DIVISION OF MEDICINE

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PhD thesis

Investigating immunological and metabolic pathways involved in the pathogenesis of chronic liver disease

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Declaration of Authorship

I, Katrin Schoelzel, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated.

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Date:

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List of Abbreviations

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
AAV2	adeno-associated virus 2
ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide-1-β-d-ribofuranosid
AIH	autoimmune hepatitis
AILD	autoimmune liver disease
ALD	alcoholic liver disease
AMBRA 1	autophagy and beclin 1 regulator 1
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated kinase
APC	antigen-presenting cell
α-SMA	α smooth muscle actin
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCLC	Barcelona Clinic Liver Cancer
bMAIT	blood-derived MAIT cell
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C	Celsius
CaMKKβ	Ca^{2+}/calmodulin-dependent protein kinase β
CC	Compound C
CCL	CC motif chemokine ligand
CCR	CC motif chemokine receptor
CD	cluster of differentiation
CD	Crohn's disease
cDNA	complementary deoxyribonucleic acid
CKI	cyclin-dependent kinase inhibitor
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4

CXCL	CXC motif chemokine ligand
CXCR	CXC motif chemokine receptor
DAMP	danger-associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
dNTP	deoxyribose nucleoside triphosphate
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FGF	fibroblast growth factor
FXR	farnesoid X receptor
g	gravitational force
g	gram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GWAS	genome wide association studies
h	hour
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HGF	hepatocyte growth factor
HIF-1a	hypoxia inducible factor-1a
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HRP	horseradish peroxidase
HSC	hepatic stellate cell
IBD	inflammatory bowel disease
IFNγ	interferon γ
IGF	insulin-like growth factor
IL	interleukin

iNKT cell	invariant natural killer T cell
kDa	kilo Dalton
kPA	kilo Pascal
l	litre
LAL	liver-associated lymphocytes
LC3	microtubule-associated protein light chain 3
liMAIT	liver MAIT cell
LKB1	liver kinase B1
LOX	lysyl oxidase
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
M	molar
MAdCAM-1	mucosal vascular addressin cell adhesion molecule-1
MAIT cell	mucosal-associated invariant T cell
MCP-1	monocyte chemoattractant protein-1
MDSC	myeloid-derived suppressor cell
MEF	mouse embryonic fibroblasts
mg	milligram
μg	microgram
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
min	minute
ml	millilitre
μl	microliter
ml	millilitre
μl	microliter
mM	millimolar
μM	micromolar
mm	millimetre
μm	micrometre
MR1	MHC class I-related molecule 1
MS	multiple sclerosis
mTOR	mammalian target of rapamycin

NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor-κB
nm	nanometre
NSCL	non-small cell lung cancer
PAMP	pathogen-associated molecular pattern
PBC	primary biliary cholangitis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death protein-1
PDGF	platelet derived growth factor
PDGF-BB	platelet derived growth factor isoform BB
PE	phycoerythrin
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PKB	Akt/protein kinase B
PLZF	promyelocytic leukaemia zinc finger
PRR	pathogen recognition receptor
PSC	primary sclerosing cholangitis
PVDF	polyvinylidene fluoride
qPCR	quantitative real-time polymerase chain reaction
raptor	regulatory associated protein of mTOR
rcf	relative centrifugal force
RNA	ribonucleic acid
RORγT	factor retinoic acid-related orphan receptor γT
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
shRNA	small hairpin RNA
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism

SREBP1	sterol regulatory element-binding protein-1
STAT	signal transducer and activator of transcription
STK11	serine/threonine kinase 11, also called liver kinase B1 (LKB1)
SV 40	simian virus 40
TACE	transarterial chemoembolization
TAK1	TGFβ-activated kinase 1
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
TGFβ	tumour growth factor β
Th cell	T helper cell
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TIMP	tissue inhibitor of matrix metalloproteinase
TLR	Toll-like receptor
TNFα	tumour necrosis factor α
Treg	regulatory T cell
TSC1	tuberous sclerosis 1
UC	ulcerative colitis
UDCA	ursodeoxycholic acid
ULK1	Unc 51-like kinase 1
UV	ultraviolet
VEGF	vascular endothelial growth factor
wt	wild type

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Abstract

Chronic liver disease affects a large number of people with a rising incidence, and is characterized by the development of liver fibrosis, eventually resulting in cirrhosis, Treatment options for end-stage liver disease are limited and liver cirrhosis represents the main risk factor for hepatocellular carcinoma (HCC), highlighting the need for novel therapeutic approaches.

In this thesis, metabolic as well as immunological pathways contributing to the development of liver fibrosis and subsequent HCC have been investigated.

Hepatic stellate cells (HSCs) are considered as the key players in fibrosis development, as their activation results in excessive extracellular matrix deposition, leading to the establishment of liver fibrosis. In addition, tumour-stromal interactions between HSCs and HCC play a role in HCC pathogenesis, and are therefore considered as a potential target for novel HCC therapy. In this thesis, the role of the AMPK pathway in tumour-stromal interactions between HSCs and HCC has been investigated, with a special focus on pharmacologically targeting AMPK in both HSCs and HCC.

Moreover, the development of liver fibrosis and cirrhosis is accompanied by a chronic inflammatory immune response, fostering a pro-fibrogenic environment, especially in the context of autoimmune liver disease (AILD). Mucosal-associated invariant T cells (MAIT cells) are a recently discovered subset of innate like T cells that represent up to 40% of all liver lymphocytes. Besides being crucial for anti-microbial defence, MAIT cells contribute to autoimmune diseases and are thought to play an important role in tissue inflammation in the liver, as they secrete pro-inflammatory cytokines upon activation. Therefore, the role of MAIT cells for the development of liver fibrosis in AILD was explored. More specifically, the MAIT cell subset was characterized in human patients with AILD, and mechanisms of MAIT cell activation, as well as MAIT cell – HSC interactions were investigated.

Chapter 1

1. Introduction

1.1 The liver

The liver is the largest organ in the human body and fulfils a variety of functions. Besides its important metabolic tasks in carbohydrate, lipid and nitrogen metabolism, the liver secrets bile, thereby facilitating the digestion of dietary fats, as well as the excretion of cholesterol, bilirubin, metabolites and toxins. Moreover, the liver produces acute phase and serum proteins and, as a secondary lymphoid organ, plays an important role for the immune system [1]. The functions of the liver are facilitated by its unique architecture and dual blood supply. While only 30% of blood is supplied by the hepatic artery, 70% is derived from the portal vein containing nutrients, food-derived antigen, microbes and microbial products from the gut [2]. In the liver, the blood flows through the hepatic sinusoids, which are only 5-7μM in diameter, leading to a slow intrahepatic blood flow that facilitates not only optimal metabolism of nutrients, but also interactions between immune cells and liver cells [3, 4]. The liver sinusoids are lined by liver sinusoidal endothelial cells (LSEC), which form a discontinuous endothelium that is lacking a basal membrane and characterized by open pores, so-called fenestrae, allowing for the passage of metabolites and the extravasation of immune cells into the space of Disse [5]. The space of Disse marks the space between LSEC and hepatocytes, and is rich in hepatic stellate cells (HSCs), liver pericytes that are important for the maintenance of the three dimensional structure of the liver, Kupffer cells, the liver-specific macrophages, and other immune cells [4]. The liver parenchyma is formed by hepatocytes, the main cell type in the liver, which is responsible for the synthesis of proteins, metabolism of drugs and toxins, as well as for the secretion of bile, thereby ensuring the normal function of the liver [1].

1.2 Liver cirrhosis

Liver cirrhosis is the result of a chronic wound healing response that results from chronic liver injury and is characterized by progressive accumulation of fibrillar extracellular matrix (ECM) associated with nodular regeneration of the liver parenchyma. Cirrhosis results in progressive loss of normal liver function, leading eventually to liver failure and death, if untreated [6, 7].

In Europe, liver cirrhosis is the fourth most common cause of death and an increasing cause for morbidity and mortality in developed countries [8, 9]. In the United Kingdom, the prevalence of liver cirrhosis was 76.3 per 100,000 aged over 25 in 2001, and over 50% higher in men than in women [10]. Excessive alcohol consumption is the strongest risk factor for cirrhosis [11], although viral hepatitis resulting from chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as fatty liver disease are important risk factors as well [9]. The frequency of risk factors varies regionally, thus alcohol abuse, HCV infection and fatty liver disease are most common in western countries, whereas HBV infection is the main risk factor in Asia [9, 12]. Furthermore, liver cirrhosis can evolve from a chronic immune-mediated damage as a result of autoimmune liver disease (AILD), such as primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH) [13, 14] (detailed description in chapter 3.1.4). Other risk factors include Wilson's disease (copper overload), haemochromatosis (iron overload) and α1-antitrypsin deficiency, while some cases are cryptogenic [15, 16].

Of note, liver fibrosis, and even cirrhosis can be reversed when the harmful agent or stimulus is removed [17, 18]. Nevertheless represents liver cirrhosis the main indication for over 5000 liver transplantations in Europe per year [9, 16], which currently is the only curative treatment for end-stage, decompensated liver cirrhosis. However, liver transplantation is not eligible to all cirrhotic patients and there is a lack of donor organs, underscoring the need for novel therapeutic strategies.

1.2.1 The pathophysiology of liver cirrhosis

Chronic liver injury leads to the development of liver fibrosis and subsequently cirrhosis through multiple mechanisms, and can be considered as an excessive wound healing response, characterized by hepatocyte necrosis, inflammation and excessive ECM deposition [6, 19]. Notably, the cirrhotic liver contains up to six times more ECM than a normal liver [20]. Progression to cirrhosis occurs after approximately 15-20 years of exposure to a toxic agent [19]. Besides activation of HSCs and cytokine release by immune cells (see chapter 2.1.1.2), hepatocyte death plays a major role in the pathogenesis of liver fibrosis. Hepatocytes are the main targets of hepatotoxic agents, such as hepatitis viruses, alcohol or bile acids [19]. In response to toxic agents, damaged hepatocytes release fibrogenic mediators, leading to the activation of HSCs. Moreover, hepatocyte necrosis fuels hepatic inflammation, which also contributes to creating a pro-fibrogenic microenvironment [21-23]. Once activated, HSCs develop into ECM secreting, hyper proliferative myofibroblasts and are considered the main cell type for the establishment of liver fibrosis [6, 24]. Moreover, HSCs secrete pro-inflammatory cytokines that activate immune cells, leading to a vicious circle in which mutual stimulation of inflammatory and pro-fibrogenic cells fuels the development of liver fibrosis [25].

Myofibroblasts are considered the main cell type that regulates repair in response to injury in various organs, including the liver, lungs, skin and kidney, by deposition of ECM [26]. Although HSC are the main source of myofibroblasts in the liver [27, 28], other cell types contribute to the pool of liver myofibroblasts as well. For example, portal myofibroblasts, which are located around bile ducts, play a role for the development of biliary fibrosis [29, 30]. Furthermore, bone marrow derived myofibroblasts are thought to contribute to the development of liver fibrosis [31], although their contribution in murine fibrosis has shown to be weak [32].

Notably, liver fibrosis occurs in different patterns according to their aetiology. For example, viral hepatitis shows interface hepatitis and portal-central vein bridging fibrosis, whereas alcoholic fibrosis and non-alcoholic fatty liver disease (NAFLD) are characterized by perisinusoidal or pericellular fibrosis showing a so-called chicken wire pattern [33]. Biliary cirrhosis is characterized by bile duct and portal

myofibroblast proliferation, resulting in the formation of portal-portal fibrotic septa [7, 33, 34].

Besides affecting the quantity of ECM, liver fibrosis also results in changes of ECM quality. In the healthy liver, the ECM in the space of Disse mainly consists of collagen IV and VI. During fibrosis development, ECM is replaced by fibrillary collagens, such as collagen I and III, as well as fibronectin, leading to so-called capillarization of the sinuoids [35].

When chronic liver diseases has evolved to cirrhosis, it is characterised by major structural changes including extensive capillarization of the liver sinusoids, in combination with functional abnormalities, e.g. endothelial dysfunction. These structural and functional changes are responsible for the development of portal hypertension [16], a major complication of liver cirrhosis. Other important clinical complications of cirrhosis, which largely result from portal hypertension, include ascites, variceal bleeding, hepatic encephalopathy and renal failure. Moreover, liver cirrhosis is the major risk factor for the development of hepatocellular carcinoma (HCC), as more than 80% of HCCs develop on a fibrotic or cirrhotic background [19, 36]. The development of HCC will be discussed in chapter 2.1.2.

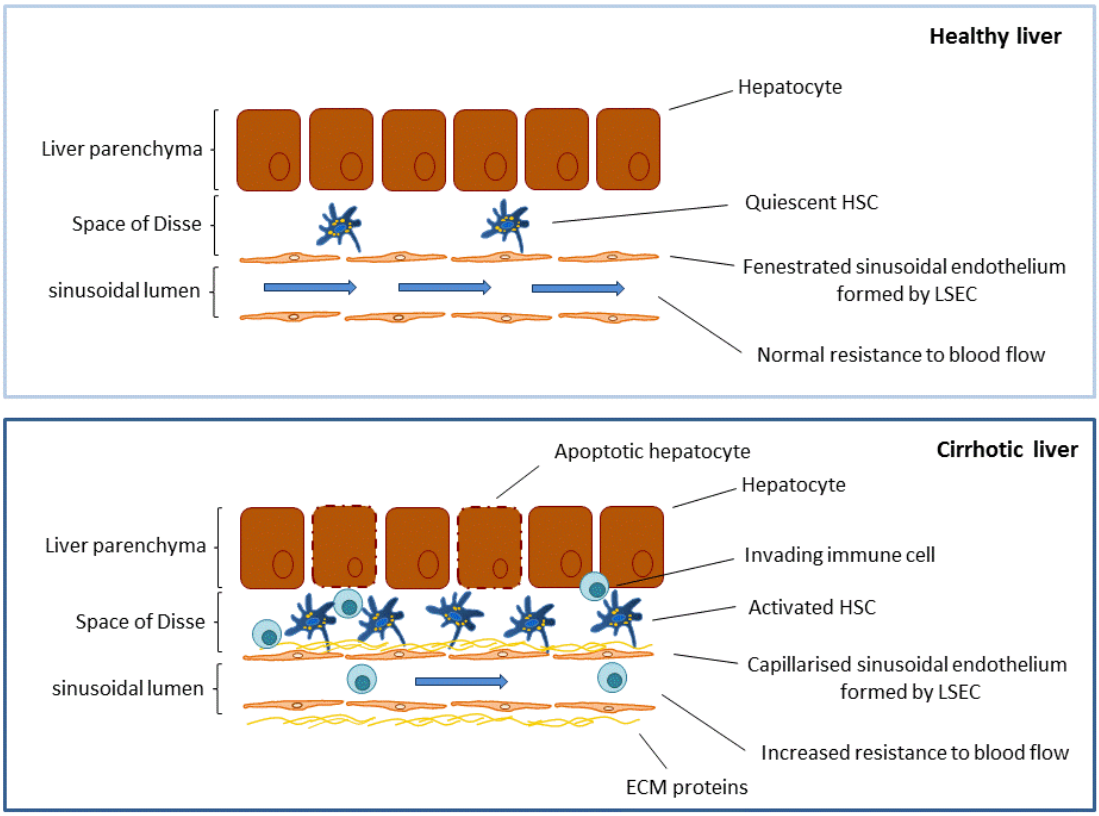


Figure 1.1: Changes in the hepatic architecture in a cirrhotic liver
Following chronic injury, hepatocytes become apoptotic and HSCs become activated, leading to their proliferation, loss of retinoids, release of pro-inflammatory cytokines and deposition of ECM proteins. LSEC lose their fenestrae and the liver sinusoids become capillarised and contraction of HSCs leads to increased resistance to the sinusoidal blood. Immune cells are invading the liver tissue.
Adapted from Bataller and Brenner 2005 [19]

1.2.2 Diagnosis and assessment of fibrosis

The presence of chronic liver disease and cirrhosis is often difficult to diagnose in an early phase as they remain asymptomatic for a long time and decompensating events, such as variceal bleeding, ascites, jaundice or hepatic encephalopathy only occur when cirrhosis is already established. Nevertheless, cirrhosis can be suspected by routine imaging obtained by ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) especially if major imaging changes are associated with poor synthetic liver function [16]. Nevertheless, liver fibrosis and cirrhosis can be

diagnosed by imaging techniques, such as ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) in combination with the detection of poor synthetic liver function [16]. Furthermore, measuring liver stiffness using transient elastography provides a valid, non-invasive tool to diagnose cirrhosis, and to distinguish early from late fibrosis stages [37, 38]. In addition, liver biopsy allows for diagnosis and staging of liver cirrhosis, and provides insight into its underlying cause [16].

Based on the information obtained by such histological examination or non-invasive techniques, liver fibrosis can be divided into different stages of severity. Different scoring systems have been developed to grade fibrosis, e.g. the METAVIR [39] and the Ishak scoring system [40], which are based on histological examination of liver tissue. Moreover, grading scales for non-invasive methods have been established [37, 38].

As liver cirrhosis of any underlying cause is the major risk factor for the development of HCC and HCC is the most common cause for death in cirrhotic patients, all patients with cirrhosis should be screened for HCC development by ultrasound every 6 months [41, 42].

1.2.3 Treatment of liver cirrhosis

It has been shown that progression of liver fibrosis and cirrhosis can be attenuated and even reversed when the harmful agent is removed [18]. Therefore, treatment of liver fibrosis and cirrhosis is based on treating the underlying cause of cirrhosis, e.g. antiviral treatment in HCV and HBV infection, immunosuppression in autoimmune hepatitis, abstinence from alcohol in alcoholic liver disease, weight loss and lifestyle change in fatty liver disease, venesection for haemochromatosis and copper chelating agents or zinc in Wilson's disease [16, 20, 43, 44].

Portal hypertension is the underlying cause of most clinical complications of liver cirrhosis and their subsequent mortality, and is therefore treated with non-selective β-blockers or endoscopic band ligation [45, 46].

In decompensated cirrhosis, or cirrhosis with HCC, liver transplantation is a therapeutic option, although the criteria for transplantation and the shortage of donor

organs only make liver transplantation available for a limited number of patients, emphasizing the urgent need for novel therapeutic approaches.

Persisting inflammation perpetuates the development and progression of liver fibrosis [22]. Thus, selective immunosuppression represents a novel strategy for treatment. To date, general immunosuppression with corticosteroids is only indicated in autoimmune hepatitis and alcoholic hepatitis [47, 48]. Moreover, reversal of HSC activation, inhibition of HSC proliferation and induction of HSC apoptosis are other strategies for the development of anti-fibrotic drugs. Along these lines, various compounds have been tested in experimental fibrosis in rodent models nonetheless evidence for successful treatment strategies in humans is still missing [19, 49, 50].

1.3 Aim of the thesis

The aim of this thesis was to investigate different pathophysiological pathways that may contribute to the pathogenesis of chronic liver diseases in humans.

In order to investigate the role of metabolic pathways in the development of HCC, the following hypothesis was proposed:

The AMPK pathway is involved in tumour stromal interactions between HSCs and HCC and represents a potential target for anti-fibrotic and anti-cancer therapy.

To test this hypothesis, the following aims have been pursued:

1. Investigating the interaction between HSCs and HCC cells
2. Determining a role for AMPK in such interaction
3. Testing the effect of pharmacological compounds that target AMPK in HSCs and HCC cells
4. Determining the mechanism of action of the employed compounds

Moreover, to test the involvement of mucosal-associated invariant T cells (MAIT cells), a subset of innate-like T cells, in fibrosis development, a second hypothesis was formulated:

MAIT cells are activated in the liver in autoimmune liver diseases and contribute to chronic inflammation and the development of liver fibrosis in patients with autoimmune liver disease.

The aims pursued to test this hypothesis were:

1. Characterizing the MAIT cell subset in patients with AILD and healthy humans
2. Investigating the functional outcome of MAIT cell activation by different mechanisms
3. Analysing the interaction between MAIT cells and HSCs

Chapter 2

Summary Chapter 2

In this chapter, interactions between the HCC cell lines HepG2 and PLC/PRF/5 and primary human HSCs were investigated *in vitro*. HSCs inhibited HCC proliferation and induced AMPK inhibition in HCC cells.
HCC conditioned media differentially affected AMPK activation and proliferation in HSCs. Whereas PLC/PRF/5 conditioned medium induced AMPK activation in HSC and did not affect HSC proliferation, HepG2 conditioned medium inhibited AMPK in HSCs and stimulated HSC proliferation and migration. Moreover, HCC cells induced the expression of pro-inflammatory genes in HSCs.
Both the AMPK activator AICAR and the AMPK inhibitor compound C were able to inhibit HCC-induced HSC proliferation by exerting cell cycle arrest. AICAR in addition inhibited the mTORC1 pathway in HSCs and reversed the pro-inflammatory gene signature induced by HCC cells in HSCs. Moreover, compound C induced autophagy in HSCs.

Main findings in this chapter

1. HSCs inhibit HCC cell proliferation

2. HCC cells exert differential effects on HSC proliferation

3. Activation of the AMPK pathway in HSC is modulated differently by HepG2 and PLC/PRF/5 cells

4. AICAR and compound C inhibit HSC proliferation through various mechanisms

2.1 Introduction

2.1.1 Hepatic stellate cells (HSCs)

2.1.1.1 Physiological functions of HSCs

HSCs are star-shaped liver non-parenchymal cells, comprising approximately 15% of liver-resident cells. They are located in the space of Dissé, i.e. the space between the sinusoidal endothelium and the basolateral side of the hepatocytes, where they are in close contact with LSEC and hepatocytes [24, 51, 52].

In the normal, healthy liver, one characteristic of HSCs is the storage of vitamin A. HSCs store 80% of total body vitamin A in cytoplasmic lipid droplets [24, 53]. Notably, vitamin A auto fluorescence allows for imaging and sorting of HSCs by flow cytometry [54-56]. Moreover, HSCs contribute to the maintenance of the three dimensional structure of the liver. Extracellular matrix (ECM) turnover is regulated by HSCs through secretion of ECM proteins and matrix degrading enzymes, i.e. matrix metalloproteinases (MMPs), as well as their inhibitors, tissue inhibitors of matrix metalloproteinase (TIMPs) [51]. Under steady-state conditions, HSCs secrete collagen type III, IV and small amounts of collagen I, as well as laminin [57].

In addition, HSCs are crucial regulators of the sinusoidal blood flow, as they can modulate the vascular diameter, owing to their star shape and contractile properties [58, 59].

HSCs also contribute to hepatic regeneration [24, 60], a unique feature of the liver. Upon resection of up to two thirds of the liver's tissue, the liver can regenerate rapidly [61]. HSCs regulate both the early and late phases of liver regeneration [62], although HSCs are not progenitor cells for hepatocytes [27].

Furthermore, HSCs show immunological features. They not only contribute to anti-viral immune surveillance by cross-allocation of MHC class I molecules to other sinusoidal cell populations [54], but also contribute to hepatic tolerance by inducing the differentiation and accumulation of regulatory T cells [63, 64], induction of myeloid derived suppressor cells [65] and by vetoing CD8 T cell activation by dendritic cells (DCs) [66].

2.1.1.2 HSC activation and liver fibrosis

HSCs become activated upon liver injury, or following exposure to several mediators, for example angiotensin II, insulin, insulin-like growth factor (IGF) or lipopolysaccharide (LPS) [67]. HSC activation follows different phases. Whereas in the initiation phase paracrine stimuli from damaged parenchymal cells drive HSC activation, in the consecutive phase of perpetuation, HSC activation is driven by both paracrine and autocrine stimuli [24, 28, 33].

Following activation, HSCs differentiate into myofibroblast-like cells; a process characterized by the loss of vitamin A droplets, increased proliferation and enhanced production of α-smooth muscle actin (α-SMA). Moreover, activated HSCs produce large amounts of ECM components, such as collagen-I, glycosaminoglycans, proteoglycans or glycoprotein [6, 51]. Besides producing excessive ECM, secretion of TIMPs, which inhibit ECM degradation by MMPs, increases. Overall, this results in an imbalanced ECM turnover and the development of liver fibrosis, subsequently leading to the establishment of liver cirrhosis [6, 28, 51].

Furthermore, HSCs secrete various cytokines and growth factors when they are activated. Of note, transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF) are the key fibrogenic mediators for HSCs [68-70]. TGF-β leads to autocrine stimulation of ECM deposition in HSCs [71, 72], and PDGF is the most potent HSC mitogen, stimulating HSC proliferation in an autocrine manner. Following liver injury, both PDGF and PDGF receptor are up-regulated in the liver [73, 74]. Moreover, immunological mediators, such as IL-1β, IL-6, IL-8 and monocyte chemotactic peptide-1 (MCP-1 or CCL2) are secreted by activated HSCs [75, 76]. The secretion of cytokines and chemokines contributes to HSC activation itself, but also perpetuates a pro-inflammatory microenvironment through recruitment of immune cells [22, 23].

In addition, activated HSCs contribute to the development of portal hypertension, as HSC activation results in increased contractility stimulated by enothelin-1 [58, 59, 77]. Increased HSC contractility leads to an impairment of the hepatic blood flow through constriction of individual sinusoids, as well as through contraction of the cirrhotic liver [78].

Furthermore, it has been shown that the interaction between activated HSCs and liver tumour cells contributes to hepatic tumour formation and progression of HCC [15], as discussed in more detail in chapter 2.1.2.2.

Importantly, chronic hepatic injury and persistent HSC activation are key factors in hepatic fibrogenesis. In fact, HSCs are the main contributors to the development of liver fibrosis, irrespective of the underlying cause [27]. Notably, after removal of the pro-fibrogenic stimulus, HSC can deactivate and return to a quiescent state, although the underlying mechanism remains elusive [22, 79]. After fibrosis resolution, HSCs remain primed and respond rapidly to recurring fibrogenic stimuli [79].

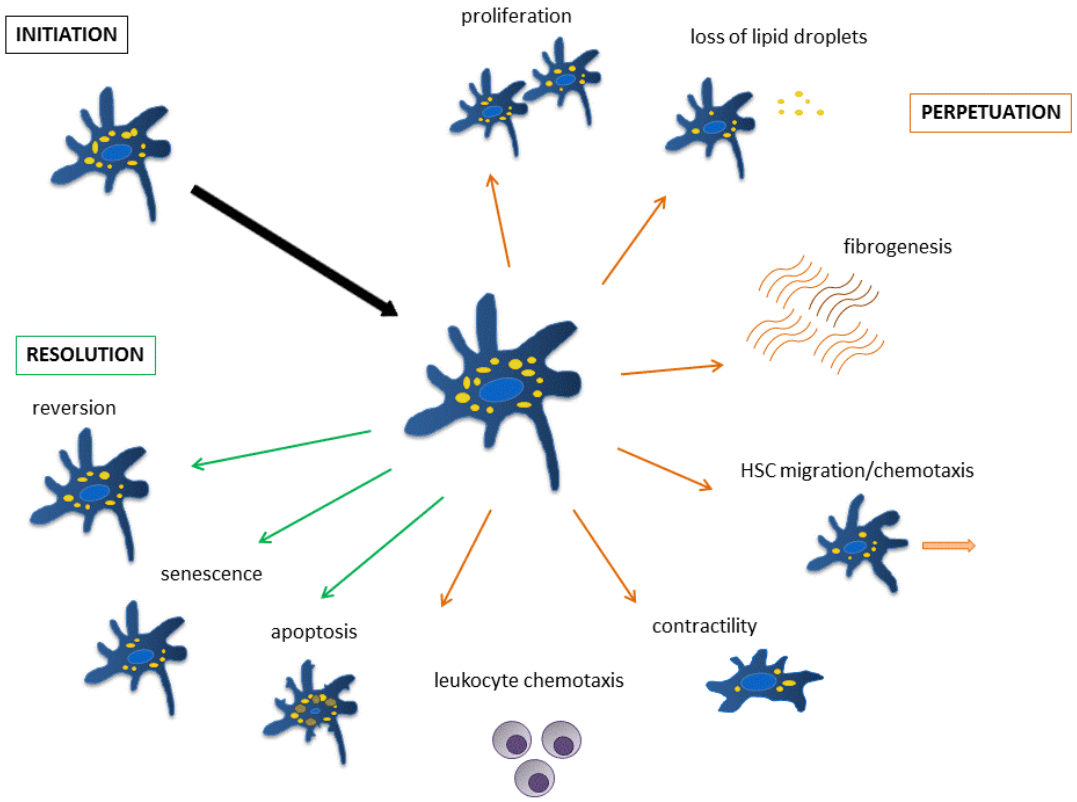


Figure 2.1: Activation of HSCs

After the initiation of HSC activation, HSCs proliferate and lose their lipid droplets. Excessive deposition of ECM proteins leads to fibrogenesis. In the perpetuation phase, HSC migration is increased. Activated HSCs become contractile and release pro-inflammatory cytokines leading to the recruitment of immune cells to the liver. After withdrawal of the harmful stimulus, HSCs return to a quiescent state and undergo apoptosis in the resolution phase.

Adapted from Friedman, S.L. 2003 [80]

2.1.2 HCC overview

HCC represents the most common subtype of primary liver malignancies, accounting for approximately 90% of all liver cancers worldwide [81, 82]. The incidence of HCC is increasing in Europe and the United States of America, and it is the fifth most common solid tumour and the second leading cause for cancer deaths worldwide [82, 83].

70-90% of HCC cases occur on a background of liver cirrhosis, making chronic liver disease the major risk factor for HCC development [84, 85]. The most frequent risk factor for HCC is chronic HBV infection, accounting for 50% of cases [86]. Other common risk factors include chronic HCV infection, excessive alcohol consumption and obesity causing non-alcoholic steatohepatitis (NASH) [82, 87]. Less common risk factors are haemochromatosis, α1-antitrypsin deficiency, autoimmune liver disease, porphyria and Wilson's disease. Recently, infection with adeno-associated virus 2 (AAV2) has been identified as a novel cause of disease, especially in non-cirrhotic individuals with HCC [88]. Of note, the incidence of risk factors varies regionally. Chronic HBV infection and Aflatoxin B1 exposure are the most common risk factors in eastern Asia and Sub-Saharan Africa, whereas in North America and Europe chronic HCV infection and alcohol abuse are most common [87]. Moreover, NAFLD and consequent NASH represent an important risk factor for HCC development in the western world [89, 90].

HCC surveillance by ultrasound is crucial in cirrhotic patients in order to diagnose and treat HCC early. Curative treatments include tumour resection, liver transplantation and tumour ablation, although they are only amenable to patients with early stage HCC and preserved liver function. In addition, chemoembolization and systemic treatment with Sorafenib have been shown to achieve a survival benefit [91, 92] .

2.1.2.1 HCC development and pathophysiology

HCC development is a complex multistep process and differs across the different aetiologies, explaining the heterogeneity of HCC [84, 93]. In each HCC nodule, a mean of 40 different mutations occur, which, in combination with epigenetic

alternations, lead to a unique genetic profile of each nodule [84]. Nevertheless, some molecular changes leading to the development of HCC are common throughout HCCs.

Following chronic liver injury, hepatocytes regenerate and form histologically normal hyperplastic nodules, which can evolve to pre-malignant dysplastic nodules and eventually HCC [84, 94]. Of note, HCC can arise from hepatocytes, but also from stem or progenitor cells [95]. The malignant transformation is accompanied by mutations in several oncogenes and tumour-suppressor genes. Mutation or depletion of the tumour suppressor gene p53 (TP53), which is important for cell cycle control, is present in 12-48% of cancers, depending on the tumour stage. Moreover, mutations of the β-catenin gene CTNNB1, which is part of the Wnt signalling pathway, are frequent, especially in HCV related HCC [96-98]. Both TP53 and CTNNB1 mutations occur in early and late HCC stages [93], whereas alterations in telomere maintenance have shown to be important for HCC initiation and promotion [98-100]. Hence, telomerase reactivation, which is required for telomere synthesis and usually not expressed in mature hepatocytes, is required for malignant transformation of hepatocytes [101]. Of note, TERT is part of the telomerase complex [99], and the TERT promoter is mutated in 59% of HCC [102]. In addition, members of the ErbB receptor family of tyrosine kinases, which are involved in various cancers, can be overexpressed in HCC. For example, ERBB1, or epidermal growth factor receptor (EGFR), is overexpressed in HCC, compared to adjacent non-malignant liver tissue. Such ERBB1 overexpression correlates with a more aggressive phenotype, characterized by a high proliferative index, de-differentiation and intra-hepatic metastasis [103]. Nevertheless, as data on alterations in ErbB receptor expression are scarce, the relevance of such alterations needs to be validated in larger cohorts. Other mechanisms contributing to HCC pathogenesis include methylation of cancer-relevant genes and genomic instability, for example caused by chromosome segregation defects and genomic alterations [93, 98]. The most common DNA amplifications related to HCC are the chromosome regions 11q13 and 6p21, which encode for the oncogenes cyclinD1 (CCND1) and fibroblast growth factor (FGF) 19 (11q13) and vascular endothelial growth factor A (VEGFA) (6p21) [84].

Mutations and alterations in the above-mentioned genes result in activation of several pathways related to cell survival and proliferation. For example, Ras and EGFR

signalling are activated in 50% of HCCs [104] and the mTOR pathway is disrupted in 40-50% of HCCs due to alterations in upstream signalling [105, 106]. Moreover, IGF signalling and the hepatocyte-growth factor (HGF) c-Met axis are frequently activated in HCC [104].

Besides the dysregulation of proliferation and growth control, oxidative stress and inflammation contribute to the development of HCC, especially if excess alcohol intake or chronic viral infections are the underlying cause [93, 98].

Depending on their genetic mutations, HCC tumours can be classified into subgroups [98]. To date, two main molecular subclasses have been identified, each of which includes approximately 50% of cases: proliferative and non-proliferative HCC [107, 108]. The proliferative subclass is associated with HBV-related HCC and a poor outcome; characteristic mutations include RAS, mTOR, IGF signalling, as well as FGF19 amplification [107, 109, 110]. In contrast, the non-proliferative class is associated with alcohol- and HCV-related HCC and characterized by CTNNB1 mutations [111].

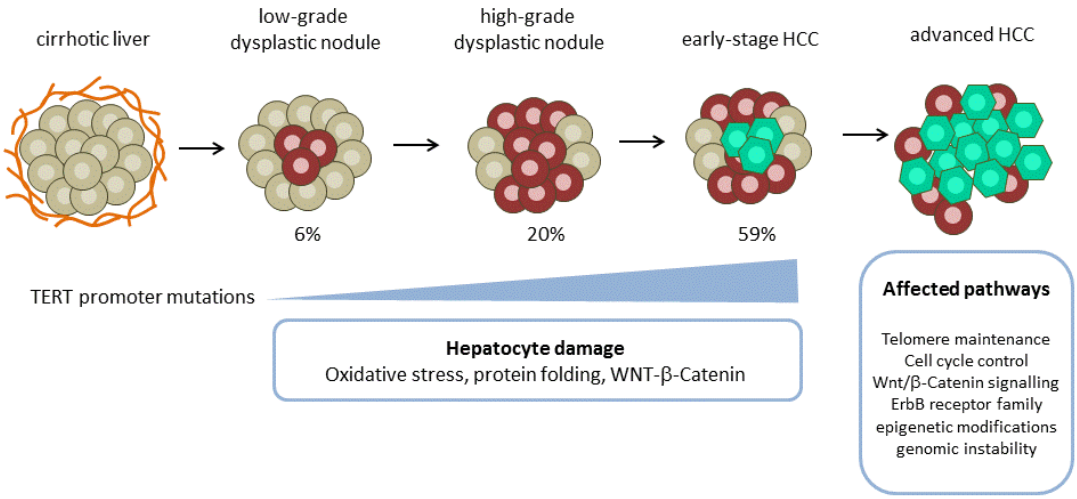


Figure 2.2: Development of HCC and driver genes
Development and progression of HCC from cirrhotic liver to advanced HCC and the involved mutations and pathways. TERT promoter mutations are involved in the progression from low-grade dysplastic nodules to early stage HCC and are present in most HCC cases in cirrhotic livers. A detailed description of molecular pathways involved in HCC development can be found in chapter 2.1.2.1.
Adapted from Llovet, J. M. et al. 2016 [84]

2.1.2.2 The tumour microenvironment in HCC development and progression

The altered microenvironment in the chronically diseased liver has been shown to contribute to cancer onset and progression. The microenvironment, composed of ECM proteins and proteoglycans, cytokines and chemokines, as well as different cell types, such as fibroblasts, myofibroblasts, endothelial cells and immune cells, can be largely modified in tumours and contribute to a pro-tumourigenic environment [84, 112].

The tumour microenvironment plays a particularly important role for HCC development, as more than 80% of HCCs develop in fibrotic and cirrhotic livers [36]. Activated HSCs are the key players in the development of liver cirrhosis and there is evidence that the bidirectional cross-talk between HSC and tumour cells is associated with HCC tumour progression. Activated HSC not only surround dysplastic nodules, but also infiltrate the HCC stroma and peritumoural tissue [113, 114]. Moreover, after curative resection, the presence of a HSC gene expression signature is a poor prognostic indicator in HCC [115]. Besides, it has been shown that activated HSC enhance HCC cell proliferation and HCC tumour growth [116] through TGF-β secretion [117]. Moreover, HSCs increase HCC cell migration and foster a pro-inflammatory and pro-angiogenic microenvironment [118].

Chronic liver disease is not only characterized by liver cirrhosis, but also by a pro-inflammatory microenvironment. HCC development is strongly linked with inflammation, as 90% of HCCs arise in chronically inflamed livers with hepatitis due to viral infection, excessive alcohol intake or accumulation of fat (NASH) [84]. Whereas infiltration of immune cells into established HCC nodules is protective [119], activation of inflammatory pathways in the liver tissue surrounding HCC is associated with a poor prognosis [120]. Thus, an inflammatory microenvironment is in particular supporting HCC onset. The development of HCC is facilitated by secretion of cytokines by different cell types of the microenvironment. The secretion of TNFα by macrophages makes hepatocytes less sensitive to apoptosis, thus favouring carcinogenesis [121]. In addition, secretion of lymphotoxin-α, lymphotoxin-β, IL-6 and HGF by the microenvironment promotes hepatocyte growth [122, 123].

Moreover, HSCs have shown to suppress immune responses in HCC, thereby contributing to a tumour permissive environment. Activated intratumoural HSCs induce T cell hypo responsiveness [124], and co-transplantation of HSCs and HCC

results in reduced lymphocyte infiltration and apoptosis of monocytes in tumours [125]. In addition, the presence of HSCs leads to increased Treg and myeloid-derived suppressor cell (MDSC) numbers in the tumour stroma [126].
Overall, tumour-stromal interactions, especially the cross-talk between HCC, HSCs and the immune system, represent an important factor in HCC development and progression. Therefore, targeting the microenvironment in HCC is considered as a potential target for novel anti-cancer therapy.

2.1.2.3 HCC treatment

The Barcelona Clinic Liver Cancer (BCLC) classification allows for staging of HCC, taking both tumour burden and liver function into account. Moreover, the BCLC classification provides recommendations for treatment according to the different stages of HCC [127, 128].
At present, different curative treatment options exist for early stage HCC, such as surgical interventions like partial hepatectomy or liver transplantation. However, surgical treatment is only eligible to approximately 40% of patients [84], in which case locoregional therapies, such as radiofrequency ablation, microwave ablation, transarterial chemoembolization (TACE), ethanol injection or cryotherapy represent alternatives for HCC treatment [128].
In addition to the previously mentioned interventions, targeted molecular therapy, such as immune therapy and systemic treatment with small molecules, play an important role in treatment of advanced, non- resectable HCC. Sorafenib, a small molecule multi-kinase inhibitor, is targeting various receptors implicated in the pathogenesis of HCC, such as vascular endothelial growth factor (VEGF) receptors 1, 2 and 3 and PDGF receptor β [91].
However, despite the variety of treatment options for different stages of HCC, survival rates are low at late tumour stages. Whereas curative treatments in early stage HCC, i.e. resection, ablation and transplantation, can achieve survival rates of up to 60 months, compared to a natural history of 36 months survival, patients treated with intermediate HCC stages and TACE face a median survival of 26 months [129, 130]. Moreover, a multicentre, double-blind, placebo-controlled study revealed only a 3

month survival benefit (11 compared to 8 months) for patients with advanced HCC treated with Sorafenib, compared to placebo [131]. Similarly, Regorafenib, which has a similar biochemical profile to Sorafenib, was shown to increase survival by 2.8 months compared to placebo in patients with progressing HCC under Sorafenib therapy [132].

