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Investigating the Effect of IL-17 on Ductular Reaction During Liver Regeneration

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ABBREVIATIONS

NAFLD	Non-alcoholic fatty liver disease
HCC	Hepatocellular carcinoma
ALF	Acute liver failure
CLD/CLI	Chronic liver disease/chronic liver injury
NASH	Non-alcoholic steatohepatitis
MCD	Methionine-choline deficient
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
MSC	Mesenchymal stem cell
HPC	Hepatic progenitor cell
cdHPC	Cholangiocyte-derived hepatic progenitor cell
DR	Ductular reaction
PHx	Partial hepatectomy

ABSTRACT

NAFLD is a highly prevalent disease, currently affecting an estimated 25% of the global population. Liver transplantation remains the only curative treatment, but this is becoming increasingly limited due to lack of donor availability, viable organs, organ rejection, and disease recurrence. Hepatic progenitor cells are activated following chronic liver damage, expanding to replace the liver's hepatocyte and cholangiocyte population. This is accompanied by ductular reaction (DR), a compensatory mechanism involving the remodelling of bile ducts. In this investigation, we demonstrate a novel pathway involving IL-17A, a proinflammatory cytokine, which drives the bipotency of these progenitor cells to promote ductular reaction and liver regeneration by stimulating expression of the stem cell marker, CD133. This is achieved through various signalling pathways, most notably NF-κB and Wnt, factors of which are significantly upregulated following IL-17A treatment of cholangiocyte-derived hepatic progenitor cells (cdHPCs). FACS analysis revealed $CD11b^+$ $Ly6G^+$ neutrophils to be the major source of IL-17A in a mouse model of fatty liver disease. This study indicates that IL-17A may act as a potential therapeutic target to mediate cdHPC-driven liver regeneration, preventing the need for liver transplantation in the future.

INTRODUCTION

1.1 Overview of Liver Diseases

Liver disease is one of the major causes of death worldwide with cirrhosis, viral hepatitis and hepatocellular carcinoma (HCC) accounting for approximately 2 million deaths every year.[1] In 2017, 1.32 million deaths worldwide could be directly attributed to cirrhosis, making it the 11th most common cause of mortality.[2] However, due to the lack of accurate mortality data for some regions where the prevalence of liver disease is high such, as Africa, it is likely that this number is far greater.[1] Liver disease consists of a range of conditions, including non-alcoholic fatty liver disease (NAFLD), fibrosis, cirrhosis, autoimmune hepatitis and HCC. Whilst these constitute chronic cases of the disease, severe acute liver injury and subsequent failure also remains a problem due to its high mortality. Acute liver failure (ALF) is a rare disorder arising from the rapid deterioration of liver function but in the absence of existing chronic liver disease. As a consequence of severe and abrupt hepatocyte injury, patients develop hepatic encephalopathy and coagulopathy, with a high mortality rate of 30%.[3-5] Paracetamol-induced hepatotoxicity is the major cause of ALF in the UK and most of the developed world, whereas viral hepatitis-induced ALF is more prevalent in developing countries.[1, 6] Other known causes of this condition include hepatic ischaemia, as a result of circulatory failure or septic shock, autoimmune hepatitis and rare factors such as Wilson's disease, Budd-Chiari syndrome and malignancies. It has been reported that drug-induced liver injury, hepatitis B infection and autoimmune hepatitis tend to have the worst survival rates.[6,7] Although chronic liver disease (CLD) and ALF have entirely separate

aetiologies, the only treatment to improve patient and survival outcomes is liver transplantation, a highly invasive and limited option due to donor availability.[5]

1.1.1 Chronic Liver Disease

Chronic liver disease (CLD) occurs as a result of the progressive deterioration of liver functions, largely due to continuous inflammation destroying the liver parenchyma. To compensate for this, the liver has an extraordinary ability to regenerate, replacing hepatic cells and restoring liver function. However, the constant cycle of destruction and regeneration leads to fibrosis and eventually, cirrhosis of the liver. The aetiological basis of CLD varies from toxin exposure and prolonged alcohol abuse, to viral infections, autoimmune disease and genetic or metabolic disorders. Liver cirrhosis is an end-stage of CLD, with widespread disruption of liver architecture, the formation of nodules, neo-angiogenesis and the deposition of extra-cellular matrix (ECM).[8] The majority of patients with cirrhosis are asymptomatic until the liver becomes decompensated, leading to ascites, hepatic encephalopathy, variceal bleeding and portal hypertension. Despite alcohol abuse accounting for most cases of CLD, 10% are due to NAFLD and also commonly associated with obesity.[9]

NAFLD can be separated into two separate conditions of the liver – non-alcoholic fatty liver (NAFL), where steatosis can occur with or without the presence of mild lobular inflammation, and non-alcoholic steatohepatitis (NASH), which can involve varying degrees of fibrosis and cirrhosis.[1] The severity of inflammation is characterised by a marked inflammatory infiltrate, consisting of monocytes,

lymphocytes and occasionally, neutrophils. The presence of Kupffer cells (KC) often correlates with necroinflammation, liver injury and the degree of fibrosis. KC hepatic infiltration is strongly linked to the pathogenesis of fatty liver disease, playing a role in mediating inflammation, regulating triglyceride storage and hepatocyte injury.[10] NAFLD is prevalent in an estimated 25% of the global population, likely as a result of the growing obesity epidemic which currently affects 2 billion people worldwide.[1] Prevalence of this disease is highest in the Middle East and South America, and lowest in Africa. In the majority of cases, NAFLD is accompanied by obesity, but there is a growing incidence of 'lean' NASH cases, particularly in Asia.[11] These findings indicate that not only obesity, but diet as well is key in the development of steatosis. In 0.5% to 2.6% of cases, NASH when coupled with cirrhosis can also lead to the development of HCC. Although this is a low incidence rate, the projected increase of NASH cases by 56% in the next 10 years strongly suggest that this chronic liver disease will become a major health service and economic burden.[11-13] Currently, the only successful treatment for cirrhosis and end-stage liver disease is to undergo liver transplantation. However, the immune response and availability issues involved with this necessitate the need for new viable treatment options and alternatives to liver transplantation.

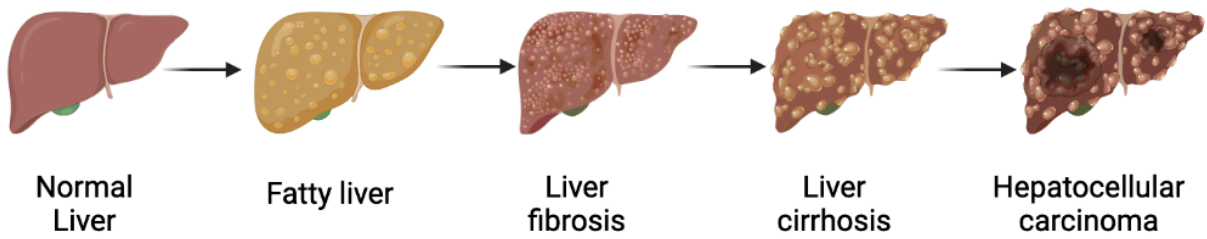

Figure 1: The progression of liver disease from healthy to hepatocellular carcinoma.
Incidences of fatty liver disease and fibrosis are considered reversible, whereas the reversal of cirrhosis and hepatocellular carcinoma relies on the availability and success of liver transplantation.

1.1.2 Animal Models of Liver Injury

MCD

The methionine-choline deficient (MCD) is the most common model to induce fatty liver disease in mice, largely due to its ability to replicate a severe phenotype of NASH in a short period of time. Steatohepatitis can occur in as little as 10 days following administration, and perisinusoidal fibrosis is induced at 8 to 10 weeks, making the MCD diet an efficient and reproducible model to investigate NAFLD. It consists of a high sucrose content (40%) and moderate fat content (10%), but the deficiencies in methionine and choline are responsible for the severe histopathology seen in this model. The lack of choline results in the inhibition of phosphatidylcholine synthesis, leading to impaired very low-density lipoprotein (vLDL) production and lipid accumulation in the liver. Deficiency of methionine, an essential amino acid, results in a lack of glutathione (GSH). A decrease in GSH, a potent antioxidant, causes oxidative stress and alterations in cytokines and adipokines, inducing liver damage. As the MCD diet does not lead to obesity, hyperlipidaemia, and peripheral insulin resistance, which are metabolic hallmarks of NAFLD, it is not suitable for investigating the multisystemic aspects of this disease but is acceptable for exploring intrahepatic developments in NAFLD.[14, 15]

$Mdm2^{flox/flox}$

The murine double minute 2 knockout ($Mdm2^{flox/flox}$) mouse model induces liver injury through a different mechanism to the MCD diet. This is controlled under hepatotropic AAV8-Cre, deleting the Mdm2 gene specifically in hepatocytes to avoid the complications of Mdm2 knockout throughout the system. In the liver, Mdm2 is responsible for regulating p53, a tumour suppressor protein, to allow for normal cell

proliferation, renewal of hepatocytes and hepatocyte metabolism.[16] The deletion of hepatocyte-specific Mdm2 leads to senescence, apoptosis and fibrosis, causing significant liver damage. The incidence of fibrosis in this model is a result of connective tissue growth factor (CTGF) synthesis promoting fibrogenesis in the liver.[17] Adipocyte-specific liver damage, however, induces chronic p53 activation and triggers adipose tissue loss. These mice develop hyperlipidaemia, hyperglycaemia, and a non-alcoholic fatty liver phenotype.[18] In non-specific $Mdm2^{-/-}$ mouse models, however, the effects are widespread and involve multiple systems, leading to cellular apoptosis throughout the body. Using this model may affect the outcomes of exploring individual systems, such as the liver, and therefore may not be a true representation. Therefore, using hepatotropic AAV8-Cre to specifically deleted Mdm2 in hepatocytes is more accurate model.

1.2 Current and Prospective Treatments

1.2.1 Whole Organ Transplantation

Liver transplantation is the gold standard treatment for almost all end-stage liver diseases, with a 1- and 5-year adult patient survival rate of 92% and 80%, respectively.[19] 5000 liver transplantations are performed annually in Europe, owing to the growing indications for transplant.[20] Patients with acute and chronic liver failure, cirrhosis, autoimmune diseases such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), as well as metabolic disorders and malignancies, are now being treated using whole organ transplantation.[21] However,

this definitive treatment has several major limitations, and therefore the need for alternative therapies has become substantial.

The growing list of patients being considered for transplantation, as well as a large proportions of these having more comorbidities (diabetes mellitus, obesity and portal vein thrombosis), far outweighs the number of donor livers available.[21] This is despite a 60% increase in donor number from 2010 to 2016.[19] In addition to this, liver transplants are associated with a variety of risks. The need for immunosuppressive treatment following transplantation, especially in cases of prolonged immunosuppressant (IS) use, is associated with increased mortality in the late phase of liver transplantations. Although IS drugs are essential in the induction and maintenance of immunosuppression, and as treatment for rejection, their use can lead to significant nephrotoxicity and subsequent renal replacement therapy. Calcineurin inhibitors (CNI) and mammalian target of rapamycin inhibitors (mTOR-is) carry a greater risk in terms of drug-drug interactions, toxicity and rejection.[22]

Aside from IS-induced renal injuries, a high frequency of renal injuries occurs in transplant recipients as a result of pre-existing hepato-renal syndrome and nephrotoxic antimicrobial agents. It has been suggested that anywhere between 17 and 94% of patients experience post-operative renal injury, increasing the occurrence of morbidity and mortality after transplantation.[23]

A further complication following liver transplantation is graft-versus-host disease (GVHD). This condition is most frequent in intestinal transplantation, with liver transplantation having the second highest incidence rate (0.5% to 2%). Despite this

very low incidence rate in comparison to other transplant-related complications, GVHD carries an 85% mortality rate, often due to delayed diagnosis and initiation of therapy.[24, 25] This complication is the result of donor immune cells recognising the recipient's antigens as foreign, inciting an immune response, and attacking healthy cells and tissues. A higher incidence of GVHD is seen in grafts with a greater proportion of immunocompetent donor lymphocytes, including bone marrow cells.[24]

The recurrence of liver disease following liver transplantation poses a further problem with this treatment, particularly in autoimmune liver diseases, where recurrence rates range from 10% to over 50%. Approximately 12.4% of patients with PSC, and between 1% and 5% of those with PBC, experience graft failure and require re-transplantation.[26] In patients with NAFLD, there is a higher incidence of peri-surgical infections, malignancies, and cardiovascular events. Those also with diabetes mellitus prior to transplant have an increased risk of cardiovascular complications, infections, graft rejection and reduced survival. Recurrent rates are particularly high in NAFLD, with 1-, 3- and 5-year incidence rates being 59%, 57% and 82% respectively. Interestingly, high rates of *de novo* NAFLD in patients post-transplantation, have been reported, as well as a 11%-14% recurrence of cirrhosis in advanced liver disease cases.[27]

1.2.2 Potential Cellular Therapies for Liver Diseases

MSC Therapy

In response to the problems associated with relying solely on liver transplantation, there have been numerous clinical trials with the aim of finding alternative therapies.

Stem cells in particular have been the subject of these trials, with researchers exploiting their remarkable ability to differentiate into specialised cell types with changes in microenvironment. Mesenchymal stem cells (MSCs; also known as mesenchymal stromal cells) are multipotent and highly immunomodulatory stem cells originally isolated from the bone marrow.[28, 29] They are capable of differentiation into various stromal cells, including adipocytes, reticular cells, and osteoblasts. As well as being bone marrow derived, MSCs can be isolated from the adult connective tissues dental pulp, adipose tissue, and peripheral blood, as well as menstrual blood, amniotic fluid, the placenta, and components of the umbilical cord.[28, 30]

MSCs are characterised in several ways, where they must be plastic-adherent in standard culture conditions and express certain surface markers. Mesenchymal lineage markers CD105, CD73 and CD90 must be expressed, whereas a lack of haematopoietic lineage markers, including CD34, CD45, CD11a and CD19, and the endothelial lineage marker, CD31. Additionally, to be classed as an MSC, human leukocyte antigen (HLA-DR) surface molecules must be absent and MSC must be capable of differentiating into osteoblasts, adipocytes and chondroblasts *in vitro.*[30-32]

MSCs have a range of immunomodulatory properties through immune cell interaction and cytokine, chemokine and growth factor secretion, allowing them to have a significant effect on the immune response.[28, 33] MSCs suppress dendritic cell (DC) generation, migration and maturation, impairing their ability to activate antigen specific $CD4^+$ T cells. Interactions between DCs and MSCs also shifts these $CD4^+$ cells from a pro-inflammatory (Th1) phenotype to an anti-inflammatory (Th2) one. MSCs have also been shown to be capable of promoting an anti-inflammatory, or

immunoregulatory, monocyte/macrophage phenotype, which further inhibits Th1 and DC differentiation. MSC production of IL-6 and hepatocyte growth factor (HGF) modulates IL-10 secretion by monocytes, which further promotes an anti-inflammatory environment through Treg expansion. [30, 34] In addition to this, MSCs suppress T cell and natural killer (NK) cell proliferation, the latter resulting in the reduced cytotoxic activity of NK cells.[28, 34]

A recent study showed that the use of mesenchymal stem cells (MSC) in NAFLD-induced mice showed a reverse in steatosis through the suppression of $CD4^+$ T lymphocytes. Improvements in lobular inflammation and liver fibrogenesis were also reported.[35] In end-stage CLD, the transplantation of bone marrow-derived MSC into alcohol-induced cirrhotic livers improved fibrosis histology and liver function, as measured by Child-Pugh and alkaline phosphatase (ALP) levels.[36] Due to their high degree of plasticity, MSC are able to differentiate into both mesodermal cell lineages such as cardiomyocytes, osteoblasts, adipocytes and chondrocytes, and non-mesodermal cells including hepatocytes.[35] Additionally, MSC can secrete anti-fibrotic and anti-inflammatory factors to reduce chronic inflammation and potentially regress fibrosis pathophysiology.[37] These properties, alongside the ability of MSCs to promote tissue regeneration and regulate immune environments, are what make MSCs an exciting treatment prospect.

Pluripotent Stem Cell Therapy

Studies involving other stem cell sources, including pluripotent embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have also had some success in

improving CLD outcomes. In CCl_4-treated mice, ESCs were potent enough to differentiate into hepatocyte-like cells that integrated into the liver parenchyma and reduced fibrosis.[37] However, reports of splenic tumorigenicity indicate that further thorough investigations into the safety of ESCs need to be made before they can even be considered a potential treatment option.[38] Interestingly, a study exploring the engraftment and regenerative capabilities of human iPSCs discovered that they exhibited similarities to that of ESCs and when differentiated, to that of human primary hepatocytes. Human iPSCs were capable of producing multistage hepatic cells that could secrete corresponding levels of human-specific liver proteins to those of hepatocytes.[39]

Hepatocyte Transplantation

Aside from stem cell-based therapies, allogeneic hepatocyte transplantation has also been considered as a potentially alternative treatment to liver transplantation. This cellular therapy is less invasive and less expensive than surgical interventions, can be performed repeatedly, and may be the basis for a regenerative response in patients with acute liver failure. Primary human hepatocytes (PHH) can be isolated from previously rejected whole donor livers, liver segments I and IV, foetal livers and donor animal livers, such as from healthy pigs.[40, 41] Successful hepatocyte transplants have been seen in a range of diseases: urea cycle defects in liver-based metabolic disorders; Crigler-Najjar syndrome; Glycogen storage disease type I, and factor VII deficiency.[40] However, the success of hepatocyte transplantation relies on a good supply of high-quality human hepatocytes; freshly isolated PHHs are the optimum choice for in vivo transplantation.[42] Unfortunately, limitations in supply and

difficulties in maintaining hepatocytes in culture mean that hepatocyte transplantation is often unsuccessful.[41, 42] Freezing hepatocytes via cryopreservation offers a solution, although freeze-thawing cycles damage mitochondria, impacting cellular respiration and may also induce apoptosis, although this is reduced when suspended in human plasma.[40, 42] Careful handling and aseptic techniques are central to preventing contamination, and all hepatocytes must be subjected to microbiological analysis before being transplanted, another potential barrier in this process. Additionally, the quality of isolated cells is usually poor and therefore are not viable for transplantation.[40] One method to overcome this that has been to generate immortal human hepatocytes via the introduction of telomerase constructs, retroviral transfection, or spontaneous transformation. However, there is an increased risk of tumour transmission or tumorigenesis with this method. Separately, although the possibility of using animals to increase hepatocyte supply has been discussed, engraftment was found to be very low at only 0.5% of the recipient liver mass under normal conditions.[41] In order for hepatocyte transplantation to become a viable treatment option, improvements in the success of cell engraftment, isolation quality and understanding of underlying immune mechanisms, are needed.

