesis and cell growth [190]. Moreover, treatment of mice suffering from lung cancer with αIL-17 could restore secretion of IFNγ and resulted in reduced tumour proliferation and angiogenesis [189].
Within human HCCs, IL-17-producing cells were found and supposed to promote tumour progression [123]. Together with expression of STAT3, IL-17^{+} cells were shown to be a negative predictor for patients' survival and post-operative recurrence [122, 123, 191]. An overexpression of IL-17 in HCC tumour cells is considered to promote angiogenesis and neutrophil recruitment by the activation of AKT signalling in HCC cells. Subsequent secretion of IL-6 by hepatocytes and induction of pSTAT3 signalling pathways increased the expression of IL-8 and VEGF, which supported the recruitment of neutrophils and tumour vascularity [122]. Elevated frequencies of neutrophils and monocytes were also found in human HCC patients, accompanied by elevated serum levels of IL-6 [192]. We also observed increased numbers of neutrophils in the early inflammation of IL-17-competent Mdr2$^{-/-}$ mice compared to IL-17-deficient mice, but the presence of neutrophils in late-stage liver inflammation and in HCC tissue was not investigated and should be aim of further studies.

Interestingly, IL-6 expression was also downregulated in liver tissue of IL-17-deficient Mdr2$^{-/-}$ mice that exhibited lower tumour burden (Fig. 3.24). Reduced levels of IL-6 together with the lack of IL-17 expression could also result in decreased levels of STAT3, although IL-17

was found to not directly induce STAT3 phosphorylation and signalling. However, IL-17 was shown to upregulate IL-6 secretion and to stabilise IL-6-mediated activation of pSTAT3 in human hepatocytes *in vitro* [122, 171]. The transcription factor STAT3 is constitutively expressed in many human cancers, like prostate cancer or breast cancer. It was shown to be excessively expressed by hepatocytes in HCC, where STAT3 is involved in anti-apoptotic signalling and activation of cell proliferation [171, 191, 193, 194]. Hence, IL-6-induced phosphorylation and activation of STAT3 is discussed to promote survival and proliferation of tumour cells [195].
In line with these findings, IL-6 deficient mice appeared to be protected from HCC development in diethylnitrosamine (DEN)-induced liver cancer, which might also be related to reduced STAT3 signalling [196].

Additionally, STAT3 is able to stimulate the expression of PD-L1 in APCs [169, 170]. Both PD-1 and PD-L1 were shown to be excessively expressed on tumour infiltrating T cells and tumour cells in livers of HCC patients and different inhibitors blocking the PD-1/PD-L1 interactions are tested in HCC therapy [197–199]. Due to the elevated levels of IL-6 in tumour-burdened Mdr2$^{-/-}$ mice, we speculated for increased expression of PD-L1 via STAT3 activation in liver tissue, which reduces the immune response against tumour cells and improves tumour cell survival. Indeed, we found increased levels of *Cd274*, but decreased expression of *Pdcd1* within livers of IL-17-competent Mdr2$^{-/-}$ mice (Fig. 3.24). In line with our findings, a positive correlation of PD-L1 and IL-17 expression in tumour tissue of breast cancer patients has been described [200]. IL-17A was shown as a direct stimulator of PD-L1 expression in different human and murine cells *in vitro* and anti-tumourigenic effects of IL-17A inhibition were associated with PD-L1 downregulation in mice [200]. On the other hand, the relation of IL-17 and T cell expressed PD-1 in tumourigenesis is not completely understood so far and still under investigation.

To identify the underlying mechanisms of the observed differences in tumour development, we analysed disease progression in our chronic mouse models over time regarding immune cell composition, liver inflammation and fibrogenesis. Interestingly, we did not observe relevant IL-17-dependent differences in the course of cholangitis over time.
Mdr2$^{-/-}$ mice are known to have a strong, acute inflammatory phase in the early months of age, decreasing with time to a milder, but chronic cholangitis [147–149]. The same development was presented by Mdr2$^{-/-}$/IL-17$^{-/-}$ mice. Only in the very early stage of cholangitis, at an age about 5 weeks, Mdr2$^{-/-}$/IL-17$^{-/-}$ mice tended to exhibit a milder inflammation, seen in the histological scoring of liver inflammation (Fig. 3.21). This phenotype was accompanied by significantly reduced neutrophil recruitment into the liver (Fig. 3.22), which was described to be based on IL-17-dependent activation of IL-8 and IL-6 in endothelial and epithelial cells [29, 104]. Usually, neutrophils are quickly recruited to the site of inflammation

to eliminate pathogens by phagocytosis. An important mechanism for neutrophil effector function is the production of reactive oxygen species (ROS) that was shown to promote fibrogenesis and might also be responsible for some degree of tissue damage during cholangitis [201].