Although many drugs have failed in phase III trials, there are a variety of novel concepts for HCC therapy promising to be effective alternatives in pre-clinical studies. Cell cycle inhibitors, as well as receptor tyrosine kinase inhibitors are currently tested in phase III trials. Moreover, a small molecule against TGF-β as well as a FGF16-specific kinase inhibitor are tested [84]. In addition, immune therapeutic approaches are under investigation. For example, the monoclonal antibody nivolumab inhibits programmed cell death protein 1 (PD1) which blocks inhibition of T cell signalling, and shows good results in patients with Sorafenib-resistant HCC [84].

2.1.3 AMPK in cancer and fibrosis development

AMPK, an evolutionary conserved energy-sensing enzyme, is a crucial regulator of cell growth and metabolism. Upon cell stress, such as low nutrients, hypoxia or prolonged exercise, decreasing levels of ATP, or increasing levels of AMP or ADP lead to activation of AMPK, resulting in promotion of catabolic pathways and inhibition of anabolic pathways in the cell [133, 134]. Besides its role in regulating metabolism, AMPK has recently been implicated in the development of different cancers, amongst them HCC [135].

A first link between AMPK and cancer was made when it was discovered that LKB1, a known tumour suppressor, is the major upstream kinase of AMPK [136]. Thus, LKB1, also known as STK11, has been shown to be mutated in Peutz-Jeghers syndrome, a rare, inherited cancer susceptibility [137], as well as in various cancers, such as lung [138, 139], pancreatic [138, 140] and cervical cancers [138]. However, there is evidence that AMPK itself plays an important role in cancer development and progression. As mentioned above, AMPK regulates the cell cycle by stabilization of p53 and p27, thus establishing a metabolic checkpoint for cell proliferation and growth under conditions of metabolic stress [141-143]. Activated p53 can, in turn,

activate AMPK through induction of transcription of the β subunit of AMPK [144], establishing a tumour-suppressive feed-back loop. Of note, it has been shown that gain-of-function mutants of p53, which are frequently occurring in cancers [145], directly inhibit AMPK by binding to the α subunit, thereby promoting uncontrolled cell growth [146].

In addition, AMPK regulates cell growth by suppression of fatty acid-, triglyceride- and cholesterol- synthesis, as well as protein synthesis as described above [147], and reprogramming of the metabolism is considered as a hallmark of cancer development [112, 148]. AMPK also exerts a so-called anti-Warburg effect. In cancer cells, the Warburg effect has been described as the switch of metabolism from mitochondrial oxidative phosphorylation to aerobic glycolysis, accompanied by rapid glucose uptake, enabling proliferation, cell growth and survival [149]. Despite enhancing glucose uptake and glycolysis in the short term, AMPK promotes oxidative metabolism by upregulation of oxidative enzymes and enhancing mitochondrial biogenesis in the long term [150, 151]. Inhibition of mTOR, which is responsible for translation of hypoxia inducible factor-1α (HIF-1α) that translates many Warburg effect relevant genes, also contributes to the anti-Warburg effect of AMPK [152, 153].

Further evidence for AMPK being a tumour suppressor originates from the observation that AMPK activating drugs inhibit cancer development and growth. For example, treatment with the anti-diabetic drug Metformin results in a lower incidence of various types of cancer, e.g. HCC, pancreatic cancer and colon carcinoma in humans [154, 155]. Moreover, cancer development is suppressed by treatment with Metformin in animal models [156, 157], and AICAR and Metformin suppress cell growth of various cancer cells *in vitro* [158-161]. Moreover, in non-small cell lung cancer (NSCL), the most common lung cancer, AMPK activation correlates with a better prognosis [162], and mutations in LKB1 result in unchecked cell proliferation due to missing AMPK activation [163]. Furthermore, activation of AMPK inhibits the metastatic potential of melanoma cells [164].

There is evidence that AMPK plays a particularly important role in the pathogenesis of HCC. It has been shown, for example, that AMPK α2 is down-regulated in HCC, leading to a poorer tumour differentiation and a worse prognosis for patients with AMPK α2 under-expressing tumours [141]. Similarly, low LKB1 expression results in greater tumour severity and shorter disease-free survival in HCC patients [165].

Moreover, pharmacological activation of AMPK in HCC cells leads to inhibition of HCC cell proliferation and colony formation, as well as to inhibition of tumour growth in animal models [141, 166].

Interestingly, AMPK also plays a role in fibrosis development in the liver in different disease settings. NASH is associated with obesity and insulin resistance and characterized by hepatic steatosis, hepatocyte damage, inflammation and fibrosis [167], and it has been shown that the AMPK activator Metformin is protective in murine model of NASH [168]. Moreover, the AMPK activators Metformin, adiponectin, AICAR and A-769662 reduce the triglyceride content of hepatocytes in rodents [169-171]. Furthermore, AMPK activation plays a role for the development of alcohol induced liver damage, as AICAR and adiponectin prevent the development of alcoholic liver disease in murine models [172-174].

The development of liver fibrosis is characterized and driven by activation of HSCs, which is described in detail in chapter 2.1.1.2. Notably, pharmacological activation of AMPK in HSCs can prevent or reverse HSC activation. For example, adiponectin inhibits proliferation, migration and the expression of pro-fibrotic genes in rat HSCs [175]. Moreover, *in vivo* administration of adiponectin inhibits HSC proliferation and prevents the development of fibrosis in murine models [174, 176]. In addition, adiponectin, as well as the AMPK activators AICAR and Metformin, inhibit PDGF-induced proliferation and migration [177, 178], as well as TGF-β induced collagen I expression of HSCs *in vitro* [179].

Of note, AMPK seems to be involved in regulating tumour-stromal interactions in HCC, as it has been shown that the AMPK activator Metformin inhibits angiogenesis in a HSC HCC *in vitro* co-culture system. This was mediated through reduced VEGF secretion by HSCs by AMPK activation [180].

The ability to survive metabolic stress is crucial to cancer cells. Thus, there is growing evidence that, in established tumours, AMPK can also act as a tumour promoter [181]. In LKB-1 deficient lung tumours, re-expression of LKB1 protects the tumour cells from cell death due to glucose starvation [182]. Moreover, AMPK supports the growth of breast cancer and astrocytic brain tumours [183, 184]. In metastatic prostate cancer, overexpression of the AMPK subunit β1 protects cell survival [185], and in osteosarcoma, AMPK protects from cell death induced by reactive oxygen species (ROS) [186, 187].

Considering the role of AMPK for fibrosis development and the initiation of tumour development, one aim of this thesis was to test whether AMPK plays a role for HCC development in the context of a fibrotic liver. More specifically, the interaction between HSCs and HCC cells and a possible involvement of AMPK in such tumour-stromal interactions was investigated.

2.1.3.1 Adenosine monophosphate-activated kinase (AMPK), structure and regulation

AMPK exists as a heterotrimer, consisting of the catalytic α subunit and the two regulatory subunits β and γ. Whereas the α and β subunits exist in two isoforms α1, α2 and β1,β2, respectively, the γ subunit occurs in 3 different subunits (γ1, γ1 and γ3) [188]. It has been shown that the different isoforms show tissue specific distribution and different subcellular localization [189, 190]. Thus, AMPKα1 is predominantly located in the cytoplasm, whereas AMPKα2 is mostly located in the nucleus, suggesting differences in signalling functions [189].

AMPK is allosterically activated by the binding of AMP to the γ subunit leading to a conformational change resulting in a 2-5 -fold increase in activity [191, 192]. In addition, AMPK is activated by the phosphorylation of AMPKα on threonine residue 172 (Thr^{172}) by upstream kinases [192]. This, in combination with the allosteric activation, this leads to a 1000 -fold increased activity [193]. Allosteric activation, in addition, protects AMPK from dephosphorylation at Thr^{172} [194].

AMPK phosphorylation at Thr^{172} is mediated by its upstream kinases liver kinase B1 (LKB1), Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) and TGFβ-activated kinase 1 (TAK1). However, it has been shown that LKB1 represents the major mediator of AMPK activation and it remains unclear whether AMPK activation by TAK1 requires LKB1 [134, 195].

Importantly, phosphorylation of AMPKα on serine residue 485 of the α1 isoform or serine 491 of α2 ($Ser^{485/491}$) leads to AMPK inactivation and even antagonizes the activation of AMPK [196]. This inhibiting phosphorylation is primarily mediated by Akt/Protein kinase B (PKB) [196, 197].

Besides being invoked by metabolic stress, AMPK is also activated by different pharmacological agents. Metformin and Phenformin, biguanides used for oral treatment of type 2 diabetes, have been shown to activate AMPK through inhibition of complex I of the respiratory chain and thereby lowering ATP levels. Notably, Metformin has been shown to activate AMPK in an LKB1-dependent manner [195, 198]. Another widely used AMPK agonist is 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranosid (AICAR). In the cell, AICAR is converted to ZMP (5-amino-4-imidazolecarboxamide ribonucleotide), an AMP analogue, that activates AMPK through binding to the γ subunit [191, 199]. Moreover, AICAR induces phosphorylation of AMPK-Thr172 independently of LKB1 though the ataxia telangiectasia mutated (ATM) protein kinase, a member of the PI3 kinase superfamily [200]. AMPK is in addition activated by the direct small-molecule AMPK activator A769662, naturally occurring components, for example resveratrol, as well as by the chemotherapeutic Pemetrexed [134]. A769662 directly activates AMPK by binding a domain in between the α and the β subunit, thereby mimicking the effect of AMP, leading to allosteric activation and protection of dephosphorylation of AMPK-Thr172 [201, 202]. Resveratrol induces phosphorylation of AMPK-Thr^{172} by CaMKKβ and increases intracellular calcium levels [203]. Pemetrexed leads to accumulation of ZMP by inhibition of purine synthesis, which then in turn activates AMPK allosterically [204].

Moreover, AMPK in the liver can be regulated by the adipokine adiponectin by induction of AMPK phosphorylation at Thr^{172} [205].

The only compound widely used as a selective inhibitor of AMPK is the cell-permeable pyrazolopyrimidine derivative Compound C (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5- a]pyrimidine). It has been used to rescue the anti-proliferative effects of Metformin and AICAR [177]. However, there is evidence that the anti-proliferative effects of Compound C (CC) are mediated through various mechanisms independently of AMPK [206, 207].

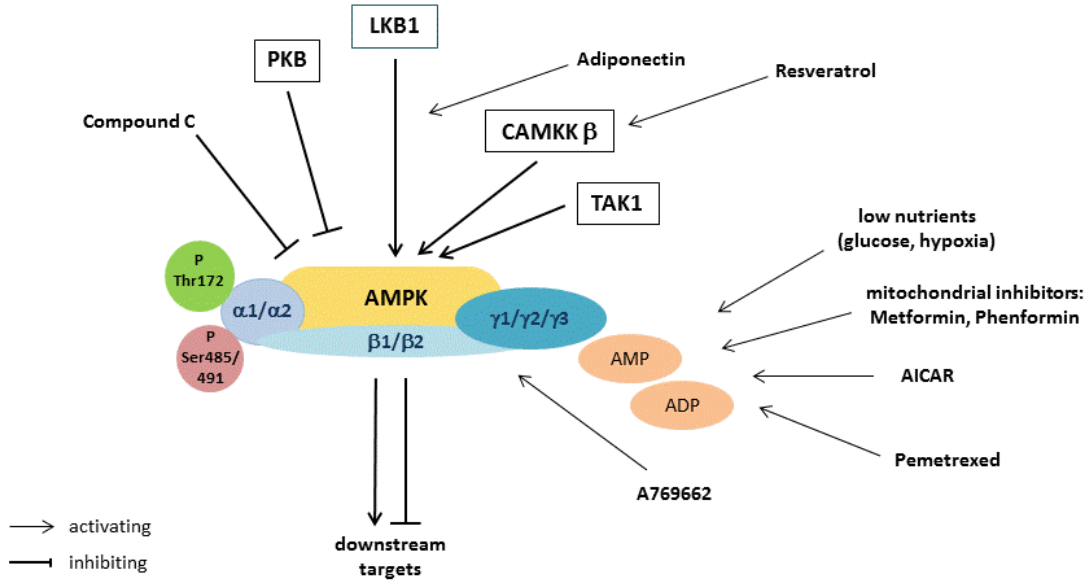


Figure 2.3: AMPK – structure and regulation
AMPK is a heterotrimeric complex consisting of the subunits a, b and g. Phosphorylation of AMPK is mediated by its upstream kinases LKB1, CAMKK β, TAK1 and PKB. Activation of AMPK is accompanied by phosphorylation of AMPK Thr^{172}, whereas AMPK inhibition is characterised by phosphorylation of AMPK a Ser$^{485/491}$. Several pharmacological compounds, such as AICAR, Metformin, Phenformin, A769662, Resveratrol and Pemetrexed can activate AMPK through different mechanisms. In addition, Adiponectin can mediate phosphorylation of AMPK Thr^{172}. Compound C is the only known AMPK inhibitor.
Adapted from Mihaylova and Shaw 2011 [134]

2.1.3.2 Downstream targets of AMPK

When energy levels in the cell are low, the activation of AMPK leads to inhibition of energy-consuming pathways, whereas energy-saving pathways are encouraged, and cell growth and cell proliferation are inhibited through numerous mechanisms and pathways [134, 147].

Importantly, AMPK inhibits cell growth via suppression of the mTORC1 (mammalian target of rapamycin complex 1) pathway. It has been shown that AMPK controls mTORC1 both by phosphorylation of the tumour suppressor TSC2 upstream of mTOR, as well as by direct phosphorylation of the mTORC1 subunit Raptor

(regulatory associated protein of mTOR) [208], thereby blocking the ability of mTORC1 to phosphorylate its downstream substrates. Of note, inhibition of the mTOR pathway not only inhibits cell proliferation, but also the synthesis of proteins and other macromolecules [209]. Moreover, AMPK causes cell cycle arrest through stabilization of the tumour suppressor p53, which is achieved through phosphorylation of either the p53-deacetylase Sirtuin 1 (SIRT1) [141] or the p53-regulator MDMX [210] by AMPK.

AMPK also regulates several metabolic pathways. Thus, AMPK increases fatty acid uptake via translocation of the fatty acid transporter CD36 into the plasma membrane [211], and promotes fatty acid oxidation through inactivation of acetyl-CoA carboxylase 2 (ACC2) [212, 213]. At the same time, AMPK inhibits fatty acid- and sterol synthesis by phosphorylating the pathways' rate limiting enzymes ACC 1 and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, respectively [212, 214]. Besides such rapid, direct regulation of enzymes involved in fatty acid synthesis, AMPK exerts transcriptional control on fatty acid synthesis by inhibition of the transcription factor sterol regulatory element-binding protein-1 (SREBP1) [215] which up-regulates the expression of genes related to fatty acid and sterol synthesis, including fatty acid-synthase, ACC and HMG-CoA reductase [216].

AMPK has also been shown to increase glucose uptake via upregulation of the glucose transporters GLUT1 and GLUT4 [217, 218], and activates glycolysis though phosphorylation of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) [219]. In contrast, glycogen synthesis is inhibited by AMPK through phosphorylation of glycogen synthase [220].

Furthermore, AMPK activation can drive autophagy, a catabolic pathway activated during starvation or stress, leading to digestion of organelles or cellular macromolecules [221]. AMPK triggers autophagy by direct phosphorylation of Unc 51-like kinase (ULK1), an initiator of autophagy, as well as by inhibition of mTORC1, which in turn inhibits autophagy [222]. AMPK also regulates the cyclin-dependent kinase inhibitor (CKI) p27 through phosphorylation at Thr198, leading to stabilization and cytoplasmic location of p27, thus promoting autophagy and cell cycle arrest [143].

Moreover, AMPK has recently shown to regulate cell polarity and migration [223-225].

AMPK furthermore enhances mitochondrial function by phosphorylation of the transcription factor peroxisome proliferator-activated receptor-gamma coactivator (PGC) -1a, a master regulator of mitochondrial biogenesis [151], and stimulates the removal of defective mitochondria by mitophagy in an ULK1 dependent manner [134, 226].

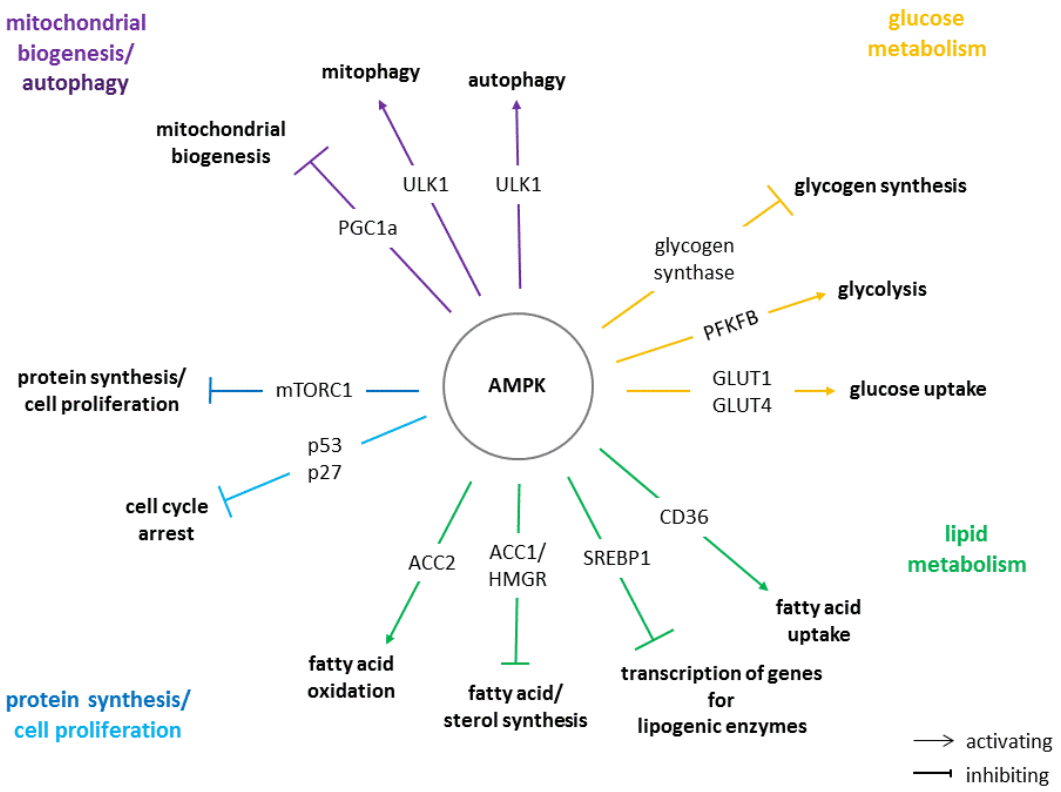


Figure 2.4: Downstream targets of AMPK
When AMPK is activated, it promotes energy saving pathways while inhibiting energy-consuming pathways. AMPK exerts effects on glucose metabolism, lipid metabolism, protein synthesis and cell proliferation, mitochondrial biogenesis and autophagy. The mechanisms through which AMPK regulates these pathways are described in chapter 2.1.3.1.
Adapted from Hardie, D. G. et al. 2012 [147]

2.2 Materials and Methods

2.2.1 Cell culture

2.2.1.1 Human HSCs

Human HSCs were isolated from wedge sections of human liver tissue, obtained from patients undergoing liver surgery at the Royal Free Hospital after giving informed consent, as described before [227, 228]. Briefly, about 10g of total human liver tissue was digested with 0.01% Collagenase type IV (Sigma Aldrich), 0.05% Pronase (Calbiochem) and 0.001% DNase I (Sigma Aldrich). The homogenate was filtered through a 100µm cell strainer (BD Falcon) and the flow-through was centrifuged at 50 x g for 2 minutes at 4°C to remove hepatocytes. After washing the supernatant, gradient centrifugation was performed at 1400 x g (no break) for 17 minutes at 20°C using an 11.5% Optiprep gradient (Sigma). Finally, the interface was collected and washed. The obtained HSCs were cultured in Iscove's Modified Dulbecco's Medium supplemented with 20% foetal bovine serum (FBS), 2 mM/l glutamine, nonessential amino acids 1x, 1.0 mM/l sodium pyruvate, antibiotic-antimycotic 1x (all GIBCO), referred to as complete HSC medium hereinafter. Experiments were performed on cells between passage 3 and 8 employing at least three different cell preparations, and cells were grown to approximately 90% confluence before using them for experiments or frozen down for long term storage. Cells were frozen down after trypsinisation and washing and resuspended in HSC freezing medium, consisting of 45% complete HSC medium, 45% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich). HSCs were cooled down to -80°C using a Mr. Frosty ^{TM} freezing container (Thermo Scientific) and then stored in a liquid nitrogen tank until required for further experiments. When used for further experiments, HSCs were thawed rapidly and cultured in a cell culture flask in complete HSC medium.

2.2.1.2 HepG2 and PLC/PRF/5 cells

HepG2 and PLC/PRF/5 are human liver cancer cell lines originating from hepatocellular carcinoma and were purchased by American Type Culture Collection (ATCC®). These two cancer cell lines show a distinct profile of mutations with HepG2 cells featuring mutations in the gene encoding for β-catenin and PLC/PRF/5 cells showing mutations in the p53 and cyclin-dependent kinase inhibitor 2A gene [229, 230].

To culture HCC cells, cells were thawed rapidly and washed before culturing them in a cell culture flask in Minimum Essential Medium α supplemented with 10% FBS, nonessential amino acids 1x, 1.0 mM/l sodium pyruvate, Antibiotic-Antimycotic 1x (all GIBCO), referred to as complete HCC medium hereinafter. Cells were passaged at least twice and grown to approximately 90% confluence before using them for experiments.

To freeze and store cells, cells were trypsinised when they were approximately 90% confluent. After washing, the cells were resuspended in complete HCC medium supplemented with 4% DMSO (Sigma Aldrich). Cells were cooled down to -80°C using a Mr. Frosty ™ freezing container (Thermo Scientific) and then stored in a liquid nitrogen tank until required for further experiments.

2.2.1.3 Mouse embryonic fibroblasts

Simian virus 40 (SV 40) large T antigen immortalized mouse embryonic fibroblasts (MEFs) were kindly provided by Dr Benoit Viollet, Institute Cochin, INSERM, Université Paris Descartes, Paris, France.

Briefly, AMPKα1/α2 $^{-/-}$ and wt MEFs were obtained from 10.5 day postcoitum embryos, the genotype was confirmed by PCR and immunoblot analysis. MEFs from the second or third culture passage were immortalized by introducing the SV 40 large T antigen using the pSV-Ori^{-} vector [231].

MEFs were cultured in complete MEF medium, consisting of Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 1mM sodium pyruvate and Antibiotic-

Antimycotic 1x (all gibco) and grown to approximately 90% confluence, and passaged at least twice before used for experiments.
To freeze MEFs down, cells were trypsinised and washed once. After washing, the cells were resuspended in complete MEF medium supplemented with 10% DMSO (Sigma Aldrich). Cells were cooled down to -80°C using a Mr. Frosty ™ freezing container (Thermo Scientific) and then stored in a liquid nitrogen tank until required for further experiments. MEFs were thawed rapidly and washed before culturing them in a cell culture flask.

All cells used were cultured under standard conditions at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was routinely renewed 3 times a week. Moreover, cells were tested regularly for mycoplasma contamination by Polymerase Chain Reaction (PCR) performed in the core facility of the Institute for Liver and Digestive Health.

2.2.2 Cell counting

All cells were counted using C-chip Neubauer improved counting chambers (Nano EnTek). 10µl cell suspension was applied to the counting chambers and cells in all 4 outer squares were counted. Cell number was determined according to the following formula:

$$\text{Cells/ml} = \text{average count per outer square} \times \text{dilution factor} \times 10^4$$

2.2.3 Conditioned medium

2.2.3.1 Preparation of conditioned medium

To obtain conditioned medium of HSC, HepG2 and PLC/PRF/5 cells, 0.6 x 10^6 HSC or 3 x 10^6 HepG2 or PLC/PRF/5 cells (unless indicated differently) were cultured in a cell culture dish (100 x 22mm) in complete HSC or HCC medium, respectively. After 24 hours, cells were washed twice with HBSS (gibco) and incubated in serum-free medium for 48 hours. Conditioned media were collected and centrifuged at 247 x g, 7 min, 21°C. The supernatant was used immediately, or frozen down and stored at -20°C until used for experiments. Conditioned medium was thawed and brought to 37°C before use.

2.2.3.2 Fractionation of conditioned medium of HepG2 and PLC/PRF/5 cells

In order to produce conditioned medium containing different fractions of proteins, conditioned medium of HepG2 and PLC/PRF/5 cells was obtained as described above and fractionated using filters with pore sizes of 10 kDa and 30 kDa. Conditioned medium was centrifuged in Amicon Ultra centrifugal filter devices (Millipore) for 20 min at 3200 x g. Because the fraction containing proteins bigger than the filter's pore size was highly concentrated, the original volume was recovered with serum free HCC medium. Fractionated conditioned medium was used for experiments immediately after centrifugation.

2.2.4 Treatment of cells

2.2.4.1 Treatment of HSC cells with conditioned medium of HepG2 or PLC/PRF/5 cells

HSC were plated on a cell culture dish (0.3 x 10^6/6 well or 0.006 x 10^6/ 96 well) in complete HSC medium. After 24 hours, cells were washed with HBSS (gibco) and serum-starved for 24 hours. Subsequently cells were treated with conditioned medium of HepG2 or PLC/PRF/5 cells for 24 hours.
Afterwards, cell proliferation was assessed, or cells were harvested for protein analysis (chapter 2.2.11) or RNA analysis (chapter 2.2.12).

2.2.4.2 Treatment of HepG2 and PLC/PRF/5 cells with conditioned medium of HSCs

HepG2 and PLC/PRF/5 cells were plated on a cell culture dish (0.3 x 10^6/6 well or 0.006 x 10^6/ 96 well) in complete HCC medium. After 24 hours, cells were washed with HBSS (gibco) and serum-starved for 24 hours. Subsequently cells were treated with conditioned medium of different HSC preparations. After 24 hours, proliferation was assessed by bromodeoxyuridine (BrdU) incorporation assay (Roche, see chapter 2.2.5), or cells were harvested for protein analysis (chapter 2.2.11).

2.2.4.3 Treatment of cells with pharmacological compounds

Primary human hepatic stellate, PLC/PRF/5 and HepG2 cells, as well as MEFs were treated with different concentrations of AICAR (0.25-4mM), Metformin (1-100mM), Phenformin (10-1000μM) or Compound C (2.5-40μM). Cells were plated in complete medium and serum-starved for 24 hours prior to treatment.
AICAR was purchased from Calbiochem, Metformin and Phenformin from Sigma. AICAR and Phenformin were reconstituted with water, Metformin was reconstituted

with serum free HSC medium or serum free HCC medium, depending on the cell type treated.

2.2.5 BrdU incorporation assay

Cell proliferation was quantified by BrdU Cell Proliferation ELISA kit (Roche). The assay is based on the replacement of thymidine by the pyrimidine analogue BrdU in proliferating cells. A colorimetric immunoreaction as a measure of BrdU incorporation into newly synthesized cellular DNA can be detected photometrically (absorbance at 370nm).

Briefly, 0.006 x 106 HSCs, PLC/PRF/5 or HepG2 cells were plated in complete medium in a 96 well plate in quadruplicates or triplicates. After 24 hours, cells were washed and serum-starved for 24 hours. Afterwards, treatment, as well as the BrdU labelling solution was added, according to the manufacturer's protocol. After 24 hours of treatment, the BrdU ELISA was developed according to the manufacturer's protocol and absorbance was detected with Fluostar Omega Plate Reader (BMG labtech).

2.2.6 MTS assay

To determine viability of cells, CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega) was used according to the manufacturer's protocol. Briefly, a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) is added to the culture which subsequently is converted into a formazan product by metabolically active cells. The quantity of formazan can be measured by the absorbance at 490 nm directly correlating with the number of metabolically active cells.

HSCs, PLC/PRF/5 or HepG2 cells (0.006 x 10^6/96 well) were cultured in complete medium for 24 hours in a 96 well plate in quadruplicates or triplicates. Afterwards, cells were washed, cultured in serum free medium for 24 hours and subsequently treated for 24 hours. MTS was added for 2 hours and absorbance was measured with Fluostar Omega Plate Reader (BMG labtech).

2.2.7 Wound healing assay

For wound healing assays, 0.09 x 10^6 HSCs were plated on a 24 wells cell culture plate. After 24 hours, cells were washed and the medium was replaced by serum free medium. After 24 hours of serum starvation, a scratch was applied with a plastic pipette tip and cells were washed before applying the treatment. The cells were imaged using a Nikon Eclipse TE200 microscope at the time of treatment, as well as after 24 hours, to monitor cell migration.

2.2.8 Cell cycle analysis

For cell cycle analysis, 0.3 x 10^6 HSCs were plated on a 6 well plate in complete medium. After 24 hours, cells were washed and cultured in serum-free medium for 24 hours, followed by treatment. Cells were trypsinised after 24 hours of treatment, washed and fixed with ice-cold 70% ethanol, which was added dropwise while vortexing the cell pellet. Fixation was carried out at 4°C for 30 min. This was followed by two washing steps and incubation with propidium iodide (50μg/ml, Promega) for 10 minutes at room temperature. Propidium iodide accumulation was analysed with BD LSR Fortessa (5L SORP) using BD FACSDiva software (version 6.2) and analysed with FlowJo (version 10.0).

2.2.9 Cell death enzyme linked immunosorbent assay (ELISA)

To analyse cell death in HSCs, Cell Death Detection ELISA^{Plus} (Roche) was used according to the manufacturer's protocol. In summary, 0.006 x 10^6 HSCs were plated on a 96 well plate in complete medium in triplicates. After 24 hours, cells were washed and serum starved for 24 hours, followed by treatment. After 24 hours treatment, supernatants were removed and cells were lysed. Nucleosomes can be quantified by an ELISA principle in cell lysates to determine apoptosis, as well as in supernatants to determine necrosis. The kit contains an anti-histone antibody specific to the histones H1, H2A, H2B, H3 and H3, and an anti-DNA antibody binding to single and double stranded DNA.

The amount of nucleosomes can be detected photometrically by an anti-DNA horseradish peroxidase. Absorbance at 420nm was measured with Fluostar Omega Plate Reader (BMG labtech).

2.2.10 Antibody-based LC3 detection with flow cytometry

In order to quantify the accumulation of LC3 in autophagosomes in HSCs after treatment with AICAR and CC, 0.05 x 10^6/well HSCs were plated on a 24 wells cell culture plate in complete HSC medium. After 24 hours, cells were washed and cultured in serum free medium for 24 hours. Subsequently, different concentrations of AICAR (0.5-2mM) or CC (5-20μM) were applied in serum free medium for 24 hours.

In order to detect autophagosomal LC3B, the Muse^{TM} autophagy LC3-antibody based kit (Meck Millipore) was used according to the manufacturer's instructions.

Briefly, cells were washed and incubated with a permeabilising agent that ensures extraction of cytosolic LC3, while maintaining autophagosomal LC3. Cells are then detached from the cell culture plate and LC3 is labelled using an Alexa Fluor 555 coupled anti-LC3 antibody. Fluorescence was detected with the Muse^{TM} cell analyser (Merck Millipore). The autophagy induction ratio is calculated by dividing the mean fluorescence intensity (MFI) of LC3 in the treated cells by the MFI of LC3 in untreated, unlabelled cells.

2.2.11 Western blot

2.2.11.1 Total protein cell lysate

To obtain total protein cell lysates, cells were washed with 1x phosphate buffered saline (PBS, gibco), then radio immunoprecipitation assay (RIPA) buffer was added and cells were scraped. RIPA buffer was homemade and consisted of 20 mM Tris-HCl pH 7.6, 150mM NaCl, 5mM EDTA, 1% NP-40 (nonyl phenoxypolyethoxylethanol), 1mM phenylmethylsulfonyl fluoride (PMSF), 1X Protease Inhibitors Mix, 1mM Na_3VO_4 and 1mM NaF.
Cell lysates were sonicated with the "Ultrasonic Processor" (Sonics, Vibra Cell) at an amplitude of 50 for 15 seconds, centrifuged at 11,000 rcf for 10 minutes at 4°C, and proteins were stored at -80°C.

2.2.11.2 Total protein isolation from conditioned medium

Because several ingredients in conditioned medium of HCC cells interfere with the bicinchoninic acid (BCA) assay used for protein quantification (see below), proteins from conditioned medium were precipitated in 100% acetone at 4°C overnight and centrifuged at 13,000 rcf for 15 min at 4°C. Afterwards, the pellet was resuspended in 2% sodium dodecyl sulfate (SDS) and stored at -80°C.

2.2.11.3 Protein quantification

To determine protein concentrations, a bicinchoninic acid (BCA) assay was used according to manufacturer's protocol (Micro BCA^{TM} Protein Assay Kit, Thermo Scientific). The method is based on the formation of a purple-coloured reaction product requiring the reduction of Cu^{2+} ions by proteins. The reaction product exhibits absorbance at 562 nm which correlates linearly with increasing protein concentrations.

2.2.11.4 SDS page

To separate proteins, 25µg of protein lysate were loaded on 10% acrylamide gels (*separating gel:* 10% acrylamide mix, 0.4 M Tris (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, 0.001% TEMED, H_2O; *Stacking gel:* 5% acrylamide mix, 0.1M Tris (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate, 0.001% TEMED, H_2O). Protein lysates were supplemented with 4x Laemmli Sample Buffer (Bio-Rad) containing 5% β-mercaptoethanol and boiled prior to loading. For electrophoresis, 100-150V was applied for 90-150 min. Mini PROTEAN® System (Bio-Rad) was used with 1x Running Buffer (Ultra-Pure 10x Electrophoresis Grade, 0.25M Tris, 1.92M Glycine, 1% SDS, GeneFlow) and Precision Plus Protein™ Standards Dual Colour (Bio-Rad) was employed as protein standard.
Proteins were blotted to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P Transfer Membranes, MilliporeTM) by wet transfer at 100V for 75 min with a transfer buffer containing 25mM Tris, 192mM Glycine and 10% Methanol.

2.2.11.5 Antibody incubation

After transfer, PVDF membranes were stained with Ponceau S solution (0.1% (w/v) in 5% acetic acid, Sigma Aldrich) to verify efficient transfer. Membranes were blocked with 3-5% Bovine Serum Albumin (BSA, Sigma Aldrich) in Tris Buffered Saline (homemade) with 0.1% Tween-20 (Sigma Aldrich). Subsequently, primary antibodies were incubated overnight at 4°C or for 1 hour at room temperature. After washing, specific secondary antibodies coupled to a horseradish peroxidase were applied for 1 hour at room temperature and SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) was used to develop signals. For following antibody incubations, antibodies were stripped with Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific). To verify equal loading of samples, expression of the house-keeping proteins β-actin or tubulin was detected. All primary antibodies are listed in table 2.1, and all secondary antibodies in table 2.2.

Antibody	Species	Company	Dilution
4E-BP1	Rabbit	Cell Signaling	1 : 1,000
phospho-4E-BP1 (Thr37/46)	Rabbit	Cell Signaling	1 : 1,000
Albumin, monoclonal HAS-11	Mouse	Sigma-Aldrich	1 : 2,500
AMPK a	Rabbit	Cell Signaling	1 : 1,000
Phospho-AMPK (Thr172)	Rabbit	Cell Signaling	1 : 1,000
Phospho-AMPK (Ser485/491)	Rabbit	Cell Signaling	1 : 1,000
β-Actin, monoclonal AC-15	Mouse	Sigma-Aldrich	1 : 5,000
LC3B	Rabbit	Cell Signaling	1 : 1,000
LKB1	Rabbit	Cell Signaling	1 : 1,000
Phospho-LKB1 (Ser428)	Rabbit	Cell Signaling	1 : 1,000
S6	Rabbit	Cell Signaling	1 : 1,000
phopsho-S6 (Ser235/236)	Rabbit	Cell Signaling	1 : 2,000
tubulin	Rabbit	Cell Signaling	1 : 1,000

Table 2.1: Primary antibodies for western blot analysis

Antibody	Company	Dilution
goat anti-rabbit IgG HRP	Santa Cruz Biotechnology	1:10,000
goat anti-mouse IgG HRP	Santa Cruz Biotechnology	1:20,000

Table 2.2: Secondary antibodies for western blot analysis

2.2.12 Quantitative real-time PCR

RNA was isolated from cells employing RNeasy mini Kit (Qiagen) according to the manufacturer's protocol, purity and RNA concentration were measured with Nanodrop spectrophotometer (Thermo Scientific). cDNA was synthesized with

MultiScribe reverse transcriptase, random primers, deoxyribose nucleoside triphosphate (dNTP) mix and RNase inhibitor (all Applied Biosystems) according to the following protocol, using Quanta Biotech Q Cycler II: 2 min 50°C, 10 min 95°C, followed by 40 cycles of 15 seconds 95°C and 60 seconds 60°C.

Gene expression was measured via quantitative real time PCR (qPCR) using Taqman gene assays (table 2.3) and 7500 Fast Real Time PCR System (all Applied Biosystems). To quantify gene expression, the comparative C_T method was used as described previously [232] using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control.

gene	**assay ID**	**company**
TIMP-1	Hs00171558_m1	Applied Biosystems
Col1A1	Hs00164004_m1	Applied Biosystems
CCL2	Hs00234140_m1	Applied Biosystems
LOX	Hs00942480_m1	Applied Biosystems
MMP2	Hs01548727_m1	Applied Biosystems
IL-1β	Hs01555410_m1	Applied Biosystems
IL-8	Hs00174103_m1	Applied Biosystems
IL-6	Hs00985639_m1	Applied Biosystems
GAPDH	Hs02758991_m1	Applied Biosystems

Table 2.3: Taqman gene assays used for real-time PCR

2.2.13 Statistical analysis

Statistical analysis was performed using Microsoft Excel or Graph Pad Prism. Values are expressed as mean +/- standard deviation (SD), mean +/- standard error of the mean (SEM), or mean +/- 95% confidence interval as indicated. Statistical significance was analysed with unpaired, two-tailed, parametric Student T-test when significance between 2 groups was analysed. ANOVA was employed in order to test statistical significance between more than 2 groups.

2.3 Results

2.3.1 Conditioned medium of HSCs inhibits HCC cell proliferation and modifies the AMPK pathway in HCC cells

In HCC, the tumour microenvironment, consisting of HSCs, myofibroblasts, cancer-associated fibroblasts and immune cells, is considered to play an important role for cancer development [84, 233] and may provide a novel target for anti-cancer therapy in HCC. It has been shown that especially the cross-talk between activated HSCs and HCC cells creates a microenvironment favouring HCC progression [118].

An established method to investigate tumour-stromal interactions *in vitro* is treating one cell-type with the supernatant, also referred to as conditioned medium, of the other cell type, and thereby analysing the paracrine effect of one cell type on the other one [116, 234, 235]. In this thesis, HCC cells were incubated with conditioned medium of different preparations of activated primary human HSCs and vice versa. Because human HCC is characterized by a vast heterogeneity in terms of genetic aberrations [98, 107], two different HCC cell lines, i.e. PLC/PRF/5 and HepG2, which show a different genetic profile [229, 230], were chosen for the experiments. Whereas PLC/PRF/5 cells show mutations in cell-cycle regulating genes p53 and cyclin-dependent kinase inhibitor 2A, HepG2 cells show mutations in the CTNNB1 gene encoding for β-catenin, a protein of the Wnt signalling pathway [229, 230], all of which represent frequent mutations in human HCC [84].

HCC cells and HSCs are cultured in different media with, for example, different glucose concentrations. Thus, as a first experiment, the effect of differences in glucose concentrations on cell proliferation was investigated in order to validate the *in vitro* system. PLC/PRF/5 and HepG2 cells were treated with serum free HSC medium (containing 4500mg/l glucose) and serum free HCC medium (containing 1000mg/l glucose) for 24 hours and proliferation was measured by BrdU incorporation assay. As depicted in figure 2.5a, different glucose concentrations did not affect HepG2 and PLC/PRF/5 cell proliferation. Moreover, proliferation of HepG2 and PLC/PRF/5 cells was not dependent on serum in cell culture media, as proliferation was unchanged when cells were incubated in serum free HCC medium, compared to complete HCC medium (figure 2.5a).