1.2.3 Pharmacological Interventions

A variety of pharmacological therapies have been explored to treat cholestatic disease and improve patient outcomes. Ursodeoxycholic acid (UDCA), a bile acid used to treat primary biliary cholangitis (PBC) has shown to be hepatoprotective with

improved liver function and reduced inflammation, but long-term trials have shown little benefit in reversing hepatic steatosis. Metformin, which is most commonly used in the treatment of Type 2 diabetes, is known to improve insulin resistance, a key feature in NASH aetiology. Despite this, the use of metformin failed to exhibit any histological benefits.[43] Other studies have explored the potential role of glucagon-like peptide-1 (GLP-1) analogues, which are also used in Type 2 diabetes treatment to control glycaemia and reduce weight.[44] GLP-1 is an intestinal hormone, playing a central role in regulating glycaemia, promoting β–cell proliferation and inhibiting glucagon release. As a result of this, gastric emptying is delayed, increasing satiety, and limiting food intake.[45, 46] Patients with NAFLD have downregulated GLP-1 receptor production, and therefore poor glycaemic control.[47] It has been suggested that hyperglycaemia induces hepatic steatosis, likely through aberrant metabolic pathways leading to excess fat accumulation. GLP-1 receptor agonists (GLP-1RA), such as liraglutide, proved effective in reducing hepatic inflammation, steatosis and fibrosis, as well as showing histological improvements.[44, 48] These benefits and a good safety profile make GLP-1RAs an attractive treatment in the resolution of NAFLD-associated steatosis and inflammation.[47]

1.3 Liver Regeneration

The liver has a remarkable capacity to regenerate as a result of hepatic tissue injury or partial hepatectomy (PHx). Under these circumstances, the remaining hepatocytes undergo hypertrophy and proliferate to restore either the resected liver mass or replace damaged tissue. However, in cases of chronic insult, such as NAFLD, cirrhosis and autoimmune hepatitis, the ability of hepatocytes to self-renew becomes overwhelmed, impairing hepatocyte-driven regeneration. This is often accompanied by widespread hepatocyte senescence, which can lead to liver function insufficiency or even failure.[49] Instead, is it thought that a compartment of bipotent hepatic progenitor cells (HPCs), also known as oval cells, become activated and undergo a massive expansion to repopulate the liver. These progenitor cells are capable of differentiating into both hepatocytes and the biliary epithelial cells, or cholangiocytes, that line the biliary tree.[50-52] Cholangiocytes can also act as facultative liver stem cells, dedifferentiating into HPCs which can then differentiate into hepatocytes and cholangiocytes. These newly formed cells proliferate rapidly to restore the injured tissue, with some cholangiocytes reverting back to their progenitor-like state to replace the HPC population.[53] Ko et al have shown that this biliary-driven liver regeneration may be regulated by bromodomain and extraterminal (BET) proteins throughout the dedifferentiation, HPC proliferation, hepatocyte proliferation and maturation process.[54] BET proteins are involved in regulating the expression of genes associated with immunity through epigenetic modification. This modification, through recognising acetylated histones and recruiting transcription factors, such as RELA, is essential in activating gene transcription for cytokines, chemokines, and other immune response mediators. It may be that BET-related

modifications are involved in liver stem cell activation as part of the immune response.[55] It is also thought that hepatocytes are able to transdifferentiate into cholangiocytes or biliary-like cells following cholangiocyte damage.[50] Whether HPCs differentiate into hepatocytes or cholangiocytes has been shown to rely on antagonistic interactions between Wnt, Notch and Yap signalling pathways. Biliary cell fate is specified as a result of the Notch-expressing HPCs and Jagged1-expressing myofibroblasts, whereas Wnt3a-induced suppression of Notch signalling in HPCs favours the hepatocyte lineage. As Notch acts to signal biliary promotion during development, it is likely to play a role in driving HPC differentiation into cholangiocytes. Using genetic lineage tracing, it has been suggested that *Sox9*, *Foxl1*, and *Lgr5* are the key expression markers of progenitor cells that are able to give rise to both types of hepatic cells *in vivo*.[56] TROP2 and EpCAM have also been proposed as human HPC markers, although EpCAM and SOX9 are also expressed by cholangiocytes.[52]

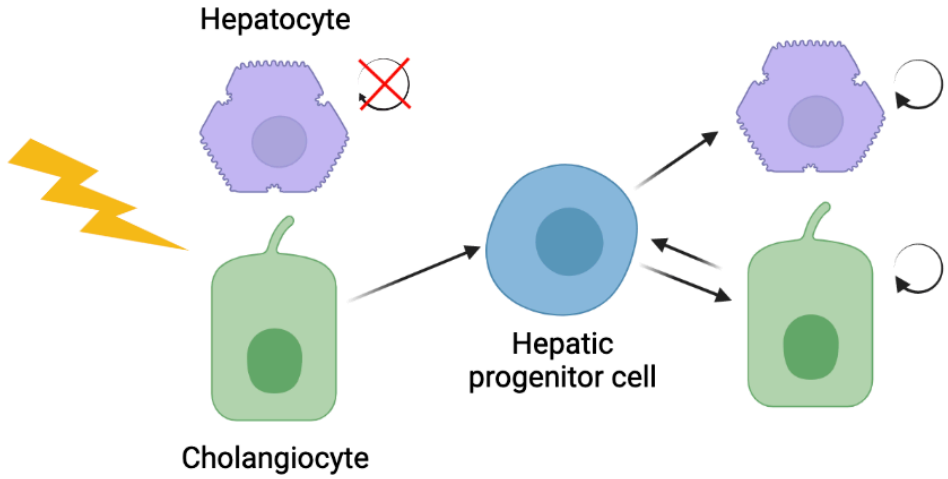

Figure 2: The proposed mechanism of liver regeneration in chronic liver injury. Chronic damage to the liver exhausts the ability of hepatocytes to self-renew, leading cholangiocytes to transdifferentate into a progenitor-like cell, capable of renewing both cholangiocytes and hepatocytes. Cholangiocytes are then able to differentiate back into progenitor cells, maintaining this population.

1.3.1 Ductular Reaction

As a by-product of HPC activation, a compensatory mechanism known as ductular reaction (DR) occurs.[57] Characterised by the proliferation of reactive bile ducts, DRs emerge following chronic liver injury or biliary disorders, and consist of cells expressing both biliary epithelial cell and hepatocyte features.[52, 58] Their shared morphology and keratin expression suggests that these cells are putative HPCs, able to give rise to hepatocytes and cholangiocytes. However, it is difficult to discern the direction of cell fate and functional role of HPCs due to limitations in lineage tracing. Therefore, they may be derived from biliary epithelium, or via the de-differentiation of hepatocytes, undergoing ductal metaplasia to contribute to DRs.[51] The resulting reactive lesion is accompanied by a complex of stroma, inflammatory cells and bone-marrow derived macrophages, and is commonly associated with the progression of portal fibrosis in NASH. It also appears that the expansion of HPCs precedes DR development as HPC activation is seen in NAFLD cases without the emergence of DRs or fibrosis.[59] To further explore this, it is possible to identify the direction of DRs via genetically modified mouse models and fate mapping.

1.3.2 The Role of HPCs in Liver Regeneration

There has been significant debate over the extent of HPC contribution to hepatocyte regeneration in acute and chronic liver injury. Conflicting data and the use of lineage tracing with existing mouse lines has failed to define the contribution of progenitor cells to the maintenance of homeostasis within the liver. In patients with advanced liver disease, HPC-driven liver regeneration is shown to be ineffective, especially in

cases of massive hepatic necrosis during acute liver failure.[60] Some studies have shown that HPCs do play a key role in liver mass recovery after CCl_4-induced injury or PHx in mice, whereas others have given strong evidence for hepatocytes as the main contributors to maintaining liver mass.[49] This shows a need for more accurate insights into the role of HPCs in triggering mass liver regeneration. Zebrafish models have been widely used to support the findings that cholangiocyte-derived hepatic progenitor cells (cdHPCs) are responsible for liver regeneration after extreme hepatocyte loss. Biliary cells were shown to dedifferentiate into progenitor or hepatoblast-like cells, expressing both albumin and cytokeratin-19 (CK19), which are expressed by hepatocyte and cholangiocytes respectively.[61] Cholangiocyte to hepatocyte transdifferentiation was also suggested to require Notch as a promoter of dedifferentiation and formation of a bipotential intermediate. Extensive loss of hepatocytes downregulates this Notch signal, allowing HPCs to undergo hepatocyte differentiation. Cre/loxP-based inducible lineage tracing showed that these new hepatocytes largely arose from cholangiocyte transdifferentiation as a result of Notch signalling and Sox9b activation in transgenic zebrafish models. Defective biliary cell development, however, impacted this process and led to impaired hepatocyte regeneration. This was shown through the pre-treatment of defective zebrafish larvae with γ-secretase inhibitor DAPT, which inhibits Notch signalling, and metronidazole (MTZ) for the inhibition of biliary development, showed hepatocyte regeneration to be significantly impaired in the absence of intrahepatic biliary development.[62]

In contrast, it has been argued that bipotential liver progenitor cells are mostly derived from mature hepatocytes, not cholangiocytes. Both mouse and human

models have been used to show that hepatocytes undergo metaplasia to form biliary-like progenitor cells in a reversible process and proliferate into ducts in the periportal region of the hepatic lobule. In a 3,5-diethoxycarbonyl-1,4-dihydrocollidine DDC-injury setting, *Fah*$^{-/-}$ mice were transplanted with ROSA-mTmG hepatocytes to explore the fate of mature hepatocyte in liver injury. To investigate this in human models, human hepatocyte transplantation into DDC-injured *Fah*$^{-/-}$ *Rag2*$^{-/-}$ *Il2r*$\gamma^{-/-}$ triple knockout mice was used. Additionally, hepatocyte-derived progenitors seem to be more efficient at hepatocyte differentiation when compared to biliary-derived progenitors in serial transplantation experiments.[50] Liver regeneration following toxin-induced injury indicated that hepatocytes were the main constituents of the facultative stem cell compartment.[63] When performing genetic fate tracing in mice with chronic liver injury, hepatocytes were not found to have originated from biliary epithelial cells. Instead, any hepatocytes detected were derived from pre-existing hepatocytes, indicating that progenitor cells do not contribute to regeneration.[64] Biliary-derived proliferative ducts (bilPDs) and hepatocyte-derived proliferative ducts formed during ductular reaction were functionally distinct and appeared to derive from different lineages, as shown by genome-wide expression profiling and organoid forming assays. This was despite equal expression of bile duct markers such as Sox9 and Hnf1b and similarities in morphology.[50] In human models, cdHPCs were unsuccessful in replacing the hepatocyte population without therapeutic enhancement, suggesting that the underlying molecular pathways are yet to be fully determined.[61] It is also possible that in greater injury settings, progenitor or stem-like cells may exhibit bipotency to regenerate hepatocytes.[63]

Despite the contradictory evidence on HPC-driven liver regeneration, it has been discovered that widespread hepatocyte senescence is required for cdHPCs to form hepatocytes. However, lineage tracing experiments involving mouse models do not typically resemble human liver disease as DRs are often accompanied by significant hepatocyte replication, failing to recapitulate human disease. Hepatocyte senescence increases during chronic liver damage, and therefore this needs to be accounted for when designing murine models. Recently, it has been shown that significant biliary-driven hepatocyte regeneration occurs when hepatocellular regeneration is impaired. Inhibiting hepatocyte proliferation via β1-integrin knockdown, inducible deletion of Mdm2 or p21 overexpression resulted in HPC activation and the appearance of cholangiocyte-derived hepatocytes and DR. HPC transplantation into adult mouse livers where hepatocyte proliferation was impaired let to the restoration of liver parenchyma, regenerating both biliary epithelial cells and hepatocytes.[57, 58] From this, it may be concluded that previous findings were not truly representative of human disease as hepatocyte-mediated regeneration was still occurring in the models used.

1.4 Background of IL-17

The IL-17 family of pro-inflammatory cytokines is comprised of six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F. Although there are similarities in protein structure amongst all members of this cytokine family, IL-17A and IL-17F are more closely related, sharing 50-55% homology.[65, 66]They are particularly essential in maintaining barrier immunity to bacterial and fungal infections.[67] These cytokines are almost exclusively produced by adaptive Th17 cells, a recently discovered effector $CD4^+$ T cell that is independent of the traditional lineage of Th1 and Th2 cells.[68] However, it has been proposed that some innate immune cells can also produce IL-17. Innate lymphoid cells (ILC), more specifically, IL-17-expressing Type 3 innate lymphoid cells (ILC3), have been described as the innate counterparts of Th17 cells as they do not express an antigen receptor. Eosinophils, neutrophils, monocytes, $CD8^+$ T cells (known as Tc17), $\gamma\delta$ T cells, natural killer T (NKT) and natural killer (NK) cells have also been found to contribute to IL-17 production. Furthermore, a subset of $TCR\delta\beta^+$ cells, termed 'natural' Th17 cells, have recently been shown to make this cytokine. It has been reported that myeloid cells produce IL-17, but the validity of this data has been brought into question.[69, 70]

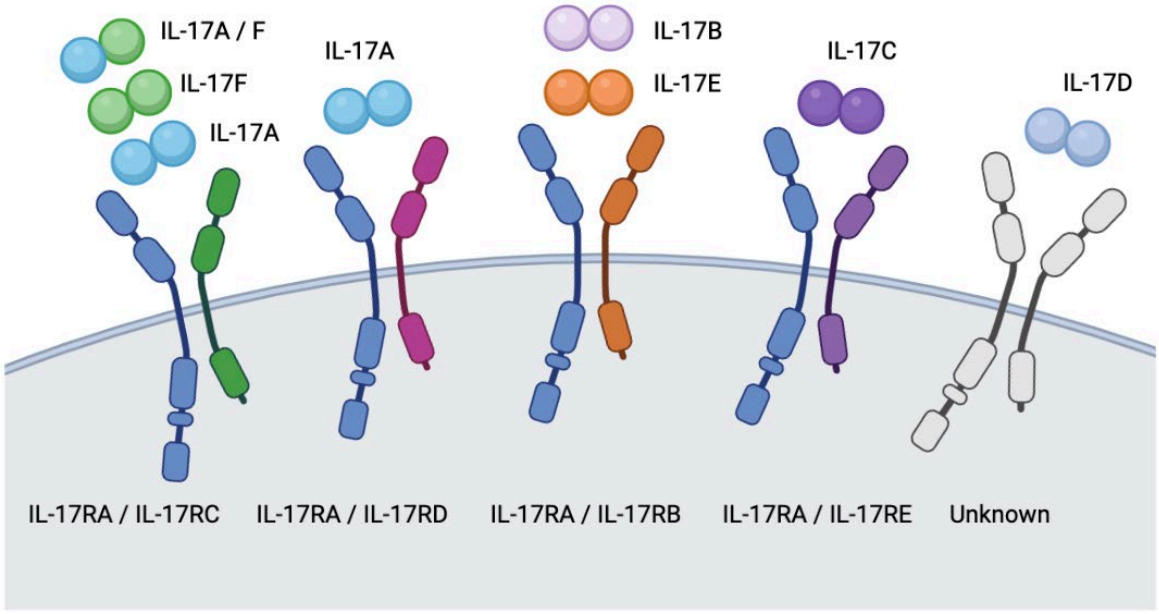

Figure 3: The IL-17 cytokine family.
Schematic showing the IL-17 family members and their corresponding receptors.

1.4.1 The Role of IL-17 in the Immune System

Th17 cells mediate autoimmunity and are involved in the body's immune defence against pathogens.[71] They form part of the $CD4^+$ T cell effector response, secreting several pro-inflammatory cytokines. Naïve $CD4^+$ T cells (Th0) differentiate into Th17 as a result of the combined actions of T cell receptor (TCR) stimulation in the presence of transforming growth factor beta (TGF-β), IL-6, IL-21 and IL-23. Signal transducer and activator of transcription 3 (STAT3) activation then leads to ROR-γT transcription factor expression.[72] Negative regulation of Th17 can occur through IL-17E and IL-27 down-regulating IL-23 expression. These helper T cells are characterised by the secretion of IL-17A and F, IL-21, IL-22, which has been implicated in hepatoprotection, and tumour necrosis factor alpha (TNF-α). The

release of these cytokines results in tissue inflammation and leukocyte recruitment via the induction of neutrophil-attracting chemokines, such as CXCL1, CXCL2 and CXCL8.[70, 73] IL-17A is also able to induce the expression of the neutrophil-recruiting protein, granulocyte colony-stimulating factor (G-CSF), as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) in NK cells.[69, 74] IL-17 signalling and the subsequent induction of cytokines and neutrophil chemokines is key to their biological effects. Dysregulated production of IL-17A, however, has been closely linked to driving inflammatory pathology in autoimmune diseases such as psoriasis, rheumatoid arthritis, multiple sclerosis and asthma.[67] This tissue inflammation occurs as a result of IL-17A-induced production of pro-inflammatory mediators by various cells, including macrophages, osteoblasts and endothelial or epithelial cells. On the other hand, IL-17A deficiency in mice has shown to cause resistance to some autoimmune diseases.[72]

The primary role of IL-17 in the immune system's inflammatory response is to upregulate inflammatory gene expression, including cytokines (G-CSF, TNF-α, IL-6) chemokines (CXCL1, CXCL2 etc.), matrix metalloproteinases (MMPs), inflammatory effectors (acute phase proteins, complement proteins) and antimicrobial proteins (mucins, defensins). The synergistic signalling capacity of IL-17A with other stimuli, such as TNF-α, lymphotoxin, IL-1 and interferon gamma (IFN-γ), facilitates its ability as a powerful inducer of inflammatory cytokines. Upregulation of inflammatory gene expression occurs either through inducing de novo gene transcription or stabilising target mRNA transcripts. These transcripts are stored in cytoplasmic granules, and can be translated or degraded rapidly, depending on the cell's need.[69]

1.4.2 IL-17 Signal Transduction

The IL-17A receptor (IL-17RA) is vital to IL-17 signalling and is widely expressed in the liver. Its surface level expression correlates with the efficiency of IL-17A signalling, as well as the effects of IL-17F. It is expressed ubiquitously and can be further induced in haematopoietic cells and fibroblasts. Deletion of IL-17RA leads to the abrogation of downstream effects of IL-17A and IL-17F, however, the mechanisms behind IL-17 signalling are not yet fully understood. After the binding of IL-17 to its receptor, the association of Act1, a unique cytosolic adaptor, with the IL-17 receptor occurs. Act1 and IL-17R form a proximal signalling complex with TRAF6, with Act1 and TRAF6 functioning as E3 ubiquitin ligases. TRAF6 polyubiquitination and subsequent autoubiquitination is essential for IL-17-induced NF- κB activation. The canonical NF-κB pathway is activated, albeit weakly, by IL-17 signalling as the majority of target genes have essential NF-κB promoter elements. NF-κB forms a complex with IκB kinase (IKK) and is later released from this complex to allow its localisation to the nucleus. Induction of inhibitor of NF-κB zeta (IκBζ) co-operates with NF-κB to drive the transcription of numerous IL-17 target genes. IκBζ can also facilitate IL-17-induced gene expression by the suppression of miR-23b, which inhibits IL-17 signalling. In addition to NF-κB activation, the MAPK (mitogen-activated protein kinases) pathways are also induced by IL-17. This includes ERK, p38 and JNK pathways, but this response varies by cell background. The weak activation of NF-κB by IL-17 and its modest ability to induce inflammatory gene transcription highlights the importance of alternative pathways to indirectly control gene expression. This can be through the control of IκBζ, or via IL-17-induced regulation of mRNA stability.[69]