On the other hand, increased recruitment of $\gamma\delta$ T cells was observed in Mdr2$^{-/-}$/IL-17$^{-/-}$ mice at the age of 5 weeks and 15 weeks (Fig. 3.22). Beside Th17 and Tc17 cells, $\gamma\delta$ T cells are an early source of IL-17 in the progress of inflammation and can be recruited via the CCR6/CCL20 axis, which vice versa can be induced by IL-17 signalling [30, 202, 203]. $\gamma\delta$ T cells that lack IL-17 expression are phenotypically associated with elevated expression of T cell-inhibiting IL-10 and TGFβ and therefore considered to have an anti-inflammatory and anti-tumoural function [204, 205]. The increased recruitment of $\gamma\delta$ T cells observed in young Mdr2$^{-/-}$/IL-17$^{-/-}$ mice further indicates a beneficial role for $\gamma\delta$ T cells in chronic cholangitis and later tumourigenesis in the liver, although the distinct phenotype and cytokine secretion of these immune cells has to be characterised in further studies.
In contrast to Kryczek et al. who observed NK recruitment correlating with Th17 recruitment, we detected increased frequencies of NK cells in IL-17-deficient Mdr2$^{-/-}$ mice [206]. In PBC patients, NK cells were found to be over-activated with increased secretion of IFNγ and upregulated expression of the liver-homing receptor CXCR6 [207]. Nevertheless, differences in immune cell recruitment were found to disappear over time together with the milder cholangitis in the Mdr2$^{-/-}$/IL-17$^{-/-}$ mice (Fig. 3.22).

Importantly, we could not observe that the constitutive lack of IL-17 was beneficial for the development of liver fibrosis in Mdr2$^{-/-}$ mice (Fig. 3.21). In contrast, ERKR1/2 inhibitors, which block IL-17A downstream signalling, were shown to reduce HSC-dependent collagen production in CCL_4 induced experimental liver fibrosis [121]. Additionally, significantly reduced deposition of collagen fibres were found in Mdr2$^{-/-}$ mice after treatment with IL-17A depleting antibodies [208]. These contrary observations might be result of the different genetic backgrounds of the Mdr2$^{-/-}$ mouse models, since Tedesco et al. used Mdr2$^{-/-}$ mice bred on the FVB background. These mice are known to exhibit a slightly different course and progression of sclerosing cholangitis compared to mice bred on the C57Bl/6 background, as used in this thesis, which might account for the altered phenotype [154, 155].

However, the mechanism for the delayed HCC development in IL-17-deficient Mdr2$^{-/-}$ mice are still not understood and further investigations are needed. An important aspect in future analyses should be the specific depiction of tumour infiltrating immune cells, especially neutrophils, macrophages, NK cells and $\gamma\delta$ T cells, their activation and cytokine secretion. Also the IL-17-dependent expression of IL-6 and STAT3, their cellular sources and their effects on tumour cells should be further investigated.

Of great interest is also a deeper characterisation of the developed tumours themselves, since different dysplastic manifestations were found in Mdr2$^{-/-}$ mice compared to only small tissue abnormalities in Mdr2$^{-/-}$/IL-17$^{-/-}$ mice. Hence, a direct comparison of tumour tissue is aggravated, but essential.
Other effects of IL-17 deficiency in the Mdr2$^{-/-}$ mouse model that might be interesting, are the expression of anti-microbial peptides and tight junction proteins in bile duct cells, as we clearly showed a direct effect of IL-17A in the activation of cholangiocytes. Especially the defective bile duct integrity is a problem that is common in cholangiopathy patients and could also play a major role in the progression of bile-toxicity-induced cholangitis in the Mdr2$^{-/-}$ mice.