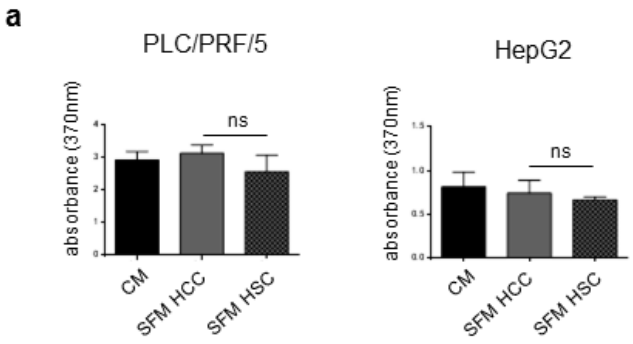


Figure 2.5: Glucose content of cell culture media does not influence cell proliferation.
(a) Cell proliferation of HepG2 and PLC/PRF/5 cells measured by BrdU incorporation after treatment with different media for 24 hours. Data represent mean +/- SD from biological triplicates, ns= not significant, (a): representative data of 3 independent experiments. CM= complete HCC medium, SFM HCC= serum free HCC medium, SFM HSC= serum free HSC medium

To validate the finding that activated HSCS promote HCC proliferation *in vitro* [116], PLC/PRF/5 and HepG2 cells were treated with conditioned medium of 3 different HSC preparations, originating from 3 different patients, for 24 hours and proliferation was measured by BrdU incorporation assay. Interestingly, in PLC/PRF/5 cells, the conditioned medium of different HSC preparations inhibited proliferation significantly (figure 2.6a). Similarly, in HepG2 cells, inhibition of proliferation or no effect on cell proliferation was observed after treatment with conditioned medium of three different HSC preparations (figure 2.6a). As this challenged the previously published data by Amann et al. [116], it was tested whether differences in the protocol, i.e. the number of HSCs used to obtain HSC conditioned medium, or the glucose content of media, influenced cell proliferation of HepG2 and PLC/PRF/5 cells. As shown in figure 2.6b, no difference was observed in HepG2 and PLC/PRF/5 cell proliferation, measured by BrdU incorporation assay, when incubated with HSC conditioned medium obtained from cultures of $2x10^6$ (protocol published by Amann et al. [116]) or $0.6x10^6$ HSCs (standard protocol in our laboratory), respectively. Moreover, HCC cell proliferation was unchanged after treatment with HSC conditioned medium obtained by incubating different numbers of HSCs in low glucose medium (figure 2.6b).

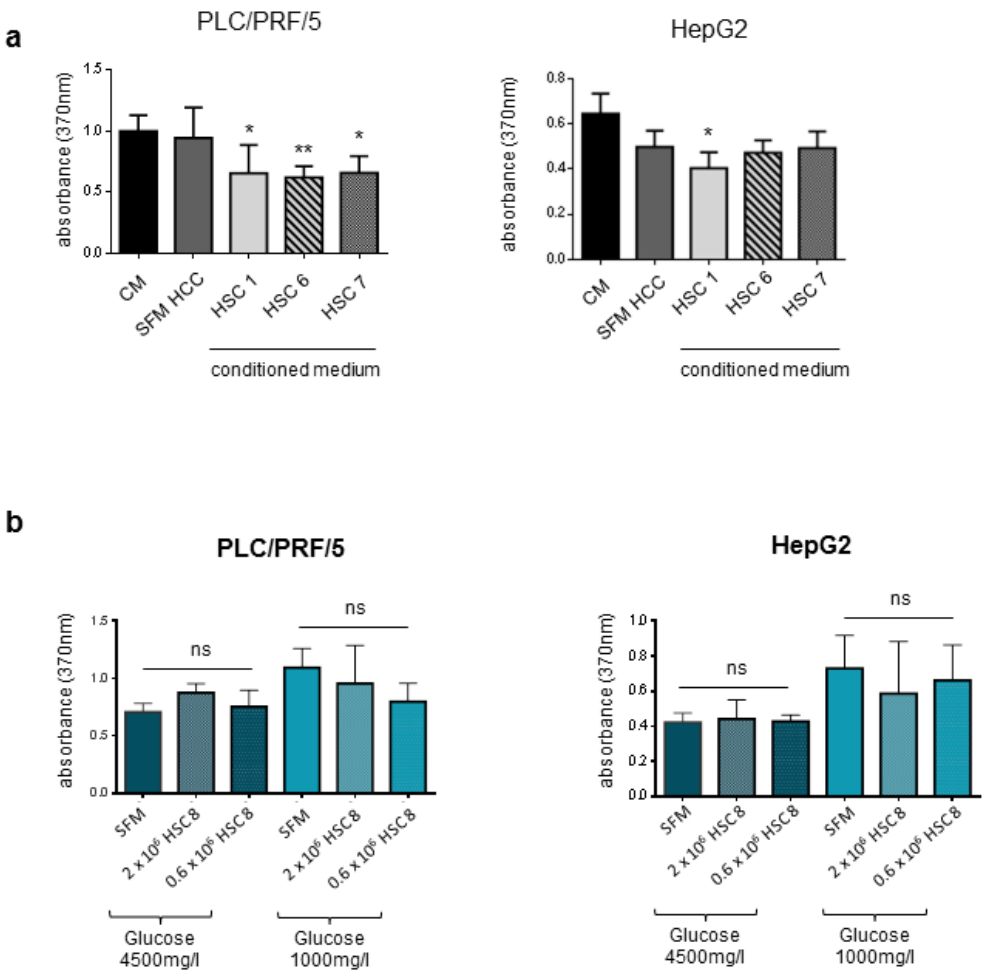


Figure 2.6: HSC conditioned medium inhibits HCC cell proliferation.
(a) Cell proliferation of PLC/PRF/5 and HepG2 cells measured by BrdU incorporation after treatment with HSC conditioned medium of 3 different HSC preparations for 24 hours. (b) Cell proliferation of PLC/PRF/5 and HepG2 cells after treatment with HSC conditioned medium obtained from HSC cultures with different cell numbers and media with different glucose content. Data represent mean +/- SD from biological triplicates, *p< 0.05, **p<0.01 vs serum free medium or as indicated, ns= not significant. (a)- (b): Representative data of 3 independent experiments. Each HSC preparation was isolated from a different patient. CM= complete HCC medium, SFM HCC= serum free HCC medium, SFM HSC= serum free HSC medium

Cell proliferation can be regulated through activation of AMPK, which in turn inhibits the mTORC1 pathway, a critical regulator of protein synthesis, cell growth and cell proliferation [134, 147]. Thus, the expression levels and phosphorylation of AMPK and its upstream kinase LKB1 in HCC cells were analysed. PLC/PRF/5 and HepG2 cells were treated with conditioned medium of three different HSC preparations for 24

hours and phosphorylation of LKB1, as well as AMPK at different phosphorylation sites, were analysed by western blot. Phosphorylation of LKB1-serine 428 (Ser^{428}) leads to the activation of AMPK through phosphorylation of AMPK-Thr^{172} [195]. In contrast, phosphorylation of AMPK-$Ser^{485/491}$ inhibits AMPK activation [236] (see supplementary figure 2.31). As shown in figure 2.7a, treatment of HepG2 cells with conditioned medium of HSCs did not induce phosphorylation of LKB1 at Ser^{428} compared to serum free HSC medium. However, down-regulation of AMPK phosphorylation at Thr^{172} was observed in HepG2 cells treated with HSC conditioned medium (figure 2.7a). In contrast, phosphorylation of AMPK at $Ser^{485/491}$ was mildly induced or not affected, depending on the HSC preparation utilized (figure 2.7a).

In PLC/PRF/5 cells, phosphorylation of LKB1 at Ser^{428} was mildly induced after treatment with HSC conditioned medium, compared to serum free HSC medium (figure 2.7b). Furthermore, a marked down-regulation of phosphorylation of AMPK at Thr^{172} and mild induction of AMPK phosphorylation at $Ser^{485/491}$ were observed (figure 2.7b).

Overall, these results indicate that HSCs can inhibit proliferation of HCC cells in a paracrine fashion. Furthermore, HSCs inhibit AMPK activity in HCC cells by downregulating the phosphorylation of AMPK-Thr^{172}.

In HCC development, mutual interactions between HSCs and HCC cells have been described [118]. Therefore, the effect of HCC cell conditioned medium on HSC activation has been analysed in the next set of experiments.

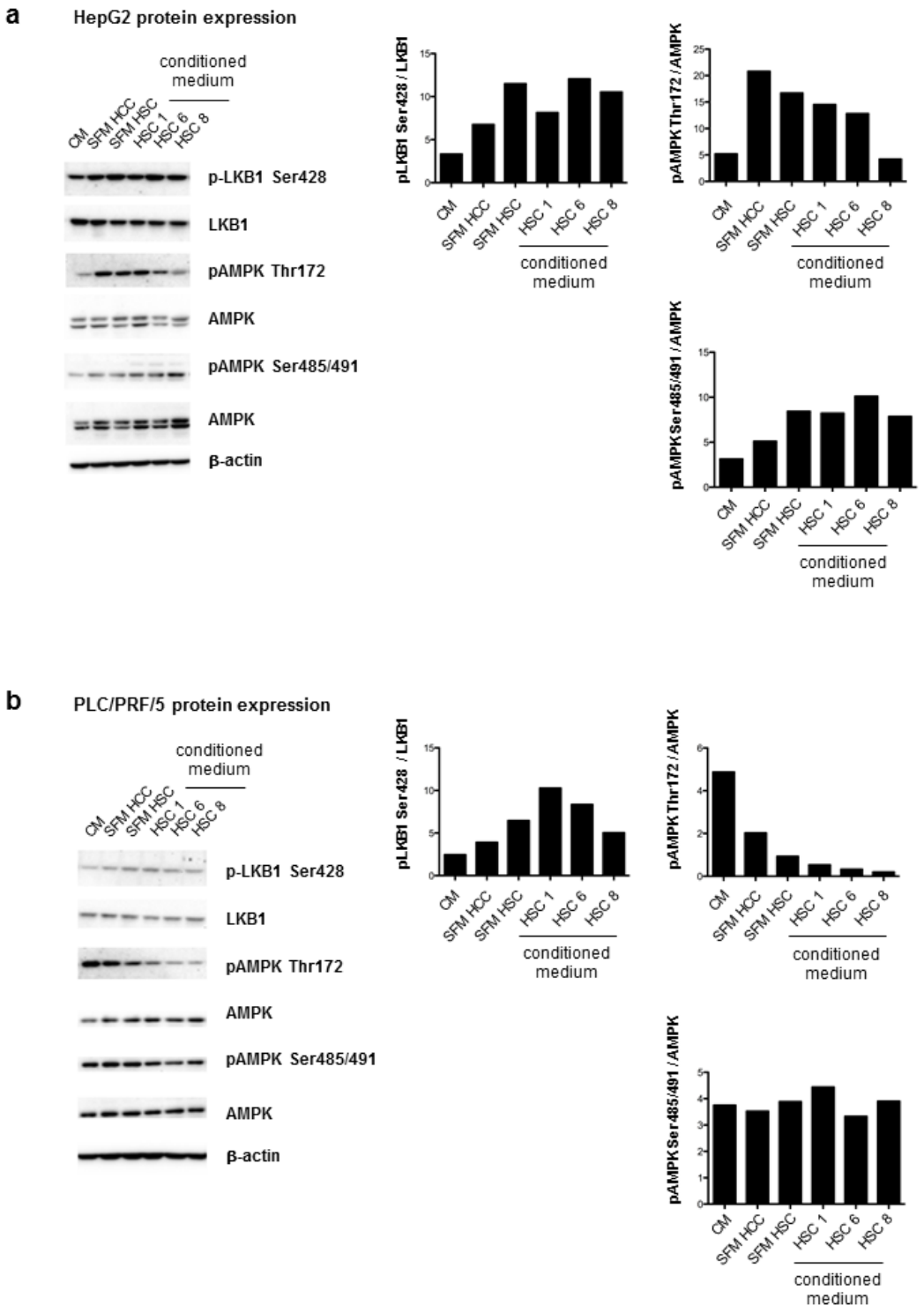


Figure 2.7: HSC conditioned medium modifies the AMPK pathway in HCC cells. Protein expression in (a) HepG2 cells and (b) PLC/PRF/5 cells after treatment with conditioned media of 3 different HSC preparations for 24 hours. (a)- (b): Representative data of 3 independent experiments. Each HSC preparation was isolated from a different patient. CM= complete HCC medium, SFM HCC= serum free HCC medium, SFM HSC= serum free HSC medium

2.3.2 Conditioned media of HCC cells induce up-regulation of pro-inflammatory and pro-fibrogenic genes in HSCs

Under physiological, steady-state conditions, HSCs are in a quiescent state and the main vitamin A storing cell type [24]. Following chronic injury, HSCs become activated and develop into myofibroblast-like cells, which largely contribute to ECM remodelling in the liver [51]. Once HSCs are activated, their proliferation and migration increases. Moreover, activated HSCs produce large amounts of ECM components, e.g. collagen I, leading to the development of fibrotic septae. An imbalance between matrix deposition and degradation eventually contributes to the establishment of fibrosis and cirrhosis. Whereas MMPs produced by HSCs are able to degrade matrix, activated HSCs also secrete inhibitors of MMPs, so-called TIMPs, which bind and inactivate MMPs, preventing the breakdown of scar tissue [6, 51].

Besides the excessive ECM deposition and dysregulated ECM homeostasis, HSCs contribute to a pro-inflammatory microenvironment in the liver, which favours fibrosis development and progression. Thus, the production of pro-inflammatory cytokines, such as IL-1β and IL-6 is associated with HSC activation, with IL-6 being a crucial cytokine in HCC carcinogenesis [237]. IL-1β contributes to the development of fibrosis, both by direct effects on HSCs, as well as by perpetuating the pro-inflammatory micro-environment [22, 23]. Moreover, activated HSCs secrete chemo attractants, such as CCL2, also known as MCP 1 and IL-8, which recruit immune cells that in turn foster the pro-inflammatory micro-environment in the liver [33].

To test whether HCC cells exert a paracrine effect on gene expression associated with HSC activation, three different primary human HSC preparations were treated with conditioned medium of PLC/PRF/5 or HepG2 cells for 24 hours, and gene expression was analysed by quantitative real-time PCR.

As shown in figure 2.8a, no significant change in expression of genes associated with excessive ECM deposition, like Collagen 1 (Col1a1), TIMP-1 and MMP-2 could be detected, compared to treatment with serum free HSC medium. In contrast, there was moderate, but significant up-regulation of the gene expression of lysyl oxidase (LOX) (figure 2.8a), which is responsible for collagen cross-linking [238].

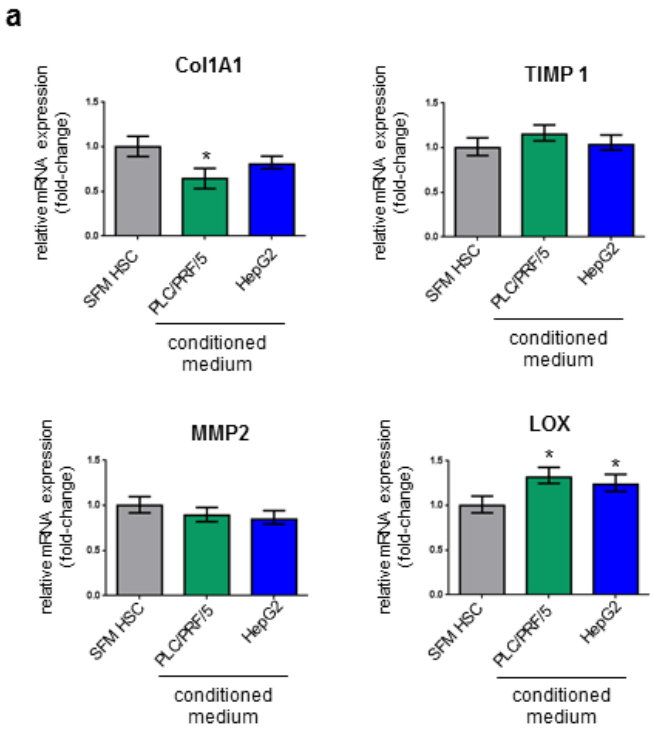


Figure 2.8: The influence of HCC conditioned medium on the expression of pro-fibrogenic genes in HSCs.
(a) Gene expression in HSCs after treatment with conditioned medium of PLC/PRF/5 or HepG2 cells for 24 hours. Gene expression is normalized to treatment with serum-free HSC medium. Data represent mean +/- 95% confidence interval, *p=0.05 vs. serum free medium, pooled data from 3 independent experiments with 3 different HSC preparations. SFM HSC= serum free HSC medium

Moreover, the cytokines CCL2, IL1-β and IL-8 were highly up-regulated after treatment with conditioned medium of HepG2 or PLC/PRF/5 cells. Notably, PLC/PRF/5 conditioned medium induced a 3-fold up-regulation of CCL2 expression, a 6-fold upregulation of IL-1β expression and a 4-fold up-regulation of IL-8 expression (figure 2.9a). HepG2 conditioned medium induced 6-fold upregulation of IL-1β expression, and approximately 1-fold up-regulation of CCL2 and IL-8 expression (figure 2.9a).

Moreover, down-regulation of IL-6 expression after treatment with HepG2 conditioned medium (figure 2.9a) as well as down-regulation of Collagen 1 expression after treatment with PLC/PRF/5 conditioned medium (figure 2.8a) were observed.

Overall, these data show that HCC cells can exert a paracrine effect on HSC gene-expression, further promoting the activated, pro-inflammatory and pro-fibrogenic phenotype of HSCs.

The present analysis of pro-fibrogenic and pro-inflammatory genes in HSCs represents a selection of the most relevant genes showing HSC activation. To analyse a wider set of genes and to screen for other pathways affected by HCC conditioned medium, a gene expression array could be used.

Moreover, the data presented in this chapter led to investigating HSC proliferation and migration, further measures of HSC activation [24, 51], following treatment with HepG2 and PLC/PRF/5 conditioned medium.

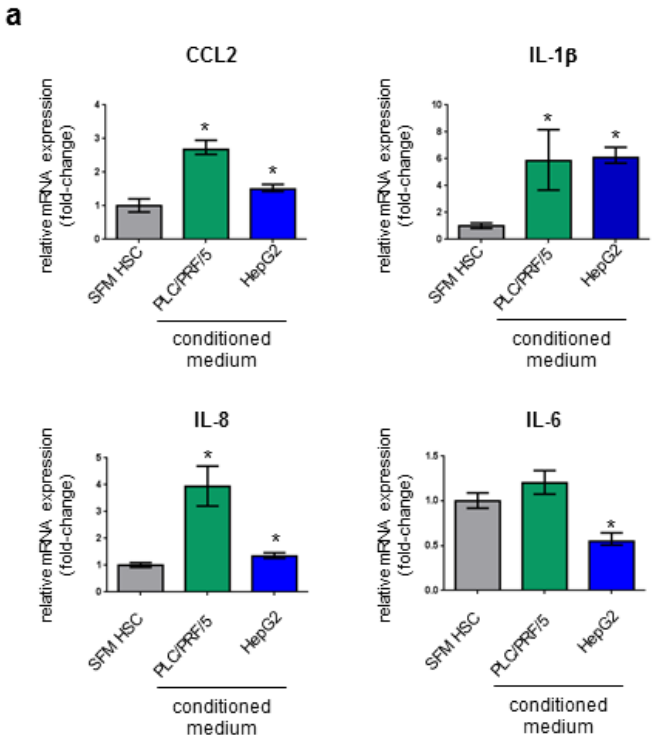


Figure 2.9: HCC conditioned medium induces up-regulation of pro-inflammatory genes in HSCs.

(a) Gene expression in HSCs after treatment with conditioned medium of PLC/PRF/5 or HepG2 cells for 24 hours. Gene expression is normalized to treatment with serum-free HSC medium. Data represent mean +/- 95% confidence interval, *p=0.05 vs. serum free medium, pooled data from 3 independent experiments with 3 different HSC preparations. SFM HSC= serum free HSC medium

2.3.3 HCC cells exert a paracrine effect on HSC proliferation and migration

It has previously been shown that rat HSCs are activated by rat HCC tumour cells as measured by increased HSC proliferation and α-SMA production after treatment with conditioned medium of rat HCC cells [114]. Moreover, HCC conditioned medium altered gene expression in human HSCs (figures 2.8 and 2.9). Nevertheless, evidence about induction of proliferation in human HSCs by human HCC tumour cells is still lacking. Hence, the influence of conditioned medium of HepG2 and PLC/PRF/5 cells on human HSC proliferation was analysed.

First, to test whether differences in glucose between serum free HCC medium (glucose 1000mg/l) and the control serum free HSC medium (glucose 4500mg/l) influenced HSC proliferation, HSCs were treated with the different cell culture media and proliferation was assessed via BrdU incorporation assay. As shown in figure 2.10a, no difference in HSC proliferation could be observed after treatment with the different serum-free media.

Next, 3 different preparations of primary human HSCs were treated with conditioned medium obtained from PLC/PFR/5 or HepG2 cells and proliferation was measured by BrdU incorporation assay. As shown in figure 2.10b, conditioned medium of HepG2 cells significantly increased proliferation of HSCs compared to serum free medium. In contrast, HSC proliferation was unchanged when treated with conditioned medium of PLC/PRF/5 cells.

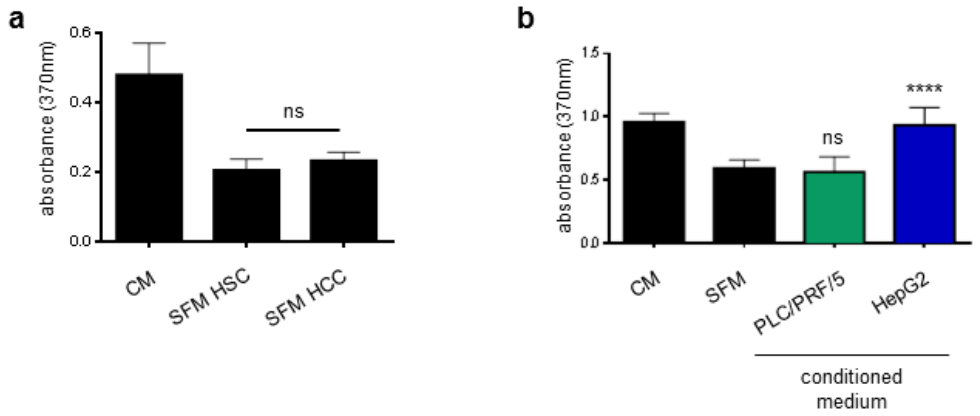


Figure 2.10: Conditioned medium of HepG2 but not PLC/PRF/5 cells induces proliferation in HSCs.
(a) Proliferation of HSCs after treatment with different cell culture media for 24 hours measured by BrdU incorporation. (b) Proliferation of HSCs after treatment with conditioned medium of PLC/PRF/5 or HepG2 cells for 24 hours measured by BrdU incorporation. (a) – (b) data represent mean +/- SD of biological triplicates, ****p<0.0001, ns = not significant vs. serum free medium or as indicated. (a)- (b) Representative data of 4 independent experiments using 4 different HSC preparations. CM= complete HCC medium, SFM HSC= serum free HSC medium, SFM HCC= serum free HCC medium.

As increased migration to sites of tissue repair is another feature of HSC activation [19, 239], the effect of HCC conditioned medium on HSC migration was investigated. Three different preparations of primary human HSCs were treated with conditioned medium of PLC/PRF/5 or HepG2 cells for 24 hours and HSC migration was assessed employing a 2D wound healing assay. Indeed, HepG2 conditioned medium induced migration in HSCs, whereas migration was not induced upon treatment with PLC/PRF/5 conditioned medium, compared to serum free HSC medium (figure 2.11). These data show that HCC conditioned medium differentially influences HSC migration. Besides the 2D wound healing assay that was used here, a Boyden chamber transwell assay, where HSCs migrate through a permeable membrane could be used in future experiments to validate the data. Compared to the 2D wound healing assay, the Boyden chamber assay allows for quantification of the migrated cells band provides a more elaborate way of assessing cell migration.

Taken together, the data presented in this chapter show that conditioned medium of HepG2, but not PLC/PRF/5 cells induces HSC proliferation and migration in a paracrine fashion, both of which are important features of HSC activation.

HSC activation seems to be regulated through the fuel-sensing enzyme AMPK, amongst others, as AMPK activation has been shown to reverse PDGF-induced activation in HSCs [178]. However, evidence about a role for AMPK in HCC cell mediated HSC activation is still missing. Therefore, the activation state of the AMPK pathway in HCC conditioned medium treated HSCs was addressed next.

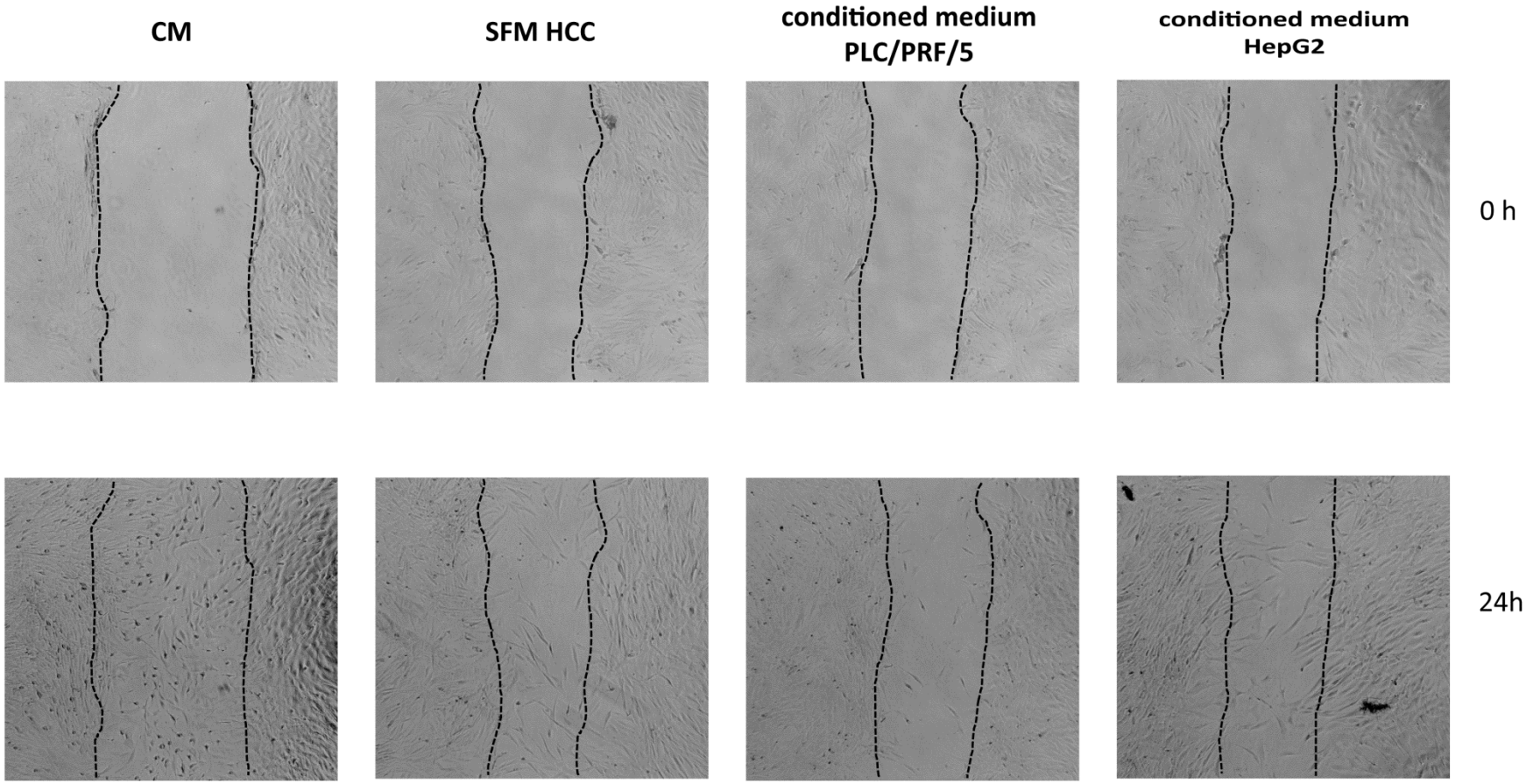


Figure 2.11: HepG2 but not PLC/PRF/5 conditioned medium induces HSC migration

(a) Wound healing assay, migration of HSCs on plastic following a scratch. HSCs were treated with CM, SFM or HCC conditioned medium for 24 hours. Representative data of 3 independent experiments using 3 different HSC preparations. CM = complete HSC medium, SFM HCC = serum free HCC medium.

2.3.4 HCC cells modify the AMPK signalling pathway in HSCs in a paracrine manner

It has previously been shown that activation of AMPK leads to inhibition of PDGF-BB-induced proliferation in HSCs [134, 178]. Moreover, cell proliferation in general is regulated by AMPK through several mechanisms. Thus, AMPK induces cell cycle control through activation of p53 [141]. Moreover, cell proliferation is regulated through inhibition of the mTOR pathway by AMPK [240].

To investigate whether the effect on HSC proliferation by treatment with HCC conditioned medium was associated with differential activation of the AMPK pathway, 3 different preparations of primary human HSCs were cultured in conditioned medium obtained from PLC/PFR/5 or HepG2 cells for 24 hours, and expression levels of AMPK and its upstream kinase LKB1 were analysed by western blot.

As depicted in figure 2.12, conditioned medium of PLC/PRF/5 cells induced a strong phosphorylation of AMPK at Thr^{172} and only mild phosphorylation at AMPK-$Ser^{485/491}$, compared to treatment with serum free medium, indicating overall activation of AMPK.

In contrast, the conditioned medium of HepG2 cells induced mild phosphorylation of AMPK at Thr^{172}, whereas a strong phosphorylation of AMPK at $Ser^{485/491}$ was observed, suggesting inhibition of AMPK activity (figure 2.12).

Surprisingly, phosphorylation of LKB1 was strongly reduced after treatment with the conditioned media of both cell lines, compared to serum free medium (figure 2.12).

Overall, these results show that HepG2 and PLC/PRF/5 cells differentially affect activation of the AMPK pathway, as well as cell proliferation and migration in HSCs. AMPK regulates both cell proliferation and migration [134, 223]. Therefore, the data presented in this chapter suggest that differential activation of AMPK by HepG2 and PLC/PRF/5 cells may cause the differences in HSC proliferation. To test this hypothesis, a genetic knockdown of AMPK in HSCs could be used in future experiments. This could be achieved by treatment of HSCs with small interfering RNA (siRNA) or small hairpin RNA (shRNA).

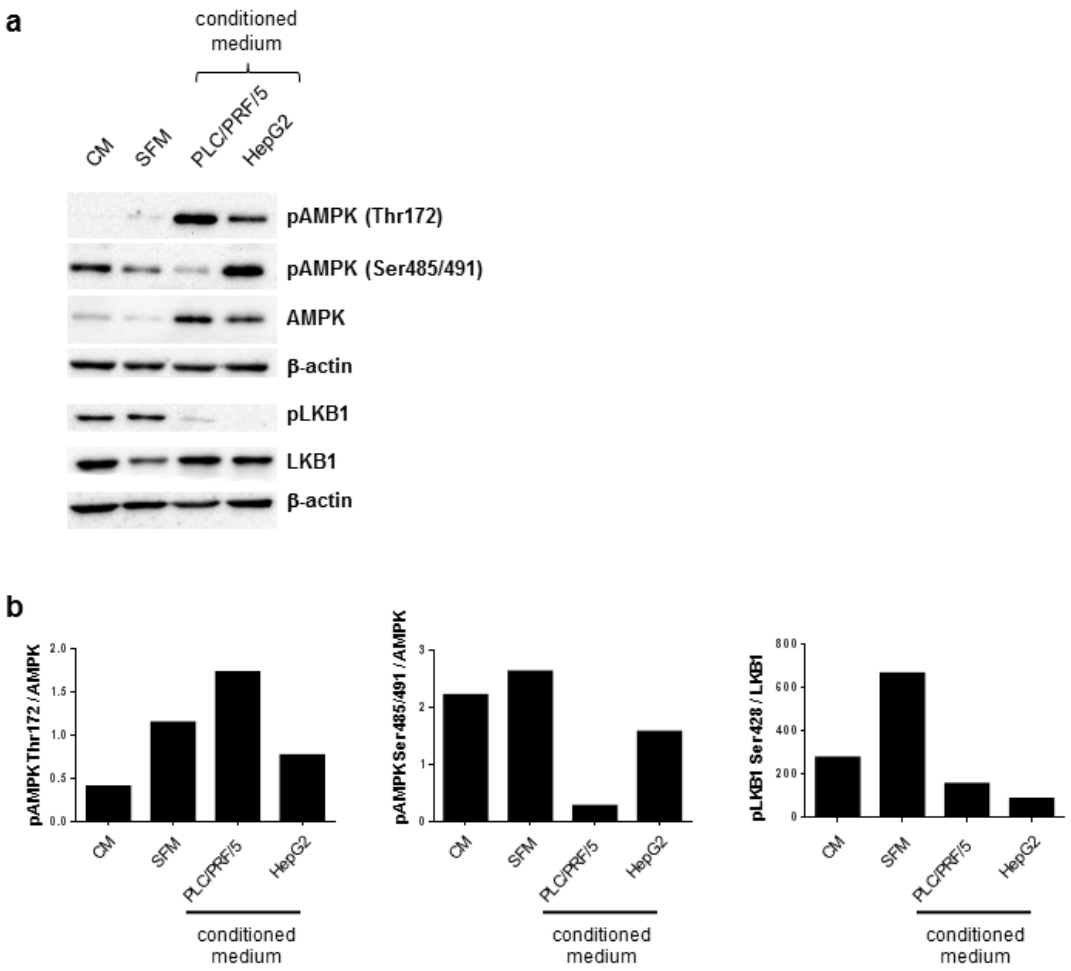


Figure 2.12: HCC conditioned medium affects the AMPK pathway in HSCs. Protein expression in HSCs after treatment with conditioned medium of PLC/PRF/5 or HepG2 cells for 24 hours (a) representative protein expression and (b) quantification. (a)- (b): Representative data of 4 independent experiments with 4 different HSC preparations. CM= complete HCC medium, SFM = serum free HSC medium

2.3.5 AICAR and CC equally inhibit HSC proliferation but exert differential effects on AMPK activation in HSCs

The pharmacological compound AICAR is a well-known activator of AMPK [199], and has been shown to inhibit PDGF-BB-induced proliferation and migration of human HSCs [178]. The only AMPK inhibitor that has been described to date is the

cell permeable agent CC, which can reverse the anti-proliferative effect of Metformin in human HSCs [178]. Here, the effect of AICAR and CC on HSC proliferation under basal conditions, as well as after treatment with HCC conditioned medium was investigated.

Firstly, to exclude a cytotoxic effect of AICAR and CC on HSCs, primary human HSCs were treated with different concentrations of AICAR (250µM – 4mM) or CC (2.5µM - 40µM) for 24 hours and cytotoxicity was measured by MTS assay. As shown in figure 2.13a, concentrations from 1 - 4mM AICAR induced a reduction of metabolic activity in HSC, which, up to 2mM, was not associated with morphological changes indicative of cytotoxicity or cell death (data not shown). Furthermore, 20-40µM CC resulted in a significant reduction of HSC metabolic activity (figure 2.13b), but no morphological signs of signs of cytotoxicity were observed for concentrations equal to or below 20µM CC (data not shown).

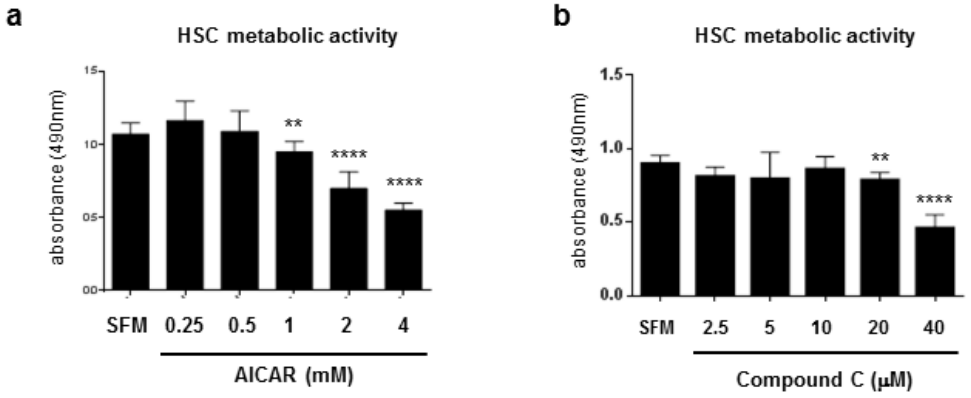


Figure 2.13: AICAR and CC inhibit HSC metabolic activity dose-dependently.
HSCs were treated with (a) 0.25-4mM AICAR or (b) 2.5-40µM Compound C for 24 hours. Metabolic activity was measured by MTS test. (a)- (b) Data represent mean +/- SD of biological triplicates, **p<0.01, ****p<0.0001, vs. serum free HSC medium. Representative data of 3 independent experiments with different HSC preparations. SFM = serum free HSC medium

To investigate whether AICAR and CC can influence HSC proliferation under basal conditions, three different preparations of primary human HSCs were treated with

different concentrations of AICAR or CC for 24 hours and proliferation of HSCs was measured by BrdU incorporation assay. As shown in figure 2.14, proliferation of HSCs was significantly inhibited in a dose-dependent manner after treatment with AICAR (figure 2.14a). Surprisingly, also CC inhibited HSC proliferation dose-dependently (figure 2.14b).

Because inhibition of cell proliferation was observed after treatment with 1mM AICAR or 10µM CC, and cytotoxicity was excluded for these concentrations, 1mM AICAR or 10µM CC were used in the following experiments.

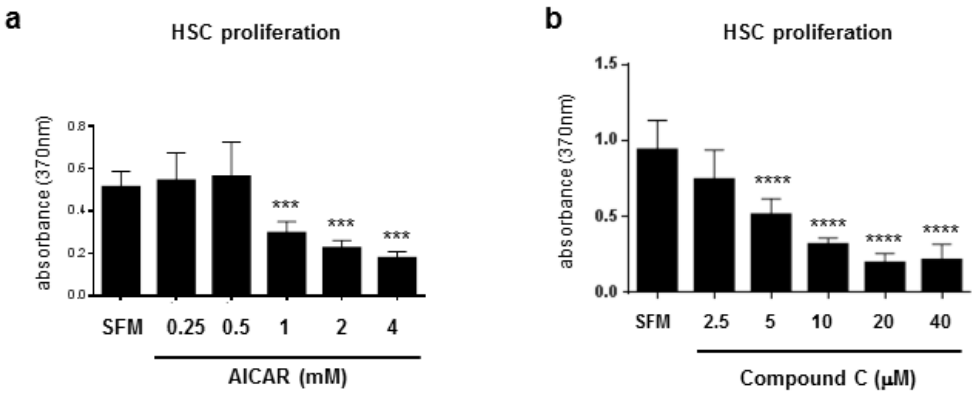


Figure 2.14: AICAR and CC inhibit HSC proliferation dose-dependently.
HSCs were treated with (a) 0.25-4mM AICAR or (b) 2.5-40µM Compound C for 24 hours. Proliferation was measured by BrdU incorporation assay. Data represent mean +/- SD of biological triplicates, ***p<0.001, ****p<0.0001 vs. serum free HSC medium. Representative data of 3 independent experiments with different HSC preparations. SFM = serum free HSC medium.

To investigate how AICAR and CC influence the activation status of AMPK in HSCs, cells were treated with 1mM AICAR or 10µM CC and phosphorylation of AMPK at Thr^{172} and $Ser^{485/491}$ was examined by western blot. As shown in figure 2.15, only treatment with 1mM AICAR, but not with 10µM CC, induced phosphorylation of AMPK-Thr^{172}, whereas both compounds induced phosphorylation of AMPK-$Ser^{485/491}$, indicating that AICAR leads to overall activation of AMPK. In contrast, CC inhibits AMPK in HSCs, suggesting that CC inhibits cell proliferation in HSCs in an AMPK-independent manner.

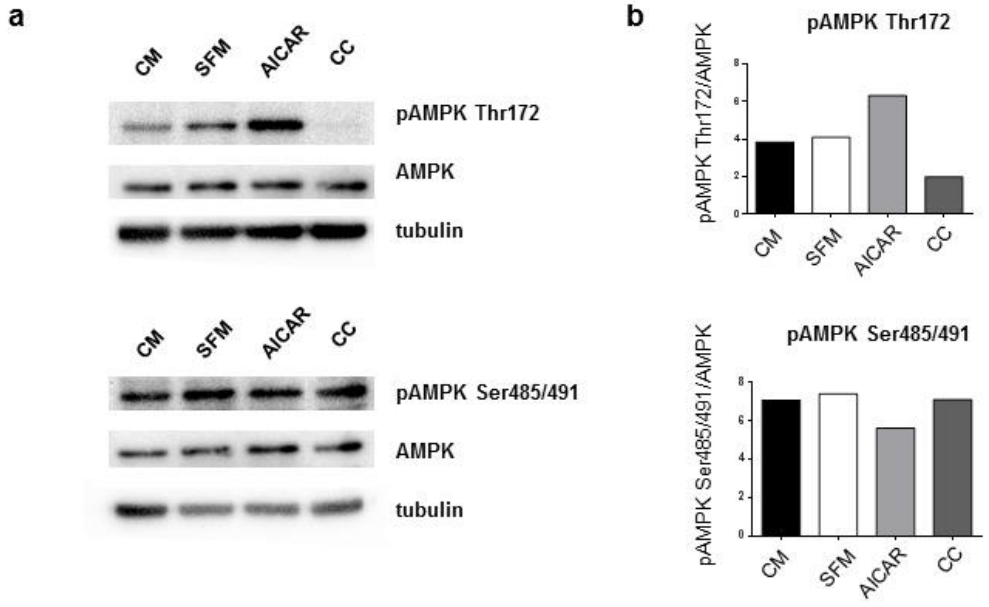


Figure 2.15: AMPK activation in HSCs by AICAR and CC.
Protein expression of HSCs treated with 1mM AICAR or 10µM CC for 24 hours.
(a) Representative western blots and (b) quantification. Representative data of 4 independent experiments with different HSC preparations.