The induction of pro-inflammatory cytokines via the synergistic actions of IL-17 and TNFα is the result of the stabilisation of inherently unstable mRNA transcripts, rather than through NF-κB-mediated activation. Stabilisation of mRNA is controlled non-canonically. The primary pathway involved is the inducible phosphorylation of Act1, independent of TRAF6.[75] Act1 binds and stabilises the mRNA that encodes key inflammatory proteins. Its SEFIR domain binds to a SBE (SEFIR-binding element) in inflammatory genes such as the chemokine gene *Cxcl1.* This forms three distinct protein-RNA complexes (RNPs) that in turn regulate three different mRNA metabolism events: mRNA is prevented from decaying in the nucleus, mRNA decapping in P-bodies is inhibited, and translation is promoted. Although this enhanced inflammation is advantageous to the clearance of pathogens, it also plays a significant role in the pathology of severe inflammatory conditions.[76]

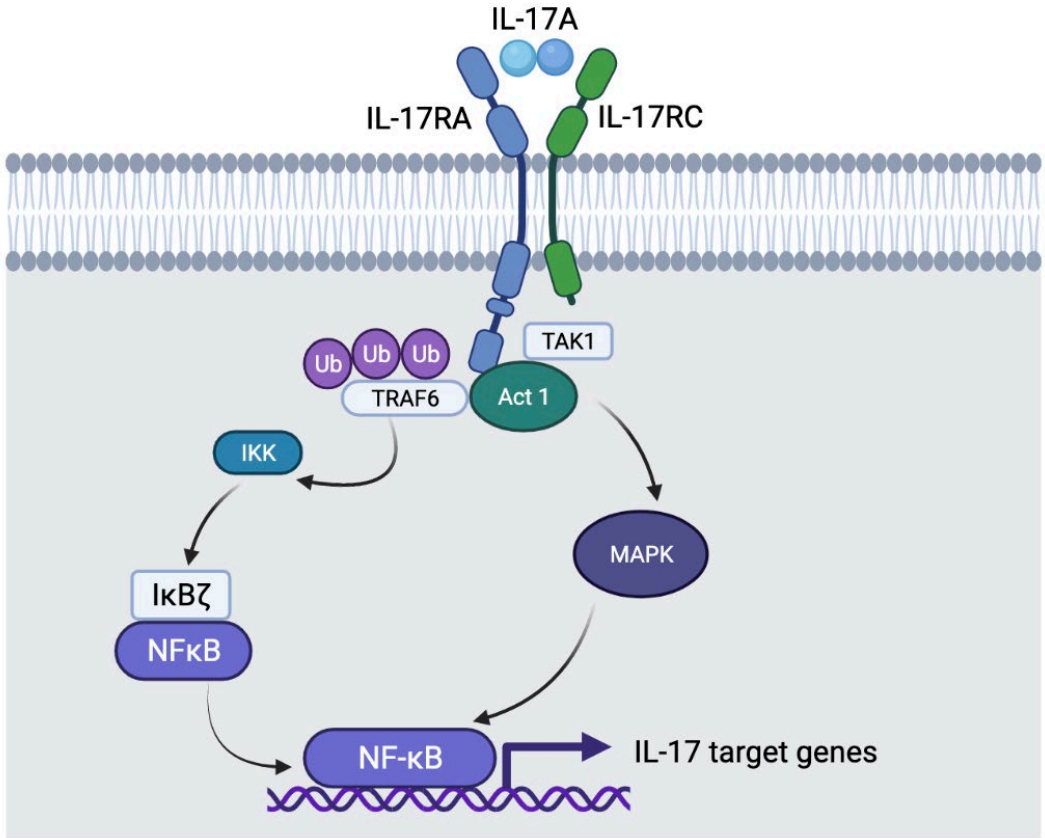

Figure 4: IL-17A and the NF- κB pathway.
Schematic representation of the mechanistic relationship between IL-17A and the canonical and non-canonical NF-Kb pathways.

1.4.3 The Role of IL-17 in Chronic Liver Diseases

IL-17 and NAFLD

The IL-17A receptor, IL-17RA, is almost ubiquitously expressed in the liver by both liver resident cells and innate immune cells.[67, 70] If production of IL-17A is dysregulated, this causes widespread IL-17RA activation and downstream signalling, resulting in an inflammatory response. This activation of the IL-17 axis may be a causal contributor to the pathogenesis of NAFLD. It has been reported that NAFLD in the context of obesity is associated with an increase in systemic and hepatic IL-17A expression. Furthermore, IL-17A has been associated with the occurrence of obesity, glucose dysmetabolism and hepatic injury. Interestingly, genetic deletion of IL-17RA in mice protected them from this glucose dysmetabolism, and combined with antibody-mediated neutralisation of IL-17A, were also protected from steatohepatitis and hepatocellular damage. In MCD-fed mice, which produce a NAFLD model, there was an increased expression of IL-17RA. This correlated with a notable increase in hepatic $CD11b^{+}F4/80^{+}$ macrophage infiltration. These macrophages also increasingly skewed towards a pro-inflammatory phenotype, producing high levels of TNF-α, IL-1β, IL-6, IL-12 and IL-23, and are characterised by their ability to phagocytose pathogens and present their antigens to T lymphocytes.[77, 78]

As neutrophilic inflammation is a component of human NASH pathology, it is likely that the release of neutrophil-attracting chemokines by IL-17 is a contributing factor. IL-17 production is also widely known to regulate the process of neutrophil infiltration; its signalling leads to a greater response to inflammatory stimuli and

neutrophil recruitment. Dietary stress, which is common in NAFLD, also promotes myeloid cell recruitment to the liver.[67] Despite this, a study into the regulation of inflammation by IL-17A showed there was no significant difference in neutrophil infiltration during short-term MCD feeding. However, this may suggest that neutrophil infiltration only occurs in a more severe pathology, and therefore requires a longer-term MCD feed, or may indicate the suitability of the animal model.[77]

IL-17A has also been shown to mediate neutrophil-dependent hepatocyte damage via ROS-driven oxidative stress. The combined effects of neutrophil infiltration and inflammation-associated ROS production induces injury in hepatic parenchymal cells. It has been suggested that steatosis sensitises hepatocytes, making them more susceptible to hepatocellular injury and fibrosis as a result of inflammation or oxidative stress.[67] NAFLD-associated inflammation is often characterised by the infiltration of macrophages and subsequent release of proinflammatory cytokines.[77] Interestingly, the dysregulation of lipid metabolism that occurs in NAFLD leads to the loss of CD4+ T lymphocytes via mitochondrial disruption, and was shown to accelerate hepatocarcinogenesis in both mouse models and human livers.[79]

IL-17A and Fibrosis

IL-17A and its receptor, IL-17RA, also play a role in the development of fibrosis. The expression of IL-6, TGF-β and alpha smooth muscle actin (α-SMA) increased in CCl_4-induced fibrosis, alongside a significant increase in Th17 cell frequency.[80] IL-17RA$^{-/-}$ mice show a critical attenuation of liver fibrosis in both bile-duct ligation and

CCl_4 models. Hepatic stellate cell (HSC) culture indicates that IL-17A directly stimulates their activation via STAT3, with Th17 cells also promoting the expression of α-SMA.[70] Significant upregulation of IL-17A gene expression in fibrotic livers may indicate IL-17A plays a role in activating hepatic stellate cells. The activation of isolated HSCs *in vitro* has been shown to be dependent on IL-17A, which acts through its receptor, IL-17RA, which is expressed on the surface of HSCs.[81] In addition to this, IL-17A enhances the response of HSCs to TGF-β through increased receptor expression, in turn upregulating production of collagen type 1, α-SMA and TIMP-1.[82] Collagen production in HSCs is stimulated by IL-17-mediated Stat3 activation. IL-17 triggers the nuclear localisation of phosphorylated Stat3, inducing collagen-α1 (I) expression, which does not occur in Stat3-deficient HSCs.[70] From this, it appears that IL-17A activation of hepatic stellate cells involves both TGF-β and Stat3.

1.4.4 The Role of IL-17 in Other Pathologies

In addition to the liver, IL-17A is essential in facilitating inflammatory responses in various other organs. IL-17A plays a pathogenic role in hypertensive kidney disease-associated inflammation. Infusion of IL-17A in mice resulted in increased blood pressure that was associated with $CD3^+$ and $CD4^+$ T helper lymphocytes and neutrophilic kidney infiltration. Kidney biopsies from hypertensive nephrosclerosis patients showed a significant presence of IL-17A positive cells, predominantly Th17 and γδ T cells.[83] In cardiovascular disease, the upregulation of the C/EBP pathway

through IL-17A and IL-17F signalling leads to atherosclerotic plaque instability, increased lesion size and inflammation through IL-6/G-CSF production, and myeloid cell recruitment. Some studies, however, argue that IL-17A can promote plaque stability by decreasing vascular cell adhesion protein (VCAM) expression and T cell infiltration.[84] This suggests that there is a fine line between the pathogenic and protective sides of IL-17 signalling. A role for IL-17A has also been shown in heart transplantation. Previous studies indicate that IL-17 contributes to the development of chronic rejection or cardiac allograft vasculopathy. Itoh et al show that IL-17A also accelerates acute allograft rejection by suppressing the expansion of regulatory T cells. In this context, γδ T cells are responsible for the production of IL-17, rather than $CD4^+$ or $CD8^+$ T lymphocytes.[85]

1.4.5 The Role of IL-17A in Liver Regeneration

Chronic liver diseases are often associated with raised levels of infiltrated immune cells, such as $IL\text{-}17^+$ cells as well as the expression of cytokeratin 19 (CK19) expressing cells. These two cell populations have been shown to co-localise and appear to increase in conjunction with each other. The number of IL-17-producing cells infiltrating the liver was also shown to correlate with the degree of DR.[86] This may indicate a role for IL-17 signalling in HPC activation and proliferation, supported by the discovery that cholangiocytes express the IL-17 receptors, IL-17RA and IL-17RC, as well as Act1.[87] Disruption of the IL-17 or IL-27 receptor genes also prevented HPC expansion and subsequent inflammation, highlighting the collaborative role of IL-17 and IL-27 in promoting HPC-driven regeneration.[86] In livers with alcoholic steatohepatitis (ASH), IL-17A-producing cells were discovered in close

proximity to HPCs, suggesting IL-17A signalling may be an important pathway in HPC biology.[88] When administered in mice following partial hepatectomy, IL-17 promoted liver regeneration, but when absent, led to reduced expression of regenerative growth factors and cell cycle regulators, slowing the regenerative process.[89] The protective, as well as pathogenic role that IL-17A plays in liver regeneration indicates there is a delicate balance that needs to be addressed when considering IL-17A as a therapeutic target. Understanding the mechanisms that underlie IL-17A signalling, and how this can be enhanced or inhibited to reach this balance is vital to achieve the aim of targeting IL-17A to promote liver regeneration.

1.5 The Role of Other Mediators in Liver Regeneration

1.5.1 Tumour Necrosis Factor-alpha (TNF-α)

Other mediators have been previously described as playing an essential role in the process of liver regeneration. Activation of the TNF-α receptor, TNFR1, induces cell death, inflammation and fibrosis. Interestingly, it also aids in hepatocyte survival and subsequent regeneration. TNF-α signalling via TNFR1 contributes to not only acute and chronic hepatic inflammation, but also remodelling of the fibrotic tissue, as well as tumorigenesis. TNF-α/TNFR2 activation can also promote these changes, although to a lesser extent.[90]

TNFR1 is expressed ubiquitously throughout the liver. Once activated by TNF-α, it can lead to pro-inflammatory, cytotoxic and apoptotic outcomes. TNFR2, however, is found primarily on haematopoietic cells, and unlike TNFR1, lacks the intracellular death domain that induces TNFR1-dependent cell death. Multiple studies have shown that TNF-α-mediated signalling through TNFR1 is vital for inducing hepatocyte proliferation and regeneration. In *Tnfr1-/- Mdr2-/-* mice, the significant upregulation of several hepatic genes has been recorded.[90] The *Mdr2-/-* knockout mouse model leads to liver injury, biliary fibrosis, and sclerosing cholangitis through failure to secrete phosphatidylcholine into the bile.[91] Compared to the *Mdr2-/-* mice, dKO mice had increased expression of *Il1b, Il23, Tgb1*, and *Il17a.* Upregulation of *IL17a,* alongside the transcription factor *Rorγt,* indicates the presence of a Th17 population in the liver. Rorγt is essential in inducing Th17 lineage fate, as well as in the regulation of *Il17a* transcription.[92]

1.5.2 Lymphotoxin-beta Receptor (LTβR)

An additional core member of the TNF receptor superfamily, lymphotoxin-beta receptor (LTβR), is also involved in the liver's unique ability to regenerate following injury. LTβR is expressed on the stromal cells of lymphoid tissues, follicular dendritic cells, monocytes and dendritic cells. The importance of its ligands, LIGHT and $LT\alpha_1\beta_2$, in regulating liver homeostasis and liver regeneration, has also been highlighted. $LT\alpha_1\beta_2$ is expressed on activated T, B and NK cells.[93, 94] Whilst TNFR1 solely signals through the rapid and transient canonical NF- κB pathway, LTβR can also act via the non-canonical pathway. This signalling route is slower, but persistent, responding to TNFR signals to carry out more specific functions. LTβR activation can induce cytokine, chemokine or adhesion molecule expression, as well as regulate cell proliferation and survival.[94, 95] Interestingly, the activation of LTβR by T cell-derived $LT\alpha_1\beta_2$ on mouse macrophages can downregulate pro-inflammatory cytokines, such as TNF and IL-6. It has been suggested that this receptor controls pro-inflammatory cytokine expression by activating a signalling pathway that prevents the exacerbation of inflammatory cytokine production.[96]

In the context of the liver, membrane bound $LT\alpha_1\beta_2$ is delivered to the regenerating liver by infiltrating T cells. Consequent LTβR signalling activates the NF-KB signalling pathways in activated B cells. Mice deficient in LTβR ($LT\beta R^{-/-}$) show a severely defective ability in surviving PHx. Marked liver damage and the inability to initiate DNA synthesis following partial hepatectomy was also noted.[94, 97] In 70% PHx mice with this deficiency, liver regeneration was significantly compromised. This led

to decreased survival of LTβR$^{-/-}$ mice when compared to WT animals, showing the important involvement of lymphocyte-restricted ligands in liver regeneration. In KO mice, the lack of Ki67 upregulation and the downregulation of cyclin D1 suggested that mice deficient in this receptor were unable to initiated synchronised hepatocyte cell division. Failure of these regeneration mechanisms may lead to a compensatory response through an increase in TNF and LTα expression. After PHx, serum TNF and IL-6 levels increase, which activate canonical and non-canonical NF-κB pathways. However, mice functionally deficient in both pathways are unable to initiate liver regeneration. Cooperative TNFR/LTβR signalling via NF-κB is therefore essential for efficient regeneration to occur.[95]

Aside from these changes in cytokine expression, LTβR$^{-/-}$ mice also exhibited an imbalance in bile acid (BA) homeostasis in their regenerating livers. Increased ALP levels indicated that deficient mice had developed intrahepatic cholestasis, although the onset of regeneration was not delayed. In WT mice, BA were more hydrophilic, which are considered to be hepatoprotective, whereas LTβR$^{-/-}$ mice had significantly more toxic hydrophobic BA.[94] Bile acid signalling is mediated by the farnesoid X receptor (FXR) and is considered essential for an efficient liver regenerative response following injury.[98] RT-PCR analysis of liver tissue samples from LTβR$^{-/-}$ and WT mice showed an upregulation in *FXR* and *TGR5* expression in KO animals. TGR5 is a G-protein coupled bile acid receptor, and the marked upregulation of its transcription factor counterpart may demonstrate a compensatory mechanism to minimise the toxic effects of BA. Additionally, the increased expression of *LTβR, TGR5* and *FXR* on cholangiocytes may indicate that LTβR-mediated signalling is important in BA homeostasis, especially during liver regeneration.[9]

1.5.3 A20

A20, also known as TNF-α protein 3, plays a key role in the regenerating liver. Originally characterised as a NF-κB inhibitory signalling protein, A20 is now referred to as ubiquitin-regulating and potentially a susceptibility gene for inflammatory diseases. It also protects cells from TNF-induced cytotoxicity.[99] Following injury, inflammation or resection of the liver, hepatic expression of A20 increases as part of a protective response. Through the inhibition of NF-κB activation and subsequent downregulation of cytokine production, A20 is able to reduce inflammation, protecting hepatocytes from apoptosis. Secondly, A20 acts to reduce oxidative stress by increasing the expression of peroxisome proliferated-activated receptor-alpha (PPARα) to enhance fatty acid oxidation, therefore also optimising energy production. A20 further induces a hepatoprotective response by promoting hepatocyte proliferation through a decrease in the cyclin-dependent kinase inhibitor p21, and by upregulating IL-6/STAT3-mediated signals.[100] The enhancement of IL-6/STAT3 signalling is achieved by decreasing suppressor of cytokine signal-3 (SOCS3) expression to promote liver regeneration. The release of IL-6 (and TNF-α) allows hepatocytes to enter the cell cycle and proliferate. This pathway is regulated by a negative feedback loop involving SOCS3, which if inhibited, leads to improved regeneration after PHx.[101]

Knockout of A20 in mice leads to sustained NF-κB- dependent gene expression in the liver, hepatocyte apoptosis and premature lethality as a result of significant multi-organ inflammation. Mice deficient of this protective protein are subjected to excessive liver damage following partial hepatectomy, as shown by increased

plasma ALT, AST and Total Bilirubin.[100] SOCS3 expression also increases, attenuating IL-6/STAT3-mediated signalling to prevent hepatocyte proliferation and liver regeneration. This was shown by a lack of $Ki67^{+}$ hepatocytes.[101, 102] $A20^{-/-}$ mice developed more significant steatosis, necrosis, and haemorrhage when compared to wild-type (WT) animals. Spontaneous development of chronic liver inflammation in mice lacking A20 expressive in liver parenchymal cells has also been reported, indicating its importance in normal liver tissue homeostasis.[102] Overexpression of A20 promotes hepatocyte proliferation and improves survival, even after severe toxic or ischaemic injuries, and radical hepatectomy.[101]

This hepatoprotective protein is an essential player in the liver regenerative response. The combination of A20 knockout and partial hepatectomy leads to insufficient upregulation of cyclin D1. Prolonged upregulation of this protein is vital in initiating regeneration.[100] Interestingly, hepatocyte-specific A20 knockout mice were also more susceptible to chemically or high fat diet-induced hepatocellular carcinoma (HCC) development.[102]

1.5.4 Hepatocyte Growth Factor (HGF) Signalling

The initiation of liver regeneration relies on the activation of the resident stem cell compartment to replace damaged hepatocytes and biliary cells following the loss of their proliferative capabilities. Hepatic progenitor cells (HPC) are widely thought to reside within the Canals of Hering, which are the terminal bile ductules of the liver.[103] Previous studies have highlighted the role of hepatocyte growth factor (HGF) and its receptor, MET, in maintaining HPC. Gene knockout studies confirmed the

upregulation of MET expression during early development and its role in liver organogenesis. MET is needed to stimulate progenitor cell proliferation, hepatocyte differentiation and apoptotic resistance.[104, 105] In MET-defective HPC, expansion did not occur, and these progenitor cells were unable to commit to a hepatocyte lineage during chronic toxic liver injury.[106]

1.5.5 Epidermal Growth Factor (EGF) Signalling

In addition to MET signalling, epidermal growth factor receptor (EGFR) is involved in mediating hepatic homeostasis.[103] Deregulation of EGFR signalling can be implicated in failure to initiate liver regeneration. *Egfr*$^{-/-}$ mice exhibited a decrease in survival after partial hepatectomy. Liver regeneration following 70% PHx relies on sufficient HPC expansion to replace lost or damaged tissue.[107] Both MET and EGFR receptors activate during this process to trigger MAPK/ERK, PI3K/AKT and STAT3 pathways. These are essential in the control of proliferation, differentiation, motility regulation and protection against apoptosis. MET in particular strongly induces STAT3 and AKT activation, promoting HPC differentiation towards a hepatocyte lineage. The expansion of HPC and subsequent differentiation into cholangiocytes relies on EGFR-mediated NOTCH1 activation. This is also required in the process of branching morphogenesis. However, the loss of *Egfr* only resulted in NOTCH1 downregulation, attenuating *in vivo* and *in vitro* HPC differentiation towards cholangiocytes without affecting NOTCH2 expression. It is unknown whether liver regeneration, or even development, is affected by other notch receptors.[103]

IL-17A has been linked to all these mediators in various ways. It has been reported that IL-17A-mediated activation of EGFR is critical in the expansion and migration of Lrig1^{+} stem cells in wound healing and skin tumorigenesis. Synergy between IL-17

and TNF-α transactivates EGFR and is also responsible for the activation of p38 MAPK and ERK pathways.[108] The interaction between IL-17A and the deubiquitinase A20 appears to be more inhibitory, particularly as A20 has been shown to interact directly with the inhibitory distal domain of IL-17RA. A mechanism for the A20-mediated feedback inhibition of IL-17RA signalling involving the E3 ubiquitin ligase TRAF6 and removal of ubiquitin from intermediates upstream of NF-κB has also been described.[109] This evidence highlights a need for further investigations into the role of IL-17A in liver stem cell activation and potential therapeutic targets to promote liver regeneration in CLD.