4.3 Final conclusions

All together, we found both protective and harmful effects of IL-17 signalling in different settings of experimental cholangitis.
In a short term mouse model of acute cholangitis, we demonstrated an important protective function of IL-17A/F through the early activation of cholangiocytes and their T cell-regulating abilities. Particularly the IL-17-dependent regulation of T cell-inhibiting PD-L1 on cholangiocytes seemed to be of major importance for the control of T cell-driven experimental cholangitis.
Although the constitutive lack of IL-17A/F did not alter the progression of chronic cholangitis or fibrosis, we could show a pathogenic role of IL-17A/F in the long term tumourigenesis of HCC in the Mdr2$^{-/-}$ mouse model. The pathogenic effects might be attributed to the altered recruitment and activation of neutrophils and $\gamma\delta$ T cells, elevated expression of IL-6 followed by STAT3 activation or the dysregulation of IL-17-dependent PD-L1 leading to inhibited anti-tumourigenic immune response.
This thesis should stimulate further research especially into the role of IL-17 in the context of cholangiocyte activation in different cholangiopathies to assure a better understanding of disease pathogenesis and therapeutic options.

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List of Abbreviations

ACK:	Ammonium-chloride-potassium
AIH:	Autoimmune hepatitis
ALAT:	Alanine aminotransferase
AMA:	Antimitochondrial antibodies
AP:	Alkaline phosphatase
APC:	Antigen-presenting cell
ASAT:	Aspartate aminotransferase
BDL:	Bile duct ligation
BSA:	Bovine serum albumin
C57Bl/6:	C57 black 6
CCA:	Cholangiocarcinoma
CD:	Cluster of differentiation
CTL:	Cytotoxic T lymphocyte
DAMP:	Damage-associated molecular patterns
DC:	Dendritic cell
DDC:	3,5-Diethoxycarbonyl-1,4-dihydrocollidine
DSS:	Dextran sulfate sodium
ECM:	Extracellular matrix
ELISA:	Enzyme linked immunosorbent assay
EpCAM:	Epithelial cell adhesion molecule
FCS:	Fetal calf serum
FoxP3:	Forkhead box protein P3
FVB:	Friend virus B-type/N

G-CSF: Granulocyte-colony stimulating factor

GGT: Gamma-glutamyl transferase

H&E: Haematoxylin/Eosin

HCC: Hepatocellular carcinoma

HSC: Hepatic stellate cell

i.v.: Intravenous

IBD: Inflammatory bowel disease

IFNγ: Interferonγ

IL: Interleukin

ILC: Innate lymphoid cell

ITS: Insulin-Transferrin-Selenium

KC: Kupffer cell

LDH: Lactate dehydrogenase

LSEC: Liver sinusoidal endothelial cell

MACS: Magnetic cell separation

MAIT: Mucosal-associated invariant T cell

MAMP: Microbial-associated molecular patterns

MAPK: Mitogen activated protein kinase

Mdr: Multidrug resistance protein

mHAI: Modified hepatitis activity ondex

MHC: Major histocompatibility complex

MRCP: Magnetic resonance cholangiopancreatography

MRI: Magnetic resonance imaging

NF-κ: Nuclear factor κB

NK: Natural killer (cell)

NKT: Natural killer-T (cell)

OCA: Obeticholic acid

OVA: Ovalbumin

PacO: Pacific orange

PAMP: Pathogen-associated molecular patterns

PBC: Primary biliary cholangitis

PC: Phosphatidylcholine

PD-1: Programmed cell death protein 1

PD-L1: Programmed cell death protein 1 - ligand 1

PMA: Phorbol 12-Myristate 13-Acetate

PRR: Pattern recognition receptor

PSC: Primary sclerosing cholangitis

q-PCR: Quantitative polymerase chain reaction

RORγt: Retinoic acid receptor-related orphan receptor γ t

ROS: Reactive oxygen species

RT: Room temperature

STI: Soybean Trypsin Inhibitor

TCR: T cell receptor

TGFβ: Transforming growth factor β

Th: T helper (cells)

TLR: Toll-like receptor

TNFα: Tumor necrosis factor α

Treg: Regulatory T cell

UC: Ulcerative colitis

UDCA: Ursodeoxycholic acid

wt: Wild-type

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