Next, it was investigated whether the previously observed paracrine effects of HepG2 or PLC/PRF/5 cells on HSC proliferation (figure 2.10b) were influenced by AMPK activation through AICAR. Therefore, different preparations of primary human HSCs were simultaneously treated with 1mM AICAR and the conditioned medium of HepG2 or PLC/PRF/5 cells for 24 hours, and HSC proliferation was measured by BrdU incorporation assay. As shown in figure 2.16a, HSC proliferation was significantly inhibited when HSCs were treated simultaneously with HCC conditioned medium and AICAR.

Moreover, it was tested if CC affected HSC proliferation after treatment with conditioned medium of PLC/PRF/5 or HepG2 cells. Therefore, primary human HSCs were treated with 10µM CC and conditioned medium of HepG2 or PLC/PRF/5 cells simultaneously for 24 hours, and proliferation was assessed by BrdU incorporation assay. As shown in figure 2.16b, proliferation of HSCs was significantly inhibited after treatment with HCC conditioned medium in the presence of CC. Notably,

inhibition of HSC proliferation by CC was comparable to inhibition after treatment with the AMPK activator AICAR (figure 2.16c).

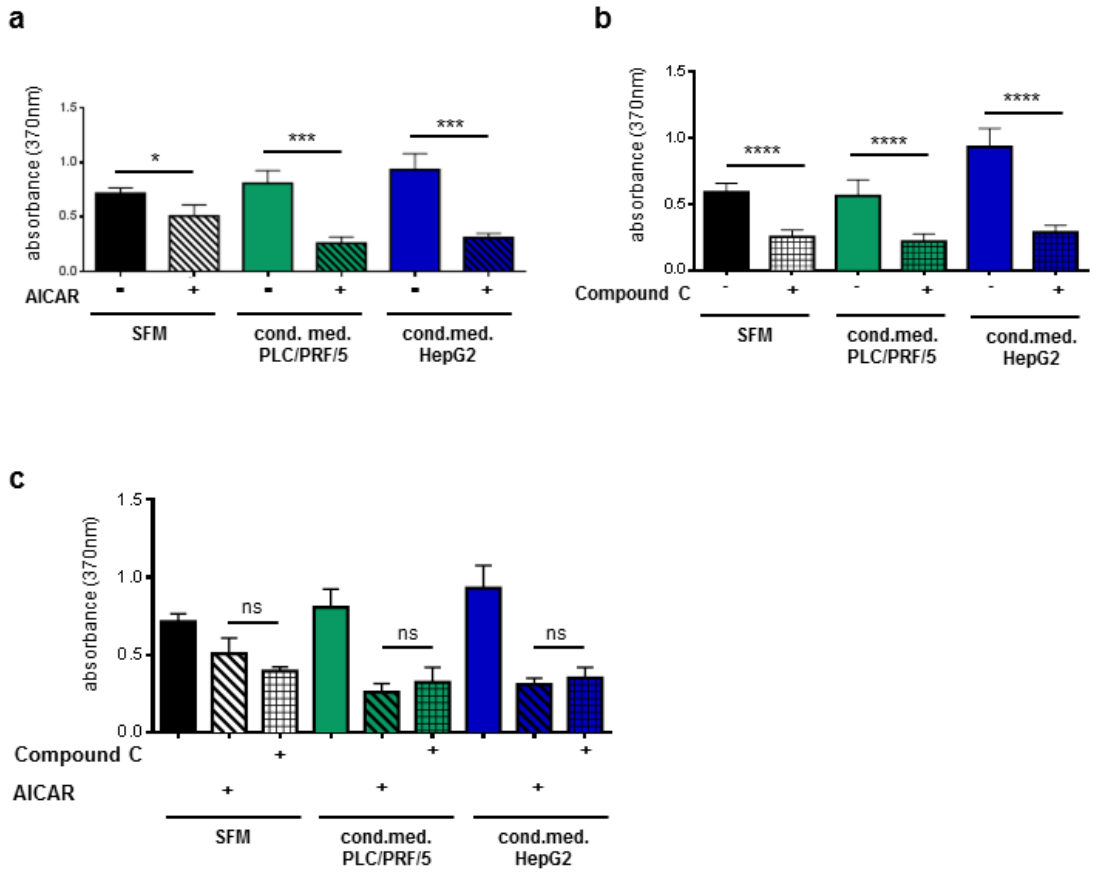


Figure 2.16: AICAR and CC are able to overcome the pro-proliferative effect of HepG2 conditioned medium on HSCs.
(a)-(c) Proliferation of HSCs after simultaneous treatment with (a), (c) 1mM AICAR or (b), (c) 10µM Compound C and conditioned medium of PLC/PRF/5 or HepG2 cells for 24 hours, measured by BrdU incorporation assay. (a)-(c): Data represent mean +/- SD of biological triplicates, **p<0.01, p<0.001, ****p<0.0001, ns = not significant, representative data of 3 independent experiments with different HSC preparations. SFM HSC= serum free HSC medium, cond.med. = conditioned medium

In order to test whether AICAR and CC also influenced HSC migration induced by HepG2 conditioned medium, three different HSC preparations were treated with HCC conditioned medium and 10mM AICAR or 10µM CC in parallel. HSC migration was assed employing a wound healing assay. Interestingly, CC but not AICAR inhibited

HepG2 conditioned medium induced HSC migration markedly (figure 2.17). Moreover, CC abrogated migration of HSCs that were treated with PLC/PRF/5 conditioned medium completely (figure 2.18).

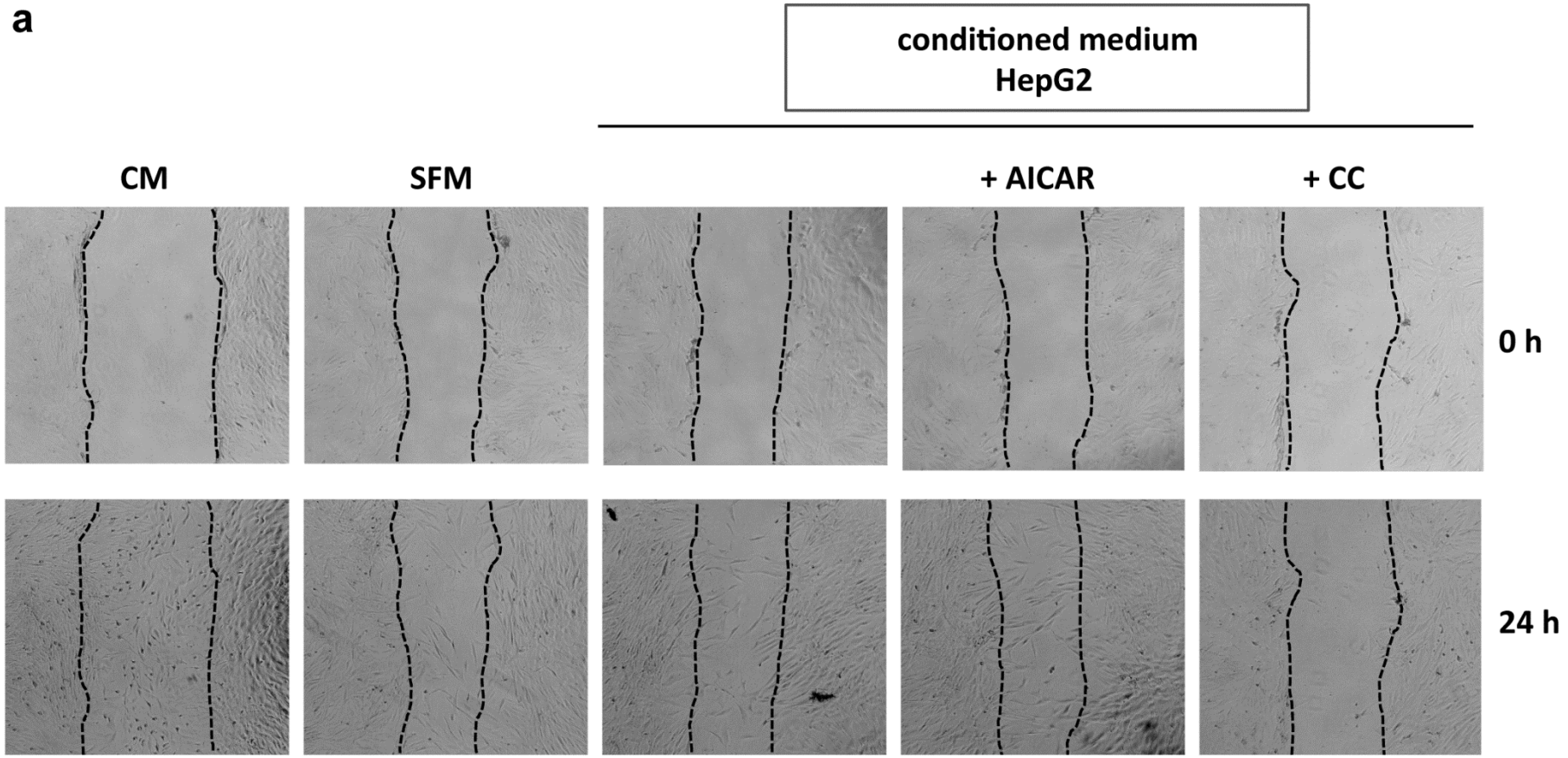


Figure 2.17: CC inhibits HepG2 induced migration in HSCs.
Wound healing assay, migration of HSCs on plastic following a scratch. HSCs were treated with (a) conditioned medium of HepG2 cells and 1mM AICAR or 10µM CC in parallel for 24 hours. 10x magnification. Representative data of 3 independent experiments with different HSC preparations. CM = complete HSC medium, SFM = SFM HCC.

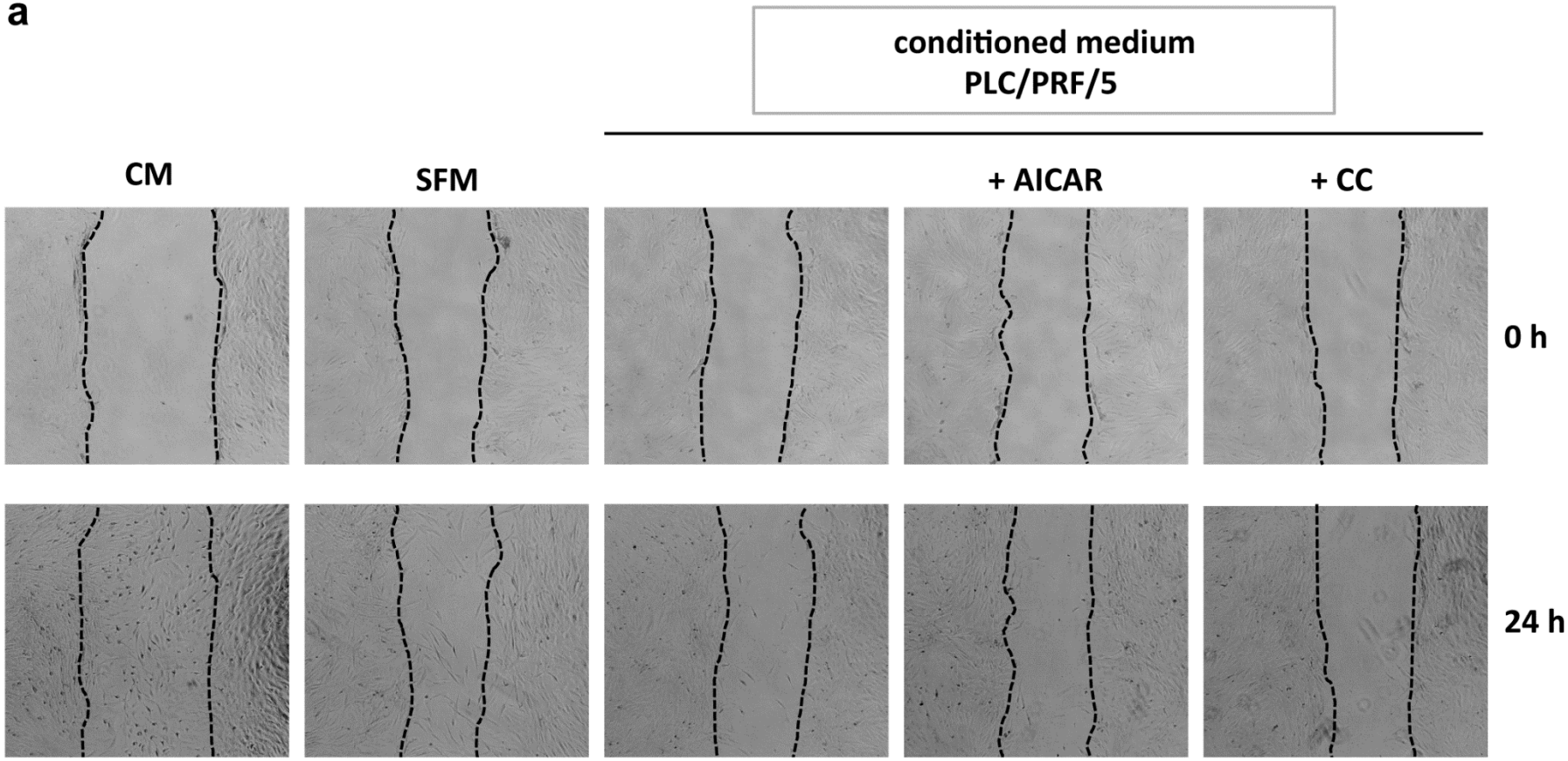


Figure 2.18: CC inhibits migration in HSCs.
Wound healing assay, migration of HSCs on plastic following a scratch. HSCs were treated with (a) conditioned medium of PLC/PRF/5 cells and 1mM AICAR or 10μM CC in parallel for 24 hours. 10x magnification. Representative data of 3 independent experiments with different HSC preparations. CM = complete HSC medium, SFM = SFM HCC.

To assess the activation status of AMPK after simultaneous treatment with HCC conditioned medium and AICAR or CC, different preparations of HSCs were treated with conditioned medium of HepG2 or PLC/PRF/5 cells and 1mM AICAR or 10μM CC for 24 hours, and AMPK activation was investigated by western blot. Interestingly, conditioned medium of PLC/PRF/5 cells induced phosphorylation of AMPK-Thr^{172} alone, or in combination with 1mM AICAR (figure 2.19), whereas phosphorylation of AMPK-Thr^{172} was considerably lower following treatment with PLC/PRF/5 conditioned medium and 10μM CC (figure 2.19). In contrast, phosphorylation of AMPK-$Ser^{485/491}$ was induced by PLC/PRF/5 conditioned medium and 10μM CC (figure 2.19).

Moreover, HepG2 conditioned medium inhibited phosphorylation of AMPK-Thr^{172} and induced phosphorylation of AMPK-$Ser^{485/491}$ compared to serum free medium, with and without AICAR and CC (figure 2.19).

Taken together, these data show that the AMPK activator AICAR and the AMPK inhibitor CC inhibit HSC proliferation. Moreover, both AICAR and CC are able to overcome the pro-proliferative effect of HepG2 conditioned medium on HSCs. Despite showing equal effects on HSC proliferation, AICAR and CC affect HSC migration and AMPK activation in HSC differently, suggesting AMPK-independent effects on HSC proliferation.

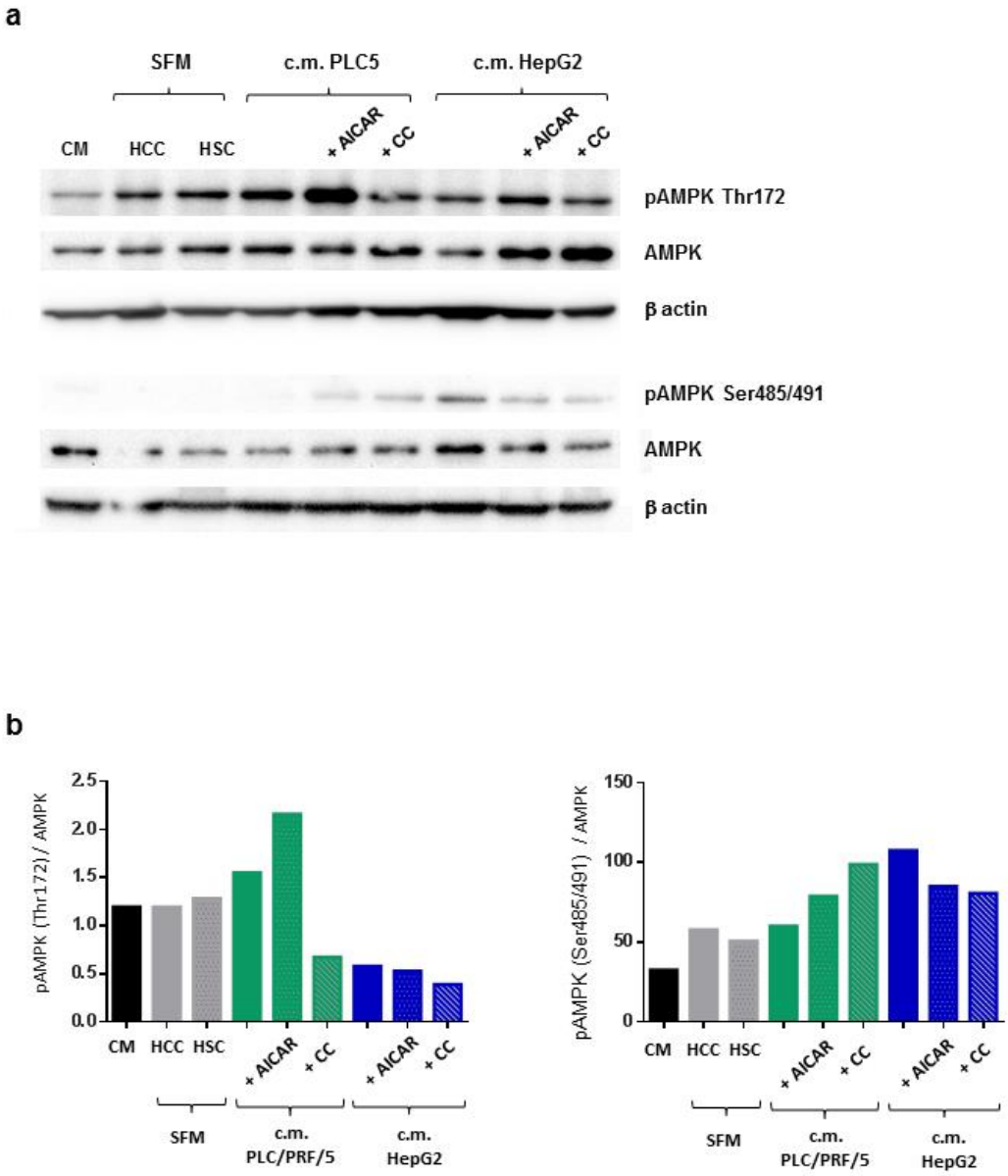


Figure 2.19: AMPK activation after treatment with AICAR or CC and HCC conditioned medium.

Protein expression in HSCs treated with conditioned medium of PLC/PRF/5 or HepG2 cells and 1mM AICAR or 10μM CC in parallel. (a) Representative western blot and (b) quantification. Representative data of 4 independent experiments with different HSC preparations.

2.3.6 Effect of AICAR and CC on pro-fibrogenic and pro-inflammatory gene expression in HSCs

Besides affecting proliferation (figure 2.10b) and migration in HSCs (figure 2.11), HCC conditioned medium exerted a paracrine effect on the expression of pro-fibrogenic and pro-inflammatory genes in HSCs (figures 2.8 and 2.9). Notably, the pro-proliferative effect of HepG2 conditioned medium was reversed by treatment with AICAR and CC (figure 2.16), and HepG2 induced migration was abolished by CC (figure 2.17). Therefore, the influence of AICAR and CC on the expression on pro-fibrogenic and pro-inflammatory genes was tested both under basal conditions and in combination with HCC conditioned medium.

To test the effect of AICAR and CC on HSCs under basal conditions, three different preparations of primary human HSCs were treated with 1mM AICAR or 10μM Compound C for 24 hours and gene expression was investigated by qPCR.

As shown in figure 2.20a, both AICAR and CC resulted in significantly lower expression of Col1a1 in HSC, compared to serum free medium. In contrast, expression of LOX was significantly increased after treatment with AICAR, but unchanged after treatment with CC (figure 2.20a). Moreover, treatment with AICAR resulted in significantly lower expression of the pro-inflammatory genes CCL2, IL-6 and IL-8, whereas expression of IL-1β was unchanged, compared to serum free medium (figure 2.20b). In contrast, treatment with CC induced the expression of pro-inflammatory genes in HSCs, resulting in significantly higher expression of CCL2, IL-1β, IL-6 and IL-8 in HSCs, compared to serum free medium (figure 2.20b).

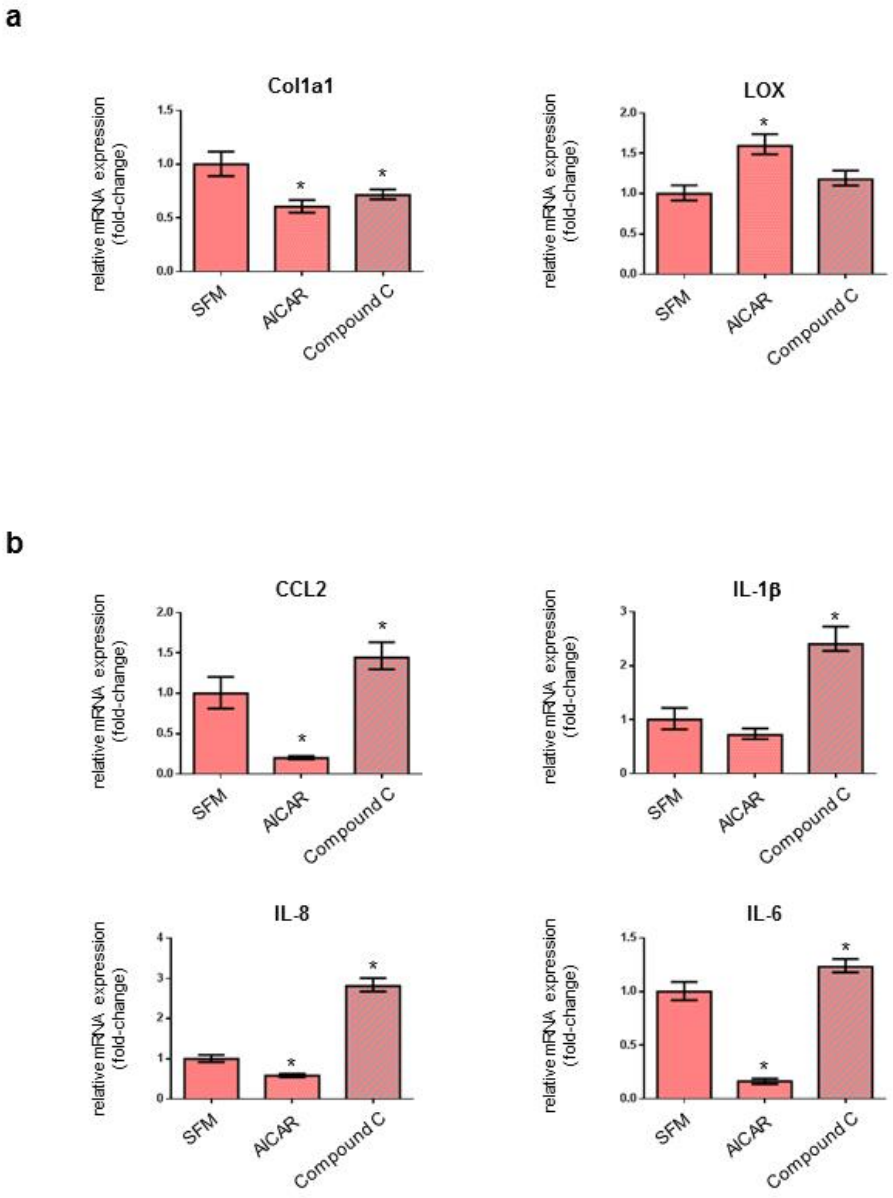


Figure 2.20: Expression of pro-fibrotic and pro-inflammatory genes in HSCs treated with AICAR or CC.

(a)-(b) gene expression in HSCs after treatment with 1mM AICAR or 10μM CC for 24 hours, gene expression is normalized to treatment with serum-free HSC medium. Data represent mean +/- 95% confidence interval, *p=0.05, pooled data from 3 independent experiments with 3 different HSC preparations, SFM = serum free HSC medium

Next, the effect of AICAR and CC on HSCs treated with HCC conditioned medium was investigated. Three different preparations of primary human HSCs were treated

with conditioned medium of PLC/PRF/5 or HepG2 cells and 1mM AICAR or 10μM CC for 24 hours. Gene expression in HSCs was analysed by qPCR.

As shown in figure 2.21a, AICAR enhanced the suppressive effect of PLC/PRF/5 conditioned medium on Col1A1 expression in HSCs. Moreover, AICAR reversed the induction of CCL2 and IL-1β expression by PLC/PRF/5 and HepG2 conditioned medium (figure 2.21b). In contrast, treatment with AICAR amplified the PLC/PRF/5 induced expression of IL-8 and IL-6 in HSCs (figure 2.21b).

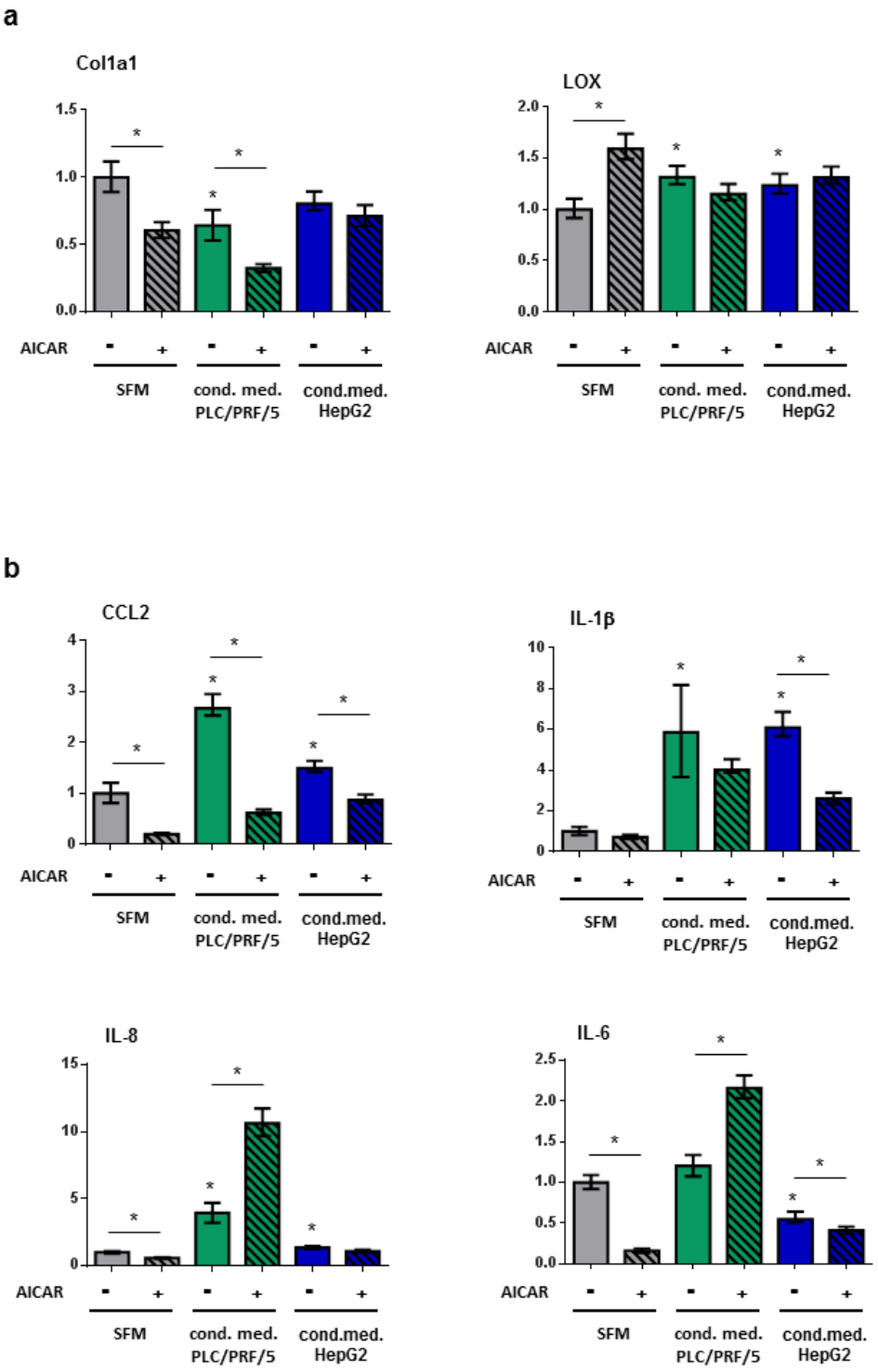


Figure 2.21: Expression of pro-fibrotic and pro-inflammatory genes in HSCs treated with HCC conditioned medium and AICAR.

(a)-(b) gene expression in HSCs after treatment with conditioned medium of PLC/PRF/5 or HepG2 cells and 1mM AICAR for 24 hours. Gene expression is normalized to treatment with serum-free HSC medium. Data represent mean +/- 95% confidence interval, *p=0.05, pooled data from 3 independent experiments with 3 different HSC preparations, SFM = serum free HSC medium.

CC abolished the induction of the collagen-crosslinking enzyme LOX induced by PLC/PRF/5 and HepG2 conditioned medium in HSCs (figure 2.22a). Moreover, CC exerted pro-inflammatory effects on HSCs in combination with HepG2 conditioned medium, as expression of CCL2, IL-1β and IL-8 was further up-regulated by CC (figure 2.22b). In contrast, the induction of IL-8 and IL-6 expression by PLC/PRF/5 conditioned medium was reversed by CC (figure 2.22b).

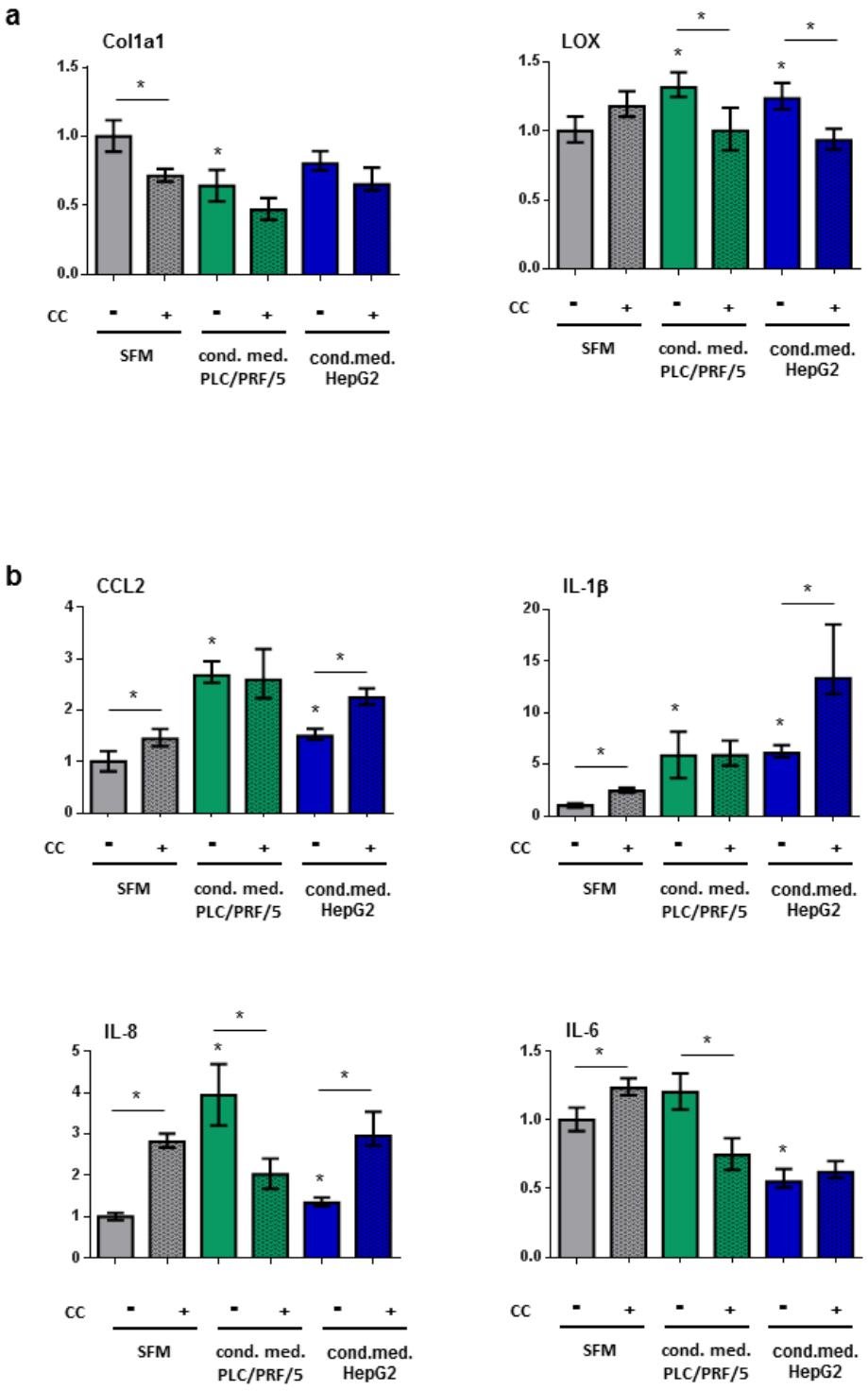


Figure 2.22: Expression of pro-fibrotic and pro-inflammatory genes in HSCs treated with HCC conditioned medium and CC.

(a)- (b) Gene expression in HSCs after treatment with conditioned medium of PLC/PRF/5 or HepG2 cells and 10μM CC for 24 hours. Gene expression is normalized to treatment with serum-free HSC medium. Data represent mean +/- 95% confidence interval, *p=0.05, pooled data from 3 independent experiments with 3 different HSC preparations, SFM = serum free HSC medium.

Neither AICAR nor CC influenced the expression of TIMP1 and MMP2 (supplementary figure 2.30, page 98).

These data show that AICAR and CC exert differential effects on gene expression in HSCs. The effects of AICAR and CC on pro-fibrogenic gene expression are mild, whereas their effects on pro-inflammatory gene expression are complex and more pronounced. While AICAR shows a rather anti-inflammatory profile, both under basal conditions and in combination with HCC conditioned medium, CC, especially in combination with HepG2 conditioned medium, simulated expression of pro-inflammatory genes in HSCs.

2.3.7 AICAR and CC inhibit cell proliferation in an AMPK-independent manner

As shown in figure 2.15, CC inhibited activation of AMPK, as shown by lacking phosphorylation of AMPK-Thr^{172} and phosphorylation of AMPK-Ser$^{485/491}$. Nevertheless, CC had the same effect on HSC proliferation as the AMPK activator AICAR (figure 2.16), suggesting AMPK independent effects of CC on HSC proliferation. Indeed, CC inhibits various kinases besides AMPK and it has been demonstrated that CC inhibits proliferation of glioma cells through various AMPK independent mechanisms [206]. Similarly, the anti-proliferative effect of AICAR has been shown to be AMPK independent, despite potent AMPK activation potential [241].

In order to confirm that AICAR and CC exert AMPK independent effects on cell proliferation, wt and AMPKα1/α2 double knockout (α1/α2 $^{-/-}$) MEFs were employed as a tool to study the effect of AICAR and CC in an AMPK knock out cell line. Such AMPKα1/α2$^{-/-}$ MEFs are lacking both isoforms of the catalytic α subunit of AMPK, resulting in a functional AMPK knockout [231]. Although differences apply between human HSCs and murine embryonic fibroblasts, MEFs are widely used to study the behaviour of activated human and murine HSCs [242-244], which are known as a "myofibroblast-like" cell [24].

First, in order to exclude a cytotoxic effect of AICAR and CC on MEFs, MEFs were treated with different concentrations of AICAR (250-1000μM) or CC (2.5-40μM) for 24 hours, and metabolic activity was assessed by MTS test. As shown in figure 2.23a, concentrations from 500μM AICAR significantly reduced metabolic activity in wt MEFs and concentrations from 250μM AICAR in AMPKα1/α2 $^{-/-}$ MEFs. Concentrations of >1000mM AICAR were cytotoxic for both wt and AMPKα1/α2 $^{-/-}$ MEFs, evident by cell detachment (data not shown). CC induced a significant reduction in metabolic activity at concentrations from 20μM (wt MEFs) and 10μM (AMPKα1/α2 $^{-/-}$ MEFs) respectively (figure 2.23b), without inducing morphological signs of cell death (data not shown).

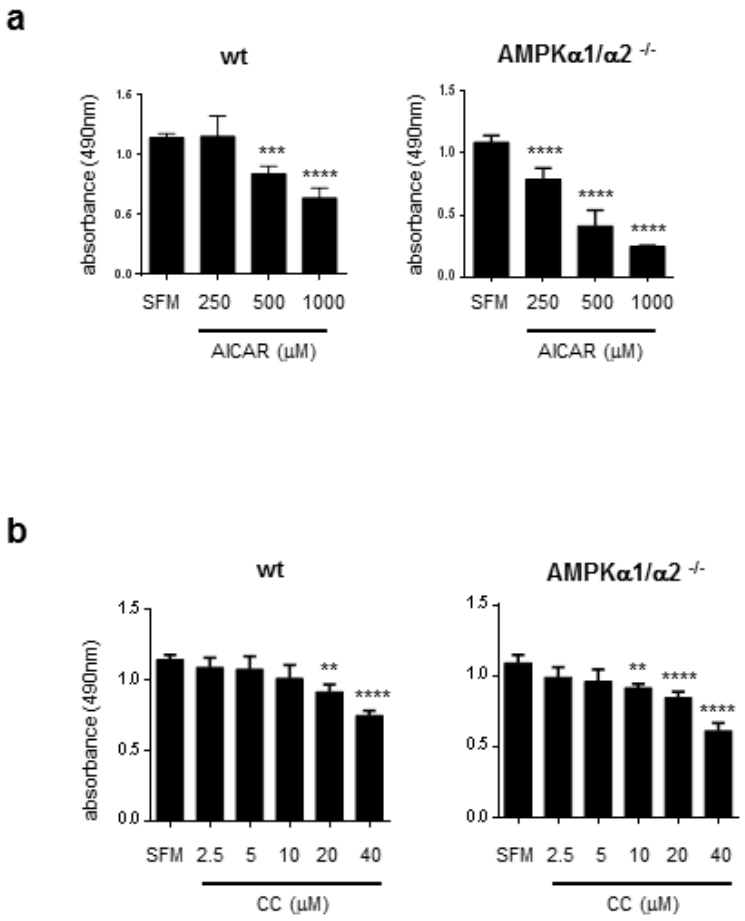


Fig 2.23: AICAR and CC inhibit metabolic activity of MEFs independently of AMPK.

Metabolic activity of wt and AMPKα1/α2 $^{-/-}$ MEFs measured by MTS assay after treatment with (a) 250-1000μM AICAR or (b) 2.4-40μM CC for 24 hours. Data represent mean +/- SD of biological triplicates. **p<0.01, ***p<0.001, ****p<0.0001 vs. serum free HSC medium. Representative data of 2 independent experiments. SFM = serum free HSC medium.

Next, the effect on cell proliferation of AICAR and CC on wt and AMPKα1/α2$^{-/-}$ MEFs was tested. MEFs were treated with different concentrations of AICAR (62.5μM – 500μM) and CC (2.5μM – 40μM) for 24 hours and cell proliferation was measured by BrdU incorporation assay. As shown in figure 2.24, both AICAR and CC inhibited cell proliferation in a dose-dependent manner in wt and AMPKα1/α2 $^{-/-}$ MEFs, suggesting that AICAR and CC inhibit cell proliferation in MEFs in an AMPK independent manner.