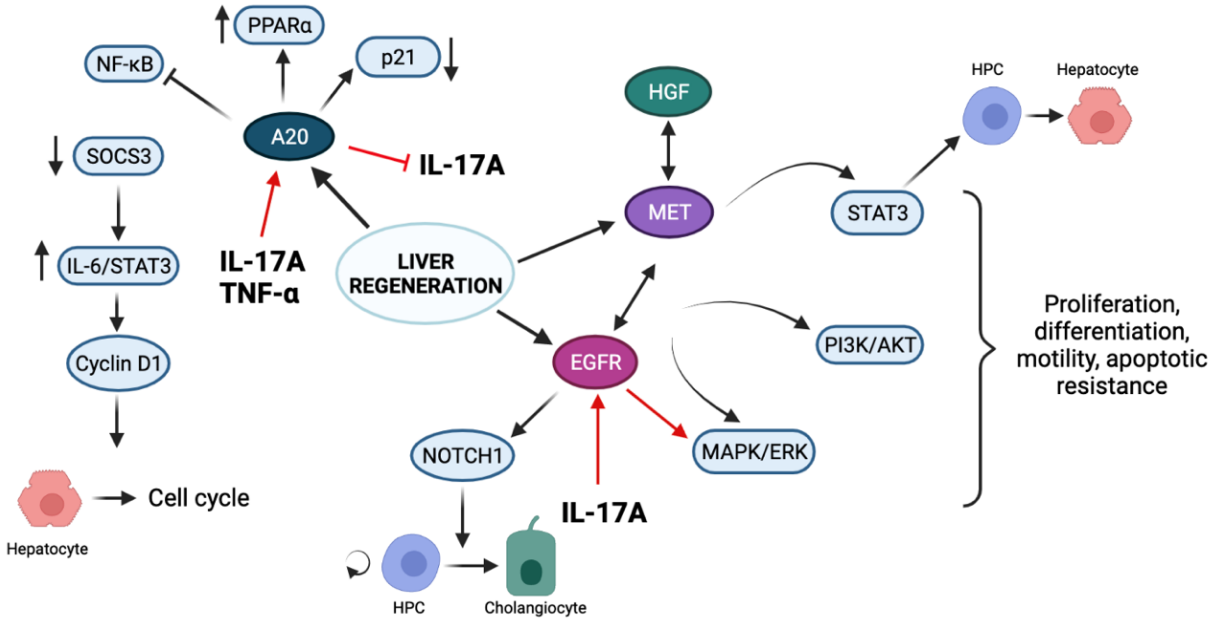

Figure 5: The relationship between IL-17A, A20, MET and EGFR.
Schematic showing the relationship between IL-17A, A20, MET and EGFR, and a summary of the potential pathways involved.

1.6 CD133 and IL-17

The relationship between IL-17A and CD133, a well-established cancer stem cell (CSC) marker, has been of recent interest. CD133, also known as PROM1, is a stem cell marker for both normal and cancerous tissues.[110] It has also been associated with processes such as regeneration, differentiation and metabolism. In addition to being an important cancer biomarker, CD133 has been implicated in normal cell growth and development. There are various factors involved in CD133 expression regulation, including hypoxia (via hypoxia inducible factors), mitochondrial dysfunction, TGF-β1, Notch, p53 and epigenetics.[111, 112] The upregulation or downregulation of CD133 can have various effects. Knockdown of CD133 in head and neck cancer initiating cells (HNCIC) was shown to reduce OCT4 and NANOG gene expression, markers of stemness. Furthermore, this led to greater epithelial differentiation and the occurrence of apoptosis.[113] Conversely, the increased expression of CD133 enhances the expression of Slug, N-cadherin and IL-1β through NF-κB activation, which are key in promoting epithelial to mesenchymal transition (EMT) in tumour progression. The expression of cell migration factors, such as Akt and Wnt, are also upregulated due to CD133 forming complexes with EGFR and β-catenin.[112] Many of these processes are comparable to those seen in the microenvironment during liver regeneration, where the activation and expansion of a hepatic progenitor cell compartment is key in replacing damaged tissue. EGFR also appears to play a role here, acting synergistically with c-Met to promote the regenerative response, and similar to the role of N-cadherins in cell communication in a tumour microenvironment, integrin signalling mediates ECM and epithelial cell communication. Additionally, the upregulation of TGF-β1 and the activation of Wnt/β-

catenin signalling pathways are common to both HPC-driven liver regeneration and CD133^{+}-associated tumour initiation.[114] In the liver, CD133^{+} cells isolated from a Huh7 hepatocellular carcinoma cell line were highly proliferative and expressed lower levels of mature hepatocyte markers than CD133^{-} cells of the same line. During early liver restoration in a 70% PHx mouse model, PROM1 was significantly upregulated.[110] A similar occurrence was found in the peripheral blood of healthy individuals following partial hepatectomy. CD133-expressing haematopoietic progenitor cells could be detected, and when cultured *in vitro*, were capable of differentiating into hepatocytes.[115] Interestingly, when co-cultured with lymphatic endothelial cells, CD133^{+} hepatoma cells promoted the expression of IL-17A at an mRNA and protein levels, as well as IL-17A secretion, when compared to CD133^{-} cells.[116] Conversely, IL-17A produced by the inflammatory tumour microenvironment was shown to stimulate the self-renewal of CD133^{+} CSCs in ovarian cancer, although the idea that IL-17A directly upregulates CD133 expression was not investigated.[117]

The appearance of DRs during liver regeneration is considered to be representative of a stem cell response, with CD133^{+}-expressing hepatic stellate cells (HSCs) playing a key role. Tube-like structures formed by CD133^{+} HSCs *in vitro* may correspond with the ductular structures formed during DR *in vivo*. Treatment of these cells with the cytokines hepatocyte growth factor (HGF), IL-6 and fibroblast-growth factor, FGF4, resulted in cells expressing typical hepatocyte markers such as alpha-fetoprotein (AFP), albumin and multidrug resistance-associated protein 2 (MRP2).[115] These findings may indicate a role for CD133^{+} HSCs in the renewal of injured liver tissue, and as a progenitor cell compartment alongside cdHPCs. Additionally, the

upregulation of CD133 expression on biliary-derived proliferative ducts (bilPDs) suggests a link between CD133 and HPCs.[50] However, the exact relationship between CD133 and IL-17A, as well as with cdHPCs, has yet to be fully established.

1.7 Wnt and IL-17

Wnt signalling is a highly conserved, complex pathway that is involved in various cellular processes throughout the body, including the regulation of stem cell survival and promotion of stemness. In the liver it has shown to be key in regulating liver development, differentiation and homeostasis, indicating its role in hepatic regeneration following injury. The canonical Wnt pathway and its central protein, β-catenin may act to promote regeneration via its target gene, Sox9. It is widely thought that hepatocytes are generated from Sox9-precursors during tissue damage, and therefore may be involved in HPC-driven liver regeneration.[118] CD133 expression has also been shown to activate the Wnt/β-catenin signalling pathway, and appears to play a functional role in renal tubular repair by regulating proliferation and controlling senescence.[119, 120] Whether this can be applied to DR during liver regeneration or not needs to be explored. In addition to this, the relationship between Wnt and IL-17A is inconclusive. There have been several studies linking the pro-inflammatory cytokine to this developmental pathway, including evidence for Wnt/β-catenin modulating IL-17A-altered macrophage polarisation.[121] However, the majority focus on the impact of IL-17A in rheumatoid diseases, rather than CLD. IL-17 reportedly decreases bone formation via dampening of Wnt signalling in osteoblastic cells and Th17-induced senescent cells show altered Wnt signalling and tissue remodelling in response to injury in osteoarthritis.[122, 123]

AIMS

- To identify the effect of IL-17A on cholangiocyte fate *in vitro*
- To investigate the expression and regulation of IL-17RA on cholangiocytes during ductular reaction
- To define the potential mechanisms in which IL-17A mediates ductular reaction

HYPOTHESIS

IL-17A drives the bipotency of cholangiocyte-derived hepatic progenitor cells to mediate ductular reaction during liver regeneration.

MATERIALS AND METHODS

Immunofluorescence staining – CK19, IL-17A, Sox9

Slides of 5μm sections from mouse liver fixed in formalin were dewaxed in xylene for 3 x 3 minutes and rehydrated through alcohols (100%/70%/65%) for 3 minutes each. Slides were then washed in dH_2O before antigen retrieval. Slides were placed in pre-warmed 0.01M Citrate Buffer pH 6.0 and heated in the microwave for 15 minutes. To prevent over-boiling and loss of tissue, there were regular breaks during the heating process. Once cooled with cold running water, slides were mounted in sequenza racks and washed with PBS. 200μl of GeneTex Trident Universal Protein Blocking Reagent (GTX30963) was then added for 30 minutes. A 1:400 dilution of rabbit anti-IL17 antibody (Cat. No. A00421-2) and 1:200 dilution of rat anti-CK19 antibody (DSHB AB_2133570) was prepared and 120μl added to each slide. For Sox9 immunofluorescence staining only, a 1:200 dilution of rabbit anti-Sox9 (Cat. No. AB5535) and a 1:500 dilution of donkey anti-rabbit (Alexa Fluor® 555, Cat. No. A21208) were used. These were incubated for 1 hour at RT, rinsed with PBS 3 times and 120μl of the secondary antibody mix was added, alongside a 1:200 dilution of DAPI. Secondary antibodies used were a 1:500 dilution of donkey anti-rabbit (Alexa Fluor® 555, Cat. No. A21208) and a 1:300 dilution of donkey anti-rat (Alexa Fluor® 488, Cat. No. A-31572). Slides were incubated for 30 minutes at RT, rinsed thoroughly and incubated for 5 minutes with 300μl of 0.5% Sudan Black B to reduce autofluorescence during imaging. 300μl of 70% ethanol was used to rinse each slide, which were then washed further with PBS and mounted with Fluoromount G®

(Southern Biotech, Cat. No. 0100-01). Slides were imaged using a Zeiss Axioskop 40 microscope, Axiocam 305 color camera and Zeiss ZEN Pro microscopy software.

IL-17A treatment, cell harvesting and qPCR

Clonal-derived hepatic progenitor cells were treated with 20ng/mL Recombinant Murine IL-17A (Peprotech, Cat. No. 210-17) or with treatment-free media (DMEM supplemented with 1% FBS and 1% penicillin/streptomycin, Life Technologies, Cat. No. 31966-021) for 2 days. Following this, cells were harvested using 300μl of lysis buffer and 400μl/sample of 70% ethanol was used for RNA extraction, which was then quantified for cDNA synthesis. RNA was converted to cDNA by two-step RT-qPCR using the Qiagen QuantiTect® Reverse Transcription Kit (Cat. No. 205310). 2μl of gDNA Wipeout Buffer was added to each sample and cDNA synthesis using a Bioer GeneTouch™ Thermal Cycler for 10 minutes. The primer Master Mix, consisting of 5μl RT Buffer Mix and 1ul RT, was then added to each sample. DNA synthesis took place for 1 hour using the thermal cycler. A 1:10 cDNA dilution was prepared using nuclease-free water, with 4μl of this solution used for each reaction. 8 Qiagen QuantiTect® Primer Assay primers were used (see Table 1); 1μl of each of these primers was mixed with 5μl SYBR™ Select Master Mix and reacted with each cDNA sample. qPCR was subsequently performed using the Roche LightCycler 480, and the data was analysed using the -2ddCt method.

Primer	Cat. No.
Mm_Ppia_1_SG	QT00247709
Mm_Prom1_1_SG	QT01065162
Mm_Gpr49_1_SG	QT00123193
Mm_Klf5_2_SG	QT01057756
Mm_Catnb_1_SG	QT00160958
Mm_Ccnd1_1_SG	QT00154595
Mm_Nfkb1	QT00154091

Table 1: List of Qiagen QuantiTect® Primer Assay primers used in qPCR of cdHPCs

Tcf/Lef H2B GFP cell line

Tcf/Lef GFP cdHPCs, which were previously generated in the lab through the transfection with a Tcf/Lef: H2B-GFP plasmid (Addgene 32610), were used. The Tcf-Lef GFP Reporter cell line was cultured and treated with either 20ng/mL Recombinant Murine IL-17 (Peprotech, Cat. No. 210-17), 20ng/mL recombinant Wnt3a (R&D Systems, Cat. No. 5036-WN) or 1μM Bay 11-7082 (Sigma-Aldrich, Cat. No. 19542-67-7) for 2 days. Some cells did not receive any treatment to act as a control. After 2 days of treatment, cells were harvested using 300μl of TryPLE and transferred to a 96-well plate. 100μl of formalin, diluted 1:1 with PBS, was added to each well. The plate was incubated in the refrigerator for 10 minutes, washed with PBS and then the plate gently centrifuged at 300G for 5 minutes. The supernatant was discarded before PBS was added. Cells were then analysed using FACS.

Lymphocyte isolation and FACS analysis

Mouse livers were harvested and cut into fine pieces with scalpels. 15mL of PBS was added and the solution was passed through a 70μm cell strainer. Hank's salt solution and 100% Percoll were prepared with 10x Hank's Salt Solution, and further diluted with PBS, of which 7.5mL was added to each sample to make up a 33% Percoll working solution. Once thoroughly mixed, they were centrifuged at 700G for 20 minutes with 2x acceleration and 2x deceleration. The supernatant was discarded, and the liver samples washed with PBS before further centrifugation at 400G for 5 minutes. The supernatant was removed again, and 1-2mL of a 1:10 Lysis Buffer solution was mixed with the samples. The volume of Lysis Buffer used was dependent on the mass of each liver. After being placed on ice for 2 minutes, 10mL of PBS was added to halt the reaction and the samples were centrifuged again at 400G for 5 minutes. The supernatant was discarded and 100μl of PBS was added to each liver sample. 80μl was transferred to each well of a V-bottom 96-well plate for FACS staining, and the remaining 20μl used as the unstained sample in separate wells. 100μl of 1:1000 Zombie NIR Live Dead antibody (Cat. No. 423106) was mixed thoroughly with each stained sample and the plate incubated at 4°C for 5-10 minutes. The plate was then centrifuged for 4 minutes at 400G, and gently tipped to leave the pellets.

Spleens were pushed gently through the 70μm strainer using a syringe plunger, topped up with 10mL PBS and centrifuged using an MSE Mistral 2000 centrifuge at 400G for 5 minutes. A 1:10 Lysis Buffer solution was prepared, and 1mL was added to each spleen for 2 minutes whilst on ice. PBS was used to prevent further reaction, and the solution was centrifuged at 400G for 5 minutes. The supernatant was

discarded and the remaining pellet was placed on ice. 700μl of PBS was added to the spleen sample and 45μl was transferred to each single-stained well. 100μl was used for the full-stained spleen and unstained spleen wells.

50μl of 2% FBS FACS Buffer was pipetted into each single-stained well. 50μl of a 1:200 antibody mix, using the below FACS antibodies outlined in Table 2, was added to each liver sample and the stained spleen sample. 0.25μl of each antibody was loaded into the individual single-stained wells. The plate was then incubated in the dark at 4°C for 30 minutes, washed with 70μl of PBS and centrifuged. The supernatant was discarded and the plate gently vortexed for subsequent FACS analysis.

Intracellular staining

To stain for IL-17A, 50μl of a 1:3 dilution of fixation/permeabilisation concentrate (ThermoFisher Scientific 00-5523-00) was added to each well and the plate incubated for 30 minutes. A 1:10 dilution of permeabilisation buffer was prepared and 100μl pipetted into the wells before being centrifuged. 50μl of a 1:200 IL-17A antibody solution was added to the full-stained liver and spleen samples, as well as the single-stained well for IL-17A. After a 30-minute incubation, the plate was washed and centrifuged. 200μl of FACS buffer was added to each well and transferred to FACS tubes.

FACS Antibodies		
Epitope	**Conjugate**	**Cat. No.**
CD3	AF700	100216
CD4	PE	100512
CD8	Percp	100731
CD45	AF488	103121
CD11b	BV510	101263
NK1.1	BV650	108736
F4/80	BV711	123147

Table 2: List of FACS antibodies used in lymphocyte analysis

Cholangiocyte isolation and FACS staining

Dissected mouse livers were minced with scissors and a scalpel until tissue pieces were very small, but not too fine as to disrupt bile duct structure. At the same time, digest media was made and pre-warmed in a water bath at 37°C. Using 0.125 mg/mL collagenase from *Clostridium histolyticum* (Sigma-Aldrich, Cat. No. C9407), 0.125 mg/mL dispase II (Life Technologies, Cat. No. 17105-041) and wash media (DMEM supplemented with 1% FBS and 1% penicillin/streptomycin, Life Technologies, Cat. No. 31966-021), 50mL of digest media was prepared. The minced tissue was collected with a 10mL serological pipette and placed in a falcon tube with cold wash media. The tube was inverted several times and then spun at 100G for 1 minute. Once the supernatant was aspirated, 15mL of digest media was added and the tube shaken to resuspend the tissue. The tube was then placed sideways in a shaker incubator and incubated for 1 hour at 37°C and 200rpm. During and after incubation, the solution was pipetted up and down with a serological pipette to ensure digestion. It was then spun again at 120G for 2 minutes, the supernatant

aspirated and resuspended in 10mL digest media. The tissue was checked under a microscope to make sure the material consisted mainly of clean small ductules. Once checked, the tissue was returned to the shaker for a further 30 minutes at a slower speed of 160rpm. A 7x TryPLE Express (Life Technologies, Cat. No. 12605-028) was prepared by mixing 1.5mL 1x and 3.45mL 10x solutions and placed in the water bath. Once incubated, the tissue was pipetted up and down, spun at 120G for 4-5 minutes and the supernatant aspirated before being resuspended in 5ml of the pre-warmed TyrPLE solution. This was pipetted up and down with a glass Pasteur pipette several times and incubated in the water bath at 37°C for 10 minutes. After 9 minutes, the tube was removed and pipetted for a further 1 minute. To stop the reaction, 30mL of wash media was added and the solution spun at 300G for 5 minutes. The supernatant was aspirated, and the remaining solution resuspended in 3ml of wash media. This was then filtered through a 40μm filter and centrifuged for 5 minutes at 320G. For MCD-injured livers, the filtering step was repeated twice. Following centrifugation, the remaining pellet could then be used for FACS staining. The same FACS protocol for lymphocyte staining was used for cholangiocytes. A 1:100 dilution of the following antibodies (see Table 3) was used to identify cholangiocytes and detect the expression of IL-17RA. Antibodies against CD45, CD31 and Ter119 were used as a lineage panel (LIN).