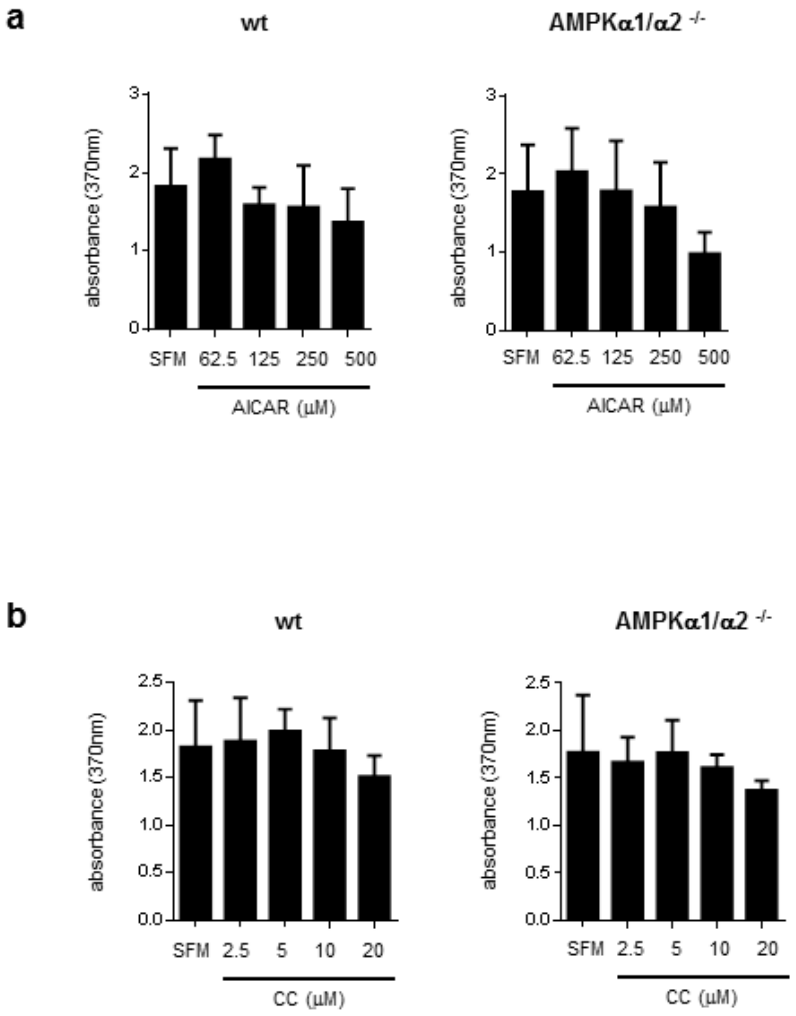


Fig 2.24: AICAR and CC inhibit proliferation of MEFs independently of AMPK. Proliferation of wt and AMPKα1/α2 $^{-/-}$ MEFs measured by BrdU incorporation assay after treatment with (a) 250-1000μM AICAR or (b) 2.4-40μM CC for 24 hours. Data represent mean +/- SD of biological triplicates. Representative data of 2 independent experiments. SFM = serum free HSC medium.

In order to elucidate the mechanism through which AICAR and CC inhibited HSC cell proliferation, several pathways were investigated.

AICAR has been shown to inhibit cell proliferation in MEFs and cancer cells by inducing cell cycle arrest independently of AMPK [241]. Similarly, CC, independently of AMPK, exerts cell cycle arrest in glioma cells [206]. Therefore, cell cycle analysis was carried out in HSCs treated with AICAR or CC. Two different preparations of primary human HSCs were treated with 1mM AICAR or 10μM CC for 24 hours, and cell cycle analysis was carried out employing propidium iodide

staining and flow cytometric analysis. Indeed, compared to serum free medium, both AICAR and CC induced cell cycle arrest in the S phase in HSCs (figure 2.25).

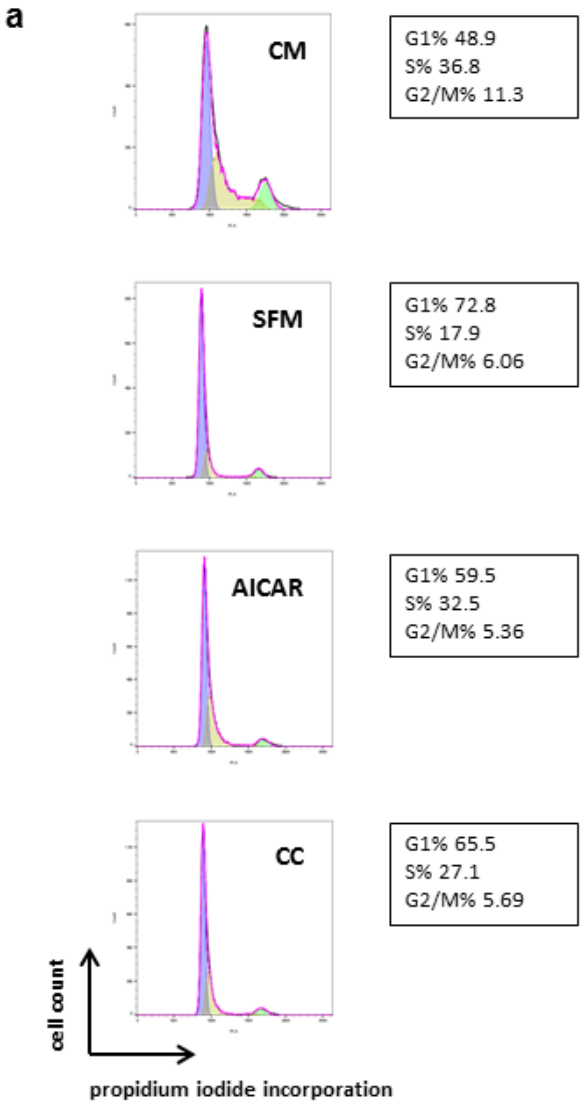


Figure 2.25: AICAR and CC induce S-phase cell cycle arrest in HSCs.
(a) Cell cycle analysis of HSCs treated with 1mM AICAR or 10µM CC for 24 hours, representative flow cytometry plots. Representative data of 2 independent experiments with different HSC preparations.

Moreover, AICAR has been shown to induce apoptosis and cell death in MEFs [241], and CC induced apoptosis and necrosis in glioma cells [206]. In order to test whether

AICAR and CC induced apoptosis or necrosis in HSCs, two different preparations of primary human HSCs were treated with different concentrations of AICAR (0.5–2mM) or CC (5-20µM) for 24 hours, and cell death was assessed by quantification of intracellular accumulation of nucleosomes (apoptosis) or in the culture supernatant (necrosis) by ELISA. Interestingly, none of the employed concentrations of neither AICAR nor CC induced apoptosis or necrosis in HSCs (figure 2.26).

Nevertheless, here, it has to be considered that analysing the release of nucleosomes represents a measure of the late stages of apoptosis. In future experiments, it would be interesting to test whether AICAR and CC affect early apoptosis by analysing cleavage of the effector caspase caspase-3, for example.

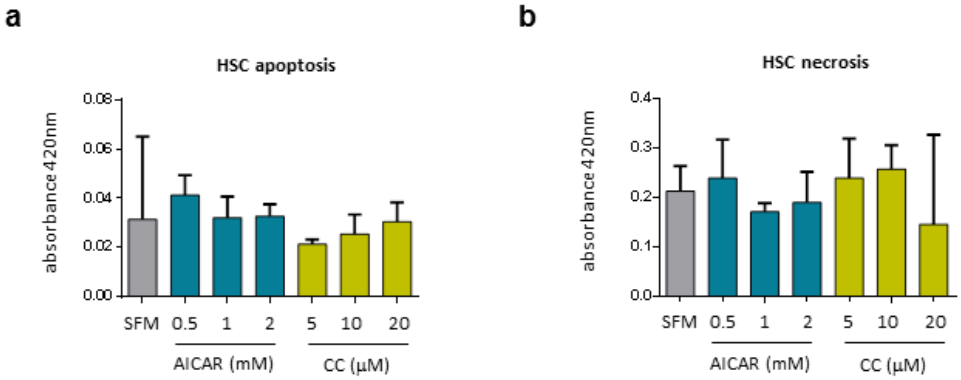


Figure 2.26: AICAR and CC do not induce apoptosis in HSCs.
Detection of nucleosomes by ELISA in HSCs treated with 0.5-2mM AICAR or 5-20µM CC for 24 hours. (a)- (b) Data represent mean +/- SD of technical triplicates. Representative data of 2 independent experiments with different HSC preparations.

The mTOR pathway is a critical regulator of cell proliferation and is regulated by AMPK. More specifically, mTOR is inhibited via phosphorylation of the tumour suppressor TSC1, which acts upstream of mTOR, or through direct phosphorylation of the mTORC1 subunit Raptor by AMPK [134]. The mTOR complex meditates its downstream effects on cell proliferation and protein translation through phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) at $Thr^{37/46}$ and the phosphorylation of the ribosomal protein S6 at $Ser^{235/236}$ by S6

kinase 1 [209]. In order to investigate the effect of AICAR and CC on mTOR signalling, 3 different preparations of primary human HSCs were treated with 1mM AICAR or 10μM CC for 24 hours, and phosphorylation of 4EBP1-Thr$^{37/46}$ and S6-Ser$^{235/236}$ were analysed by western blot. In HSCs, AICAR, but not CC inhibited the mTOR pathway as shown by lacking phosphorylation of 4EBP1-Thr$^{37/46}$ and S6-Ser$^{235/236}$, compared to serum free medium (figure 2.27).

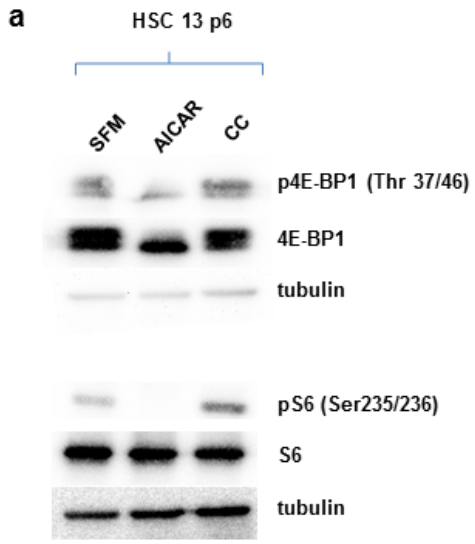


Figure 2.27: AICAR but not CC inhibits the mTORC1 pathway in HSCs.
(a) Protein expression of HSCs after 24 hours treatment with 1mM AICAR or 10μM CC. Representative western blots. Representative data of 3 independent experiments. SFM = serum free HSC medium.

Next, to test whether the inhibiting effect on mTOR by AICAR was AMPK dependent, wt and AMPKα1/α2 $^{-/-}$ MEFs were treated with 1mM AICAR and 10μM CC for 24 hours and phosphorylation of 4EBP1-Thr$^{37/46}$ and S6-Ser$^{235/236}$ was assessed by western blot. Indeed, mTOR inhibition by AICAR was AMPK-dependent in MEFs, as the lack of phosphorylation of 4EBP1 and S6 was abrogated in AMPKα1/α2 $^{-/-}$ MEFs (figure 2.28). Moreover, like in HSCs, no inhibition of mTOR by CC was evident in wt or AMPKα1/α2 $^{-/-}$ MEFs (figure 2.28).

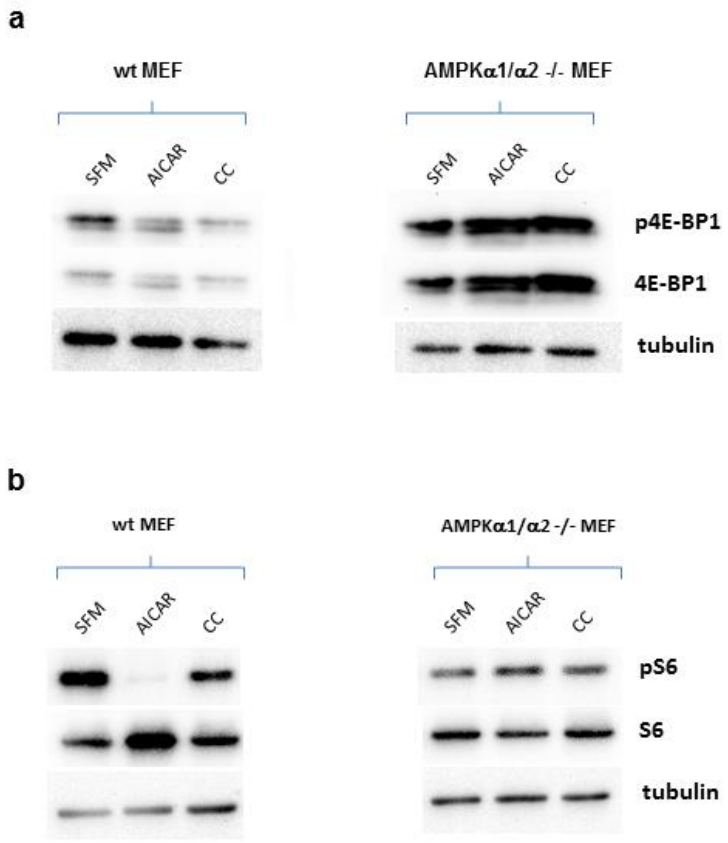


Figure 2.28: AICAR inhibits the mTORC1 pathway in MEFs in an AMPK independent manner.
(a)-(b) Protein expression of wt and AMPKα1/α2 $^{-/-}$ MEFs after 24 hours treatment with 1mM AICAR or 10μM CC. Representative western blots. Representative data of 3 independent experiments. SFM = serum free MEF medium.

Under nutrient depletion, cells can generate energy through induction of autophagy. Thus, when extracellular nutrient supply is low, cells can metabolize organelles, protein aggregates and ribosomes in order to survive [221]. During autophagy, intracellular structures are engulfed by a double-membraned autophagosome, which is then subjected to lysosomal degradation. The emerging degradation products can then be metabolized, and ATP can be generated [245]. Autophagy is regulated by AMPK through phosphorylation of ULK-1 and inhibition of mTOR, resulting in activation of autophagy [222]. Moreover, autophagy has recently been linked to cell proliferation, as both pathways are downstream of mTOR and linked by the scaffold protein AMBRA1 [246]. As mTOR inhibits autophagy and promotes cell proliferation, inhibition of mTOR results in the induction of autophagy and inhibition of cell

proliferation [209]. Therefore the effect of AICAR and CC on autophagy in HSCs was investigated.

Primary human HSCs were treated with 1mM AICAR, 10µM CC or 15µM Chloroquine as a positive control for 24 hours. Conversion of the microtubule-associated protein light chain 3B (LC3B)-I to LC3B-II, which is associated with the number of autophagosomes and widely used as a surrogate marker for autophagy [245], was analysed by western blot and flow cytometric analysis. Surprisingly, CC, but not AICAR mildly induced autophagy in HSCs as shown by increased LC3B-II accumulation (figure 2.29).

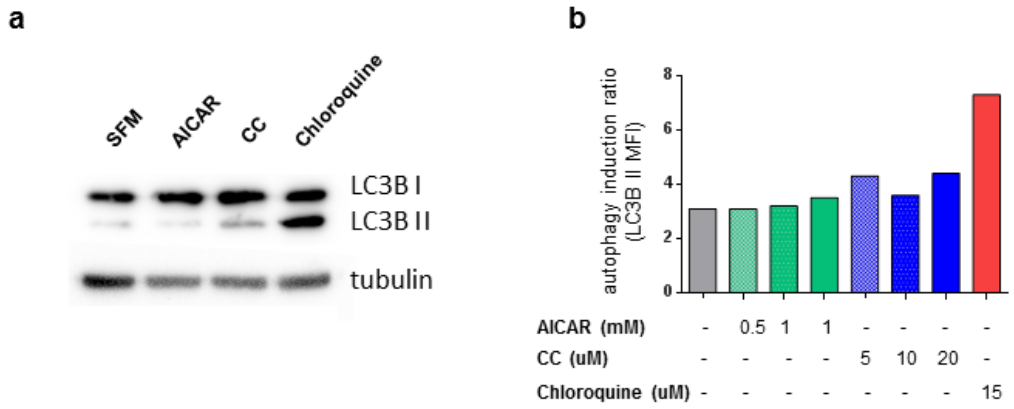
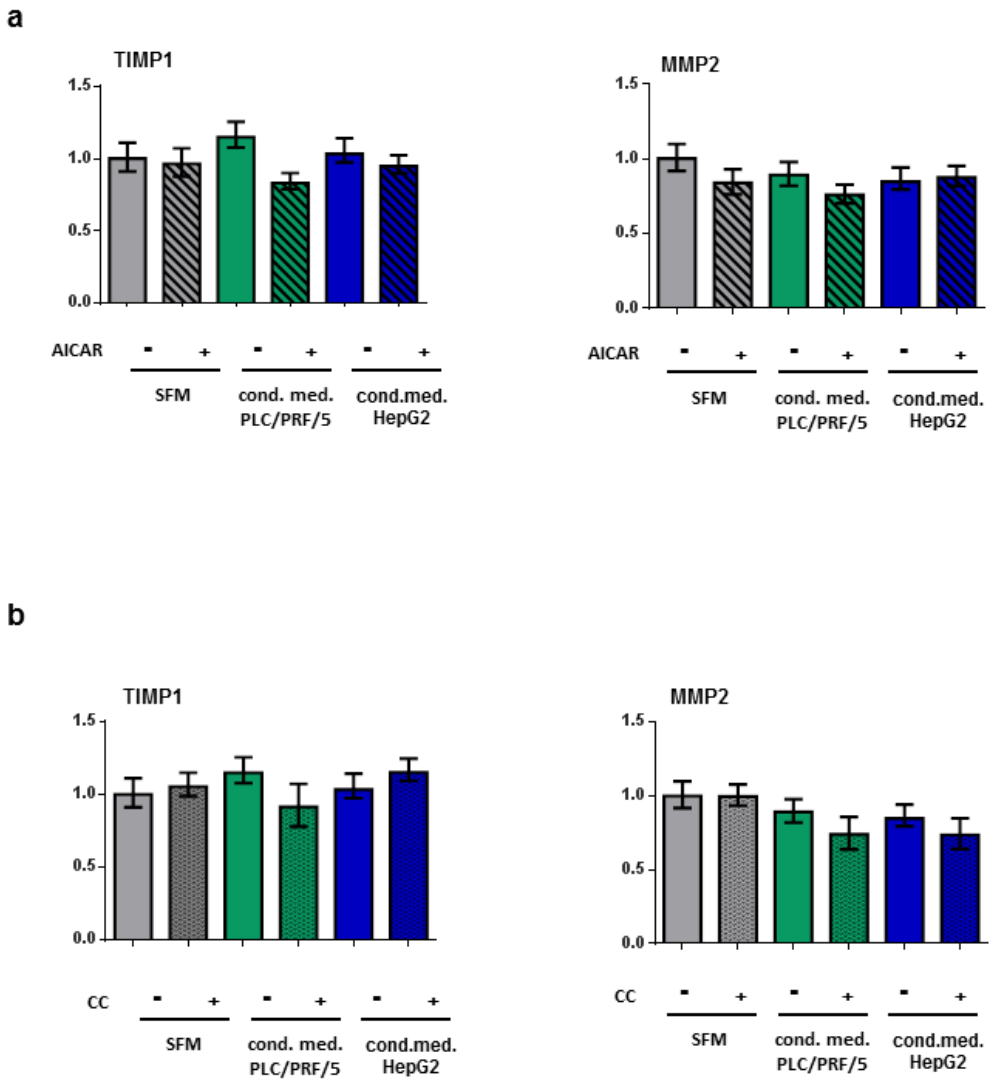


Fig 2.29: CC mildly induces autophagy in HSCs.
(a) Representative western blot of protein expression in HSCs treated with 1mM AICAR, 10µM CC or 15µM chloroquine for 24 hours. (b) Autophagy induction ratio, normalized to complete medium, in HSC treated with 0.5-2mM AICAR, 5-10µM CC, or 15µM chloroquine for 24hours measured by flow cytometry. Representative data from (a) 2 and (b) 1 experiments.

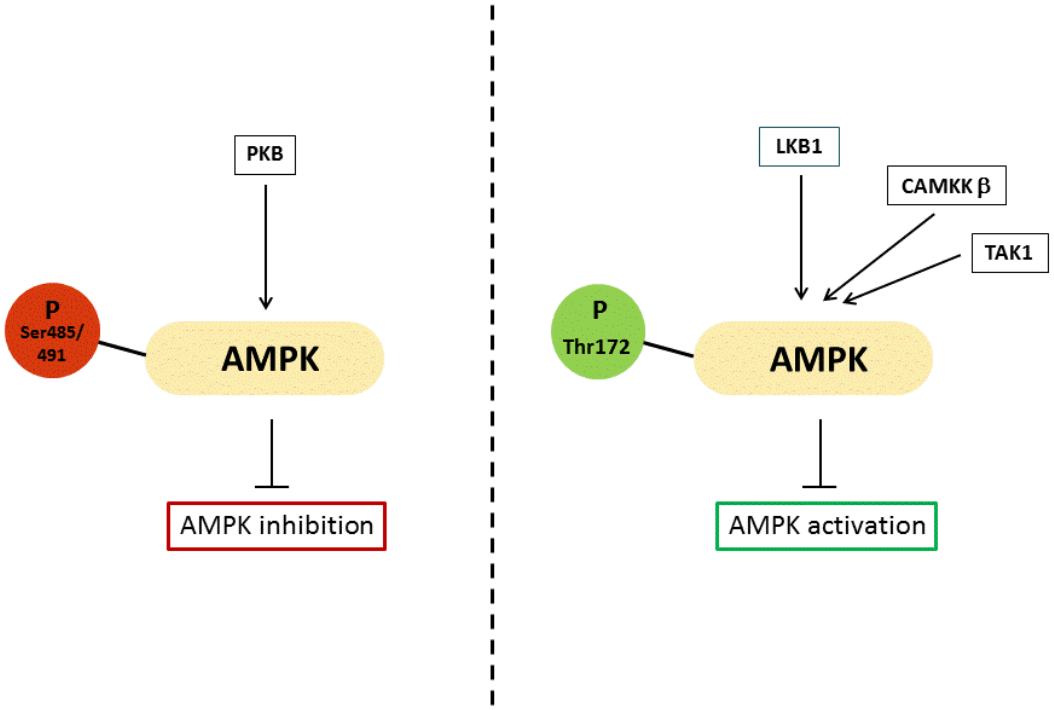
Taken together, these results show that AMPK and CC exert their anti-proliferative effects on HSCs through various, partly AMPK independent mechanisms. Whereas AICAR inhibited the cell cycle and the mTOR pathway, CC induced cell cycle arrest and autophagy in HSCs. Of note, inhibition of mTOR by AICAR was dependent on AMPK. Neither AICAR nor CC induced apoptosis or necrosis in the applied doses in HSCs.

In order to confirm the anti-proliferative effect of AMPK activation on HSCs, other AMPK activators could be tested. Expanding the analysis to A769662, which has been shown to directly bind and activate AMPK, would be particularly interesting in this context. As mentioned in previous chapters, CC is the only pharmacological AMPK activator that is commercially available at present. Taking its many off target effects into account, genetically knocking down AMPK in HSCs would represent a batter way of analysing the effects of AMPK inhibition on HSC activation.



Supplementary figure 2.30: Expression of TIMP1 and MMP2 in HSCs treated with HCC conditioned medium and AICAR or CC.

(a)-(b) Gene expression in HSCs after treatment with conditioned medium of PLC/PRF/5 or HepG2 cells and 10mM AICAR or 10μM CC for 24 hours. Gene expression is normalized to treatment with serum-free HSC medium. Data represent mean +/- 95% confidence interval, *p=0.05, pooled data from 3 independent experiments with 3 different HSC preparations, SFM = serum free HSC medium.



Supplementary figure 2.31: Regulation of AMPK through differential phosphorylation of its catalytic domain

Phosphorylation of AMPKα on $Ser^{485/491}$ mediated by Akt/PKB leads to overall inhibition of AMPK activity, whereas phosphorylation of AMPKα on Thr^{172} results in AMPK activation. The phosphorylation on Thr^{172} is mediated by its upstream kinases LKB1, CAMKKβ and TAK1. The structure and regulation of AMPK are described in detail in chapter 2.1.3.1.

2.4 Discussion

HCC is the most common primary liver cancer and one of the leading causes for cancer death worldwide [82, 83]. Treatment options are limited, as many patients are ineligible for surgical treatment due to advanced cancer stages and poor liver function [84, 91]. Besides surgical and locoregional therapies, the only approved pharmacological treatment is the multi-kinase inhibitor Sorafenib, achieving an overall survival benefit of only 3 months [84, 91, 131], emphasizing the urgent need for novel approaches for anti-cancer therapy.

In HCC development and progression, the tumour microenvironment plays a crucial role [84, 112, 233], as over 80% of HCCs have been shown to develop on the background of liver fibrosis [36].

A hallmark of the development of liver fibrosis, characterized by excessive ECM deposition, scar formation and inflammation, is the activation of HSCs [6, 51]. Moreover, there is evidence that the bi-directional cross-talk between activated HSCs and HCC cells promotes HCC cell proliferation and tumour growth [116, 118], as well as a pro-inflammatory and pro-fibrogenic microenvironment [118]. This suggests that the interaction between HSCs and HCC cells plays a crucial role in tumour development and provides a novel target for anti-cancer therapy.

The energy-sensing enzyme AMPK is a key regulator of cell metabolism and proliferation [134, 147] and has not only been shown to be implicated in cancer development and progression, but is also considered as a potential target for anti-cancer therapy [247].

In this study, an *in vitro* co-culture model employing the HCC cell lines HepG2 and PLC/PRF/5 and primary human HSCs was used to study bi-directional interactions between HSCs and HCC and the involvement of the AMPK pathway in such interaction. Furthermore, novel concepts of pharmacological HCC therapy targeting AMPK have been explored.

The findings in this study provide evidence for the involvement of the AMPK pathway in tumour-stromal interactions in HCC. Moreover, the data show that treatment with different pharmacological AMPK activators negatively regulate HSC and HCC cell proliferation, as well as the expression of pro-fibrogenic and pro-inflammatory genes in HSCs. These effects are mediated through different mechanisms and are partly dependent on AMPK.

Collectively, the data suggest that pharmacological AMPK activators could provide a novel approach for anti-fibrotic and anti-cancer therapy.

2.4.1 Validation of the experimental *in vitro* model for tumour stromal interactions between HSCs and HCC

In this study, the paracrine cross-talk between HSCs and HCC cells was investigated with an *in vitro* model employing primary human HSCs and the human HCC cell lines PLC/PRF/5 and HepG2. More specifically, to investigate possible paracrine effects between HSCs and HCC cells, conditioned medium of HSC cells was obtained for treatment of HCC cells, and vice versa.

HCC is considered to originate from a series of genetic alterations, especially in genes related to the cell cycle and cell proliferation (see chapter 2.1.2.1 and [84, 98]), resulting in uncontrolled proliferation and, eventually, malignant transformation of hepatocytes. Hepatocytes with a high capacity for proliferation and survival have a selective benefit during tumour development, resulting in characteristic alterations of oncogenic genes and pathways in each HCC tumour [98, 248]. However, some genetic alterations are shared between many tumours, amongst them p53- (12-48%), β-catenin- (5-50%) and cyclin-dependent kinase inhibitor 2A- mutations (10-60%) [84, 229, 248].

The tumour cell lines employed in this study show mutations in the genes for cell-cycle regulating genes, such as p53 and cyclin-dependent kinase inhibitor 2A (PLC/PRF/5) and β-catenin (HepG2), amongst others [229, 230]. Moreover, STK11, the gene encoding for the AMPK upstream kinase and tumour suppressor LKB1, is mutated in PLC/PRF/5 cells [229], resulting in lower expression of LKB1 on protein level (data not shown). Therefore, PLC/PRF/5 and HepG2 cells provide a valid *in vitro* tool to investigate human HCC cell behaviour in this thesis.

In the future, the analysis will be extended to a greater number of HCC cells. To test the influence of different mutations on the paracrine effect of HCC cells on HSCs, HCC cells that either show b-catenin mutations, i.e. HepG2-“like” cells, or p53 mutations (PLC/PRF/5-“like”) will be used to obtain conditioned medium and

proliferation, as well as AMPK activation, will be tested in HSCs treated with such conditioned media.

When using conditioned medium of cells to study the effect of secreted factors, it has to be taken into account that conditioned medium of both HCC cells and primary HSCs is obtained by culturing the cells in serum free medium for 48 hours. Thus, nutrients such as glutamine are likely to be, at least partly, consumed by the cells. In this study, it is difficult to exclude effects of such nutrient depletion, as cells were cultured in 100% conditioned medium following serum starvation for 24 hours. In hindsight, it would have been useful to test whether dilution of conditioned medium in fresh serum free medium would affect cell proliferation and the activation state of AMPK in order to exclude effects of nutrient depletion. Moreover, diluting conditioned medium would provide the opportunity to "titrate" the conditioned medium and therefore test the robustness of secreted factors in the employed conditioned media.

To validate the experimental system used in this study, the influence of different glucose concentrations in the employed cell culture media on AMPK activation and cell proliferation was tested. In the liver, AMPK activation is reduced by high glucose concentrations evident by reduction of phosphorylation at Thr^{172} [249]. In this study, phosphorylation of AMPK at Thr^{172} was similarly reduced in HepG2 and PLC/PRF/5 cells after treatment with serum free HSC medium (glucose 4500mg/l) compared to serum free HCC medium (glucose 1000mg/l). However, incubation with these different cell culture media did not change cell proliferation of PLC/PRF/5 and HepG2 cells. These data suggest that the observed reduction of AMPK activity in HCC cells due to high glucose in cell culture media is not sufficient to cause changes in HCC cell proliferation. Moreover, the observed effect might be due to dysregulation of HCC cell proliferation caused by mutations in cell-cycle related genes in both cell lines, as mentioned above.

Along these lines, different glucose concentrations in cell culture media had no effect on HSC proliferation, suggesting that cell proliferation of PLC/PRF/5 cells, HepG2 cells and HSCs is not influenced by differences in glucose concentrations of the cell culture media used in this study. Therefore, the effects on cell proliferation caused by conditioned media are a result of mediators secreted by the cell type employed to obtain the conditioned medium.

In conclusion, using conditioned media to study the paracrine effects between cell types provides a valid model system.

2.4.2 Interactions between HSCs and HCC cells

2.4.2.1 Activated HSCs inhibit HCC cell proliferation and affect activation of the AMPK pathway in HCC cells.

It has previously been shown that activated HSCs promote HCC cell proliferation and tumour growth [116, 118]. Amman et al. showed that HSCs support HCC tumourigenicity, both *in vitro* and in a murine *in vivo* HCC xenograft model through secretion of HGF [116]. Furthermore, it was demonstrated that the bi-directional cross-talk between HSCs and HCC cells in a co-culture model supports the establishment of a pro-inflammatory and pro-fibrogenic microenvironment [118].

In this study, the paracrine effect of human HSCs on HCC proliferation and activation of the AMPK pathway in HCC cells was investigated. Surprisingly, conditioned medium of different HSC preparations exerted an inhibitory effect on HCC cell proliferation, contradicting the previously published results by Amann et al. [116]. Of note, it was excluded that differences in protocols, with regards to HSC numbers to obtain conditioned medium, or glucose concentrations of the media were the reason for the difference between the results presented in this study and the previously published data.

Moreover, HSC conditioned medium inhibited AMPK activation in HCC cells in a paracrine manner. The catalytic AMPK subunit α can be phosphorylated at different phosphorylation sites [250]. While phosphorylation at Thr^{172} activates AMPK [194], phosphorylation at $Ser^{485/491}$ inhibits its activity and in addition favours dephosphorylation at Thr^{172} [236, 251]. In this study, conditioned medium of HSCs induced a down-regulation of AMPK α Thr^{172} phosphorylation in HCC cells. In contrast, phosphorylation of AMPK α at $Ser^{485/491}$ was mildly induced by conditioned medium of some HSC preparations compared to serum free HSC medium, suggesting overall inhibition of AMPK.

AMPK activation has been shown to inhibit cell proliferation through various mechanisms, including inhibition of the mTOR pathway [134] and stabilization of p53 [141]. Therefore one would expect induction of cell proliferation through inhibition of AMPK in HCC cells after treatment with HSC conditioned medium. As the opposite was observed, the data presented here suggest that HSC conditioned medium exerts AMPK independent effects on HCC cell proliferation. Moreover, it is possible that regulation of cell proliferation in PLC/PRF/5 and HepG2 cell lines is abnormal per se, as both HCC cell lines employed in this study, show mutations in i.e. p53 (PLC/PRF/5), cyclin-dependent kinase inhibitor 2A (PLC/PRF/5) and β-catenin (HepG2) genes [229, 230], which have been shown to be critical regulators of the cell cycle [252-254].

Low phosphorylation levels of AMPK α Thr^{172} are associated with a poor prognosis and aggressive clinical features in HCC [255]. As HSC conditioned media caused downregulation of pAMPK Thr^{172} in HCC cells, the co-culture with HSCs could enhance the tumourigenic features of HCC cells. Confirming this notion would however require a more detailed look at the features of HepG2 and PLC/PRF/5 cells treated with HSC conditioned medium *in vitro*.

Taken together, the data in this study indicate that HSCs can affect HCC cell proliferation and modify the AMPK pathway in HCC cells, suggesting a role for AMPK in tumour-stromal interactions although further experiments need to be performed to gain evidence about the exact involvement of AMPK in this interaction and its effect on HCC tumourigenicity. To test this hypothesis, AMPK could be genetically knocked out in HCC cells using siRNA or shRNA in future experiments and the effect of HSC on proliferation of such genetically modified HCC cells could be tested *in vitro*. Moreover, AMPK k.o. cells, as well as wild type HCC cells, could be co-transplanted into mice and tumour growth could be assessed *in vivo*.

2.4.2.2 HCC cells induce an activated, pro-inflammatory and pro-fibrogenic phenotype in human HSCs

It has been shown that rat HSCs are activated by HCC tumour cells [114], however data about primary HSC activation by HCC in humans are still lacking. In this study,

it was investigated whether HCC cells can activate primary human HSCs, and HSC proliferation, migration and gene expression were analysed as measures for HSC activation.

Indeed HepG2, but not PLC/PRF/5 cells, stimulate HSC proliferation and migration in a paracrine fashion, indicating activation of HSCs. As a further measure of HSC activation, expression of pro-inflammatory and pro-fibrogenic genes in HSCs was investigated after treatment with HCC conditioned medium. Expression of TIMP 1 and MMP2 was unchanged in HSCs following treatment with HCC conditioned medium for 24 hours, whereas expression of other genes was up or down-regulated. This suggests either a selective effect on up-regulation of specific genes in HSCs, or different kinetics of the up-regulation of different pro-fibrotic genes.

Indeed, Mannaerts et al. have demonstrated that upon culturing quiescent HSCs on plastic, the expression of pro-fibrogenic genes in murine HSCs starts to change between 4 and 16 hours. However, expression of some genes strikingly changes between 16 and 64 hours [256], suggesting that regulation of different genes does follow different kinetics. Although expression of some genes was unchanged in HSCs after 24 hours treatment with HCC conditioned medium, most of the analysed gene expressions had already changed after 24 hours, suggesting that HCC conditioned medium has a direct effect on HSC gene expression.

Interestingly, gene expression of LOX was upregulated in HSCs after treatment with HCC conditioned medium. In liver fibrosis, LOX is responsible for the cross-linking of collagen and elastin, converting them to insoluble fibres [238] and thereby contributing to the development and consolidation of liver fibrosis.

Furthermore, up-regulation of CCL2, IL-1β and IL-8 was observed in HSCs treated with conditioned medium of PLC/PRF/5 and HepG2 cells, showing that HCC cells induce a pro-inflammatory phenotype in HSCs. Cytokines and chemokines have been shown to play a crucial role in the development of liver fibrosis and cirrhosis, both through recruitment of immune cells or direct HSC activation [22, 23]. For example, CCL2 and IL-8 have been shown to recruit inflammatory macrophages [257, 258]. In addition, CCL2 mediates fibrosis-associated angiogenesis [257], and can further activate HSCs in an autocrine manner [259]. Moreover, IL-1β promotes progression from liver fibrosis to cirrhosis through HSC activation [260] and enhances HSC survival [261]. Overall, these data show that HCC cells can drive the expression of

pro-fibrogenic genes in HSCs, but also feed an inflammatory vicious circle resulting from up-regulation of cytokine gene expression in HSCs.

In this study, expression of IL-6, which has been shown to promote toxin-induced liver fibrosis [262], was down-regulated by conditioned medium of HepG2 cells, but not by PLC/PRF/5 conditioned medium. Moreover, PLC/PRF/5 cells, in contrast to HepG2 cells, did not induce HSC proliferation or migration, indicating differential effects of the two HCC cell lines on different features of HSC activation, which may reflect the heterogeneity that occurs in human HCC [98].

Overall, the findings in this study indicate that human HSCs become highly activated when exposed to conditioned medium of HCC cells. Such activation is characterized by increased HSC proliferation and migration, as well as production of pro-inflammatory and pro-fibrogenic cytokines. Thereby, HSCs can promote the development and consolidation of liver fibrosis, as well as the recruitment of pro-inflammatory immune cells to the liver.

2.4.2.3 The paracrine effect of HCC cells on HSC proliferation and migration – possible involvement of the AMPK pathway

Cell proliferation and migration are features of activated HSCs [24, 51], and were induced in HSCs by conditioned medium of HepG2 cells. Because AMPK is a regulator of cell proliferation [134, 240] as well as cell migration [223], it was tested whether activation of AMPK plays a role for the modulation of cell proliferation and migration in HSCs by HCC conditioned medium.

HepG2 conditioned medium induced inhibition of AMPK in HSCs, shown by only mild phosphorylation of AMPK-Thr^{172} in combination with strong phosphorylation of AMPK-Ser$^{485/491}$. In contrast, no induction of HSC proliferation or migration was observed after treatment with PLC/PRF/5 conditioned medium. In line with this, PLC/PRF/5 conditioned medium induced activation of AMPK as shown by marked phosphorylation of AMPK-Thr^{172} and only mild phosphorylation of AMPK-Ser$^{485/491}$.

These results show that activation of AMPK in HSCs correlates with a less proliferative phenotype, suggesting that the AMPK pathway is a critical regulator of HCC induced cell proliferation in HSCs. Moreover, in line with published data

showing that AMPK inhibits cell migration [223], the results presented in this study suggest that AMPK may be a regulator of HSC migration.

Importantly, the data also suggest that HCC cells can modulate the activation of the AMPK pathway in HSCs in a paracrine manner, thus influencing HSC proliferation and migration. This hypothesis is supported by previous reports demonstration that activation of AMPK with pharmacological activators inhibits PDGF-induced proliferation in human and rat HSCs [177, 178]. In addition, the anti-proliferative effect of pharmacological AMPK activators was abolished after transduction of HSCs with an AMPK silencing vector, and the pro-proliferative effect of PDGF on HSC proliferation was prevented by AMPK overexpression [177].

However, to prove that AMPK regulates HSC proliferation and migration induced by HCC cells, AMPK should be silenced in primary HSCs in order to investigate the effect of HCC conditioned medium on proliferation and migration in these cells.

Nevertheless, the results in this thesis overall suggest that the AMPK pathway is important in bi-directional tumour stromal crosstalk between HCC and HSCs, and that AMPK represents a potential target for both anti-cancer and anti-fibrotic therapy.

2.4.2.4 Elucidating a mechanism for HSC activation and modulation of the AMPK pathway in HSCs by HCC cells

Conditioned medium of different HCC cell lines shows a characteristic secretome [263]. More specifically, HepG2 and PLC/PRF/5 cells have been shown to secrete a vast amount of plasma proteins, amongst them albumin and α-Fetoprotein [264, 265], as well as different growth factors, for example IGF II [266, 267], VEGF [268] and PDGF-BB [267], the most potent HSC mitogen [69, 269]. The identification of mediators secreted by PLC/PRF/5 and HepG2 cells that modulate cell proliferation and migration, as well as the AMPK pathway in HSCs, could lead to the development of novel drugs for anti-fibrotic and anti-cancer therapies.

In order to gain insight into the composition of the conditioned medium of HepG2 and PLC/PRF/5 cells, preliminary experiments were performed. Conditioned media of

HCC cell lines were fractionated using 30kDa pore size filters and primary human HSCs were treated with different fractions of conditioned medium. Interestingly, treatment of HSC with low molecular weight fractions of either conditioned medium led to significant decrease of HSC proliferation (figure 2.32). In contrast, application of the high molecular weight fractions induced a significant increase in HSC proliferation (figure 2.32), suggesting that HSC stimulating and inhibiting mediators are carefully balanced in the conditioned medium of both HCC cell lines.

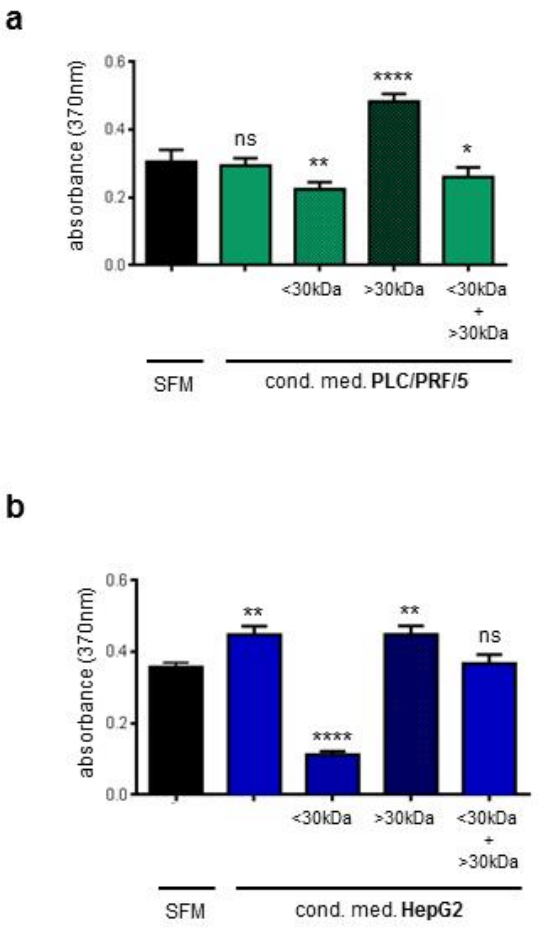


Figure 2.32: HCC conditioned medium contains activating and inhibiting mediators altering HSC proliferation.
Proliferation of HSCs after treatment with fractionated conditioned medium of (a) HepG2 cells or (b) PLC/PRF/5 cells for 24 hours measured by BrdU incorporation. Data represent mean +/- SD from biological quadruplicates, *p< 0.05, **p<0.01, ****p<0.0001, ns = not significant vs. serum free HSC medium. Representative data of 3 independent experiments, using different HSC preparations. SFM = serum free HSC medium, cond.med. = conditioned medium

Growth factors are mostly of small molecular size, e.g. PDGF (27kDa), IFG II (7.5kDa) and VEGF (21kDa), which seems to contradict the finding of low molecular weight fractions inhibiting HSC proliferation. However, growth factors can occur as di- or trimers or in oxidised forms both of which lead to higher molecular weights [270-272]. Further, it has been shown that tumours, e.g. HCC, secrete high molecular weight forms of IGF-2 [273], and that tumour-derived IGF-2 is prone to binding to high molecular weight plasma proteins [274]. All these modifications could lead to retention of growth factors in the high molecular weight fraction by the employed filters, explaining the inhibition of proliferation by the mall molecular weight fraction and the pro-proliferative effect of the high molecular weight fraction. Besides growth factors, numerous unknown tumour-derived peptide or lipid mediators, as well as micro-vesicles or other micro-particles secreted by HCC cells could mediate the observed effects on HSC activation [275, 276].

Identifying the mediators secreted by HCC cells that inhibit or enhance HSC activation could be of great importance to develop novel targets for anti-cancer or anti-fibrotic therapy. Therefore, mass spectrometry analysis of the HCC conditioned media will be performed.

2.4.3 Therapeutic concepts – targeting AMPK in HSCs and HCC

AMPK has been linked to cancer development and progression, not only because its upstream kinase, LKB1, is known to be a tumour suppressor [137], but also because of its anti-proliferative and anti-anabolic effects (see chapter 2.1.3.2 and [247]). Further evidence for AMPK being a tumour suppressor was provided by that fact that the incidence of many tumour types, including HCC, is lower in patients treated with the AMPK activator and oral anti-diabetic drug Metformin [277]. Moreover, there are several studies showing anti-cancer properties of other AMPK activators in HCC, both *in vitro* and in animal models. There is evidence that HCC cell proliferation is inhibited by pharmacological activation of AMPK with AICAR [141, 166], and Metformin augments the anti-proliferative effect of cisplatin on HCC cells in an AMPK-dependent manner [255].

Furthermore, AMPK activators, like AICAR, Metformin and Phenformin, have been shown to inhibit HSC proliferation after treatment with PDGF-BB, and to reverse the activated phenotype of HSCs [177, 178]. This suggests that AMPK activation may be beneficial for tumour-stromal interactions in HCC, as it can target both HSCs and HCC cells. However, in published studies, the benefit of AMPK activation in HSCs has only been shown in PDGF-induced HSC proliferation, and evidence about AMPK activation in HSCs under basal condition is missing. Moreover, to our knowledge, there are no data about pharmacological AMPK activators and their effect on HCC-induced proliferation in HSCs.

In this study, the dose-dependent anti-proliferative effects of AICAR, Metformin and Phenformin on HepG2 and PLC/PRF/5 cells were confirmed (figure 2.33). Importantly, the AMPK inhibitor CC inhibited HCC proliferation significantly and in a dose dependent manner as well (figure 2.34).

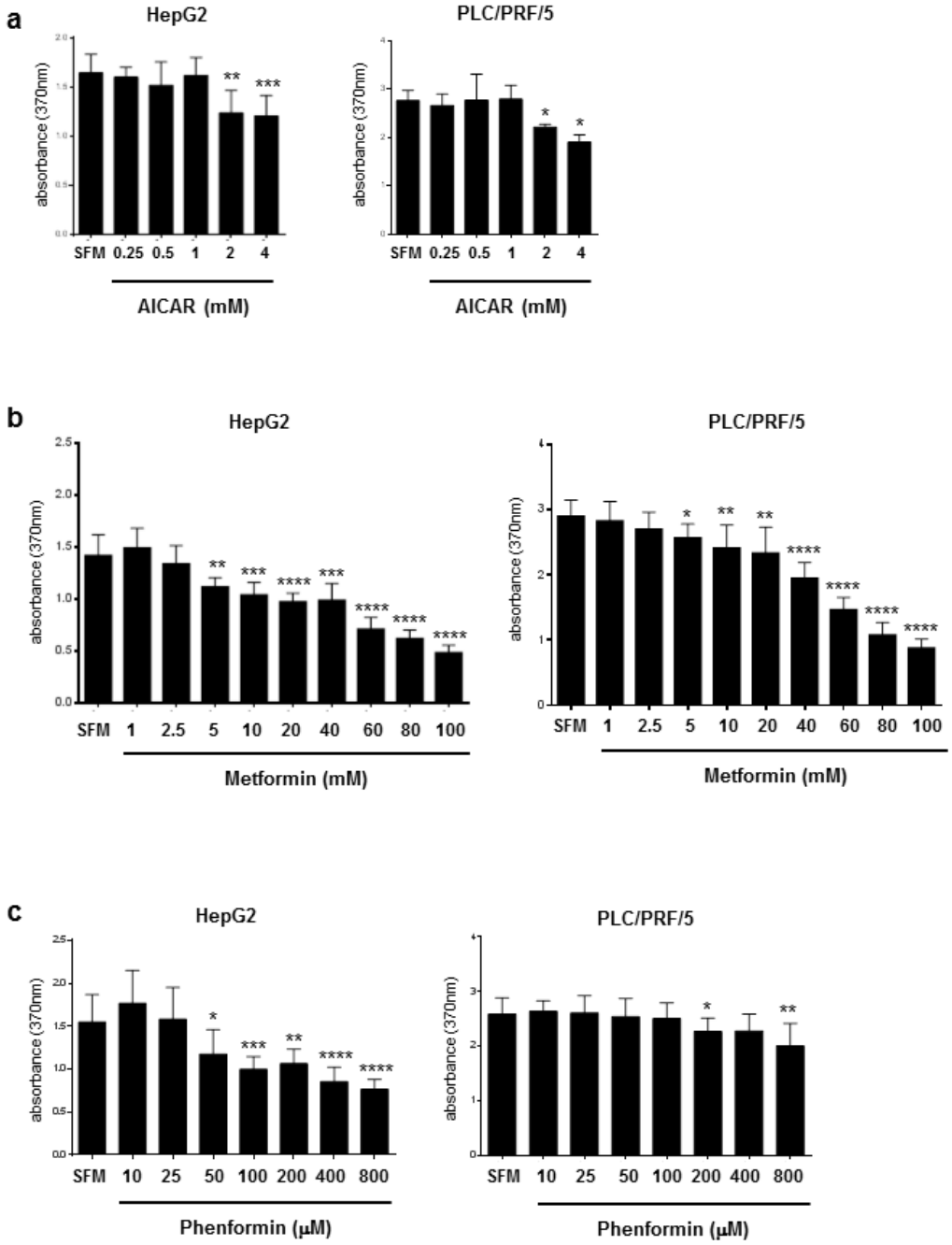


Figure 2.33: Proliferation of HepG2 and PLC/PRF/5 cells is inhibited by the pharmacological AMPK activators AICAR, Metformin and Phenformin.
Proliferation of HepG2 and PLC/PRF/5 cells after treatment with (a) AICAR (0.25-4mM) (b) Metformin (1-100mM) and (c) Phenformin (10-800μM) for 24 hours, measured by BrdU incorporation. (a)-(c): Data represent mean +/- SD of biological triplicates, *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. serum free HSC medium, representative data of 3 independent experiments. SFM = serum free HCC medium

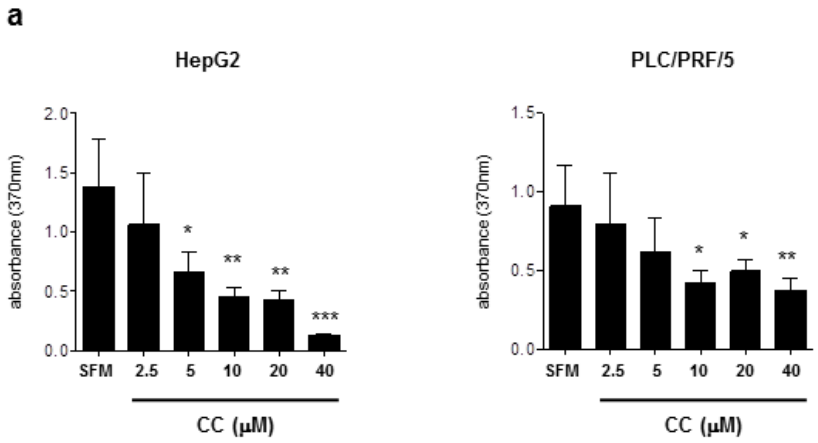


Figure 2.34: Proliferation of HepG2 and PLC/PRF/5 cells is inhibited by CC.
(a) Proliferation of HepG2 and PLC/PRF/5 cells after treatment with CC (2.5-40µM) for 24 hours, measured by BrdU incorporation. Data represent mean +/- SD of biological quadruplicates, *p< 0.05, **p<0.01, ***p<0.001 vs. serum free HSC medium, representative data of 3 independent experiments. SFM = serum free HCC medium

Similarly, proliferation of primary human HSCs was inhibited in a dose-dependent manner after treatment with different AMPK activators (figures 2.14 and 2.35), shown both under basal conditions and, more importantly, after treatment with HCC conditioned medium.

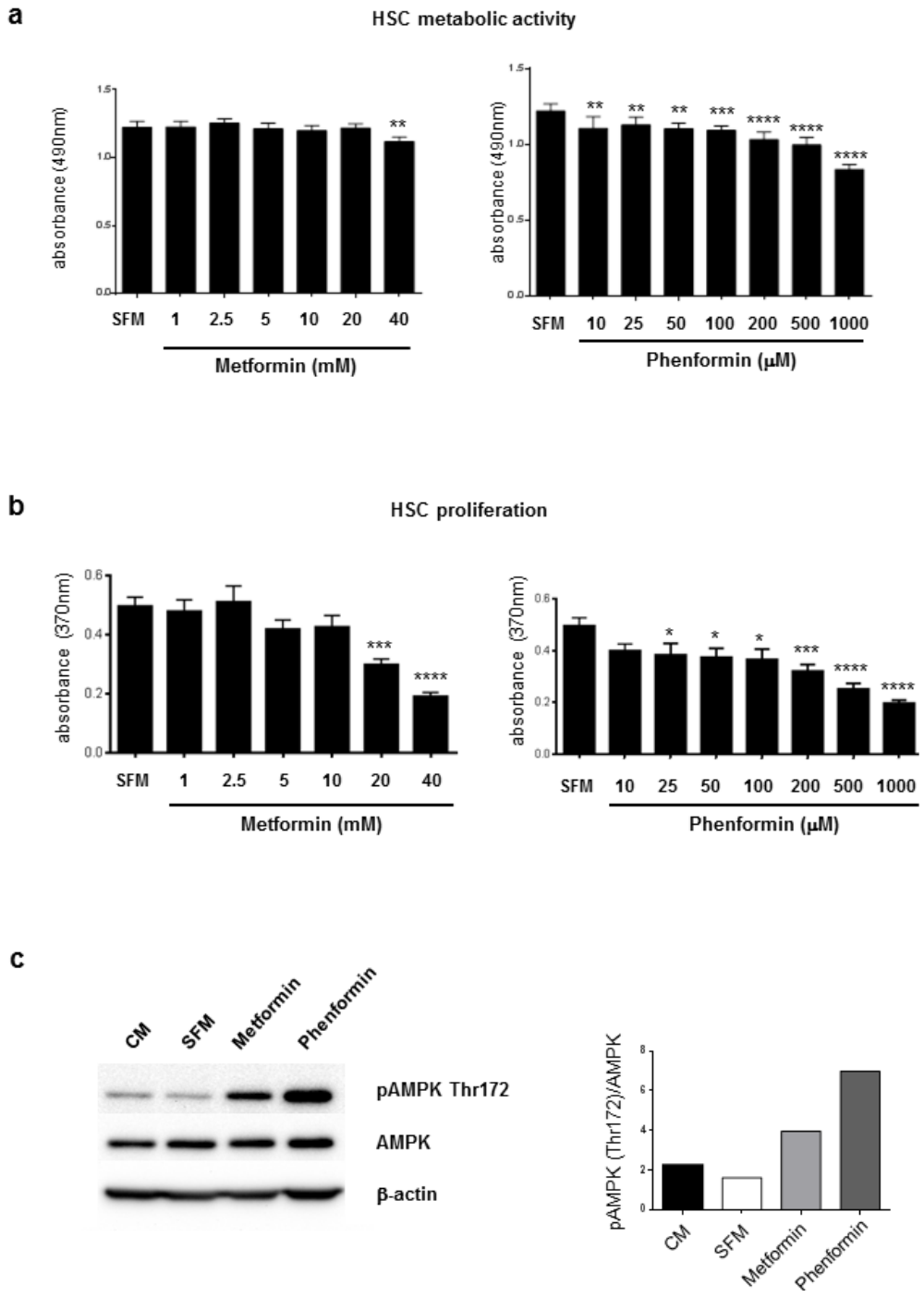


Figure 2.35: Metformin and Phenformin activate AMPK in HSCs and inhibit proliferation dose-dependently.

(a) Metabolic activity measured by MTS assay and (b) proliferation of HSC measured by BrdU assay following treatment with Metformin (1-40mM) or Phenformin (10-1000mM) for 24 hours. (c) Protein expression in HSC after treatment with Metformin (20mM) or Phenformin (50μM) for 24 hours, representative western blot and quantification. (a) Data represent SD +/- SD of technical triplicates. (b) Data represent mean +/- SEM, pooled data of 3 independent experiments. (a) and (c) representative data of 3 independent experiments. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001

vs. serum free HSC medium. CM = complete HSC medium, SFM = serum free HCC medium.

Treatment with AICAR also significantly inhibited the expression of pro-fibrogenic and pro-inflammatory genes in HSCs. Importantly, AICAR was even sufficient to reverse the induction of CCL2 and IL-1β expression by conditioned medium of PLC/PRF/5 and HepG2 cells in HSCs. This shows that AICAR can reverse the activated, pro-fibrogenic and pro-inflammatory phenotype of HSCs induced by HCC cells. The results presented in this thesis therefore provide evidence for a benefit of pharmacological AMPK activation in tumour-stromal interactions between HSCs and HCC. Considering the important role of activated HSCs in HCC development and progression [84, 118], targeting both HSC activation and HCC proliferation through pharmacological activation of AMPK represents a novel concept to break the vicious cycle of HSC activation and cancer progression.

2.4.4 CC and AICAR – Mechanisms of action in HSCs

Surprisingly, treatment of HSCs with CC, that was described to be an AMPK inhibitor and was therefore expected to induce HSC proliferation, showed significant, dose-dependent anti-proliferative effects on HSCs without being cytotoxic. Besides, CC inhibited proliferation of HCC cells in a dose-dependent manner. Importantly, CC inhibited HCC-induced HSC cell proliferation even to the same extent as the AMPK activator AICAR, suggesting that CC does not actually inhibit AMPK, or that CC exerts AMPK-independent effects on cell proliferation in HSCs. Analysis of the activation status of AMPK however showed that CC indeed inhibits AMPK in HSCs by strong phosphorylation of the inhibiting phosphorylation site $Ser^{485/491}$ and lack of phosphorylation at Thr^{172}.

There is recent evidence supporting the hypothesis that CC exerts AMPK independent anti-proliferative effects in different cancers, such as induction of apoptosis, inhibition of the Akt and mTOR signalling pathway, as well as induction of cell cycle arrest

[177, 206]. In addition, despite being considered a selective inhibitor for AMPK, CC was shown to inhibit a number of other kinases besides AMPK [278]. Overall, this supports the hypothesis that CC exerts its effects on HSC proliferation in an AMPK-independent manner.

Moreover, this assumption is supported by the fact that pre-incubation with CC abolished the inhibiting effect of AICAR and Metformin on PDGF-BB induced proliferation in HSCs [177], but did not reverse the inhibitory effect of AMPK activators on HSC proliferation under basal conditions (data not shown), suggesting that CC specifically primes HSCs to respond to PDGF-BB induced proliferation.

There is evidence that also the AMPK activator AICAR inhibits cell proliferation in MEFs in an AMPK-independent manner, e.g. by induction of cell cycle arrest [241]. Therefore, MEFs lacking both isoforms of the AMPK catalytic subunit α, i.e. AMPK $\alpha1/\alpha2^{-/-}$ MEFs, were used as a tool to confirm AMPK independent effects of AICAR and to test whether CC exerted AMPK independent effects on cell proliferation in this model system. Indeed, both AICAR and CC inhibited proliferation of MEFs in an AMPK independent manner.

Therefore, the question through which mechanisms AICAR and CC inhibited cell proliferation in HSCs emerged. In order to identify a mechanism by which CC inhibits HSC proliferation, different pathways were tested. Unlike in glioma cells and colorectal cancer cells [206, 207], CC did not induce apoptosis or necrosis in HSCs, nor did CC inhibit the mTOR pathway. However, treatment of HSCs with CC resulted in accumulation of cells in the S phase of the cell cycle. Given that CC also inhibited HSC proliferation significantly, these data show that CC induces cell cycle arrest in the S phase in HSCs. A similar effect was observed in glioma cells, where CC inhibited the cell cycle in the G2/M phase [206], as well as in colorectal cancer cells [207] and skin cancer cells [279].

Moreover, CC mildly induced autophagy in HSCs, which has been shown to be a feature of HSC activation, as attenuation of autophagy leads to decreased proliferation and reduced expression of pro-fibrogenic genes, such as collagen I, PDGF receptor β and MMP2 in mouse and human HSCs [280, 281]. Moreover, inhibition of autophagy reduces fibrosis in a murine toxin-induced liver fibrosis model [280]. Importantly, autophagy also contributes to the development of kidney and lung fibrosis, suggesting

that autophagy is a core pathway of fibrogenesis [280]. Induction of autophagy by CC in HSCs is therefore not favourable and shows that CC can possibly exert pro-fibrogenic stimuli on HSCs, although it inhibited HSC proliferation and migration.
In contrast to the pro-fibrogenic effect on HSCs, autophagy reduces pro-fibrogenic signals in hepatocytes and Kupffer cells. In addition, autophagy limits the pro-inflammatory phenotype of macrophages, which is characterized by increased IL-1β expression [282, 283] and protects hepatocytes from apoptosis [284], showing that autophagy seems to have a dual role in fibrosis development [285]. Of note, autophagy is thought to play a dual role in HCC development as well [286], emphasising the need for further studies in a model system in order to attribute a beneficial or unfavourable role to autophagy induction by CC.
Autophagy is regulated by activated AMPK through inhibition of mTOR as well as through phosphorylation of ULK-1 [222]. As CC inhibited AMPK in HSCs, it is very likely that induction of autophagy by CC is mediated independently of AMPK, although further studies need to be performed in order to elucidate the underlying mechanism. Moreover, in order to test whether the induction of autophagy and inhibition of the cell cycle by CC is mediated independently of AMPK, HSCs with silenced AMPK need to be treated with CC.

Furthermore, the results presented in this thesis show that treatment of HSCs with AICAR also induced cell cycle arrest in the S phase. Until now, it still needs to be tested whether the effect of AICAR on the cell cycle in HSCs is AMPK-dependent. However, it has been published that AMPK stabilizes the tumour suppressor p53 [141], which can induce cell cycle arrest at various checkpoints, including the S-phase checkpoint [287], suggesting that AMPK activation by AICAR could indeed result in S-phase cell cycle arrest. In contrast, there is evidence that AICAR induces cell cycle arrest independently of AMPK in glioma cells [288], showing that further experiments are required to elucidate the mechanism though which AMPK causes cell cycle arrest in primary HSCs.
Moreover, treatment with AICAR resulted in inhibition of the mTORC1 pathway in HSCs, and treatment of wt and AMPK $\alpha1/\alpha2^{-/-}$ MEFs revealed that AICAR inhibited the mTORC1 signalling pathway in MEFs in an AMPK dependent manner. This suggests that mTOR inhibition in HSCs may also be AMPK dependent, although this

requires further experiments to confirm. Thus, primary HSCs with silenced AMPK need to be treated with AICAR, in order to analyse AMPK dependency of cell cycle arrest or inhibition of the mTORC1 pathway. Overall, AICAR inhibits HSC proliferation through different mechanisms, which may at least in part be AMPK independent.

Taken together, the results in this thesis show that there is a bi-directional cross-talk between primary human HSCs and human HCC tumour cells that affects cell proliferation and activation of the AMPK pathway in both cell types, indicating a role for AMPK in tumour stromal interactions. Moreover, the data show that pharmacological AMPK activation, in cooperation with AMPK-independent effects mediated by the anti-proliferative drugs AICAR, Metformin, Phenformin and CC could represent a novel approach for anti-cancer and anti-fibrotic therapy (summarised in figure 2.36).

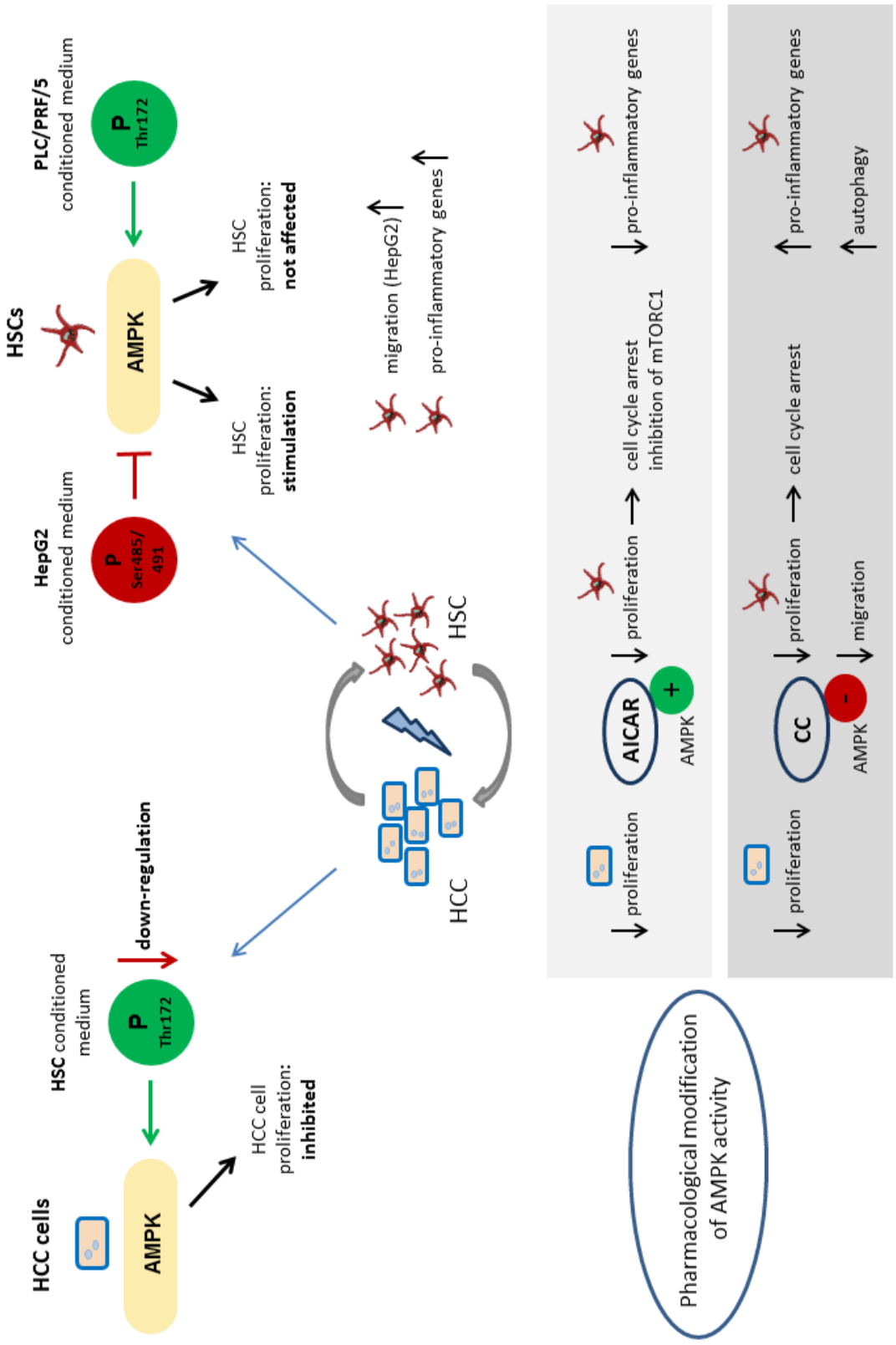


Figure 2.36: Interactions between HSCs and HCC cells and their pharmacological modification

Figure 2.36: Interactions between HSCs and HCC cells and their pharmacological modification

HSC conditioned medium induces downregulation of pAMPK-Thr^{172} in HCC cells and inhibits their proliferation. HCC cells show differential effects on HSC proliferation and AMPK activation. HepG2 conditioned medium induces HSC proliferation and migration and inhibits AMPK (pAMPK Ser$^{485/491}$). In contrast, PLC/PRF/5 conditioned medium induces AMPK activation (pAMPK Thr^{172}) and does not affect HSC proliferation. Conditioned medium from both HepG2 and PLC/PRF/5 cells induced up-regulation of pro-inflammatory genes in HSCs.

The AMPK activator AICAR and the AMPK inhibitor CC both inhibit HCC-induced HSC proliferation by causing cell cycle arrest. AICAR in addition inhibits the mTORC1 pathway. CC inhibits migration and induces autophagy in HSCs. Whereas AICAR shows an anti-inflammatory effect on HSCs, CC acts rather pro-inflammatory. AICAR and CC inhibit HepG2 and PLC/PRF/5 proliferation.

Appendix chapter 2

The work presented in this chapter has been presented at various national and international meetings in the last three years:

"Hepatocellular carcinoma differentially modulates AMPK activity and induces autophagy in hepatic stellate cells in a paracrine manner"
Poster presentation, The International Liver Congress 2016, European Association for the Study of the Liver, Barcelona, Spain

"Hepatocellular carcinoma differentially modulates AMPK activity and induces autophagy in hepatic stellate cells in a paracrine manner"
Poster presentation, Division of Medicine Research Retreat 2016, University College London, London, UK

"Modulating Tumour-Stromal Interactions by Targeting AMPK in Human Hepatic Stellate Cells and Hepatocellular Carcinoma"
Poster presentation, The International Liver Congress 2015, European Association for the Study of the Liver (EASL), Vienna, Austria

"The paracrine effect of hepatocellular carcinoma (HCC) on AMPK in human hepatic stellate cells and its pharmacological modulation"
Poster presentation, Graduate Student Day 2015, Division of Medicine, University College London, London, UK
Poster prize "The academic's choice"

"The paracrine effect of hepatocellular carcinoma (HCC) on AMPK in human hepatic stellate cells and its pharmacological modulation"
Poster presentation, Division of Medicine Research Retreat 2015, University College London, London, UK

"Modulating Tumour-Stromal Interactions by Targeting AMPK in Human Hepatic Stellate Cells and Hepatocellular Carcinoma"
Oral presentation, Graduate Student Day 2014, Division of Medicine, University College London, London, UK

"Modulating Tumour-Stromal Interactions by Targeting AMPK in Human Hepatic Stellate Cells and Hepatocellular Carcinoma"
Oral presentation, 6th Meeting of the European Club for Liver Cell Biology (ECLCB) 2014 in Treviso, Italy

"Modulating Tumour-Stromal Interactions by Targeting AMPK in Human Hepatic Stellate Cells and Hepatocellular Carcinoma"
Poster presentation, Annual meeting of the British Association for the Study of the Liver (BASL) 2014, Newcastle, UK

"Modulating Tumour-Stromal Interactions by Targeting AMPK in Human Hepatic Stellate Cells and Hepatocellular Carcinoma"
Poster presentation, Research Retreat 2014, Division of Medicine, University College London, London, UK

"Modulating Tumour-Stromal Interactions by Targeting AMPK in Human Hepatic Stellate Cells and Hepatocellular Carcinoma"
Poster presentation, The Liver Meeting 2014, American Association for the Study of Liver Diseases (AASLD), Boston, Massachusetts, USA

Chapter 3

Summary chapter 3

In this chapter, the abundance and phenotype of mucosal-associated invariant T cells (MAIT cells) were characterised in peripheral blood and liver tissue from patients with different forms of AILD, i.e. PSC, PBC and AIH. Furthermore, the ability of MAIT cells to produce pro-inflammatory cytokines such as IFNγ and IL-17 following different types of MAIT cell stimulation e.g. mimicked TCR-signalling and cytokine-mediated stimulation was investigated.

The data presented in this thesis show that MAIT cell numbers are severely reduced in peripheral blood and liver tissue of patients with AILD, irrespective of the type of AILD. Furthermore, the data provide evidence that MAIT cells from AILD patients are functionally exhausted, likely resulting from persistent activation *in vivo*. Nevertheless, the response to cytokine-mediated stimulation was intact in MAIT cells from AILD patients, which responded with IL-17 secretion to repetitive stimulation with the cytokine IL-12. Moreover, the interaction between MAIT cells and HSCs was explored, and there is evidence that MAIT cells are able to stimulate HSC proliferation *in vitro*, suggesting that activated MAIT cells might contribute to fibrosis development in AILD.

Main findings presented in this chapter:

1. MAIT cells are significantly reduced in peripheral blood and liver tissue of patients with different forms of AILD, such as PSC, PBC and AIH.

2. Circulating MAIT cells from AILD patients show signs of exhaustion.

3. Cytokine expression in response to cytokine-mediated stimulation is maintained in MAIT cells from AILD patients

4. MAIT cells from healthy controls and AILD patients produce IL-17 in response to repetitive stimulation with IL-12.

5. MAIT cells are able to promote HSC proliferation *in vitro.*

3.1 Introduction

3.1.1 The immune system

The immune system is crucial for the body's defence against pathogens, such as viruses, bacteria, parasites and fungi. Moreover, the immune system is responsible for the elimination of toxins and malignant cells. In order to establish an optimal and rapid immune response, two systems have evolved: innate and adaptive immunity.
The innate immune system is able to generate immediate responses to pathogens, owing to the recognition of conserved foreign structures with specialised receptors [289]. In contrast, the adaptive immune system is specialised to respond specifically to individual pathogens, as highly variable antigen-receptors generated by gene rearrangement allow for recognition of foreign structures [290]. The adaptive immune system itself is subdivided into two distinct systems. B cells produce soluble antibodies in order to inactivate pathogens, representing humoral immunity. T cells represent cellular immunity and are able to recognize peptide antigen, whereupon they either differentiate into cytotoxic effector cells that are able to kill infected cells, or activate other immune cells.
Both B cells and T cells can contribute to immunological memory. After the clearance of a pathogen, only a limited number of immune cells survive and differentiate into memory cells, which are able to recognize and eliminate a known antigen more rapidly and effectively [291, 292].

3.1.1.1 Activation of the innate immune response

The innate immune system constitutes the first line defence to invading pathogens. An effective innate immune response is based on the recognition of pathogen-associated molecular patterns (PAMPs), highly conserved pathogen-derived structures, or danger-associated molecular patterns (DAMPs), which are derived from altered host cells. DAMPs and PAMPs can be recognized by pattern-recognition receptors (PRRs)

expressed on innate immune cells, such as macrophages, neutrophils and dendritic cells (DCs) [293, 294]. The activation of PRRs results in the expression of several pro-inflammatory cytokines regulated by the transcription factor nuclear factor-κB (NF-κB), such as type I interferons, TNFα, IL-6, IL-12, IL-18 and IL-1β [295]. Upon the secretion of these pro-inflammatory cytokines, innate as well as adaptive immune cells are recruited and activate other immune cells, thereby establishing inflammation. To date, a number of PRRs are known in humans, including Toll-like receptors (TLRs), Nod-like receptors and C-type lectin receptors [293].

Of note, besides being expressed by innate immune cells, TLRs are expressed by hepatocytes and non-parenchymal cells in the liver, such as HSCs and LSEC, and contribute to the development of liver disease [296, 297].

3.1.1.2 T cell mediated immunity

T cells represent the effector cells of cellular adaptive immunity. They recognize antigen with a specific T cell receptor (TCR) when it is presented in the context of major histocompatibility complex (MHC) molecules, expressed by other cells. Thus, T cells do not recognize free antigen or fight pathogens directly, but exert effector function on other cells. In addition, TCRs need either of the TCR co-receptors CD4 and CD8 in order to guarantee optimal function. According to their co-receptor expression, T cells can be classified into two different subsets: $CD4^+$ T cells and $CD8^+$ T cells [298]. Whereas $CD4^+$ T cells recognize peptide antigen presented on MHC class II (MHC II), $CD8^+$ T cells respond to peptide antigen presented on MCH class I (MHC I) [299]. Of note, both T cell subsets exert distinct functions. $CD4^+$ T cells activate other immune cells, such as B cells and macrophages, and are therefore called T helper (Th) cells [300]. In contrast, $CD8^+$ T cells are able to kill infected cells, and are therefore also called cytotoxic effector T cells, short cytotoxic T lymphocytes (CTL) [301].

The differentiation into CTL requires interaction with antigen-presenting cells (APCs), such as macrophages, B cells or DCs, which provide three signals. The first signal is derived from the specific interaction between the TCR and the peptide:MHC I complex. Moreover, activating signals from co-stimulatory receptors, such as CD28

(signal 2), and soluble cytokines, such as IL-12 (signal 3), are necessary for the differentiation into CTL, as well as for an optimal T cell response [302, 303]. Moreover, IL-12 is important for the generation of memory T cells [304, 305]. The differentiation of naïve $CD8^+$ T cells into CTL results in proliferation, clonal expansion and differentiation of CTLs, allowing for the control of infection in peripheral tissues. Moreover, CTL differentiation is associated with morphological changes, i.e. the cells become bigger, highly polarized and develop cytotoxic granules during the differentiation process. CTL can kill their target cells by different mechanisms, including lysis of the target cell by release of lytic proteins, such as granzyme B or perforin, or induction of receptor-mediated apoptosis [306]. Besides, CTL produce effector cytokines, such as IFNγ and TNFα, which support both the elimination of infected cells, as well as the activation of other immune cells [307].

3.1.1.3 Innate like T cells

Innate-like T cells express a TCR, but also resemble innate immune cells, as they can rapidly respond to infection or inflammatory signals. Due to their diverse function, such innate-like T cells are important for host defence, but have also been implicated in autoimmunity [308, 309].

Invariant natural killer T (iNKT) cells express a semi-invariant TCR and, unlike conventional T cells, recognize lipid-antigen presented on CD1d molecules, which are expressed for example by DCs, macrophages and B cells[310]. Moreover, iNKT cells are able to respond to cytokines and danger signal in an innate-like manner. Thus, in response to microbial infection, iNKT cells can rapidly produce cytokines, resulting in activation and recruitment of other immune cells, thereby orchestrating both adaptive and innate immune responses [311].

Besides iNKT cells, MAIT cells and γδ T cells belong to the family of innate-like T cells. γδ T cells are not restricted to antigen-presentation by MHC I or MHC II and it still remains unknown to which ligands they respond [312]. However, they rapidly release IFNγ, TNFα and granzymes and contribute to anti-viral, as well as anti-bacterial immune responses [312]. Furthermore, γδ T cells are considered as a

potential target for anti-cancer immunotherapy, owing to their ability to release IFNγ and TNFα, as well as their cytotoxic effector function against tumours [309, 313]. MAIT cells will be discussed in detail in chapter 3.1.3.

3.1.2 The liver as an immunological organ

Besides its important task as a metabolic organ, the liver is also considered as an immunological organ [3, 4]. This is owing to the liver's unique blood supply: whereas only 30% of blood is derived from the hepatic artery, 70% of blood reaching the liver is supplied by the portal vein and originates from the gut. Therefore, the liver is constantly exposed to food-derived antigen, but also to bacteria, bacterial metabolites and PAMPs [2]. Because of its constant exposure to antigen, the liver is considered a tolerogenic organ [314]. For example, APCs in the liver are tolerant against the chronic exposure to PAMPs, preventing the establishment of inflammation in the liver [2-4]. Moreover, anti-inflammatory cytokines, such as IL-10 and TGF-β are produced by hepatocytes, LSEC and Kupffer cells, and suppress the function of APCs, which in turn inhibits T cell activation in the liver [4]. In addition, both LSEC and HSCs are able to prevent T cell activation by DCs [66, 315].

Owing to the venous blood flow, in combination with the small diameter of the hepatic sinusoids (5-7μm), the sinusoidal blood flow is very slow. This allows not only for efficient metabolism of passing molecules, but also for extravasation of lymphocytes into the liver parenchyma, where they interact with local APCs [3, 4]. This is in addition facilitated by the special architecture of the liver sinusoidal endothelium, which is formed by LSEC. Of note, the sinusoidal endothelium lacks a basal membrane and forms pores, so-called fenestrae, through which molecules and immune cells can pass into the space of Disse, i.e. the virtual space between LSEC and hepatocytes [5, 316]. In sinusoids, as well as in the space of Disse, lymphocytes encounter local professional and non-professional APCs, such as Kupffer cells, DCs, HSCs and LSEC [4, 317]. Moreover, lymphocytes can directly interact with hepatocytes through fenestrae in the sinusoidal endothelium [318].

Notably, besides their metabolic function, hepatocytes display immunological properties. They produce acute phase and complement proteins, which are able to activate the innate immune system [319]. Moreover, despite their low expression of MHC I molecules, hepatocytes are capable of presenting antigen to $CD8^+$ T cells [320].

LSEC represent the largest population of non-parenchymal APCs in the liver. They are characterized by the ability to rapidly take up soluble antigen [321-323], which they present via MHC II molecules to CD4+ T cells. Furthermore, LSEC are able to cross-present exogenous soluble antigen via MHC I molecules to $CD8^+$ T cells [324]. Notably, cross-presentation is a feature of professional APCs. Nevertheless, LSEC as non-professional APCs cross-present antigen very efficiently, their cross-presentation capacity is even superior to splenic DCs [322, 324, 325]. Cross-presentation of antigen to naïve T cells by LSEC results in T cell proliferation and a short effector phase, in which the T cells produce pro-inflammatory cytokines and are cytotoxic [326, 327]. This is however followed by the up-regulation of inhibitory signals, resulting in a resting phenotype of LSEC-primed T cells, in which they are no longer able to carry out effector function [327, 328]. Moreover, antigen-presentation by LSEC to CTLs results in inactivation and apoptosis of CTLs [329].

Liver-resident DCs also exert tolerogenic properties. They are characterized by an immature phenotype, hindering optimal activation of T cells [317]. Moreover, they produce the inhibitory cytokine IL-10 [330].

Kupffer cells, liver-resident macrophages, play an important role in host defence against infection. They release pro-inflammatory cytokines, such as IL-1, IL-6 and TNFα. However, they also contribute to the tolerogenic microenvironment in the liver by releasing IL-10 [331] and through the inhibition of DC-induced antigen-specific T cell activation [332].