FACS Antibodies		
Epitope	**Conjugate**	**Cat. No.**
CD45	AF488	103121
CD31	AF488	102414
Ter119	FITC	116205
CD133	BV421	141213
EpCAM	APC	118214
IL17RA	N/A	MAB4481

Table 3: List of FACS antibodies used in cholangiocyte analysis

Cholangiocyte cell culture and FACS staining

Cholangiocytes were cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) with 10% FCS. To prepare for FACS staining and analysis, plates were rinsed with PBS and incubated with 300μl TryPLE for 10 minutes. Following incubation, cell plates were washed gently to prevent cell loss and cholangiocytes were collected in an Eppendorf. The solution was centrifuged for 5 minutes at 300G and transferred to a 96-well plate. The FACS staining protocol for cholangiocytes was then used, with the above antibodies outlined in Table 3.

Migration/Wound healing/Scratch Assay

Cultured cdHPCs were divided into three groups of 6 wells, with each acting as a control, treated with 20ng/mL IL-17A or Recombinant Murine TNF-α (PeproTech, Cat. No. 315-01A). Prior to the scratch assay, 5μg of mitomycin C was added into 12 wells for 2 hours to inhibit proliferation. A 1:1000 dilution was used for both IL-17A and TNF- α, and 300μl of these solutions were pipetted into each well. Cells were treated for 2 days before washing and replacement with treatment-free media. The

base of each well was then scratched using a P200 pipette tip to imitate a wound and images of these cells were taken at four timepoints: 0h, 4h, 8h and 24h.

Mitomycin C Treatment and MTT Assay

Cholangiocyte-derived hepatic progenitor cells were grown to 50% to 70% confluence, transferred to a 96-well plate and incubated in 10µg/mL Mitomycin C (Alfa Aesar, Cat. No. 2J63193) for 2 hours. For cells pre-treated with IL-17A, this was done for 2 days at a concentration of 20ng/mL. After incubation, cells were washed with PBS and cultured for 48h to 72h in DMEM + 10% FCS culture medium. A separate plate was used for time zero analysis. Following the treatment period, 10µl of 0.5 mg/mL MTT labelling reagent (Tocris, Cat. No. 5224) was added to each well and incubated at 37°C5% CO_2 for 4h. 100µl of DMSO was then added to each well and pipetted to ensure thorough mixing. The plate was incubated in the dark for 15 minutes and pipetted again to fully dissolve the MTT formazan. The plate was then read using a Bio-TEK Synergy HT plate reader at 570 and 650nm.

Animal Models – MCD and $Mdm2^{-/-}$

C57BL/6J mice were used for *in vivo* experiments and to produce the MCD and Mdm2 knockout models. Male wild-type (WT) mice were given the MCD diet for 3 weeks before being culled. $Mdm2^{fl/fl}$ mice received $1x10^{11}$ GC of AAV8-Cre and culled at day 7. Murine studies were performed under the project licence of Dr Wei-Yu Lu (PPL number: P546F8E91), with the help of Dr Lu and Dr Naruhiro Kimura.

Antibody	Cat. No.
Rabbit anti-IL17	A00421-2
Rat anti-CK19	DSHB AB_2133570
Rabbit anti-Sox9	AB5535
Donkey anti-rabbit, Alexa Fluor® 555	A21208
Donkey anti-rat, Alexa Fluor® 488	A-31572
Zombie NIR Live Dead	423106
CD3 AF700	100216
CD4 PE	100512
CD8 Percp	100731
CD45 AF488	103121
CD11b BV510	101263
NK1.1 BV650	108736
F4/80 BV711	123147
CD45 AF488	103121
CD31 AF488	102414
Ter119 FITC	116205
CD133 BV421	141213
EpCAM APC	118214
IL17RA	MAB4481

Table 4: Summary table of antibodies used in this investigation

RESULTS

IL-17A expression is increased during liver injury in Mdm2$^{flox/flox}$ mouse livers

Liver sections from healthy and Mdm2 knockout mouse livers were stained with antibodies against CK19 and IL-17. Mdm2$^{flox/flox}$ mice received PBS as the control for this experiment to ensure accurate staining. The number of IL-17A^{+} cells in each group, as shown by orange fluorescence, were counted to signify the extent of IL-17A expression by immune cells localised to hepatocytes and cholangiocytes, as indicated by green fluorescence. There was a small number of IL-17A^{+} cells in healthy mouse livers, however a significant increase in cells positive for IL-17A was seen in Mdm2 knockout mouse livers compared to both control and healthy livers.

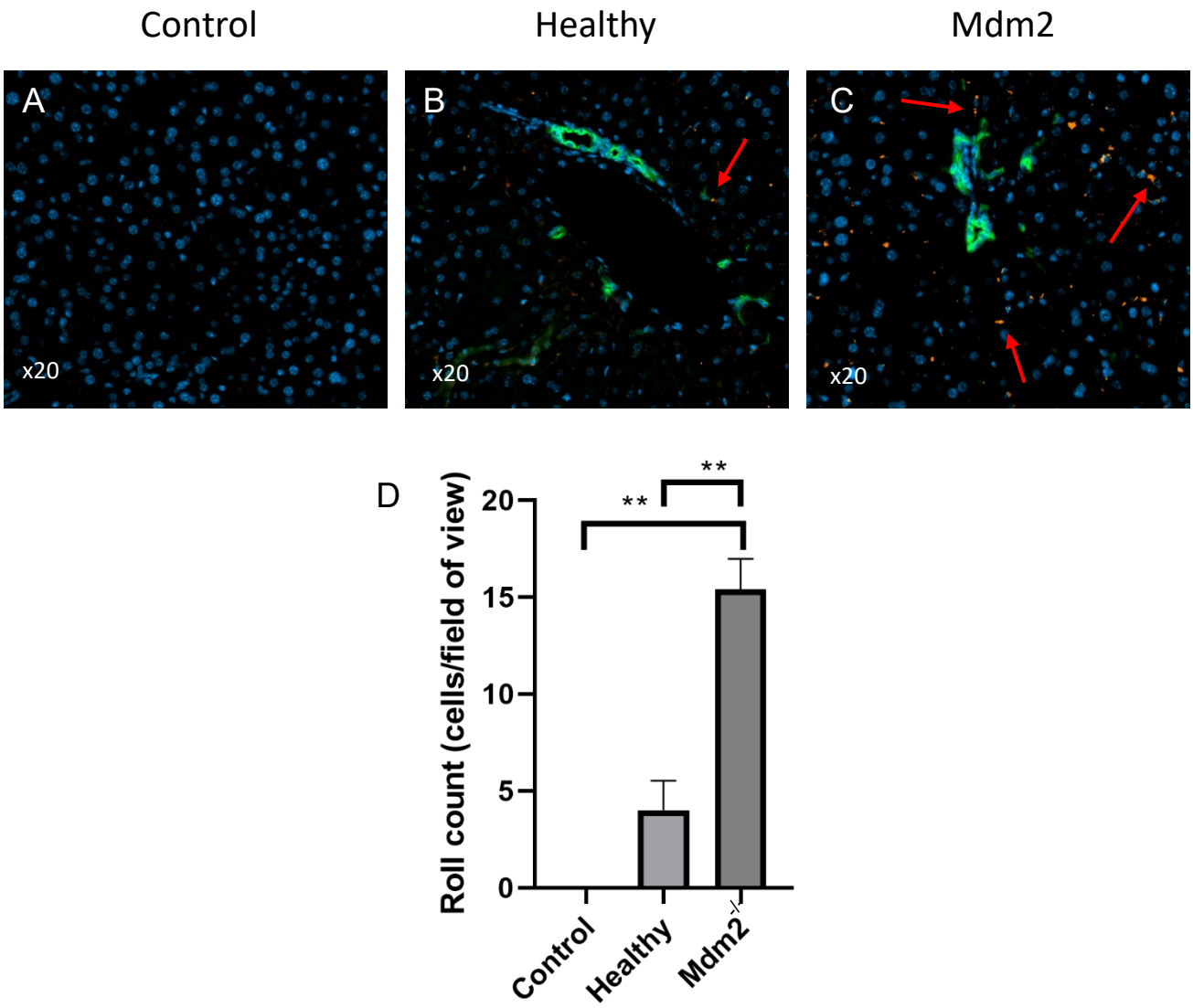

Figure 6: Greater expression of IL-17A is seen in Mdm2$^{-/-}$ mouse livers

Immunofluorescent staining of 5μm liver sections from healthy and Mdm2 knockout mice using anti-CK19 and anti-IL17 antibodies, as shown by green and orange fluorescence, respectively. DAPI nuclear staining is shown by blue fluorescence. **A)** Control liver section showing no antibody control, with DAPI staining only. **B)** Healthy liver section stained with DAPI, anti-CK19 and anti-IL17 show several areas of IL-17A^{+} cells. **C)** Liver section from Mdm2$^{-/-}$ mice showing an increase in the number of IL-17A^{+} cells. **D)** Graph showing the cell count positive for IL-17A in control, healthy and Mdm2$^{-/-}$ livers, and a significant increase in livers from Mdm2$^{-/-}$ mice. Control vs Mdm2$^{-/-}$, p=0.002; healthy vs Mdm2$^{-/-}$, p=0.0030; error bars, ± SEM.

IL-17A expression increases in multiple immune cell subsets during liver disease

The expression of IL-17A by a range of lymphocyte populations in healthy mice, as well as mice fed an MCD diet to mimic liver disease, was quantified using flow cytometry and subsequent FACS analysis. The gating strategy of IL-17A was based on unstained controls and Fluorescence Minus One (FMO. A significant upregulation in IL-17A expression by $CD11b^{Hi}$ $F480^{-}$ monocytes and $CD11b^{+}$ $Ly6G^{+}$ neutrophils could be seen in the diseased fatty livers of MCD-fed mice, when compared with healthy mouse livers. Neutrophil expression of IL-17A was almost non-existent in a healthy setting (0.43%), but in the context of liver disease in mice, 6.53% of this population were shown to express IL-17A. Although monocyte expression of IL-17A was upregulated in a disease setting, with 3.88% of this population expressing IL-17A, the greatest expression of IL-17A was seen in the neutrophil population in fatty livers. Expression levels of IL-17A did not change significantly in $CD4^{+}$ T-cells, $CD8^{+}$ T-cells or $CD11b^{lo}F480^{+}$ macrophages when comparing healthy and MCD-fed mice.

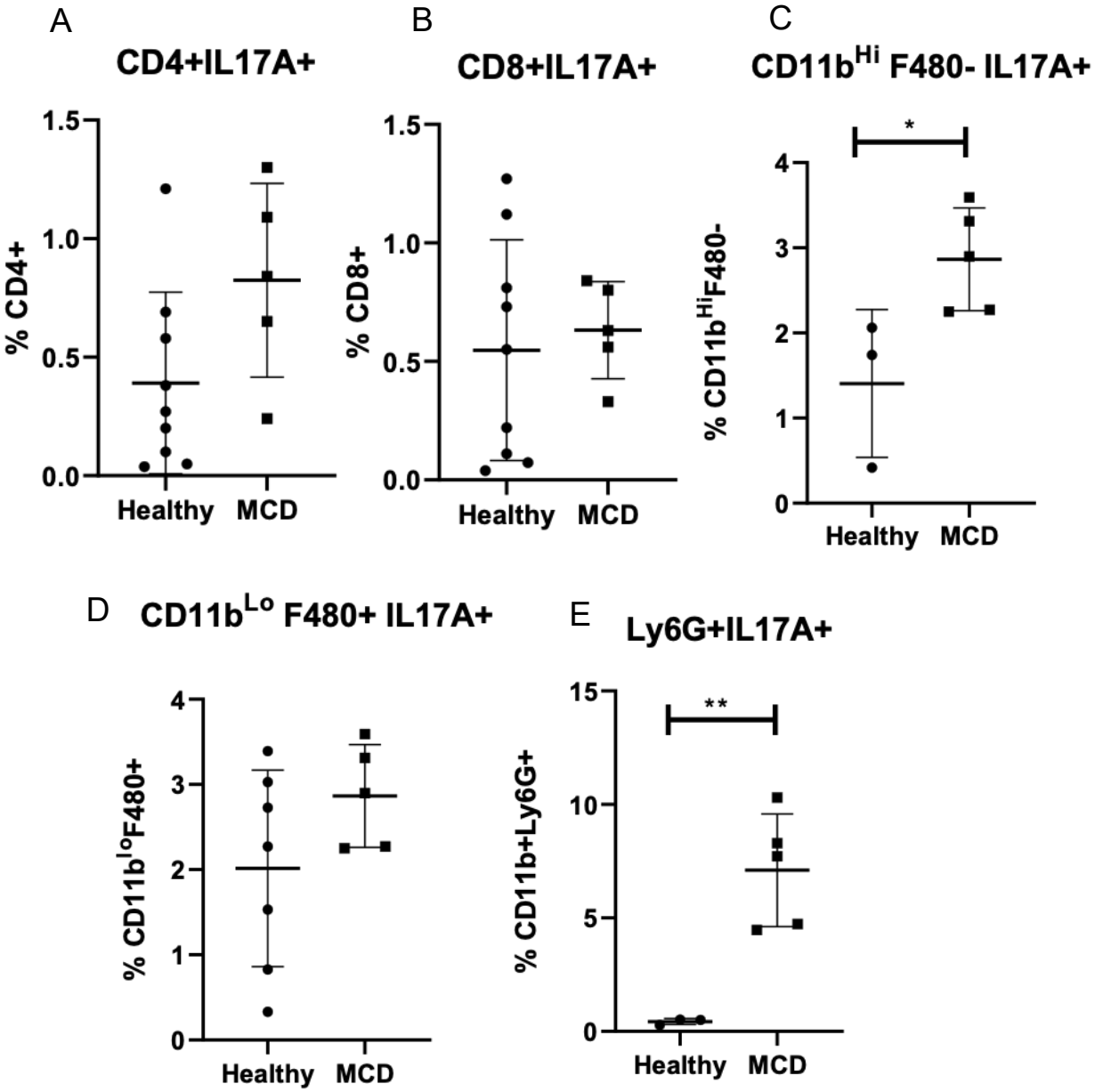

Figure 7: IL-17A expression by CD11b^{Hi} F480^{-} monocytes and CD11b^{+} Ly6G^{+} neutrophils is greater in MCD mice.

A) Upregulation of IL-17A expression by CD4^{+} cells in MDC-fed mice, compared to healthy mice was not significant. **B)** No difference in expression of IL-17A by CD8^{+} cells between healthy and diseased animals. **C)** A significant increase in IL-17A expression by CD11b^{Hi} F480^{-} cells in MCD-fed mice was seen, p=0.0297. **D)** No significant upregulation of IL-17A was seen in CD11b^{Lo}F480^{+} macrophages in MDC mice compared to healthy. **E)** IL-17A expression by Ly6G^{+}IL17A^{+} neutrophils was significantly upregulated in the MCD population, p=0.0041. Error bars, $\pm$ SEM. Healthy cohort, n= 3; MCD cohort, n=5.

IL-17RA expression increases in CDHPCs isolated from diseased livers

To investigate whether cholangiocytes express the receptor for IL-17A, the expression of the dominant IL-17 receptor, IL-17RA was investigated. Cholangiocytes were isolated by FACS from the livers of healthy mice and the fatty liver disease model, MCD. LIN (CD45, CD31, Ter119) antibody staining was used to exclude haematopoietic cells and endothelial cells, and the hepatic progenitor cells within the cholangiocyte population were isolated based on $EpCAM^+$ and $CD133^+$. From FACS analysis, the percentage of the non-parenchymal, LIN- population, which includes cholangiocytes, was higher in the MCD-treated mice, when compared to healthy mouse livers. Approximately 66.0% of cells were LIN^-, whereas only 25.8% were LIN^- in healthy mice, suggesting a general increase in non-parenchymal cell populations, such as stellate cells and cholangiocytes during liver injury (Fig. 8A). The percentage of $EpCAM^+CD133^+$ cells was similar in both healthy and MCD-fed mice. Considering that livers from MCD-fed mice have an influx of a LIN^- non-parenchymal cell population, the proportion of $EpCAM^+CD133^+$ was greater in the livers of MCD-fed mice, compared to the healthy group after normalising to the sample's total cell number (Fig. 8C). The expression of IL-17RA in healthy and fatty livers is similar, with approximately 40% of cholangiocytes expressing the IL-17 receptor. This level remained unchanged with injury (Fig. 8D). To further explore whether IL-17RA is preferentially expressed on the previously reported liver progenitor cells that express EpCAM and CD133, this data was also normalised to the total cell number in the sample. This showed that after injury, these progenitor cells increase surface expression of IL-17RA, suggesting the involvement of IL-17RA *in vivo* during liver progenitor cell activation (Fig. 8E).