Furthermore, HSCs are capable of presenting antigen to NKT cells, and induce the expansion of regulatory T cells (Tregs) [63]. Notably, antigen-uptake by HSCs is low and they lack the ability to cross-present antigen to $CD8^+$ T cells [54, 333]. However, HSCs exchange MHC I molecules with LSEC through a mechanism called trogocytosis and thereby contribute to antiviral immune surveillance in the liver [54]. Under inflammatory conditions, HSCs induce the differentiation of monocytes into

myeloid-derived suppressor cells, which inhibit T cell proliferation and effector function [65, 334].

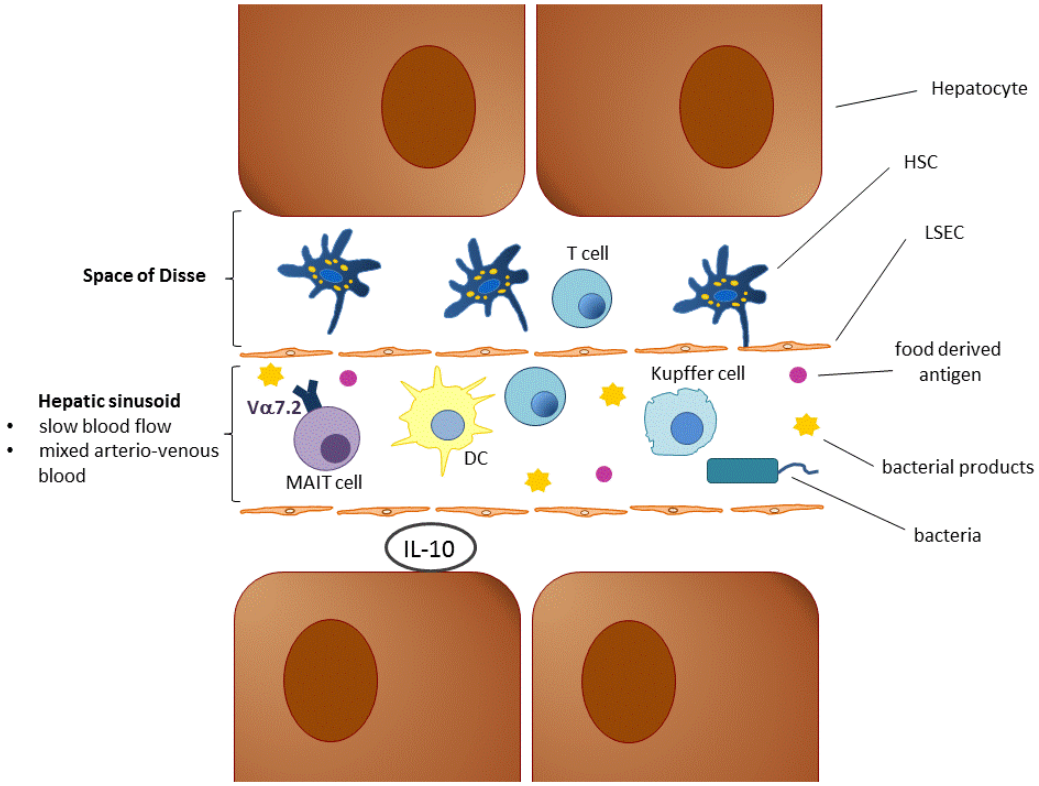


Figure 3.1: The immunological environment in the liver sinusoids
In the liver sinusoids, the slow blood flow allows for interactions between immune cells, such as T cells, Kupffer cells, dendritic cells and MAIT cells and other sinusoidal cell populations, e.g. LSEC and HSCs, but also hepatocytes. Portal venous blood reaching the liver contains food-derived antigen, but also bacteria and bacterial products. Therefore, the liver is a rather tolerogenic environment, preventing continuous immune activation by such antigen and danger signals.
Adapted from Thomson and Knolle 2010 [4]

3.1.3 Mucosal-associated invariant T (MAIT) cells

MAIT cells are the most abundant T cell population in the liver, where they represent up to 50% of T lymphocytes [335]. MAIT cells are a novel subset of innate-like T cells, which were first described about 20 years ago [336, 337]. In humans, they are equipped with a semi-invariant TCR, composed of the invariant α chain Vα7.2-Jα33 and a limited set of β chains [336], which restricts MAIT cells to antigen-presentation by the non-polymorphic MHC class I-related molecule MR1 [337, 338]. In 2013, vitamin B metabolites, derived from bacteria or yeasts, were identified as a novel class of antigen, presented to MAIT cells via MR1 [339]. Both the MAIT cell TCR, as well as MR1 are highly conserved among mammals [340, 341]. Therefore, also considering their abundance in peripheral blood and particularly the liver, MAIT cells are thought to play an important role in hepatic tissue inflammation and immunity.

3.1.3.1 Antigen presentation by MAIT cells

In humans, the majority of MAIT cells express the TCR α chain Vα7.2-Jα33, although Vα7.2 can also be paired with Jα12 and Jα20. The invariant α chain is preferentially combined with Vβ2 and Vβ13.2, although the use of β chains can vary. In mice, MAIT cells express Vα19 in combination with Vβ6 and Vβ8 [342, 343]. The MAIT cell TCR binds metabolites of riboflavin (vitamin B2) and folic acid (vitamin B9) when presented on MR1 [339]. Notably, it has been shown that, despite the expression of a semi-invariant TCR, the response of MAIT cells to different antigens shows some plasticity. To date, several activating as well as inhibiting MAIT TCR ligands have been identified. 5-OP-RU (5-(2-oxopropylideneamino)-6-D-ribitylaminouracil), an intermediate of riboflavin synthesis, is the most potent identified MAIT cell activator [344, 345]. Accordingly, MAIT cells respond to bacterial species capable of riboflavin synthesis [346-348]. The plasticity of the MAIT cell response to different ligands originates from different MR1 binding and stabilizing properties of ligands, as well as varying affinities of ligands to MR1 [345,

349]. Besides, a degree of TCR plasticity through the use of different β chains has been reported [337, 343], allowing for distinct responses to different pathogens [350].
Identification of MAIT cell TCR ligands allows for the generation of antigen-loaded tetramers enabling the precise identification of MAIT cells in human and mice [343, 344, 351].
Of note, a novel subset of MR1 restricted T cells has recently been described and defined as "non-classical MAIT cells". These cells do not express the TCR Vα7.2 chain and respond to folic acid metabolites [352].
MR1 is ubiquitously expressed in humans [340, 353], however, cell-surface expression is low, and requires the presence of antigen in order to be up-regulated [354]. In absence of antigen, an incompletely folded form of MR1 is present in the endoplasmic reticulum, only trafficking to the cell surface and being assembled in the presence of MR1 ligands [349, 355]. Moreover, MAIT cell development requires MR1 expression, as MAIT cells are absent in MR1 deficient mice [338].

3.1.3.2 Tissue distribution of MAIT cells in mice and human

In humans, MAIT cells represent 1-10% of T cells in peripheral blood, whereas they are particularly enriched in peripheral tissues like the liver (20-50% of T lymphocytes), owing to their expression of tissue-homing receptors, such as CCR6, CXCR6 and CCR9 [335, 356]. CCR6 binds to CCL20 and CXCR6 to CXCL16, both of which are highly expressed in the liver [357, 358] whereas CCR9 and its ligand CCL25 are responsible for cell trafficking to the gut [359]. Moreover, MAIT cells are present in the lungs (2-4% of T cells) [360] and the intestine, where different frequencies have been reported for the jejunum (60% of $CD4^-$ T cells), ileum (1.5% of T cells), colon (10% of T cells) and rectum (2% of T cells) [343, 361-363].
In mice, MAIT cells are rare, especially under steady-state conditions. In C57BL/6 mice, MAIT cells represent 3.3% of T cells in the lungs and 0.6% and 0.7% of T cells in the liver and lamina propria, respectively [351]. However, MAIT cell frequency has been shown to increase in mice after infection with bacteria or administration of 5-OP-RU in the presence of a toll-like receptor (TLR) agonist [364, 365].

3.1.3.3 MAIT cell development

MAIT cells develop in the thymus, where they undergo positive selection by MR1 expressing $CD4^+/CD8^+$ thymocytes [338]. Recently, a 3-stage maturation process for MAIT cell development in mice and humans has been proposed [366]. While stage 1-2 MAIT cells express a naïve phenotype, stage 3 MAIT cells show a mature phenotype and are able to produce cytokines, resembling peripheral MAIT cells. In humans, a small amount of stage 2 MAIT cells is found in the umbilical cord blood [366-368] showing that stage 2 MAIT cells exit the thymus and undergo extrathymic maturation. Moreover, MAIT cell development is dependent on MR1, the transcription factor promyelocytic leukaemia zinc finger (PLZF) protein and the presence of bacteria, as MAIT cells are absent in germ-free mice [338, 366]. Consistent with their maturation and development of reactivity to microbial-derived antigen, MAIT cells are characterized by an effector memory phenotype ($CD45RA^-$ $CD45RO^+$ $CD95^{hi}$ $CD62L^{lo}$) [335, 369, 370].

3.1.3.4 MAIT cell phenotype and effector function

Expression of TCR co-receptor expression has been characterized extensively in MAIT cells, with the majority of MAIT cells being $CD8^+$ or $CD4^-/CD8^-$ double negative (DN). MAIT cells, in contrast to conventional T cells, predominantly express CD8αα, but there are a small number of CD8αβ MAIT cells [367]. Moreover, MAIT cells express high levels of CD161, a C-type lectin, which in combination with positive staining for Vα7.2, allows for their identification [343, 351]. Functionally, cross-linking of CD161 results in reduced cytokine production, whereas the cytotoxic response is unaffected [371].

MAIT cells express high levels of cytokine receptors, which are commonly responsible for Th17 cell differentiation, i.e. IL-1β receptor and IL-23 receptor [372]. Moreover, they express high levels of IL-12 receptor (IL-12R) and IL-18 receptor (IL-18R), allowing for the induction of IFNγ production following stimulation with the innate cytokines IL-12 + IL-18[335, 356, 373]. Notably, IL-12 + IL-18 mediated stimulation is an important, TCR independent mechanism of MAIT cell stimulation.

Both stimulation with TLR agonists, as well as stimulation with non-riboflavin-synthesising bacteria are dependent on IL-12 + IL-18 [373, 374].

Furthermore, MAIT cells produce IFNγ and TNFα upon TCR-mediated stimulation, although such stimulation requires additional co-stimulatory signals or inflammatory cytokines [364, 375]. *In vitro*, MAIT cells also respond to stimulation with the mitogen phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore Ionomycin [335, 376].

Apart from IFNγ and TNFα, MAIT cells can secrete large amounts of IL-17, as well as IL-22, endowing them with a mixed Th1/Th17 cytokine expression pattern [335, 361, 372, 377]. In fact, MAIT cells are the largest IL-17 producing cell population in the liver [335, 372, 377], owing to their high constitutive expression of the transcription factor retinoic acid-related orphan receptor γT (RORγT), which is associated with IL-17 expression [335, 377-379].

In addition, MAIT cells show cytotoxic properties, as they are able to lyse bacterially infected cells by secretion of granzyme B and perforin in an MR1 dependent manner [380].

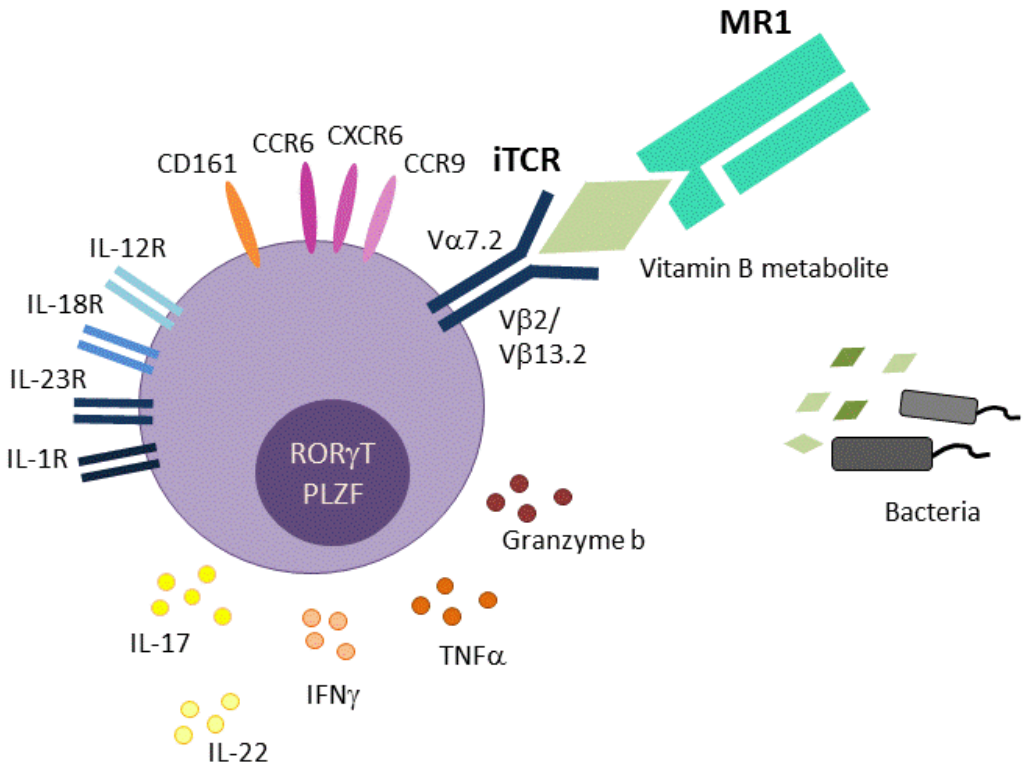


Figure 3.2: MAIT cells respond to bacteria derived vitamin B metabolites.
MAIT cells express a semi-invariant TCR (iTCR) consisting of an invariant α chain (Vα7.2) and varying b chains. They respond to bacterial derived vitamin B metabolites presented on MR1. MAIT cells also express high levels of cytokine receptors, such as IL-12R, IL-18R, IL-23R and IL-1R, as well as the tissue homing receptors CCR6, CXCR6 and CCR9. Moreover, MAIT cells express CD161 and the transcription factors PLZF and RORγT. Upon TCR stimulation or cytokine mediated stimulation, MAIT cells secrete the cytokines IL-17, IL-22, IFNγ and TNFα, and release of cytotoxic granules containing granzyme B enables them to lyse bacterially infected epithelial cells and APCs.
Adapted from Le Bourhis, L. et al. 2013 [376]

3.1.3.5 MAIT cells in the context of disease

3.1.3.5.1 Bacterial infections

MAIT cells not only respond to bacterial-derived antigen and are able to kill bacterially infected cells *in vitro*, but have also been shown to be crucial for anti-bacterial defence in mice and humans *in vivo*.

Murine studies show that MAIT cells are needed to control intraperitoneal infection with *Klebsiella pneumonia* [381] and *Escherichia coli*, as well an intravenous infection with *Mycobacterium abscessus* [347]. Moreover, MAIT cells are critical for early containment of pulmonary infections, and can control pulmonary infection with a live vaccine strain of *Francisella tularensis*, even in the absence of conventional T cells [365, 382]. Besides, MAIT cells are important for the establishment of an intact adaptive mucosal immune response in pulmonary infection models [365, 382].

Moreover, MAIT cells rapidly translocate to sites of infection, as they are decreased in peripheral blood of patients infected with *Mycobacterium tuberculosis* [346, 347], and are recruited to the lungs upon pulmonary infection [364]. Of note, MAIT cells easily become exhausted during infection. For example, MAIT cells in peripheral blood of *Mycobacterium tuberculosis* infected patients show an exhausted phenotype and their cytokine response is impaired [383, 384]. Moreover, MAIT cells are likely to contribute to infection control, as septic patients with low MAIT cell numbers are susceptible to nosocomial infections [385].

3.1.3.5.2 Viral infections

Besides contributing to antibacterial defence, MAIT cells are important during viral infections. In influenza and dengue virus infection, MAIT cells are depleted in peripheral blood and highly activated, and the degree of MAIT cell activation correlates with disease severity in acute dengue infection [386]. Moreover, MAIT cells are severely depleted in peripheral blood of patients with human immunodeficiency virus (HIV) and HCV infection [362, 387-390], despite the lack of infection of $CD8^+$ MAIT cells by HIV [362]. The loss of MAIT cells occurs early

after infection and does not recover after antiretroviral therapy in adults [362, 390, 391]. In contrast, MAIT cell numbers recover after early antiretroviral therapy in perinatally infected children [389]. Moreover, MAIT cells in HIV infected patients show an exhausted phenotype, characterized by loss of effector function and high expression of inhibitory molecules on MAIT cells [390]. Despite these reports on alterations within the MAIT cell compartment in viral infections, the exact contribution of MAIT cells for the establishment, severity and chronicity of viral disease, as well as a potential contribution of MAIT cells to immunopathogenesis in chronic HCV infection remains to be elucidated.

3.1.3.5.3 Metabolic disease

In patients with type 2 diabetes and obesity, MAIT cell frequency is significantly reduced in peripheral blood. Nevertheless, MAIT cells are activated and respond with increased cytokine production upon stimulation with PMA and Ionomycin. Moreover, in obese patients, MAIT cell frequency is increased in adipose tissue, compared to peripheral blood, and the tissue infiltrating MAIT cells produce large amounts of IL-17. Interestingly, MAIT cell frequency is restored in obese patients after bariatric surgery [392, 393].

3.1.3.5.4 Autoimmune diseases

The MAIT cell compartment is altered in various autoimmune diseases. For example, MAIT cell frequency is declined in peripheral blood of patients with the multi-system autoimmune disease systemic lupus erythematosus (SLE) [394], as well as in rheumatoid arthritis, i.e. autoimmune inflammation of the joints [395]. The loss of MAIT cells is accompanied by decreased IFNγ secretion and increased PD-1 expression, whereas IL-17A expression is unchanged [394]. In rheumatoid arthritis, MAIT cells are enriched in synovial fluid, suggesting contribution of MAIT cells to local tissue inflammation [394].

Multiple sclerosis (MS) is characterized by autoimmune damage of myelin, resulting in neurological deficits. The contribution of MAIT cells to the development of MS is controversial [396]. In experimental autoimmune encephalomyelitis (EAE), a murine model of MS, MAIT cells are protective, as overexpression of the murine MAIT TCR results in amelioration of the disease [397]. Contrariwise, a study in humans shows an increased frequency of MAIT cells in peripheral blood of MS patients. These circulating MAIT cells show a pro-inflammatory phenotype characterized by high expression of IL-17, suggesting a pathogenic role for MAIT cells in MS [398]. Moreover, MAIT cells are similar in frequency and cytokine profile in patients with relapsing-remitting MS and healthy volunteers, but express higher levels of tissue-homing receptors specific to the brain [399].

Inflammatory bowel diseases (IBD) include Crohn's disease (CD) and ulcerative colitis (UC), both of which are characterized by chronic inflammation of the gut [400]. Of note, IBD and AILD are strongly associated, and a shared pathogenic mechanism has been proposed [370]. In IBD, MAIT cells are reduced in peripheral blood and accumulate in the inflamed gut mucosa. Furthermore, MAIT cells display a pro-inflammatory phenotype, secreting high amounts of IL-17, whereas IFNγ expression is impaired [361, 401].

Similar to the contribution of MAIT cells to viral pathologies, it remains unclear how MAIT cells mechanistically contribute to the establishment of autoimmune disease and whether their activation in such diseases is protective or rather harmful.

3.1.3.6 MAIT cells in the liver

3.1.3.6.1 MAIT cells in the steady state liver

As discussed above, MAIT cells express liver-specific tissue homing markers [335, 356] and are therefore particularly enriched in the uninfected, non-inflamed liver (20-50% of T lymphocytes), where they reside around the bile ducts [356, 372]. Under such steady state conditions, MAIT cells in the liver (liMAITs) show an effector memory phenotype and predominantly express the TCR co-receptor CD8 [356, 372].

Moreover, liMAITs express a similar gene expression profile to blood-derived MAIT cells (bMAITs), which is clearly distinct from conventional $CD8^+$ T cells [372].
Furthermore, liMAITs are able to respond to bacterial antigen presented on MR1 with IFNγ expression [372]. In response to PMA and Ionomycin stimulation, liMAITs express higher levels of IFNγ, TNFα, IL-2 and IL-17. As discussed above, MAIT cells represent the largest population of IL-17 producing cells in the liver, with MAIT cells representing 60% of $CD3^+$ IL-17-producing cells [372]. In contrast, cytokine expression is much weaker and similar to bMAITs after TCR-mediated stimulation with CD3/CD28 beads [372]. However, TCR mediated cytokine expression can be highly enhanced by addition of IL-7, a cytokine released by hepatocytes [372].
Notably, liMAITs show an activated phenotype characterized by high expression of activation markers, such as CD69, CD38 and HLA-DR, despite being in a non-proliferative state as shown by low expression of Ki67 [372].

3.1.3.6.2 MAIT cells in liver disease

In chronic liver disease, such as NASH, alcoholic liver disease, PBC and PSC, liMAITs are significantly reduced, and, like in healthy livers, are localised around bile ducts. Although $CD8^+$ liMAITs represent the largest subset of MAIT cells in diseased livers, $CD4^+$ MAIT cells are relatively increased, owing to a decrease in $CD8^+$ liMAITs. The liver-homing markers CCR6 and CXCR6, as well as gut-homing molecules, such as CCR9 and integrin α4β7 are expressed to the same extent on liMAITs from diseased and healthy livers. Likewise, cytokine receptor expression is similar on liMAITs from diseased and healthy livers [356].
LiMAITs from diseased livers express high levels of IFNγ and TNFα *ex vivo*, whereas IL-17 expression is moderate. Expression of IFNγ and TNFα can be enhanced by stimulation of liMAITs from diseased livers with bacterial antigen presented by professional APCs or biliary epithelial cells [356].
Furthermore, in HCV infection, MAIT cell frequency in peripheral blood is significantly reduced, with the remaining MAIT cells showing an activated phenotype, as well as high expression of exhaustion markers [402-404]. Notably, circulating MAIT cell frequency does not recover after anti-HCV therapy [403].

3.1.4 Autoimmune liver disease overview

The term AILD comprises three different, clinically distinct diseases: PSC, PBC and AIH. The pathogenesis of these diseases still remains poorly understood and although they are distinct in terms of clinical presentation, histological picture and type of liver damage, they are all characterized by progressive liver tissue fibrosis, eventually leading to cirrhosis and end-stage liver disease [13, 14, 405]. Even though AILD are rare, there is an unmet need for new treatment options as no effective treatment is available at the moment, and AILD result in significant morbidity and mortality [13]. Due to the lack of curative treatment, liver transplantation remains the only treatment option for AILD patients once end-stage liver disease has evolved.

PSC is an immune-mediated cholangiopathy, affecting the medium-sized intra- and extrahepatic bile ducts. Histologically, PSC is characterized by so-called onion-skin like fibrosis, which evolves around the bile ducts and results in bile duct strictures and destruction [406]. In PBC, biliary epithelial cells are the target of autoimmune damage as well, although only the small, intrahepatic bile ducts are affected [407]. In contrast, AIH is characterized by hepatocyte destruction, resulting in hepatitis characterized by portal inflammation and interface hepatitis with lymphocytic infiltrates [408]. While AIH and PBC affect mainly women, PSC occurs predominantly in men, with a male to female ratio of 2:1 [409]. As mentioned, AILD are relatively rare, with PSC having a prevalence of 0-16.2/100,000, the prevalence of PBC is 1.91-40.2/100,000 [409], and the prevalence AIH is 10-15/100,000 [410]. Nonetheless, as AILD are chronic, lifelong conditions, they are associated with significant morbidity and mortality. In PSC for example, the mean time from diagnosis to death or liver transplantation is 9.6-12 years, accompanied by a lifetime risk for cholangiocarcinoma of 10-15% [405]. Notably, successful transplantation is no final cure, as recurrent disease occurs in 8-42% of cases after transplantation, underscoring the need for novel therapies [411].

3.1.4.1 Pathogenesis of AILD

The pathogenesis of AILD is poorly understood. As a vast number of environmental and genetic alterations predispose to the disease, it is likely that environmental

triggers in combination with genetic aberrations in immunoregulatory pathways result in a break-down of self-tolerance in the liver, which leads to uncontrolled autoimmune liver damage [14, 412].

3.1.4.1.1 Genetic variations

Genome wide association studies (GWAS) have revealed a large number of both HLA and non-HLA risk loci for AILD. Thus, HLA-B*08 is associated with and increased risk for PSC, HLA-DR8 with PBC and CDRB1*03-CRB1*04 with AIH [413, 414]. Moreover, variations in genes related to immunological pathways are associated with an increased risk of all three AILD. For example, PBC development is associated with variations in genes regulating antigen presentation and T cell development, like IL-12, IL-12RB and STAT4. In PSC, genes related to autoimmunity, such as IL-2 and IL2RA, but also CD28 are altered, whereas in AIH, SH2B3, a negative regulator of cytokine signalling has been identified as a risk locus [13, 14].

3.1.4.1.2 Environmental factors

Several protective, as well as predisposing environmental factors for the development of AILD have been identified. Protective factors for PSC include smoking and coffee consumption [415]. In PBC, hormonal contraception has been proposed as both protective and harmful [416, 417]. Other predisposing factors for PBC are cigarette smoking, urinary tract and vaginal infections, as well as frequent use of nail varnish [417, 418], while frequent use of antibiotics is a risk factor for the development of AIH [419].

Moreover, molecular mimicry are involved in the initiation and exacerbation of AILD. Molecular mimicry show sequence or structural homology with self-antigens, thus antibodies generated against molecular mimicry become self-reactive. For example, anti-mitochondrial antibodies (AMA), found in a high percentage of PBC patients, recognize a sequence of the pyruvate dehydrogenase complex (PDC–E2), and cross-react with several microbes, including *E. coli*, *Mycoplasma pneumonia* and *Novosphingobium aromaticivorans* [14]. In addition, xenobiotics, like acetaminophen

or compounds used in perfumes, lipsticks and food flavourings can modify self or non-self proteins, so that that they may induce immune responses [14].

3.1.4.1.2 Immunopathogenesis

AILD are strongly associated with IBD, as 60-70% of individuals suffering from PSC also develop UC [420] and PBC often coincides with coeliac disease [370]. Therefore, a common pathogenic mechanism of AILD and IBD has been suggested. AILD are characterized by inflammatory infiltrates dominated by T cells, amongst them IL-17 producing T cells, but also include B cells, NK cells and macrophages [421, 422]. It has been hypothesized that a key pathogenic mechanism of AILD in the context of IBD is the aberrant recruitment of gut-primed lymphocytes to the liver, with consequent establishment of hepatobiliary inflammation [405]. Indeed, in PSC, mucosal T cells activated during colitis are recruited to the liver by a TNFα and VAP-1-induced aberrant expression of the gut-restricted homing receptors mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1) and CCL25 in the liver [423, 424]. Moreover, expression of MAdCAM-1 is increased in patients with AIH and PBC [425, 426]. In addition, increased expression of the homing-receptors in PSC leads to increased recruitment of lymphocytes to the liver, particularly to the biliary epithelium [425, 427].

Furthermore, intestinal inflammation is accompanied by impaired epithelial integrity and increased intestinal permeability, resulting in bacterial translocation from the gut to the liver [428, 429]. However, the liver is not only exposed to intestinal microbes, such as E coli and candida, which are found in the bile of PSC patients [430], but also to microbial products, like endotoxin and bacterial metabolites. In the liver sinusoids, LSEC, HSCs, Kupffer cells, hepatocytes and DCs can recognize such PAMPs and respond with increased cytokine production, leading to recruitment of inflammatory cells to the liver. This contributes to a pro-inflammatory environment favouring the development of liver fibrosis [296, 297, 431].

Moreover, IL-17 producing Th17 cells are implicated in the pathogenesis of AILD. Th17 cells produce pro-inflammatory cytokines, e.g. IL-17 and TNFα, through which they recruit lymphocytes contributing to hepatic inflammation [421, 432-434].

Besides, IL-17$^+$ lymphocytes localise around the bile ducts in PSC patients, where they stimulate biliary epithelial cells to secrete IL-1β, IL-6 and IL-23, which contribute to both Th17 cell maintenance and the development of periductular fibrosis [435, 436]. Moreover, a reduced number of Tregs and impairment of Treg function contribute to the breakdown of tolerance in AILD [437, 438].

3.1.4.2 Therapy

Without treatment, patients with AILD are very likely to develop liver cirrhosis, eventually leading to end-stage liver disease which may require liver transplantation. In PBC, the risk of developing cirrhosis depends on the histological stage at presentation and ranges from 31% when presenting with stage 1 to 68% when presenting with stage 3 [439]. In PSC, the mean time from diagnosis to death or liver transplantation is 9.6-12 years [440, 441]. Moreover, the 5-year survival rate in untreated AIH patients is 50% [405, 442].

Whereas AIH responds to immunosuppression with corticosteroids and other immunosuppressant agents like azathioprine [405], first-line treatment in PBC includes administration of ursodeoxycholic acid (UDCA), which improves bile acid secretion, changes bile acid composition to less toxic properties and protects hepatocytes against bile acid-induced apoptosis [443]. The survival benefit of UDCA in PSC remains unclear, although biochemical improvement can be achieved [444, 445]. Dominant bile duct strictures are common in PSC, as they affect 10-50% of PSC patients and can be treated with endoscopic dilatation [405, 446]. Once end-stage liver disease has occurred, liver transplantation remains the only option for patients with AILD. In Europe, transplantations for AIH represent 2% of all liver transplantations, whereas 4% and 5% of liver transplants are for PBC and PSC, respectively [411].

Novel therapeutic approaches include inhibition of bile-acid secretion by agonists of farnesoid X receptor (FXR), inhibition of lymphocyte recruitment to the liver by CXCL10 blockade or by antibodies against MadCAM-1, CCR9 and integrin α4β7. Moreover, antibodies against IL-12 and IL-23, or the co-stimulatory molecule CD80 are thought to interfere with the immunopathogenesis of AILD [405].

3.2 Material and Methods

3.2.1 Study participants

Human samples used in this study were obtained from patients attending the outpatient clinic for AILD at the Royal Free Hospital NHS Foundation Trust, London, or from healthy volunteers.
Details of the study participants are listed in table 3.1.
Liver tissue was obtained from patients undergoing liver surgery at the Royal Free Hospital NHS Foundation Trust, London, or from healthy explanted livers considered unsuitable for transplantation. Moreover, liver tissue was obtained from explanted PSC livers.

In all cases, the study was fully approved by the Royal Free Hospital ethical board and all study participants gave written informed consent before sample collection. Long-term storage of samples was in accordance with the Data Protection Act 1998 and the Human Tissue Act 2004.

etiology	number of cases	age (years)	male	female	immunosuppression	IBD	fibroscan	fibrosis stage	liver stiffness (kPA)
		median (range)	number (%)	number (%)	number	number	number	number	median (range)
PSC	46	47.5 (24-82)	35 (76)	11 (24)	1	31	45	F0-1: 16	8.9 (2.5 - 75)
					corticosteroid (1)	UC (29)		F2: 10	
						CD (2)		F3: 7	
								F4: 12	
PBC	41	54 (30-78)	2 (5)	39 (95)	1	1	35	F0-1: 17	7.6 (4.3 - 46)
					corticosteroid (1)	CD (1)		F2: 3	
								F3: 6	
								F4: 9	
AIH	21	48 (24 - 71)	7 (33)	14 (67)	20	1	20	F0-1: 9	7.6 (3.5 - 38)
					corticosteroid (14)	UC (1)		F2: 3	
								F3: 5	
								F4: 3	
Healthy	17	31 (25 - 46)	12 (71)	5 (29)	0	0	0	n/a	n/a

Table 3.1: Study participant characteristics

3.2.2 Human primary cell isolations

3.2.2.1 Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral venous blood taken from healthy volunteers or patients with autoimmune liver disease after obtaining informed consent. 30-50ml of blood was taken using 10ml BD vacutainers® containing EDTA. PBMCs were purified immediately by gradient centrifugation using Ficoll-Paque Plus gradient (GE Healthcare) as described previously [447]. Briefly, 25-30ml blood was layered on 12ml Ficoll-Paque Plus gradient solution and centrifuged at 870 x g at room temperature for 20 min using minimal acceleration and break. After centrifugation, the cell layer was collected and washed with RPMI 140 medium (gibco) without supplements (referred to as RPMI in the following) twice at 700 x g for 15min. PBMCs were used for *in vitro* assays or flowcytometric analysis directly, or cryopreserved for later analysis.

PBMCs were frozen down in FBS supplemented with 10% DMSO. To cool down cells to -80°C, Mr. Frosty ™ freezing container (Thermo Scientific) was used. Cells were stored in liquid nitrogen for long term storage.

If required for experiments, cells were thawed rapidly and washed in T cell medium, consisting of RPMI 1640 medium supplemented with 10% FBS, 2mM glutamine, 1 x antibiotics/antimycotics (all Gibco) and 0.25% glucose (Sigma Aldrich).

3.2.2.2 Liver-associated lymphocyte isolation

Liver-associated lymphocytes (LALs) were isolated from cirrhotic liver tissue derived from explanted livers of patients with autoimmune liver disease, or from non-cirrhotic tissue derived from livers unsuitable for transplantation, as well as from wedge sections of patients undergoing liver surgery at the Royal Free hospital. All patients have given informed consent prior to sample collection.

For LAL isolation from cirrhotic tissue, 20g of liver tissue were cut into small pieces and digested with 0.5µg/ml collagenase type IV (Sigma Aldrich) for 20 min at 37° C.

The tissue was then passed through a metal mesh, followed by collagenase type IV digestion (0.5µg/ml) for 10 min at 37° C. Non-cirrhotic tissue was passed through a metal mesh and digested with 0.5µg/ml collagenase type IV at 37° for 10min. The cell suspension obtained from either cirrhotic or non-cirrhotic tissue was then centrifuged at 30 x g for 2 minutes to remove hepatocytes and cell clumps. The supernatant was incubated with DNase I (0.5µg/ml, Sigma Aldrich) for 10 min at 37° C. Afterwards, the cell suspension was washed in T cell medium and LALs were enriched with a Ficoll-Paque Plus and 40% Percoll (both GE healthcare) gradient. 5ml Ficoll-Paque Plus was over layered with 5ml 40% Percoll, which was over layered with 5ml cell suspension and centrifuged for 30 min at 900 x g at room temperature, using minimal acceleration and break. The cell layer was collected and washed by centrifugation in T cell medium. Cells were used for *in vitro* assays or floe cytometric analysis immediately.

3.2.3 Multi-colour flow cytometry

3.2.3.1 Staining of surface markers

PBMCs or purified MAIT cells were stained in a 96 well plate by adding surface marker specific antibodies diluted in PBS (gibco) for 30 min on ice. To exclude dead cells, cells were labelled with live/dead fixable UV dead cell stain kit (Invitrogen) in a 1:1000 dilution for 30 min on ice.
Afterwards, cells were washed (centrifugation at 515 x g 2min, 4° C) and analysed in PBS, or intracellular staining was performed.
All surface antibodies are listed in table 3.2.

3.2.3.1 Staining of intracellular proteins

After staining for surface markers, cells were fixed in 100µl IC fixation buffer (ebioscience) for 30 min at room temperature and washed once in 1x permeabilisation buffer (ebioscience) at 515 x g, 2min, 4° C.
Antibodies were diluted in 1x permeabilisation buffer (ebioscience) and intracellular proteins were stained for 30 min at room temperature. Cells were washed by centrifugation in permeabilisation buffer and resuspended in PBS for analysis.
All intracellular antibodies are listed in table 3.3.

Flow cytometric analysis was conducted by acquisition of cells with LSRFortessa (5L SORP, BD) using the FACSDiva version 6.2 software (BD). Analysis of flow cytometric data was done using FlowJo version 10.0 software.

Antibody	**Fluorochrome**	**Clone**	**Dilution**	**Company**
CD3	FITC	HIT3a	1 : 100	ebioscience
CD4	PE	OKT4	1 : 100	ebioscience
CD4	PE-CF594	RPA-T4	1 : 200	BD
CD8	APC	SK1	1 : 100	ebioscience
CD161	brilliant violet 605	HP-3G10	1 : 150	biolegend
CD161	PE	191B8	1 : 50	Miltenyi
Vα7.2	brilliant violet 421	3C10	1 : 100	biolegend
Vα7.2	PE	3C10	1 : 100	biolegend
CD279 (PD-1)	PE	EH12.2H7	1 : 50	biolegend
HLA-DR	FITC	G46-6	1 : 100	BD
CD69	PE dazzle 594	FN50	1 : 100	biolegend
CD38	PE dazzle 594	HIT2	1 : 50	biolegend
TIM3	PE	344823	1 : 25	R&D systems
CD39	brilliant violet 421	A1	1 : 100	biolegend
CD152 (CTLA-4)	brilliant violet 786	BNI3	1 : 100	BD
CD212 (IL-12Rβ)	APC	2.4 e 6	1 : 25	BD
CD218 (IL-18Rα)	PE	H44	1 : 50	biolegend

Table 3.2: Antibodies for surface markers

Antibody	Fluorochrome	Clone	Dilution	Company
IFN g	APC-Cy7	4S.B3	1 : 100	biolegend
IL-17A	PerCP/Cy5.5	BL168	1 : 100	biolegend
IL-22	PE-Cy7	22URTI	1 : 100	ebioscience

Table 3.3: Antibodies for intracellular proteins

3.2.3.2 Determining absolute MAIT cell numbers

To determine absolute numbers of MAIT cells, venous blood was taken from patients with AILD and healthy volunteers in 5ml BD Vacutainers® containing EDTA. For analysis, 300ul blood was transferred to a polystyrene tube and red blood cell lysis was performed. 1ml lysis buffer (homemade, 16.58g NH_4Cl, 2g $KHCO_3$, 74.4mg EDTA, 2000ml H_2O, pH 7.2-7.4) was added for 5 min. Afterwards, the cell suspension was washed and cells were resuspended in 1ml PBS (gibco) for surface staining with MAIT cell specific antibodies (as described in chapter 3.2.3.1).
Shortly before acquisition at BD LSRFortessa, 50µl CountBright™ Absolute Counting Beads (Molecular Probes) were added to the cell suspension. A minimum of 1000 beads were acquired and absolute cell number was determined after analysis with FlowJo version 10.0 as follows:

$$\text{Cells}/\mu l = A/B \times C/D$$

A = number of MAIT cell events in gate
B = number of bead events in gate
C = assigned bead count of the lot (beads/50µl)
D = volume of the sample (µl)

3.2.4 Cell sorting using magnetic beads

Magnetic bead cell separation is a cell sorting method, which allows for surface-marker based separation of cells from a single cell suspension. Cells are labelled with a specific antibody, which is attached to a paramagnetic bead and can be separated by application of a magnetic field.

To separate MAIT cells, PBMCs were labelled with phycoerythrin (PE) coupled anti-human Vα7.2 antibody (clone 3C10, biolegend), diluted 1:100 in MACS buffer (PBS, supplemented with 1% and 2mM EDTA) for 30 min on ice.

After washing cells twice (515 x g, 7min), anti-PE beads (Miltenyi biotec) were added to the labelled cells in MACS buffer (2µl beads per 1 x 10^6 total cells) and incubated for 15 min at 4° C. Cells were counted as described in chapter 2.2.2. To remove free beads, cells were washed at 289 x g, 5min and resuspended in 500µl MACS buffer.

Subsequently, cells were enriched using separation columns (MS columns, Miltenyi biotec) for positive selection, attached to a magnetic stand. After pre-loading MS columns with 500µl MACS buffer, the cell suspension was applied on the columns. While unlabelled cells pass through, the labelled cells are retained in the column, which is subsequently washed three times with 500µl MACS buffer. The columns were then removed from the magnetic stand and the cell suspension was recovered from the columns by flushing with 1ml MACS buffer.

Cell were washed in MACS buffer and used for experiments immediately.

3.2.5 *In vitro* stimulation of MAIT cells

For *in vitro* stimulation assays, 1 x 10^6 PBMCs or LALs, or 0.2 x 10^6 magnetic bead-sorted blood-derived MAIT cells, were stimulated and cultured in T cell medium. Cells were counted as described in chapter 2.2.2. T cell medium consisted of RPMI 1640 medium supplemented with 10% FBS, 2mM glutamine, 1 x antibiotics/antimycotics (all gibco) and 0.25% glucose (Sigma Aldrich).

Cells were stimulated for 16-72 hours with either 50ng/ml phorbol 12-myristate 13-acetate (PMA) and 1μM Ionomycin (both Sigma Aldrich), or different combinations of the cytokines IL-1β (R&D systems), IL-12 (Miltenyi) and IL-18 (R&D systems), 50ng/ml each.