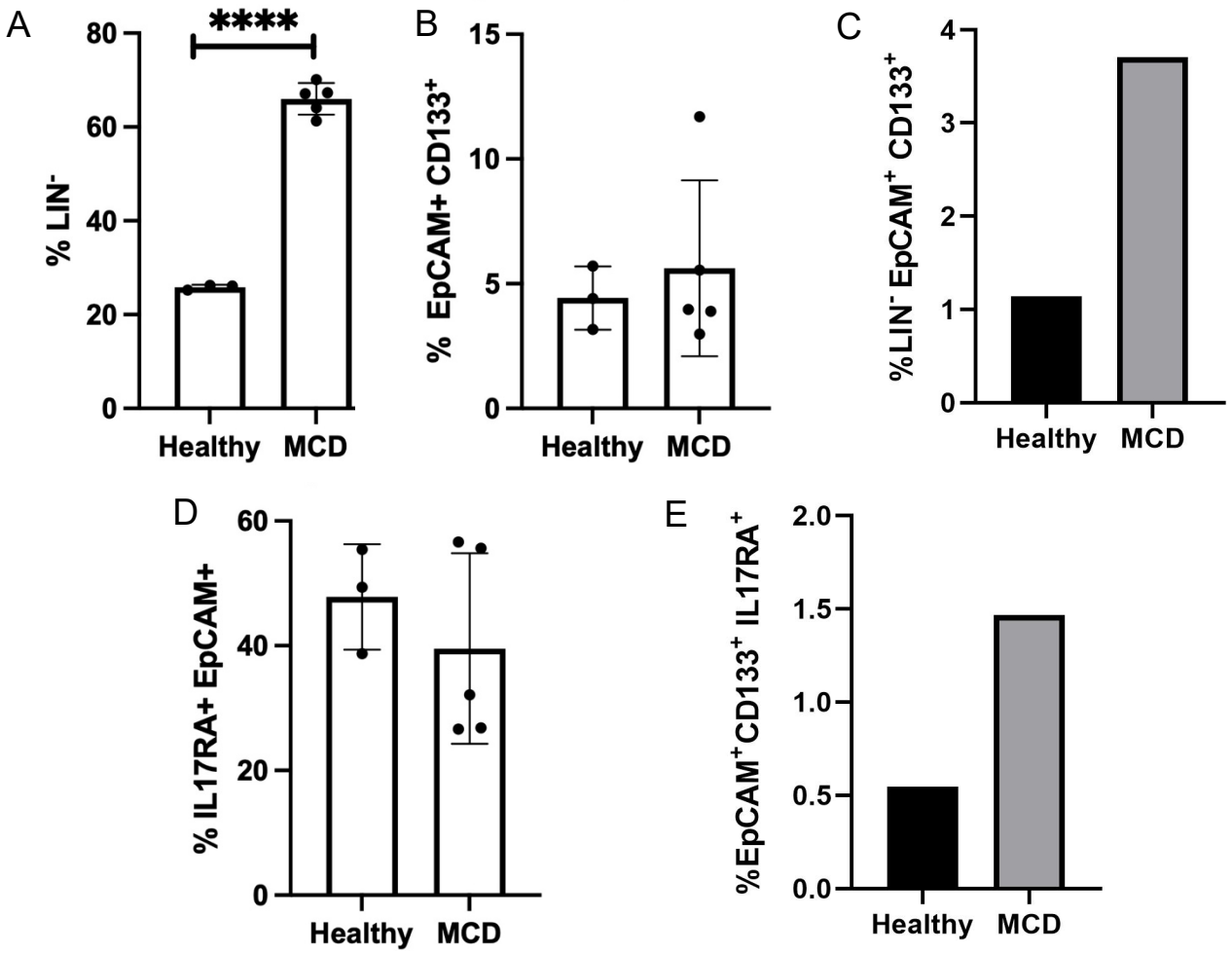

Figure 8: Expression of IL-17A is greater in CDHPCs isolated from MCD-fed mice.

A) Significant increase in LIN^-cells isolated from the livers from MCD mice, $p \leq 0.0001$ **B)** No significant change in % of $EpCAM^+CD133^+$ cells was seen in healthy and MCD-treated livers. **C)** Pooled response rate analysis showing the increase in the % of LIN^- $EpCAM^+CD133^+$ cells in MCD livers compared to healthy livers. **D)** No significant change in % of $IL17RA^+EpCAM^+$ was seen in healthy and diseased livers. **E)** Pooled response rate analysis showing the increase in the % of $EpCAM^+CD133^+IL17RA^+$ cells seen in MCD livers compared to healthy liver expression. Error bars, $\pm$ SEM. Healthy cohort, n= 3; MCD cohort, n=5.

Maintenance of cholangiocyte-derived hepatic progenitor cells (cdHPCs)

To investigate the effect of IL-17 on hepatic progenitor cells, I used the previously established hepatic progenitor cell line, cdHPC from Lu et al.[45] To ensure the cells used in subsequent experiments maintain a hepatic progenitor cell (HPC) phenotype, they were expanded in vitro and imaged using Brightfield microscopy. These cells were characterised by immunofluorescent staining with antibodies against progenitor markers CK19 and Sox9. CK19 is a key marker for cholangiocytes and alongside Sox9, the earliest and most dominant transcription factor that controls bile duct development with Sox4. The expanded cells maintain the morphology of epithelial cells, and the expression of CK19 and Sox9 indicates the expanded cells are maintained as HPCs in vitro (Fig. 9A, B, C). This could then be used as the basis for an in vitro model investigating the effect of IL-17 on biliary epithelial cells and cholangiocyte derived hepatic progenitor cells.

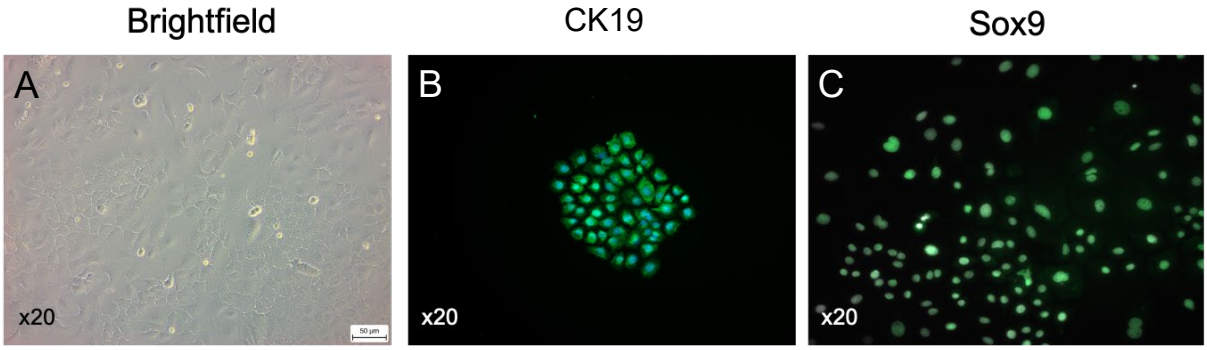

Figure 9: Brightfield and immunofluorescent images of expanded mouse cholangiocytes.

Brightfield and immunofluorescent microscope images of *in vitro* expanded mouse cholangiocytes. Cultured cells were imaged using light microscopy and fluorescent antibodies for CK19 and Sox9. **A)** Brightfield image of cultured, unstained mouse cholangiocyte-derived hepatic progenitor cells. **B)** Immunofluorescence staining of cholangiocyte marker CK19. **C)** Immunofluorescence staining of cholangiocyte marker Sox9. Objective = x20; scale: 50μm.

IL-17 enhances CD133 expression and upregulates cdHPC proliferation

cdHPCs were cultured and treated either with DMEM media as a control or DMEM media containing 10ng/mL IL-17 for 2 days. Following IL-17 treatment, cells were harvested, and qPCR analysis was performed. This analysis showed the upregulation of several key proliferative markers following treatment with IL-17, including *Ki67*, C*cnd1,* accompanied by the upregulation of hepatic progenitor cell-related genes *Sox9* and *Klf5* when compared to untreated cells. Significant upregulation was seen in *Ccnd1*, a gene responsible for controlling cell cycle progression through G1 phase, and *Klf5*, a key transcription factor, which is involved in various roles, from controlling stem cell activation and proliferation, to cell cycle progression. More importantly, treatment of cdHPCs with IL-17 resulted in a five-fold increase in *Prom1* expression, the mouse homologue of CD133, which is shown to be a marker for cell stemness. This indicated that the presence of IL-17A upregulates CD133 at transcription level, which might determine the cell fate of HPCs.

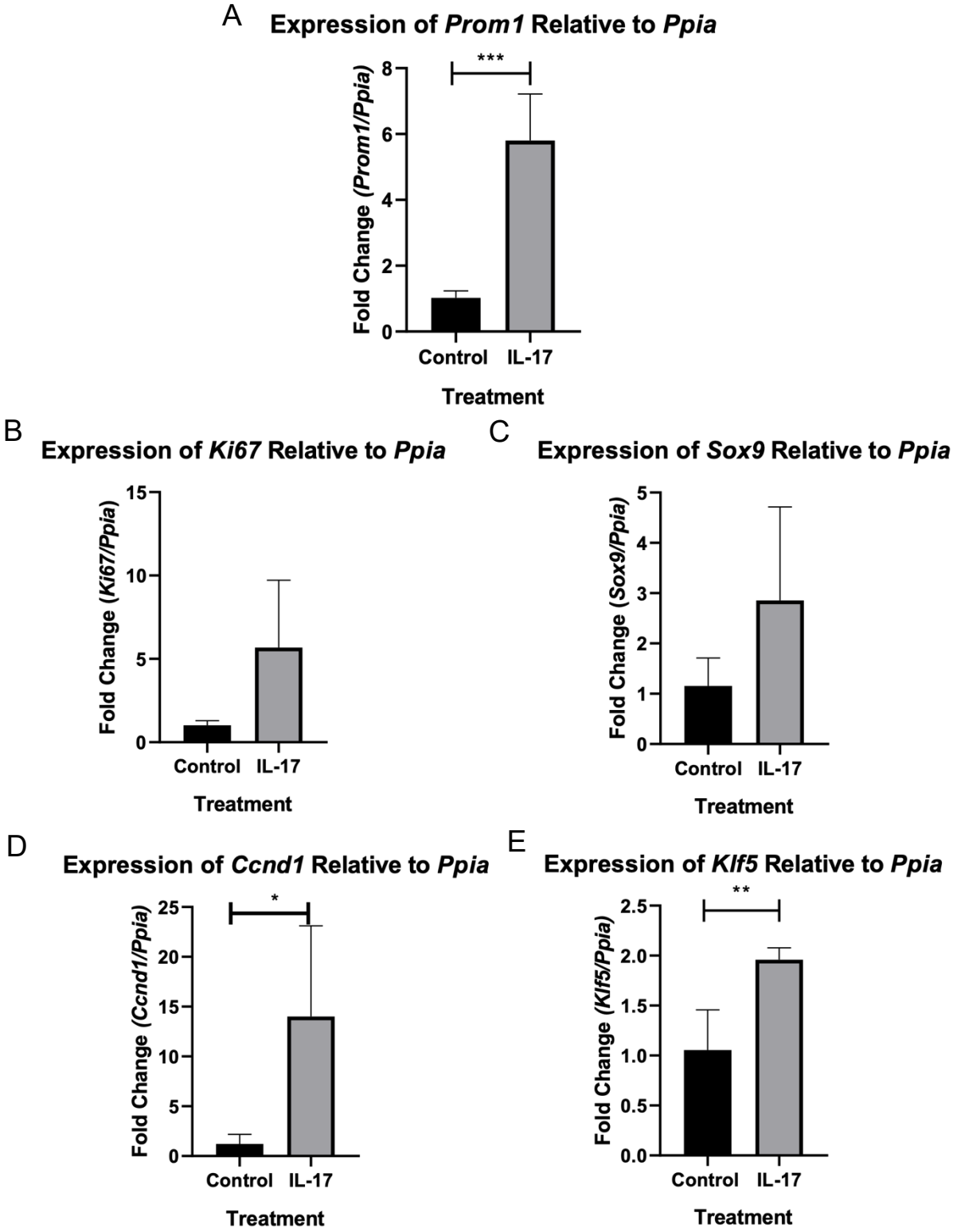

Figure 10: IL-17 treatment upregulates CD133 expression and proliferative markers in cdHPCs.

cdHPCs were treated with IL-17 for 2 days and analysed using qPCR primers for Prom1 and various proliferative markers**. A)** The expression of *Prom1* (CD133) to *Ppia,* as shown by the fold change. Significant upregulation of *Prom1* was seen in IL-17A-treated cells compared to the control, p=0.0005 n=4. **B)** Expression of *Ki67* was greater in cells treated with IL-17A but was not statistically significant, n=4. **C)** Nonsignificant upregulation of *Sox9* in cdHPCs treated with IL-17A, n=4. **D)** Increased expression of *Ccnd1* relative to the housekeeping gene, *Ppia* in the IL-17 group, p=0.0312, n=4. **E)** Significant upregulation of Klf5 in treated cells, compared to untreated cells, p=0.005, n=4. Error bars, $\pm$ SEM.

IL-17 upregulates factors involved in the Wnt signalling pathway

In addition to IL-17-related upregulation of proliferative markers, factors involved in the Wnt signalling pathway were also upregulated as a result of IL-17 treatment. *Nfkb1*, *Gpr49*, *Catnb1* and *Rela* expression increased, with significant upregulation seen in *Nfkb1*, which is responsible for crosstalk with Wnt/β-catenin pathway in inflammation, and *Gpr49*, the gene for a G protein-coupled receptor, which encodes the Wnt co-receptor Lgr5 that controls stem cell activation in epithelial organs. Although the upregulation of *Catnb* and *Rela* in cells receiving IL-17 treatment, which codes for the production of the Wnt signal transducer β-catenin and the p65 subunit of the NF-κB complex, respectively, was not statistically significant, qPCR analysis indicates that IL-17 could control Wnt and NF-κB signalling in in vitro expanded HPCs.

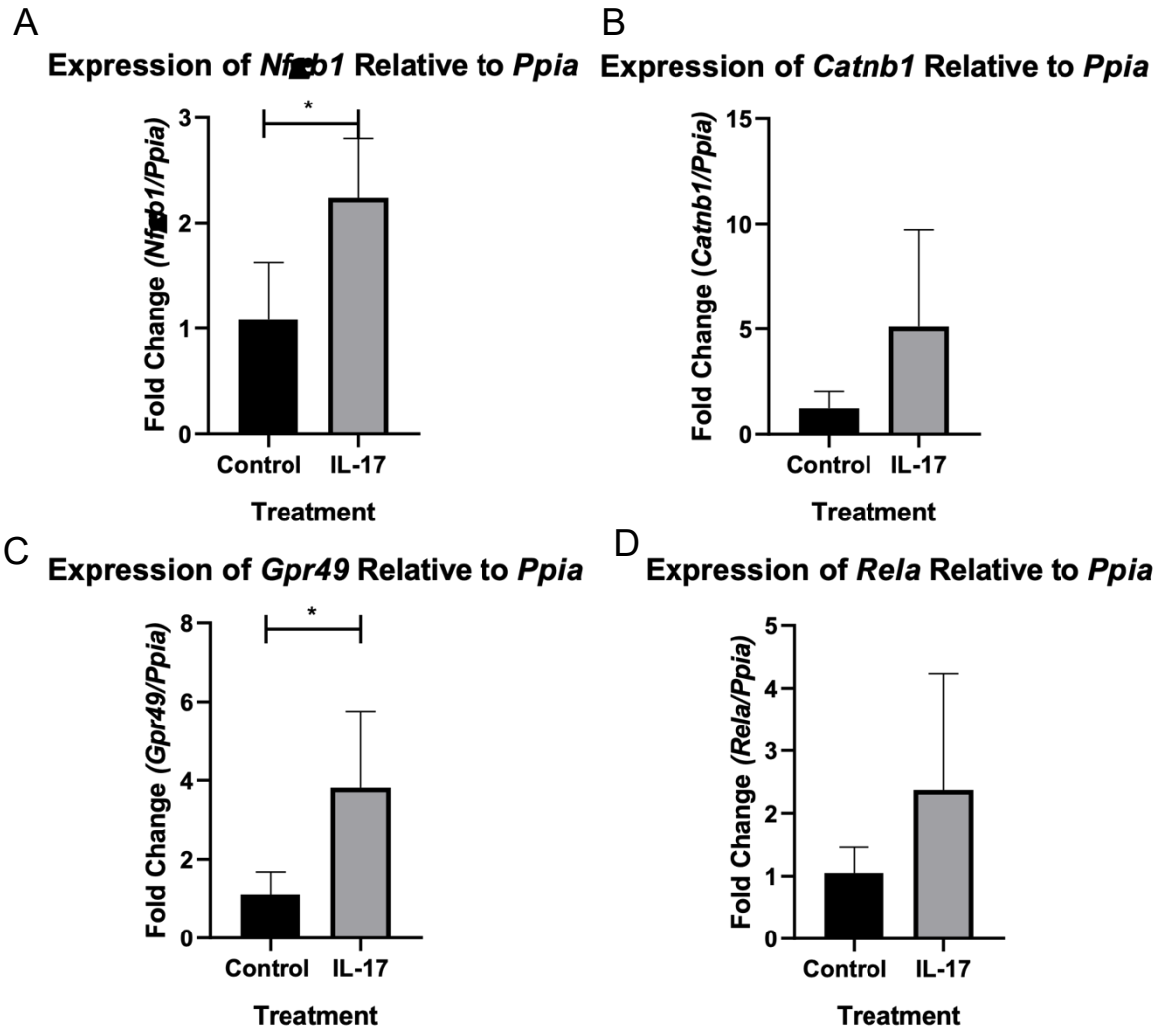

Figure 11: IL-17 treatment upregulates the expression of Wnt signalling factors in cdHPCs.

cdHPCs were treated with IL-17 for 2 days and expression of markers related to the Wnt signalling pathway was detected using qPCR analysis. **A)** Significant increase in *Nfκb* expression in IL-17-treated cells, p=0.025, n=4. **B)** Upregulated expression of *Catnb1* seen in the treated group, but not statistically significant, n=4. **C)** Marked upregulation of *Gpr49* was detected in IL-17 -treated cells, when compared to the control group, p=0.0373, n=4. D) Nonsignificant expression increase of *Rela* in treated cells, n=4. Error bars, $\pm$ SEM.

IL-17A treatment enhances CD133 expression in cdHPCs

To further show the upregulation of CD133 caused by IL-17A supplementation at a translational level, a modification of the cdHPC line was used. This established line in the laboratory was transfected with TCF-LEF GFP plasmid that allows the visualisation of Wnt signalling activation through its GFP expression. Flow cytometry analysis of the TCF-LEF GFP reporter cell line revealed that 8.50% of cdHPCs expressed both CD133 and GFP, whilst 3.91% are Wnt inactive, as indicated by the lack of GFP expression. All hepatic progenitor cells were treated either with control or IL-17A, to establish their effects on CD133 expression. Following IL-17A treatment, an almost 2-fold increase in cells that express the CD133 protein, when compared to the untreated cells, was observed (Fig. 12B). To further investigate whether the expression of CD133 is linked to the level of Wnt signalling, GFP expression was investigated in the $CD133^{+}$ expressing population. In $CD133^{+}GFP^{+}$ cells, there was an improved response to IL-17A treatment, when compared to the GFP cells alone (2-fold vs 1.5-fold) (Fig. 12C, D). These show that Wnt^{+} cells respond better to IL-17A treatment.

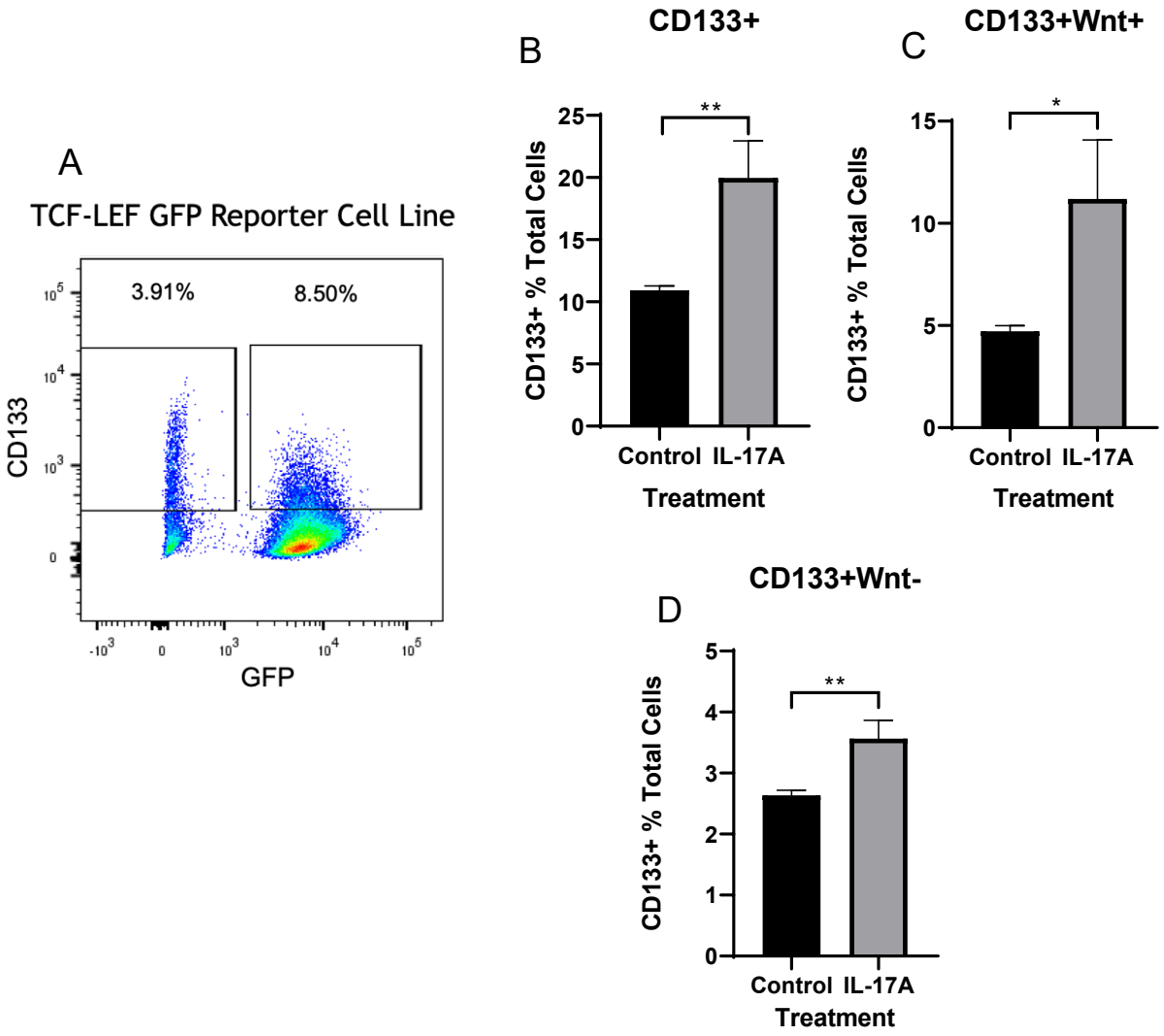

Figure 12: IL-17 treatment of cdHPCs upregulates CD133 expression with enhanced response in Wnt⁺ cells.

A TCF-LEF GFP reporter cell line was treated with IL-17A, IL-17A + NFκBI and Wnt and analysed using FACS and qPCR. **A)** Representative analysis of the gating. **B)** Percentage of CD133⁺ cells after treatment with IL-17A, p=0.0064, and IL-17A + NFκBI, p=0.0009. **C)** Percentage of GFP⁺CD133⁺Wnt⁺ cells , p=0.0179, and in IL-17A + NFκBI treated cells, p=0.0084. **D)** Percentage of cells that respond to IL-17A treatment, p=0.0067, and IL-17A + NFκBI, p=0.0134, was seen in Wnt⁻ cells. Error bars, ± SEM.

cdHPCs treated with IL-17A have a slower cell migration/healing response

A wound healing, or scratch assay, was performed in untreated cdHPCs to investigate the effect of IL-17A on cell migration. Cells were treated with either IL-17A or TNF-α to assess the impact of IL-17A on cellular proliferation and/or migration over a 24-hour period. At both 4h and 8h, cells treated with IL-17A exhibited a smaller % change in migration than cells in the TNF-α and control groups. By 4h, the wound in untreated cdHPCs had healed almost halfway (44%), whereas TNF-α -treated cells and IL-17A -treated cells had only healed by 37% and 27%, respectively. By 8h, untreated, TNF-α -treated and IL-17A-treated cells had healed by 61%, 51% and 42%, respectively. Despite IL-17A -treated progenitor cells having a slower healing response, by 24h, the % change from starting for all cells was approximately the same.

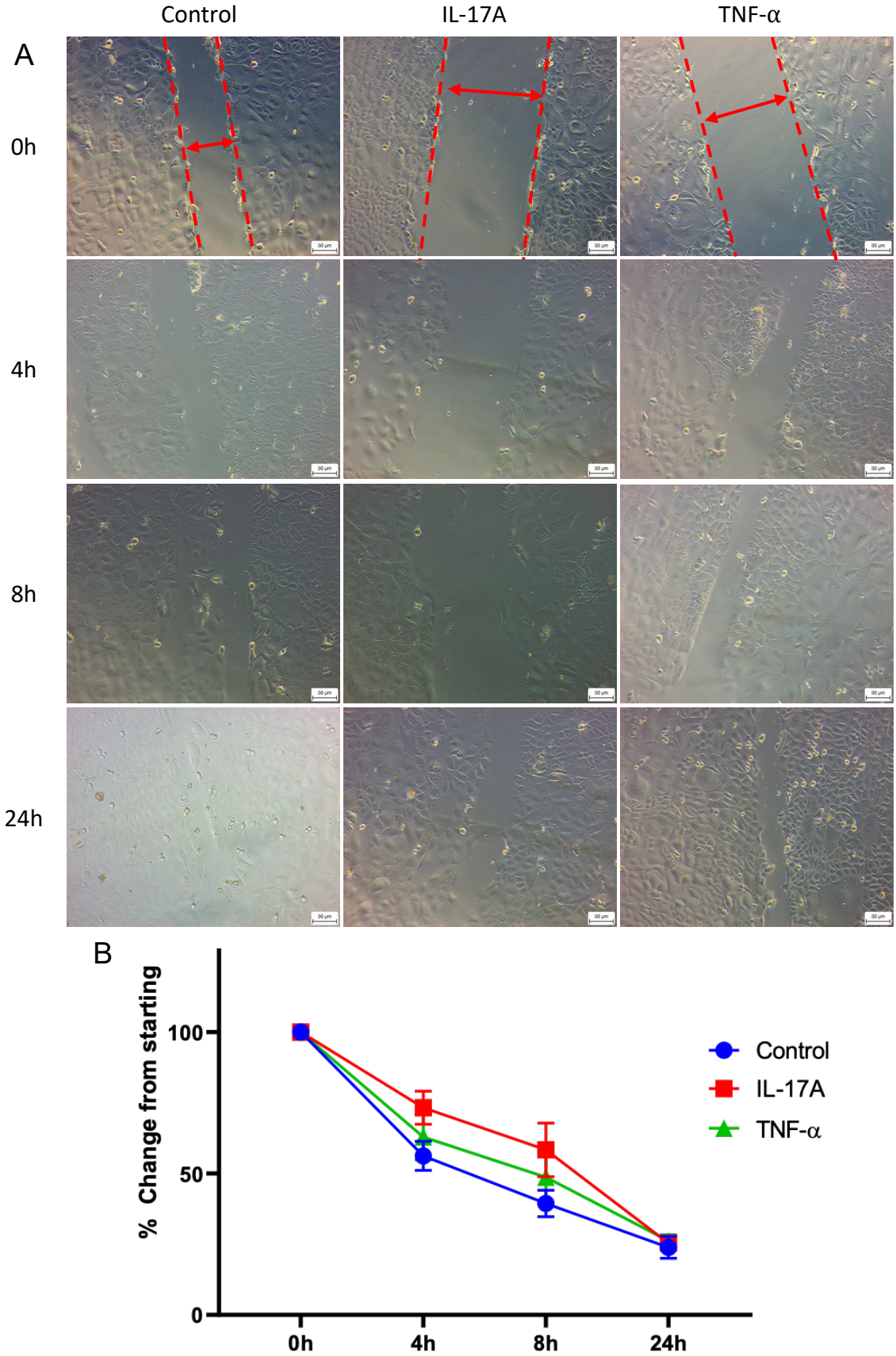

Figure 13: IL-17A treatment slows the cell migration/wound healing response in cdHPCs.

cdHPCs were treated with either IL-17A or TNF-α for 2 days, or left untreated, and then the well surface scratched to create a wound. The healing response was monitored by measuring the wound diameter at 0h, 4h, 8h and 24h. The percentage change in diameter was then calculated. **A)** Brightfield images of cdHPC wound healing response over a 24h period. **B)** Graph showing that IL-17A-treated cells exhibited a slower healing response than cells in the TNF-α and untreated groups. By 24h, wounds had healed to a similar point in all three groups. Objective = x10; scale: 50μm.

Mitomycin C treatment inhibits cdHPC proliferation

Untreated cells and those treated with mitomycin C and left to culture for 48 hours were also treated with MTT formazan to perform an MTT assay. The relative absorbance in cdHPCs post-mitomycin C treatment was lower than in the untreated cells of the control group. This shows that treating cdHPCs with mitomycin C leads to a decrease in cellular proliferation.

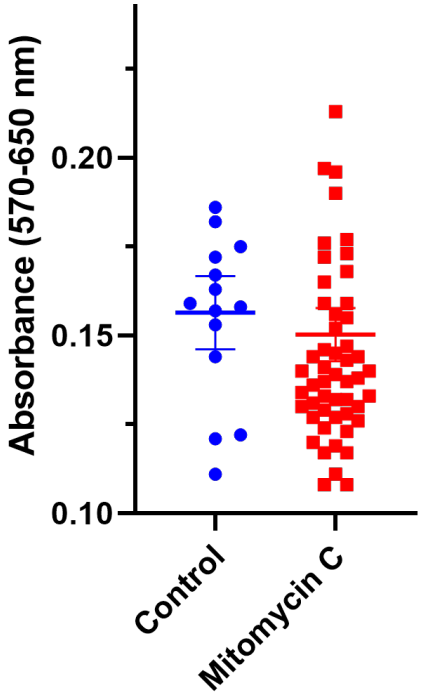

Figure 14: cdHPC proliferation is inhibited following mitomycin C treatment.

An MTT assay of cdHPCs treated with mitomycin C for 48h, and untreated cells, was analysed using absorbance levels at time zero and following MTT formazan treatment. Mitomycin C-treated cells showed a greater cell loss and lack of proliferation when compared with the control group.

IL-17A treatment promotes cdHPC proliferation

An MTT assay was also performed on cdHPCs pre-treated with IL-17A to show the effect IL-17A has on the proliferation of these progenitor cells. Following IL-17A supplementation, the absorbance levels were significantly higher than those in the untreated control group. Greater absorbance in IL-17A-treated cdHPCs indicates the cell number in these wells increased and were therefore proliferating.

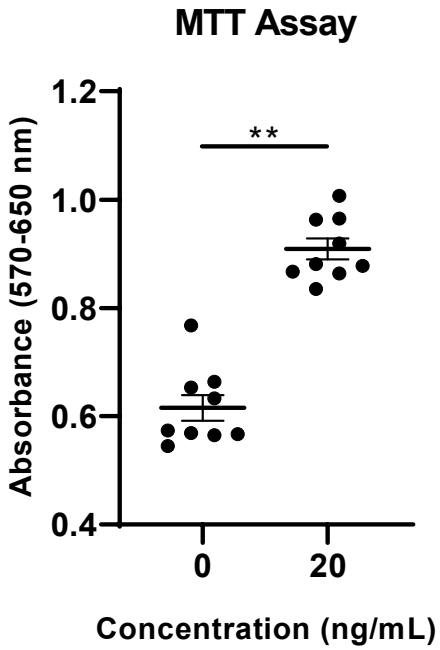

Figure 15: cdHPC proliferation increases following IL-17A treatment.

An MTT assay of cdHPCs treated with IL-17A for 2 days prior and compared to untreated cells. Greater absorbance levels in IL-17A-treated cells indicate an increase in cell number due to cdHPCs proliferating, $p \leq 0.01$, n=9.

Investigating the optimum dose of BAY 11-7082 (NF-κB inhibitor) on cdHPCs

Prior to carrying out experiments to determine the relationship between IL-17A and Wnt signalling, the optimum dose of NF-κB inhibitor was investigated to prevent cell death. This was needed after the occurrence of cell death following treatment with 5/10μM (data not shown). Hepatic progenitor cells were treated with a range of concentrations: 0, 40nM, 200nM, 1μM and 5μM and cultured for 24h, with regular check-ups. When compared to untreated cells, as seen in Fig. 16, 40nM, 200nM (data not shown) and 1μM BAY did not appear to cause any cell disintegration and death in cdHPCs. However, a dose escalation to 5μM BAY resulted in cell apoptosis and subsequently, death.

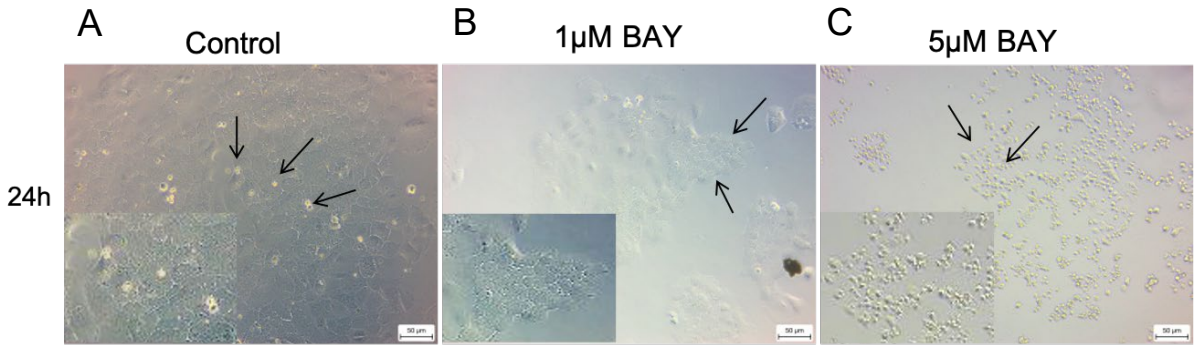

Figure 16: Dose finding experiment to determine the optimum dose of BAY on cdHPCs.

A) Brightfield microscope image of untreated cdHPCs after 24h, showing cell morphology unchanged. **B)** Image of cdHPCs after treatment with 1μm BAY showed little change in morphology. **C)** Altered morphology and apparent disintegration of cell structures shown using light microscopy in CDHPCs treated with 5μm BAY. Objective = x10; scale: 50μm.

Inhibiting NF-κB downregulates CD133 protein expression in cdHPCs

To investigate the effect of inhibiting NF-κB on the protein expression of CD133 in cdHPCs, cells were treated with IL-17A and the NF-κB inhibitor, BAY117082 and analysed using flow cytometry. A low percentage of CD133+ cells was detected in the untreated control group, however, when cells were supplemented with IL-17A, the protein expression of CD133 increased significantly, resulting in a three-fold increase in expression with approximately 30% of cells being $CD133^{+}$. In the presence of NF-κB inhibitor, the expression of CD133 was dampened in cdHPCs. Increasing the treatment concentration of NF-κB inhibitor in cdHPCs led to greater downregulation of CD133 expression. At 10μM of BAY117082, the percentage of cdHPCs expressing CD133 was comparable to control levels, and therefore IL-17A-induced CD133 protein expression was inhibited by treating cells with NFκBI. This indicates that both IL-17A and the NF-κB pathway are required for CD133 expression in cdHPCs.

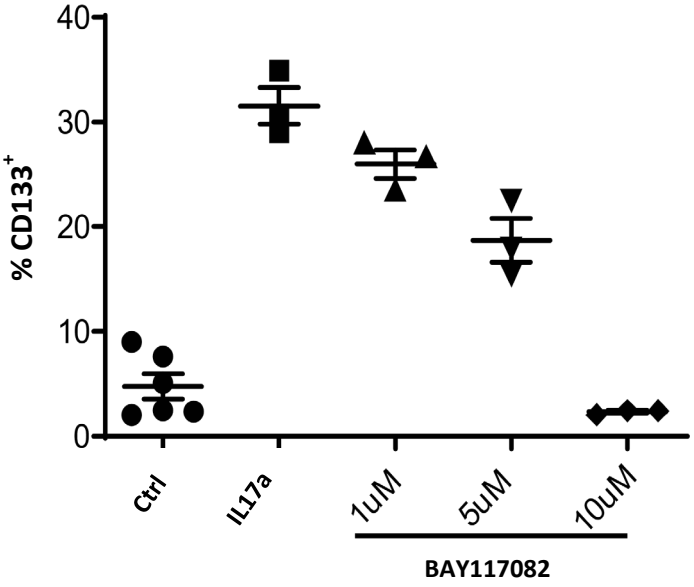

Figure 17: CD133 protein expression following IL-17A and NF-κB inhibitor treatment of cdHPCs.

FACS analysis of cdHPCs treated with IL-17A and NF-κB inhibitor showed increased CD133 protein expression after IL-17A treatment, but downregulation in the presence of NF-κB inhibitor, when compared to control groups. Control: n=5; treated groups: n=3.

DISCUSSION

From this investigation, it is clear there is a substantial role for IL-17A in the activation of liver stem cells and subsequent liver regeneration. This role appears to be varied and involve several pathways, including NF-κB, Wnt/β-catenin and a pathway involving the cancer stem cell biomarker, CD133. Using an $Mdm2^{-/-}$ mouse model to induce p53-driven apoptosis and p21-driven hepatic senescence, IL-17A expression was shown to increase during liver damage when compared to a healthy liver model. The upregulation of IL-17A during liver damage indicates that IL-17A secretion is injury-dependent and is an important part of the immune response involved in regeneration. The immunofluorescence staining also showed that the immune cells secreting IL-17A were situated close to hepatocytes and cholangiocytes, supporting the suggestion that IL-17A is associated with the hepatocyte/cholangiocyte regenerative response. Staining of liver sections from MCD-fed mice would give a more accurate representation of IL-17A upregulation during liver stem cell activation in NAFLD. However, as the MCD model results in fatty deposits, which can be seen throughout stained sections, this can obscure the expression of IL-17A. Therefore, using the $Mdm2^{-/-}$ model, whilst not producing the same liver damage response associated with steatosis, is preferable to ensure IL-17A staining can be visualised.

In vitro treatment of cholangiocyte-derived hepatic progenitor cells with IL-17A promotes the proliferation of these cells, an essential component of any regenerative response. The upregulation of *Ki67*, *Sox9*, *Ccnd1* and *Klf5* in CDHPCs exposed to IL-17A indicates that cdHPC proliferation is a consequence of the IL-17-induced

inflammatory response, which occurs during chronic liver damage. As the IL-17A receptor is expressed on cholangiocytes, it is likely that IL-17A acts via these receptors. This proliferative response supports the hypothesis that cdHPCs are key in regenerating injured hepatocytes and cholangiocytes. Expansion of this progenitor cell population is needed for the transdifferentiation of cdHPCs into hepatocytes and cholangiocytes to replace these cell populations, whilst also retaining a progenitor cell compartment. As shown by qPCR analysis, multiple points in the cell cycle are upregulated to promote this expansion. Ki67 is a well-established proliferative marker of cholangiocytes, and therefore its presence is not unexpected. The upregulation of *Sox9*, a cholangiocyte marker, is also expressed by so-called ductular reactive cells (DRCs). In long-term DDC treatment, a growing number of SOX9+ hepatocytes and hepatobiliary cells were observed, presumably arising from DRCs.[124] Additionally, Furuyama et al showed $Sox9^{+}$ precursors were involved in liver regeneration and concluded that Sox9 expression was an indicator of progenitor status. Using cell-specific genetic lineage tracing following tamoxifen injection into adult mice and subsequent liver damage, this group showed that hepatocytes were differentiated from the $Sox9^{+}$ precursor population in the biliary tree. They also concluded that Sox9-expressing hepatic progenitors were capable of self-renewal.[125] This capacity to self-renew relies on a marked proliferative response, as shown in the qPCR analysis from this investigation. Furthermore, it would be interesting to explore the effects on this population following IL-17 manipulation.

Previously, Klf5 has been shown to be important in regulating the function of the intra and extrahepatic biliary tract.[126] qRT-PCR analysis has also revealed the expression of *Klf5* in the liver and its marked upregulation following DDC injury, alongside

Epcam, a cholangiocyte marker. Interestingly, *Klf5* expression was predominantly found in the compartment containing EpCAM^{+} biliary epithelial cells, or cholangiocytes. This supports the finding here that IL-17-induced injury in cdHPCs is accompanied by a significant increase in *Klf5* expression. Whilst knockout of Klf5 alone did not cause significant differences in cholangiocyte *Klf5* expression between animals, the addition of DDC led to increased mortality, cholestasis, and the suppression of ductular reaction, showing *Klf5* plays an important role in activation of the regenerative response.[127] In a *K5-Cre*; *Klf5*$^{fl/fl}$ mouse model with a background of PSC, histological analysis of livers revealed intrahepatic bile duct proliferation, peribiliary fibrosis, inflammation, and hepatocyte necrosis. This was accompanied by the infiltration of T cells into the liver parenchyma, as well as a progressive increase in Sox9^{+} parenchymal cells. These Sox9-expressing cells were initially found near the biliary tract, before expanding their expression throughout liver parenchyma as the disease progressed.[126] A role for Klf5 in the self-renewability of colon cancer progenitor cells has also been identified. Klf5-driven activation of Ascl2 was essential for the self-renewal of these progenitor cells, which were also CD133+, indicating that Klf5 may also play a role in driving cholangiocyte to cdHPC transdifferentiation through the expression of CD133.[128] These findings may explain the upregulated expression of Klf5 and Sox9 in cholangiocyte-derived hepatic progenitor cells when subjected to IL-17 treatment, as well as the importance of Klf5 for ductular reaction and Sox9 for cholangiocyte to hepatocyte transdifferentiation as part of the injury response. However, there is lack of research into the roles of both Sox9 and Klf5 in liver stem cell activation, and the results from these investigations, as well as this study, indicate that these factors play an essential role in liver regeneration.

The upregulated expression of *Ccnd1* in cdHPCs following IL-17A treatment may suggest its relevance in the liver's regenerative response, but evidence for this is limited. *Ccnd1* activation has been shown in three separate liver regeneration models in rats with early transcriptomic changes in cell cycle-associated genes, including *Ccnd1*.[129] Following portal vein branch ligation and subsequent liver regeneration, *Ccnd1* was upregulated, alongside G2/M phase cyclins and *Cdkn1a*, indicating a programmed proliferative response whereby the proliferation during regeneration is controlled to prevent aberrant cell division.[130] Despite this, there has been no insight into whether Ccnd1 plays a more significant part in regeneration, or its relationship with IL-17A.

The interaction between IL-17 and CD133 (Prom1) has predominantly been explored in a tumour setting. Exposing cdHPCs to IL-17A has been shown to upregulate *Prom1* expression in this investigation. FACS analysis also revealed that a greater percentage of cdHPCs were CD133+ when cultured in the presence of IL-17A. This signifies there is an IL-17/CD133 axis in the liver than needs to be explored. Cancer stem-like cells (CSLCs) in ovarian cancer stimulated and transfected with IL-17 had a greater tumorigenesis capacity, exhibiting enhanced growth and sphere formation in organoid cultures, and a remarkable capacity to self-renew. Inhibition of NF-κB/MAPK signalling pathways also resulted in the inhibition of this IL-17-promoted self-renewal of CSLCs, highlighting these as potential pathways for the IL-17/NF-κB/CD133 axis in cdHPCs.[117] A role for IL-17 and CD133 in liver tumorigenesis has been supported by the correlation between IL-17^{+} cells and increased CD133 expression in hepatic progenitor cells from preneoplastic livers.[131] This indicates that IL-17 is elemental in promoting the stem cell-like

properties of stem/progenitor cells. However, the regulation between controlled tissue repair mechanisms and dysregulated regenerative mechanisms, which may cause tumorigenesis, needs to be further investigated.

The relationship between IL-17 and Wnt signalling also appears to be of importance in liver stem cell activation during liver regeneration. CdHPCs cultured with IL-17 exhibited higher expression levels of various factors involved in the Wnt signalling pathway, including *Nfκb1*, *Catnb1*, *Gpr49* and *Rela*. Crosstalk between the Wnt/β-catenin and NF-κB pathways are essential in modulating the inflammatory and immune responses, which can be positive or negative and depends on the cellular or tissue context.[132] The increased expression of *Nfκb1* and *Catnb1* suggests IL-17A can activate, or induce, the interaction of these pathways as part of the injury response in the liver. RelA (p65) forms a heterodimer with the transcription factor NF-κB in the canonical pathway and has been shown to form another complex with both β-catenin and NF-κB1 (p50 subunit) to target gene expression in human breast and colon cancers. During intestinal tumorigenesis in mouse models, the interaction between NF-κB, RelA and β-catenin was demonstrated to induce the dedifferentiation of intestinal epithelial cells to tumour-initiating cells, with a stem-like ability. Furthermore, the binding of RelA/p50 to a β-catenin/TCF (T-cell factor) complex led to the upregulation of several stem cell signature genes, including *Lgr5* and *Sox9* in the initiation of intestinal tumorigenesis.[133]

The importance of IL-17A and one of its downstream effectors, NF-κB, which is upregulated following IL-17A treatment of cdHPCs, on CD133 expression has also been observed. FACS analysis of cells supplemented with IL-17A revealed an

increasing in CD133 protein expression by cdHPCs. This upregulation, however, was inhibited with the addition of the NF-κB inhibitor, BAY117082, showing that NF-κB inhibition is detrimental to CD133 protein expression of cdHPCs. Both IL-17A and NF-κB are therefore key in the promotion of the $CD133^+$ stem-like phenotype of cdHPCs, and in driving the bipotency of these progenitor cells. Several other studies have emphasised the relationship between NF-κB and CD133, showing that $CD133^+$ cells have a more active NF-κB pathway than $CD133^-$ cells in pancreatic cancer. NF-κB activation was also shown to mediate CD133-driven EMT and tumour cell invasion.[134] Similarly, in the CD133+ cancer stem cells (CSCs) of non-small cell lung cancer (NSCLC) and ovarian cancer, NF-κB inhibition prevented EMT and the self-renewal capability of these cells, respectively.[117, 135] In addition to this, blocking the IKK-NF-κB signal, alongside NOTCH, partially inhibited the $CD133^+$ phenotype of skin CSCs.[136] From this, it is clear that NF-κB is a major factor in the expression of a $CD133^+$ phenotype, which may also be true for cdHPCs as well.

GPR49, also known as LGR5, is a signature stem cell and cancer-like stem cell (CLSC) marker that acts through the regulation of Wnt signalling. The Wnt co-receptor, Lgr5, which is coded for by GPR49, is responsible for controlling stem cell activation in epithelial organs.[137] LGR5 is an important marker of progenitor cells of multiple lineages, including the small intestine, colon, stomach, and hair follicles. Huch et al showed that, during liver homeostasis, *Lgr5* was not expressed. Following liver damage, however, there was a high level *Lgr5* expression. Lineage tracing also revealed that damage-induced Lgr5+ cells were capable of generating both hepatocytes and bile ducts *in vivo.*[138] Although significant upregulation of LGR5

was observed *in* vitro in this investigation, this increased expression may signify that liver regeneration is promoted through regulation of LGR5 and Wnt. Additionally, its status as a cancer stem cell marker may suggest a link between GPR49 and CD133, but this has not been explored further. The upregulation of these markers in cdHPCs following IL-17 treatment indicates that IL-17 not only plays an important role in promoting the cross-regulation of signalling pathways involved in mediating inflammation, but also in driving the bipotency of cdHPCs.

The use of a TCF-LEF GFP plasmid to transfect cdHPCs allowed for the visualisation of Wnt signalling activation via GFP expression. IL-17A treatment of these cells led to twice as many cells expressing the CD133 protein, when compared to the untreated control group. Cells positive for both CD133 and GFP (Wnt+) were more responsive to IL-17A, alluding to an interaction between IL-17 and Wnt signalling that is also associated with CD133 protein expression. The relationship between Wnt signalling and immune cells is implicated in a variety of settings, such as cancer progression, fibrosis, and maintenance of the tumour microenvironment. Canonical and non-canonical Wnt pathways are important in macrophage-mediated tissue injury, repair of major organs, cell fate decision and proliferation, key processes in liver regeneration.[139] Notch and Wnt signalling are also involved in directing the specification of hepatic progenitor cells by interacting with macrophages or activated myofibroblasts to maintain Numb expression within HPCs. β-catenin, which has also shown to be upregulated in the presence of IL-17, is stabilised, translocating to the nucleus to bind TCF and LEF transcription factors to target genes. As the expression of Catnb1 was found within hepatic HPCs, this indicated a significant role for Wnt signalling in HPC to hepatocyte differentiation. However, in

the absence of hepatic macrophages, bile ducts were shown to form, suggesting this pathway is also important in ductular reaction.[140]

In a study focusing on the association between Wnt expression and cholestatic liver injury in mice, the presence of Sox9 and EpCAM in the periportal area and not just the portal tract (PT) region indicated hepatocytes had developed a biliary-like phenotype. The increased expression of Wntless in this area alluded to EpCAM+ cells being the primary Wnt-producing cells in the PT following cholestatic liver injury.[141] During cholangiocyte to hepatocyte transition in ductal organoid models, the activation of the Wnt/β-catenin pathway led to these cholangiocytes developing progenitor-like features, indicating the importance of Wnt and β-catenin in the transdifferentiation of cholangiocytes in severe liver injury.[142]

The expression of IL-17A by neutrophils remains controversial and widely debated among scientists. Several studies have failed to reproduce the expression of IL-17A, and other IL-17 family members, by human neutrophils despite stimulation via agonists such as IL-6 and IL-23.[143] IL-17 is known to be a vital mediator in neutrophil recruitment migration through the expression of G-CSF, IL-6, IL-1β, TNF-α, and the chemokines CXCL1, CXCL2 and CXCL5.[144] However, the reversal of this mechanism, whereby neutrophils are a source of IL-17 production, is undetermined. In this investigation, it was shown that $CD11b^{+}$ $Ly6G^{+}$ neutrophils were the main contributors, along with $CD11b^{Hi}$ $F480^{-}$ monocytes, of IL-17A expression in the fatty diseased livers of MCD mice. $CD4^{+}$ T-cells and $CD8^{+}$ T-cells are often considered to be key sources of IL-17A in inflammatory diseases[145] but it was shown that their contribution did not significantly change between the livers of healthy and diseased

mice. Interestingly, in human liver fibrosis, neutrophils were found to be one of the main sources of IL-17. Together with IL-22, this was critical in driving TGF-β-dependent liver fibrosis.[146] This indicates that neutrophil production of IL-17 relies on other factors outside of its usual stimulants, IL-6 and IL-23, and may be specific to the disease setting. Indeed, neutrophils were shown to be major contributors to IL-17 expression in hepatic ischaemia-reperfusion injury (IRI), attracting inflammatory cells to infiltrate the hepatocytes and sinusoidal endothelial cells.[147] In kidney IRI, neutrophils, rather than CD4+, CD8+ or NK1.1+ cells, were also found to be the major source of IL-17A production.[144] It has also been suggested that neutrophils express IL-17A during synovial inflammation.[147] In addition to hepatic and renal IRI, neutrophils stimulated with IL-6 and -23 in human peripheral blood and murine bone marrow expressed both IL-17A mRNA and protein.[148] These studies support the novel findings in this investigation, whereby neutrophils are capable of being the major source of IL-17 in disease settings, including in the liver. Targeting neutrophil recruitment, or IL-17 signalling on the epithelium, may be investigated as ways to mediate the actions of IL-17A in chronic liver disease.

In addition to neutrophils, $CD11b^{Hi}$ $F480^{-}$ monocytes were shown to contribute to IL-17 expression in MCD-fed mice. Monocytes activated *in vivo* in inflamed human tissue induced intracellular IL-17 expression without IL-1β and TNF-α.[149] Despite this, there have been few studies investigating monocyte expression of IL-17A. This investigation indicates a need to explore the range of immune cell subsets that express IL-17, particularly in liver disease. Using different models, whether dietary or genetic, may provide different outcomes in response to different immune cell subsets that secrete IL-17, however.

The expression of the IL-17A receptor, IL-17RA, on cdHPCs, and the role it plays in liver regeneration has not been previously identified. The percentage of hepatic progenitor cells isolated from cholangiocytes using FACS was found to be greater in the fatty diseased livers of MCD-fed mice that in livers from healthy mice, supporting the hypothesis that cdHPCs are key contributors in the injury response. When the percentage of EpCAM+CD133+ cells were directly compared between healthy and MCD groups, there was not a significant difference between the two; this was also seen when comparing the percentage of these progenitor cells expressing IL-17RA. However, normalising the data to the sample's total cell number showed that the livers of MCD-fed mice had a greater influx of this LIN^- non-parenchymal cell population than the livers of healthy mice. The surface expression of IL-17RA was also considerably more in the EpCAM+CD133+ progenitor cells of fatty MCD livers, when compared to IL-17RA expression in healthy livers. These findings are significant in showing that hepatic progenitor cells expand in the liver following damage as part of the liver regeneration process. Similarly, IL-17RA has been shown to be important in the proliferative priming of hepatocytes during liver regeneration, regulating the expression of IL-6 and promoting residual hepatocyte proliferation.[150] Although chronic injury to the liver exhausts the ability of hepatocytes to self-renew, and therefore biliary-derived progenitor cells play a more central role, this study supports the observation that IL-17RA are key players in the injury response to drive hepatic regeneration.

This investigation also highlighted a role for IL-17A in the wound healing and proliferative response of cdHPCs. It was shown that cdHPCs supplemented with IL-

17A had a slower healing/migratory response, when compared to TNF-α-treated and untreated cells. There is contradictory evidence on the effect IL-17 has on wound healing. In skin and tendon injuries, IL-17A has been shown to delay the wound healing response, even leading to degeneration.[151, 152] However, in a separate study on skin tumorigenesis, IL-17A was shown to mediate the activation of EGFR to promote wound healing via Lrig1^{+} stem cell expansion and migration.[108] In IL-17A$^{-/-}$ mice, wound-healing defects were also observed in epidermal injuries.[153] This may be an indication that there is fine balance between the reparative and degenerative effects of IL-17A, depending on disease setting and other unknown factors, as discussed previously in the introduction of this investigation. Another suggestion is that IL-17A has a more significant part in promoting the proliferation of cdHPCs, than in their migration from bile ducts to the liver parenchyma. cdHPCs treated with mitomycin C and analysed using an MTT assay exhibited impaired proliferation compared to control (untreated) cells. However, when treated with IL-17A, there was a greater proliferative response in cdHPCs than in untreated cells. This indicates that IL-17A can promote proliferation, despite its impairment of the wound healing response. Guillot et al reported a similar finding; *in vitro* treatment with IL-17 led to increased proliferation in bipotent murine oval liver cells.[86] IL-17A has also been found to drive proliferation of keratinocytes, nasopharyngeal carcinoma cells and in breast cancer cells.[154-156] However, there needs to be further investigation into the extent of the role IL-17A plays in cdHPC proliferation, whether there is a balance between promoting this and inhibiting it, and the importance of IL-17 inhibiting a migratory response in these cells.

CONCLUSION

Limitations

There are several limitations that impact the outcomes of this investigation. Histological analysis of IL-17 expression in MCD-fed mice was not possible due to fatty deposits obscuring IL-17 immunofluorescence. Although using an $Mdm2^{-/-}$ mouse model is useful in showing IL-17A expression in generalised hepatic injury, a 3,5-Diethoxycarbonyl-1,4-Dihydrocollidine (DDC) model would show the expression of IL-17 in the presence of ductular reaction, the compensatory component of liver stem cell activation. Additionally, qPCR analysis of proliferative markers and Wnt signalling factors in healthy and MCD mice may provide greater insight into the crossover between IL-17 expression and fatty liver disease. This would also strengthen the association between NAFLD, IL-17 and the pathways involved in promoting liver regeneration. The wound healing and MTT assays also proved a challenge in this investigation. As the initial wound size was not consistent amongst treatment groups, it was difficult to assess whether this affected the healing or migratory response. Bias was easily introduced in the measuring of this response, as some sections were clearly narrower, or wider, than others. This measurement could be changed or interpreted differently as a result to fit the hypothesis of this experiment. Ensuring a standardised measurement here may resolve this limitation. For analysis of the MTT, time zero data was not used to indicate the base level of cdHPC proliferation, and how mitomycin C and IL-17A affected that cell population, due to inaccurate data collection. Due to the limited research period of this investigation, it was not possible to repeat this experiment and provide a wider analysis of effects of mitomycin C and IL-17A on cdHPC proliferation.

Future directions

It is evident that IL-17A plays a key part in the processes behind liver regeneration, whether it's protective or pathogenic, and further investigation is needed to fully understand this role. Using a blocking antibody to neutralise IL-17 and its effects in both healthy and diseased animal models may give greater insight into the role of IL-17 in liver homeostasis and during liver regeneration. This may involve modulation of processes such as liver inflammation and tissue remodelling. Alternatively, injection of IL-17 in mice following partial hepatectomy, MCD or DDC, could be used to explore the direct effects of IL-17A during liver regeneration. Aside from targeting the IL-17A cytokine itself, a transgenic mouse model can be used to delete its receptor, IL-17RA, from cholangiocytes, for example: $Krt19Cre^{ERT2}$ $IL17Ra^{fl/fl}$. Deletion of IL-17RA would prevent IL-17A from binding and inhibit the activation of any downstream pathways that may be involved. From this investigation, the IL-17A/IL-17RA complex has been implicated in the NF-κB and Wnt signalling pathways, which may be downregulated following IL-17RA deletion.

In addition to using alternative mouse models to further explore the role of IL-17A and IL-17RA, the balance between the regenerative effects and the occurrence of fibrogenesis needs to be addressed. IL-17 may prime stem cell activation, but it can also lead to liver fibrosis. The regenerative effects of IL-17A may be limited or influenced by other factors that drive IL-17 to develop a destructive function.

Concluding remarks

The research and results of this investigation strongly suggest IL-17A is key in the expansion of a hepatic progenitor cell compartment during liver regeneration

following chronic liver injury. IL-17A mediates the transdifferentiation of cholangiocytes to cdHPCs, stimulating them to express the stem cell marker, CD133. IL-17A acts through various pathways, most notably the NF-κB and Wnt signalling pathways, to upregulate CD133 expression and drive the bipotency of these cholangiocyte-derived hepatic progenitor cells.

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