For repetitive stimulation, cells were stimulated with different combinations of IL-1β, IL-12 and IL-18 (50ng/ml each). Every 24 hours, the medium was replaced and cytokines (50ng/ml) were added for up to 72 hours.

In order to inhibit secretion of cytokines and chemokines, 1x Brefeldin A and Monensin (both ebioscience) were added to the culture for the last 4 hours of stimulation.

All *in vitro* stimulation assays were incubated under standard conditions at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

After *in vitro* stimulation, PBMCs were washed and used for flow cytometric analysis. Staining of surface markers and/or intracellular proteins was performed according to the protocol described in chapter 3.2.3.

3.2.6 Co-culture of MAIT cells and HSCs

In order to analyse HSC proliferation in the presence of stimulated and unstimulated MAIT cells by BrdU proliferation assay (Roche, see chapter 2.2.5), HSCs were isolated as described in chapter 2.2.1.1. and cultured in a flat bottom 96 well plate at 0.006×10^6 HSCs per well in complete HSC medium for 24 hours. Cells were counted as described in chapter 2.2.2.

PBMCs were isolated from peripheral blood of healthy controls (see chapter 3.2.1.1) and purified by magnetic bead separation (see chapter 3.2.4). MAIT cells were added to HSCs in different ratios in T cell medium. Moreover, anti-CD3/CD28 beads (Dynabead® Human T-activator CD3/CD28, Invitrogen) were added in a ratio of MAIT cell: anti CD3/CD28 beads = 1:1 and BrdU labelling solution was added to the culture as indicated in the manufacturer's protocol.

After 48 hours of co-culture, supernatants and MAIT cells were removed and HSCs were washed extensively with Hank's Balanced Salt Solution (HBSS, gibco).

Afterwards, the BrdU assay was developed according to the manufacturer's protocol and absorbance was measured at 370nm was measured photometrically, using a Fluostar Omega Plate Reader (BMG labtech).

3.2.7 Liver stiffness measurement by transient elastography

In order to measure liver stiffness, transient elastography was performed using FibroScan® (Echosens). The 5 MHz ultrasound transducer probe was placed perpendicularly to the skin between the ribs over the right lobe of the liver, with the patient lying in dorsal decubitus with the right arm in maximal abduction. All patients underwent the test after a fasting period of at least 3 hours. The M or XL probe was used according to patients' physical constitution. Liver stiffness was expressed in kilopascal (kPa) and a test was considered valid, when at least 10 successful measurements with a success rate of at least 60% and an interquartile range (IQR) <30% were obtained.

The fibrosis stage was defined according to the previously described cut-offs of liver stiffness, with F0-1: stiffness <7.1kPa, F2: 7.1-9.4 kPa, F3: 9.5-12.4 kPa, F4: >12.5kPa [37, 38].

3.3 Results

3.3.1 MAIT cells are significantly reduced and phenotypically altered in patients with AILD

MAIT cells belong to the family of innate-like T cells and express a semi-invariant TCR which enables them to respond to bacterial-derived metabolites of folic acid and riboflavin [339, 448]. Besides protecting against bacterial infections [365], MAIT cells have recently been shown to be altered in HIV infection [362, 390], as well as in various non-infectious and autoimmune diseases, including obesity, type 2 diabetes [392], multiple sclerosis [396], type 1 diabetes [449] and IBD [361]. For example, MAIT cell frequency is significantly reduced in peripheral blood of patients with IBD, whereas activated MAIT cells accumulated in the inflamed intestinal mucosa [361]. Moreover, MAIT cells have recently been shown to be changed in chronic liver disease. In particular, a significant decline of both circulating and liver-resident MAIT cells was observed in patients with alcoholic liver disease (ALD), NASH, PSC and PBC [356]. In this context, it should be taken into account that Jeffery et al. were investigating a rather small number of samples from patients with PSC and PBC, which served as an example of chronic liver disease amongst other aetiologies. Besides, no samples from patients with AIH were investigated in their study [356]. Furthermore, MAIT cells are significantly decreased in peripheral blood of patients with chronic HCV infection [388, 402, 403].

These data suggest that alterations of MAIT cell frequency and phenotype in these disease states might contribute to disease development and/or progression, although this has not been investigated yet. Furthermore, considering the rather small sample size in the study of Jeffery et al. [356], it remains unclear whether similar changes in the MAIT cell compartment are observed for other liver diseases such as AILD.

To test whether MAIT cell frequency or phenotype are altered in patients with AILD, i.e. PSC, PBC and AIH, MAIT cells were isolated from peripheral blood of patients with AILD and healthy controls, and assessed by flow cytometric analysis. All patients were recruited from the AILD clinic at the Royal Free hospital NHS foundation trust, London UK and blood was collected after obtaining informed consent. Patient characteristics are illustrated in table 3.1.

As described before [450], MAIT cells were identified within the $CD3^+$ T cell compartment by staining of the invariant MAIT-TCR α chain Vα7.2, in combination with high expression of CD161, a C-type lectin (figure 3.3a), hereafter referred to as $CD3^+$ $V\alpha7.2^+$ $CD161^{++}$ cells or MAIT cells. As shown in figures 3.3b and 3.3c (see also [335, 343]), MAIT cells comprise about 1-10% of $CD3^+$ T cells in healthy individuals. Interestingly, both frequency (figures 3.3b and 3.3c) and absolute number (figure 3.3d) of MAIT cells were significantly reduced in peripheral blood of patients with PSC, PBC and AIH. This decline in cell numbers was specific to MAIT cells, but not conventional T cells, as $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell numbers were unchanged in patients compared to healthy controls (figure 3.3e). Notably, no significant difference was observed in MAIT cell frequency (figure 3.3c) and absolute number (figure 3.3d) between patients with PSC, PBC and AIH.

These data suggest that MAIT cells are reduced in peripheral blood of AILD patients, similar to the alterations observed for other liver diseases such as HCV [356, 402, 403].

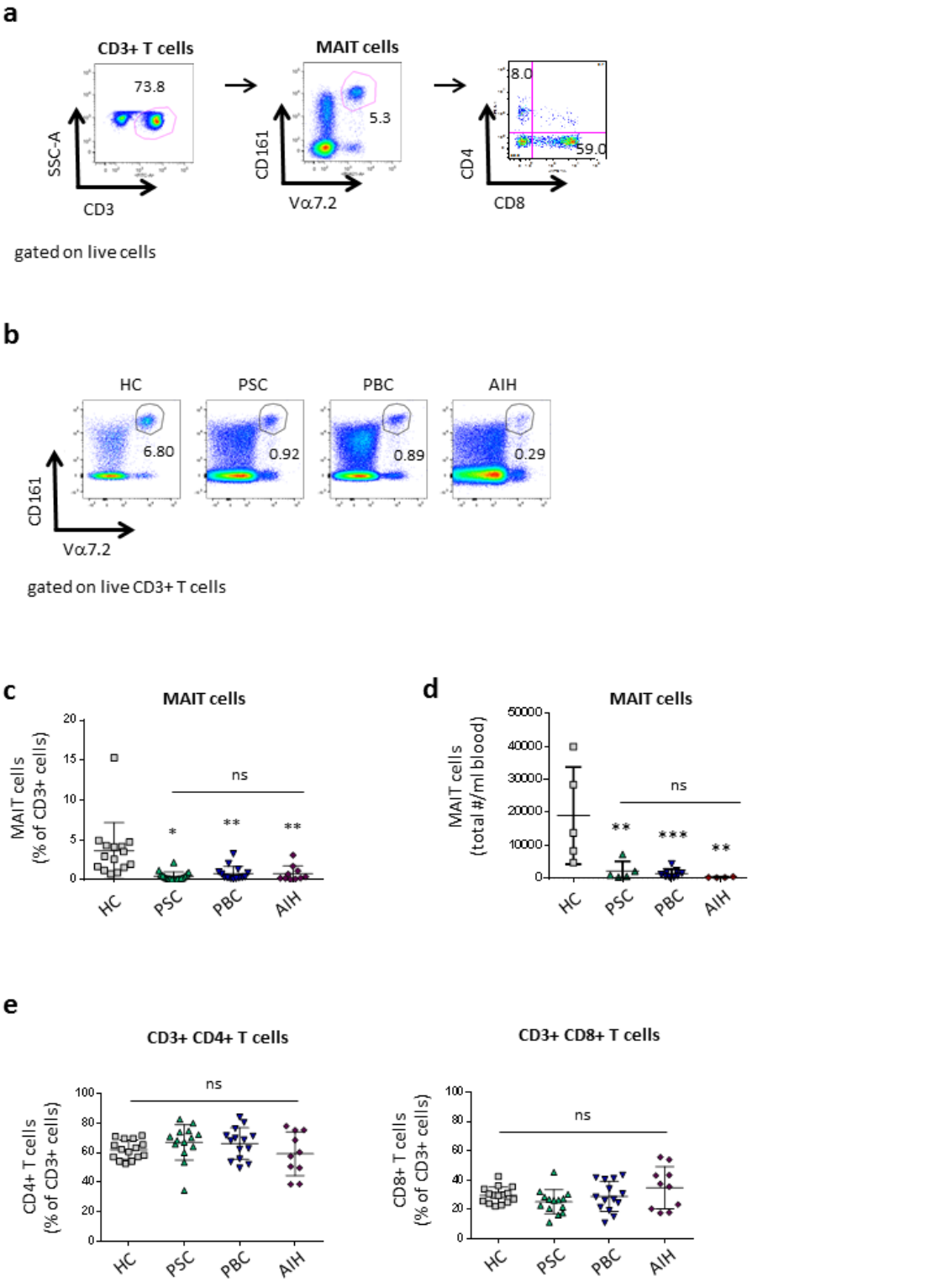


Figure 3.3: MAIT cell frequency is significantly reduced in peripheral blood of patients with AILD.

PBMCs were isolated from peripheral blood of healthy controls and analysed by flow cytometry *ex vivo*. (a) Gating strategy for MAIT cells. MAIT cells are defined as live $CD3^{+}$ $CD161^{++}$ $V\alpha7.2^{+}$ cells. (a) – (b) Representative flow cytometry plot and (c)

summary data of MAIT cell frequency in peripheral blood. (d) Absolute number of MAIT cells/ml in peripheral blood. (e) Frequency of conventional $CD3^+CD4^+$ cells and $CD3^+CD8^+$ T cells, summary data. In (c) – (e) each symbol represents data from one individual patient. Data represent mean +/- SD *p< 0.05, **p<0.01, ***p<0.001 vs. healthy controls, ns = not significant, pooled data from 4 independent experiments. HC = healthy control

All AILD are characterized by the development of liver fibrosis and cirrhosis [13, 14]. The degree of liver fibrosis and cirrhosis can clinically be assessed non-invasively through measuring the liver stiffness by transient elastography. It has been shown that liver stiffness correlates with the degree of fibrosis and kPa values are commonly used to categorize patients into four different stages of fibrosis, ranging from no or mild fibrosis (F0 - F1) to cirrhosis (F4) [37, 38]. Interestingly, the frequency of MAIT cells in peripheral blood of patients with AILD negatively correlated with the fibrosis stage (figure 3.4a). However, no significant correlation between MAIT cell number and liver stiffness was observed in the same patient cohort (p=0.0607) (figure 3.4b). This suggests that the number of MAIT cells in peripheral blood declines with disease progression in AILD.

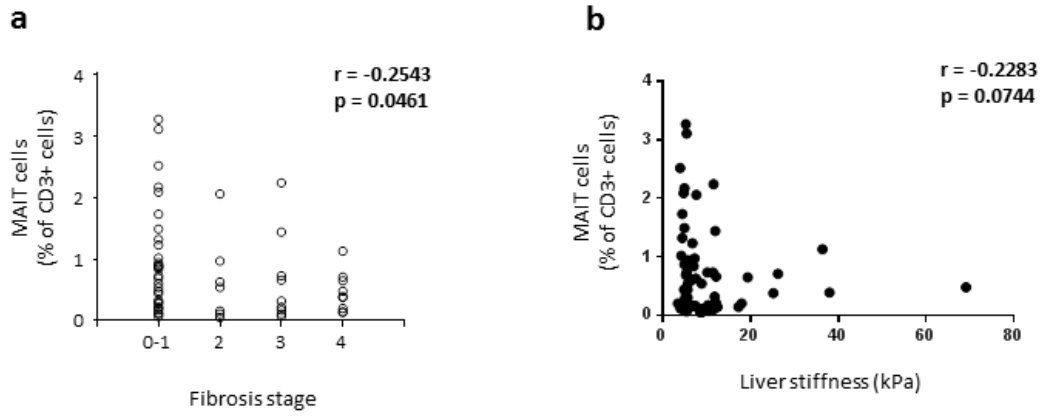


Figure 3.4: MAIT cell frequency in peripheral blood of patients with AILD negatively correlates with the fibrosis stage.
PBMCs were isolated from peripheral blood of patients with AILD and MAIT cell frequency was analysed by flow cytometry. MAIT cells were defined as living $CD3^+$ $CD161^+$ $V\alpha7.2^+$ cells and Spearman Rank correlation between MAIT cell frequency and (a) fibrosis stage (F0-1 = no or mild fibrosis: stiffness <7.1kPa, F2 = moderate fibrosis: 7.1-9.4 kPa, F3 = severe fibrosis: 9.5-12.4 kPa, F4 = cirrhosis: >12.5kPa) and (b) liver stiffness, both assessed by transient elastography, was analysed. Pooled data from >5 experiments, n = 60. Each symbol represents data from one individual patient.

MAIT cells can be divided into four different subsets based on expression of the TCR co-receptors CD4 and CD8. In healthy humans, the majority of MAIT cells express the TCR co-receptor CD8 or are double negative for CD4 and CD8 expression, with only a small number of MAIT cells being $CD4^+$ or CD4/CD8 double positive (figure 3.3a, see also [343, 367, 450]). In AILD, all subsets of MAIT cells were significantly reduced in peripheral blood, compared to healthy controls (figure 3.5a). Of note, a relative increase of $CD4^+$ MAIT cells was observed in patients with AILD, which was significant in patients with PSC and marked, but non-significant in patients with PBC and AIH (figure 3.5b).

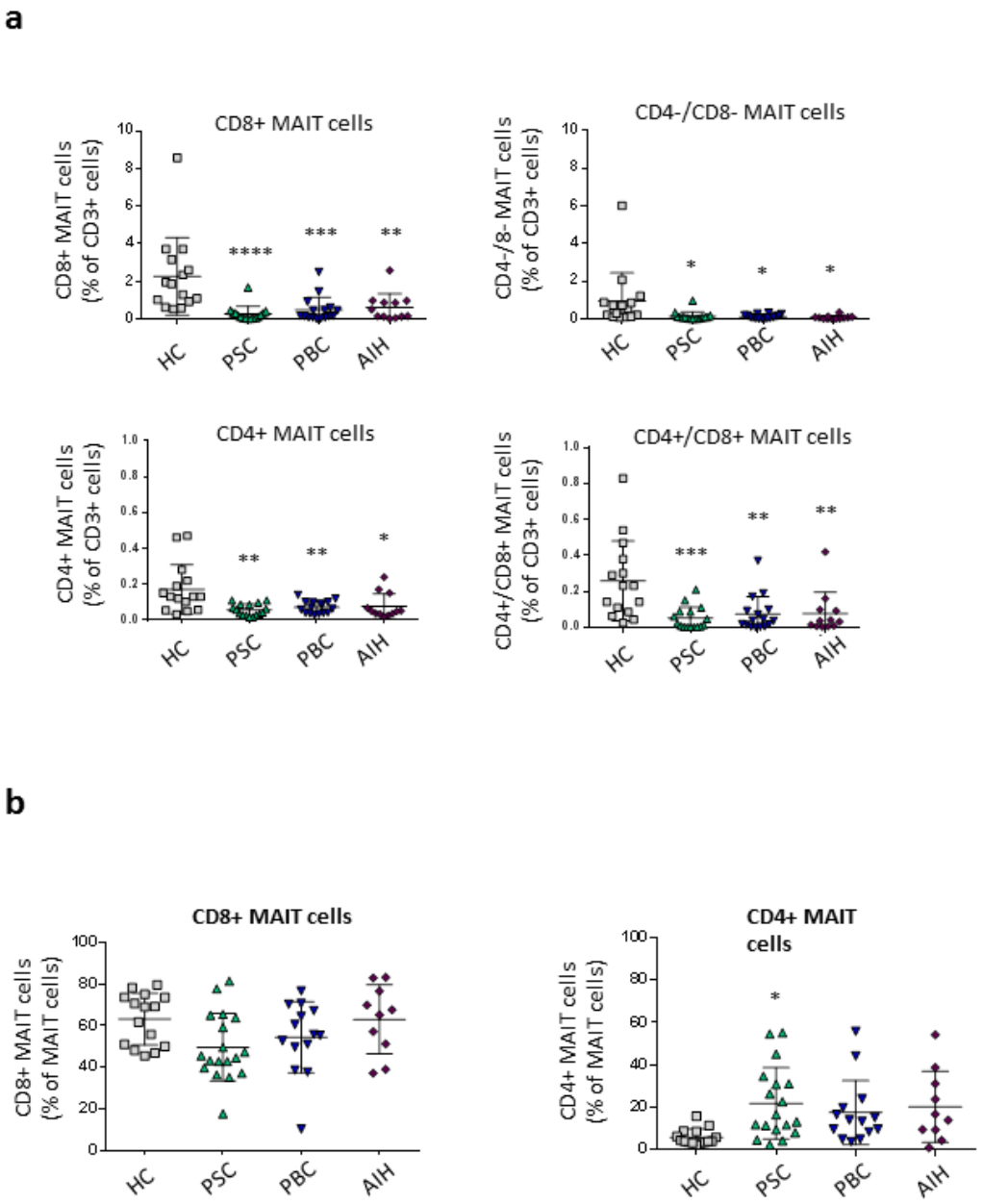


Figure 3.5: CD4$^+$ MAIT cells are relatively increased in peripheral blood of patients with AILD.

PBMCs were isolated from peripheral blood and analysed by flow cytometry *ex vivo*. (a) Frequency of CD4$^+$, CD4$^-$/CD8$^-$, CD8$^+$ and CD4$^+$/CD8$^+$ MAIT cells as % of CD3$^+$ cells in peripheral blood, summary data. (b) Frequency of CD8$^+$ and CD4$^+$ MAIT cells as % of MAIT cells, summary data. (a)- (b) Each symbol represents data from one individual. Data represent mean +/- SD *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. healthy controls, pooled data from 5 independent experiments, HC = healthy control

MAIT cells have been shown to express IL-12 receptor (IL-12R) and high levels of IL-18 receptor (IL-18R) [356, 373], making them susceptible to cytokine-mediated, TCR-independent activation by cytokines such as IL-12 and IL-18 [373].

To analyse expression levels of IL-12R and IL-18R on MAIT cells from patients with AILD, PBMCs were isolated from peripheral blood of AILD patients and healthy controls, and IL-12R and IL-18R expression was analysed by flow cytometry. As depicted in figure 3.6, MAIT cells from patients with PSC, PBC and AIH showed high surface expression of both IL-12R (figure 3.6a) and IL-18R (figure 3.6b), at levels similar to healthy controls.

Taken together, these data demonstrate that MAIT cells are severely reduced in peripheral blood of patients with AILD and phenotypically altered with a relative increase of $CD4^+$ MAIT cells. Moreover, the decline of MAIT cells is more pronounced in patients with advanced disease.

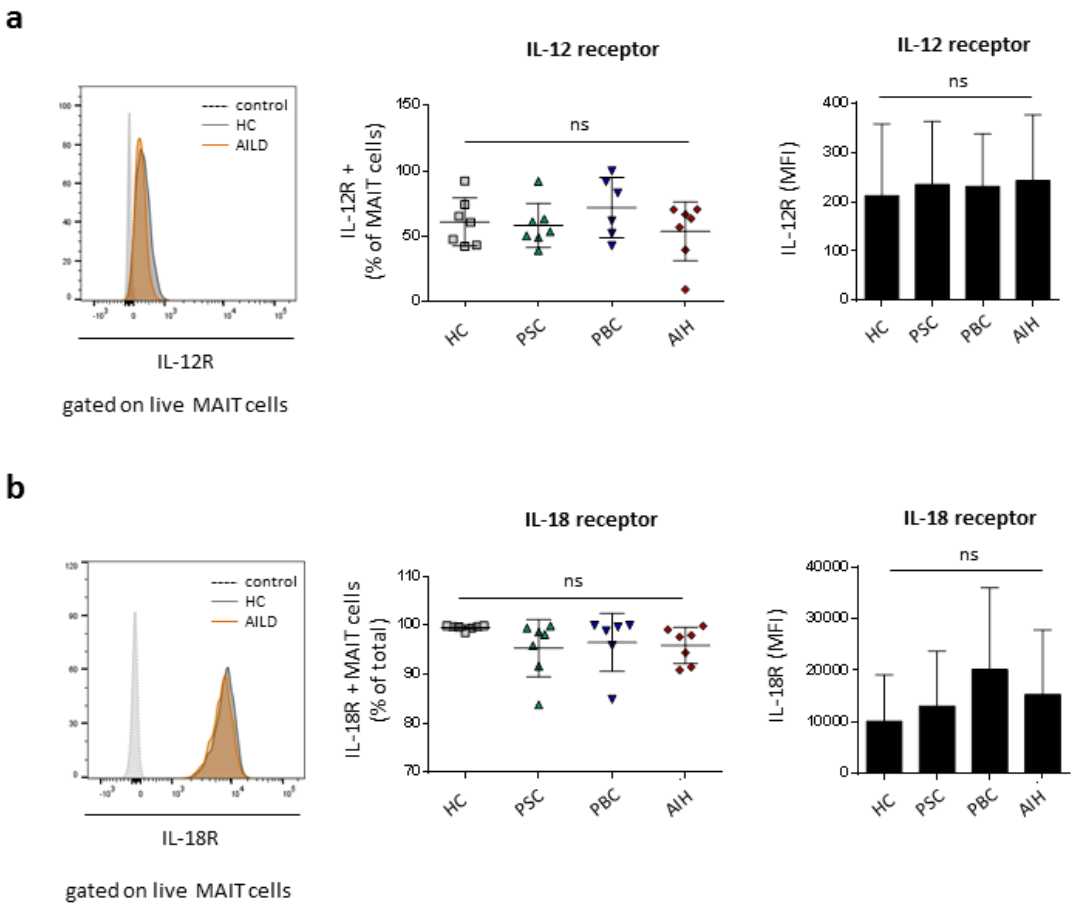


Figure 3.6: IL-12R and IL-18R surface expression is unchanged in MAIT cells from peripheral blood of patients with AILD.
PBMCs were isolated from peripheral blood and analysed by flow cytometry *ex vivo*. (a) IL-12R and (b) IL-18R expression in MAIT cells. Representative flow cytometry plots, summary data and mean fluorescence intensity (MFI). Each symbol represents data from one individual. Data represent mean +/- SD, ns = not significant, HC = healthy control.

3.3.2 MAIT cells from patients with autoimmune liver disease show features of chronic activation and exhaustion

It has previously been reported that MAIT cells are significantly reduced in peripheral blood of patients with chronic HIV and/or HCV infection, similar to the decline of circulating MAIT cells in patients with AILD described here (figure 3.3). In addition

to these quantitative changes, the reduced numbers of MAIT cells found in HIV patients seem to be functionally exhausted as a result of chronic activation, evident by a reduced ability to produce the cytokine IFNγ in response to stimulation and elevated expression of activation markers such as CD38 and HLA-DR as well as upregulation of inhibitory receptors, e.g. PD-1, TIM-3 and CLTA-4 [390, 402]. However, whether MAIT cells in AILD patients show a similar degree of exhaustion or remain unaffected despite their reduced number has not been investigated so far. Therefore, MAIT cells isolated from peripheral blood of patients with AILD were probed for features of exhaustion.

As T cell exhaustion is a stepwise process characterized by loss of effector function, followed by upregulation of inhibitory cell surface molecules and apoptotic cell death [451], surface expression of the inhibitory molecules PD-1, CTLA-4 and TIM-3 on MAIT cells from AILD patients and healthy controls was measured by flow cytometry. Whereas surface expression of PD-1 was comparable in MAIT cells from patients and healthy controls, levels of CTLA-4 and TIM-3 were higher in MAIT cells from patients with AILD (figures 3.7a and 3.7b). Interestingly, CD39, which has recently been described as a marker of terminal and irreversible $CD8^+$ T cell exhaustion [452], was also highly expressed in MAIT cells from patients with AILD, compared to healthy controls (figures 3.7a and 3.7b). Similar to an increased expression of markers typically associated with exhaustion, MAIT cells isolated from peripheral blood of patients with AILD showed significantly higher levels of the activation marker CD38, and marked but non-significant upregulation of CD69 and HLA-DR expression (figures 3.7c and 3.7d).

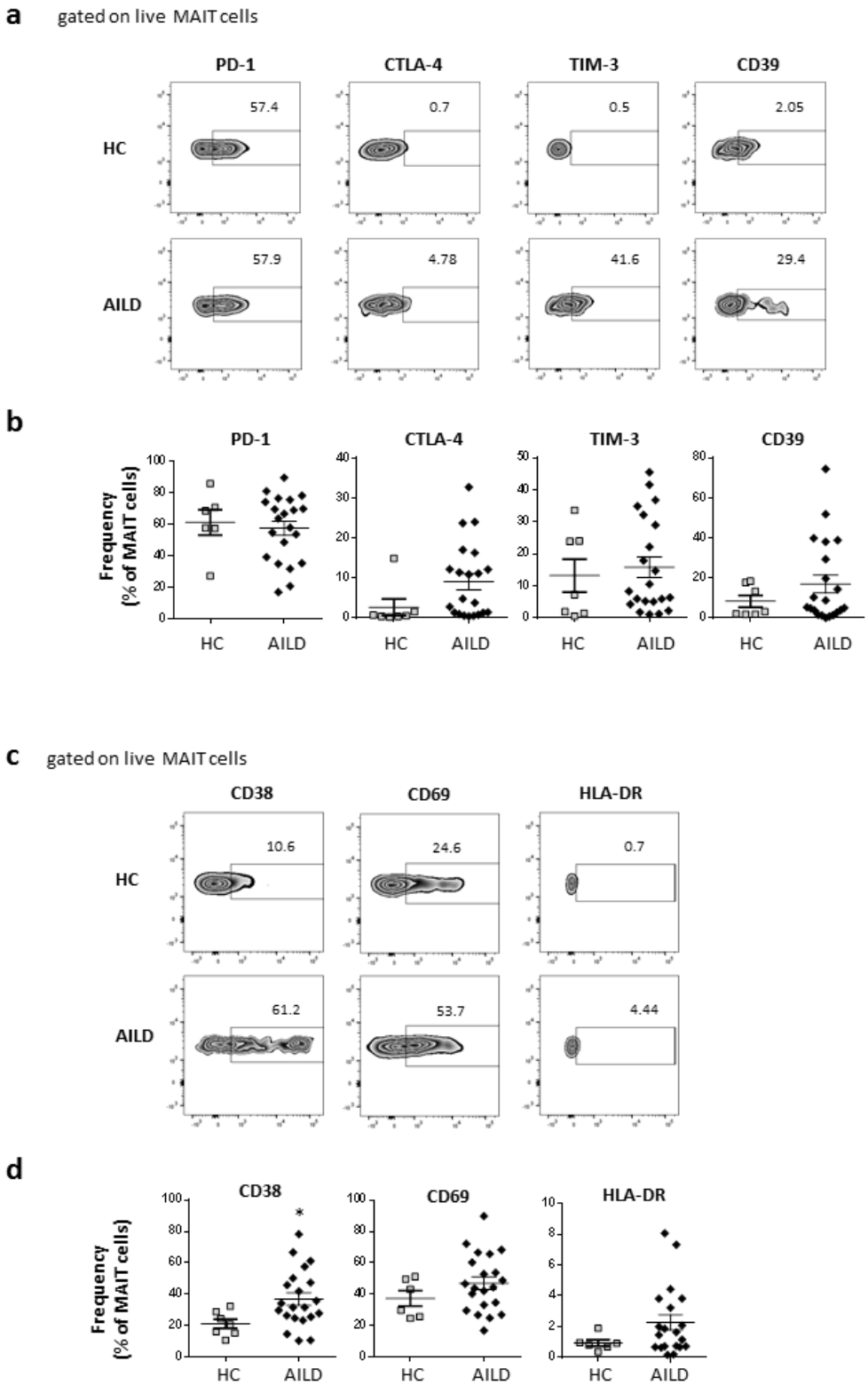


Figure 3.7: Inhibitory receptors and activation markers are upregulated in MAIT cells from peripheral blood of patients with AILD.

PBMCs were isolated from peripheral blood and analysed by flow cytometry *ex vivo*. (a) Representative flow cytometry plots and (b) summary data of PD-1, CTLA-4, TIM-3 and CD39 expression in MAIT cells. (c) Representative flow cytometry plots

and (d) summary data of CD38, CD69 and HLA-DR expression in MAIT cells. (a)-(d) Pooled data of patients with PSC (n=7), PBC (n=7) and AIH (n=7), each symbol represents data from one individual. Data represent mean +/- SEM *p< 0.05 vs. healthy controls, pooled data from 3 independent experiments. HC = healthy control, AILD = autoimmune liver disease

Moreover, analysis of the correlation between exhaustion marker expression and loss of circulating MAIT cells revealed a negative correlation between CTLA-4 (figure 3.8a), TIM-3 (figure 3.8b) and CD39 (figure 3.8c) expression and MAIT cell frequency in patients with AILD. These data indicate that the reduced frequency of circulating MAIT cells in patients with AILD might result from exhaustion.

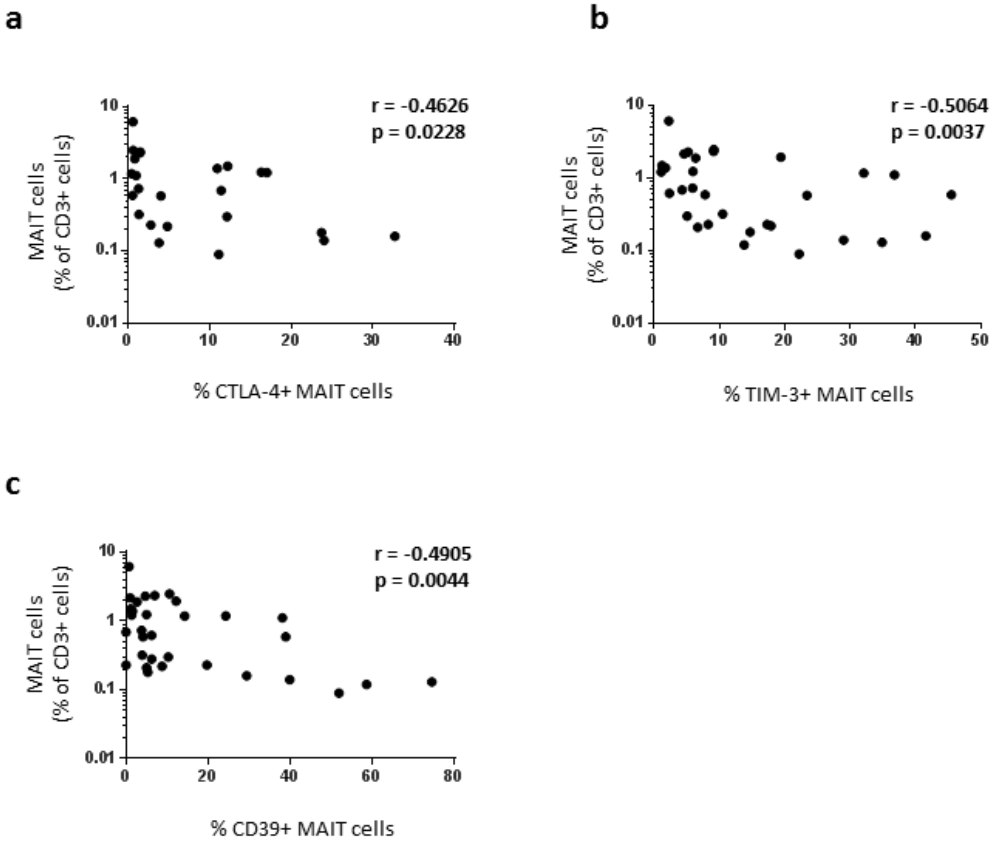


Figure 3.8: Expression of exhaustion markers negatively correlates with MAIT cell frequency in peripheral blood of patients with AILD.
PBMCs were isolated from peripheral blood of patients with AILD. *Ex vivo* expression of CTLA-4 TIM-3 and CD39 was in MAIT cells was analysed by flow cytometry. MAIT cells were defined as living $CD3^+$ $CD161^+$ $V\alpha7.2^+$ cells and Spearman Rank correlation between MAIT cell frequency and (a) CTLA-4, (b) TIM-3 and (c) CD39 was analysed. Pooled data from 5 experiments. Each symbol represents data from one individual patient.

To investigate cytokine production of MAIT cells from peripheral blood of patients with AILD in response to unspecific stimulation, PBMCs from patients with AILD and healthy controls were stimulated with PMA (50ng/ml) and Ionomycin (1μM) for 16 hours *in vitro,* and MAIT cell cytokine expression was measured by intracellular cytokine staining and flow cytometric analysis.

Whereas IL-17A production in response to such stimulation remained unchanged in MAIT cells from patients with AILD compared to healthy controls (figure 3.9a), IFNγ production was significantly impaired in MAIT cells from patients with PBC, and

markedly, but not significantly, reduced in MAIT cells from patients with PSC and AIH (figure 3.9b).

These data strongly suggest that MAIT cells in patients with AILD are not only reduced in their total number, but are also highly activated and become functionally exhausted.

In this study, IFNγ expression of MAIT cells has been employed in order to determine MAIT cell effector function. It is, however, important to note that this represents only one way of measuring MAIT cell effector function. MAIT cells express a variety of effector cytokines, amongst them TNFα and IL-2 [335]. In hindsight, a more unbiased and wider approach to test MAIT cell effector function in the context of AILD could be the analysis of a large number of cytokines, for example using flow cytometry based cytokine bead arrays. Moreover, testing the ability of MAIT cells to lyse and kill bacterially infected cells could give a more functional insight to MAIT cell effector function and could be addressed in in future experiments.

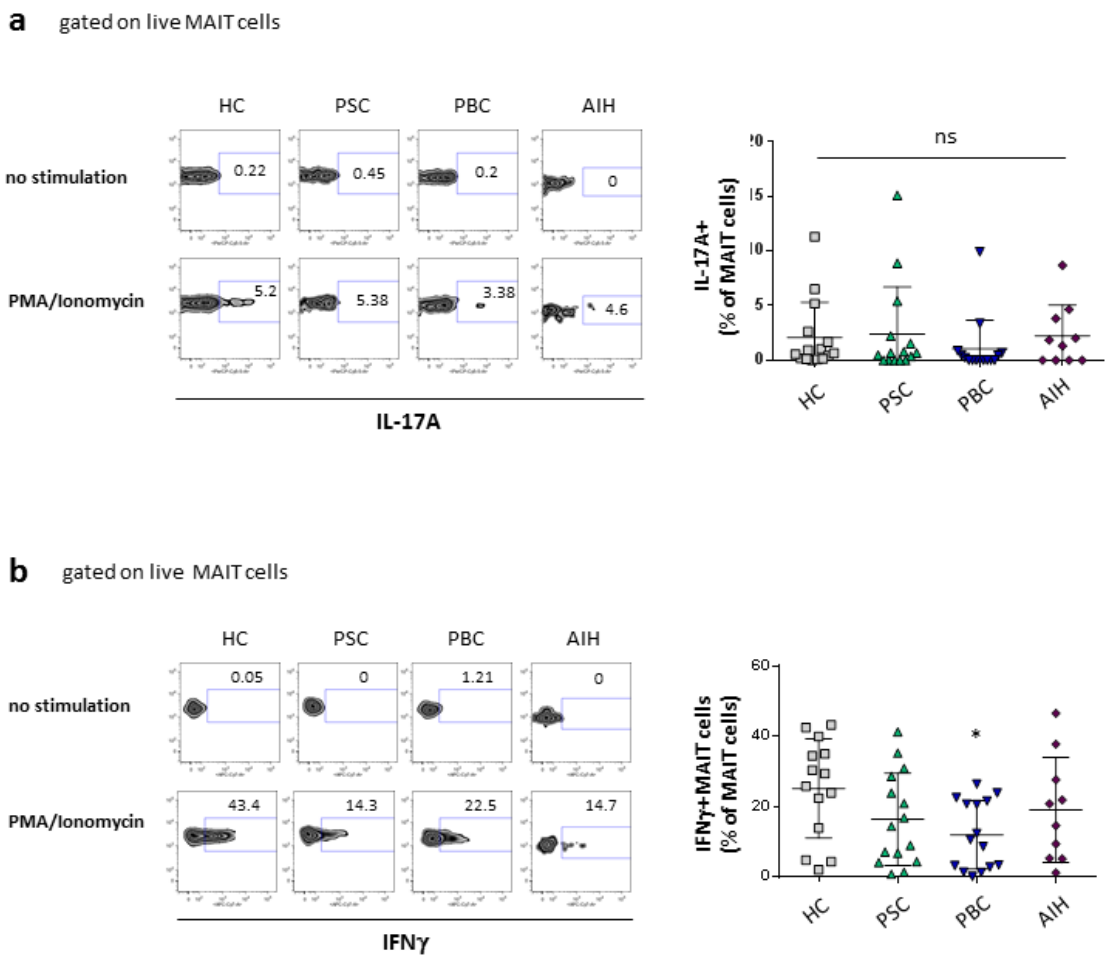


Figure 3.9: IFNγ response to PMA and Ionomycin stimulation is impaired in MAIT cells from peripheral blood of patients with AILD.
PBMCs were isolated from peripheral blood and stimulated with PMA (50ng/ml) and Ionomycin (1μM) for 16 hours. Intracellular cytokine expression was analysed by flow cytometry. Representative flow cytometry plots and summary data of (a) IL-17A expression and (b) IFNγ expression in MAIT cells. Each symbol represents data from one individual. Data represent mean +/- SD *p< 0.05 vs. healthy controls, pooled data from 4 independent experiments, HC = healthy control.

3.3.3 Repetitive stimulation of MAIT cells changes their cytokine expression pattern

MAIT cells have been shown to recognize vitamin B derived microbial metabolites presented on the MHC-like molecule MR1 [339, 448], allowing for MAIT cell activation by antigen derived from different species of bacteria *in vivo* [347, 364] and

in vitro [347, 371, 453]. Such MR1 mediated MAIT cell activation results in secretion of pro-inflammatory cytokines, e.g. IFNγ, IL-17A and TNFα [356, 373, 453]. Furthermore, MAIT cells are able to release the content of cytotoxic granules containing the proteins perforin and granzyme B, which enables them to lyse bacterially infected epithelial cells and APCs [371, 380]. In addition to MR1:TCR-mediated activation, the expression of IL-12R and IL-18R by MAIT cells allows for TCR-independent stimulation by the cytokines IL-12 and IL-18 which leads to IFNγ secretion [373]. To our knowledge, to date there is no evidence of IL-17A secretion by MAIT cells following cytokine mediated stimulation.

Interestingly, pro-inflammatory cytokines, such as IL-12, IL-18 and IL-1β have been shown to play an important role for the development of liver fibrosis. In particular, IL-12 has been shown to contribute to fibrosis development in a murine model of autoimmune biliary cirrhosis [454, 455], and IL-18 contributes to acute and chronic liver injury in several murine models [456-458]. Moreover, IL-1β is contributing to the development of liver fibrosis through HSC activation and recruitment of inflammatory cells [458, 459]. Besides, IL-1β has been shown to induce IFNγ secretion in NK cells in combination with IL-12 [460] and is a critical regulator of IL-17 secretion in Th17 cells [461].

Therefore, it was tested whether different combinations of the cytokines IL-1β, IL-12 and IL-18 can induce IFNγ and IL-17A production in MAIT cells. PBMCs isolated from healthy controls were stimulated for 24h with different combinations of IL-1β, IL-12 and IL-18 (50ng/ml each) and IFNγ by intracellular staining, followed by flow cytometric analysis. Indeed, IL-12+IL-18 specifically induced a robust IFNγ response in MAIT cells, however addition of IL-1β did not enhance IFNγ production in MAIT cells (figures 3.10a and 3.10c). None of the other cytokine combinations, or IL-1β, IL-12 and IL-18 alone were able to evoke IFNγ production in MAIT cells (figures 3.10a and 3.10c). Interestingly, IL-1β, IL-12 and IL-18 neither alone, nor in combination, were sufficient to induced IL-17A production in MAIT cells after 24 hours of stimulation (figures 3.10b and 3.10d).

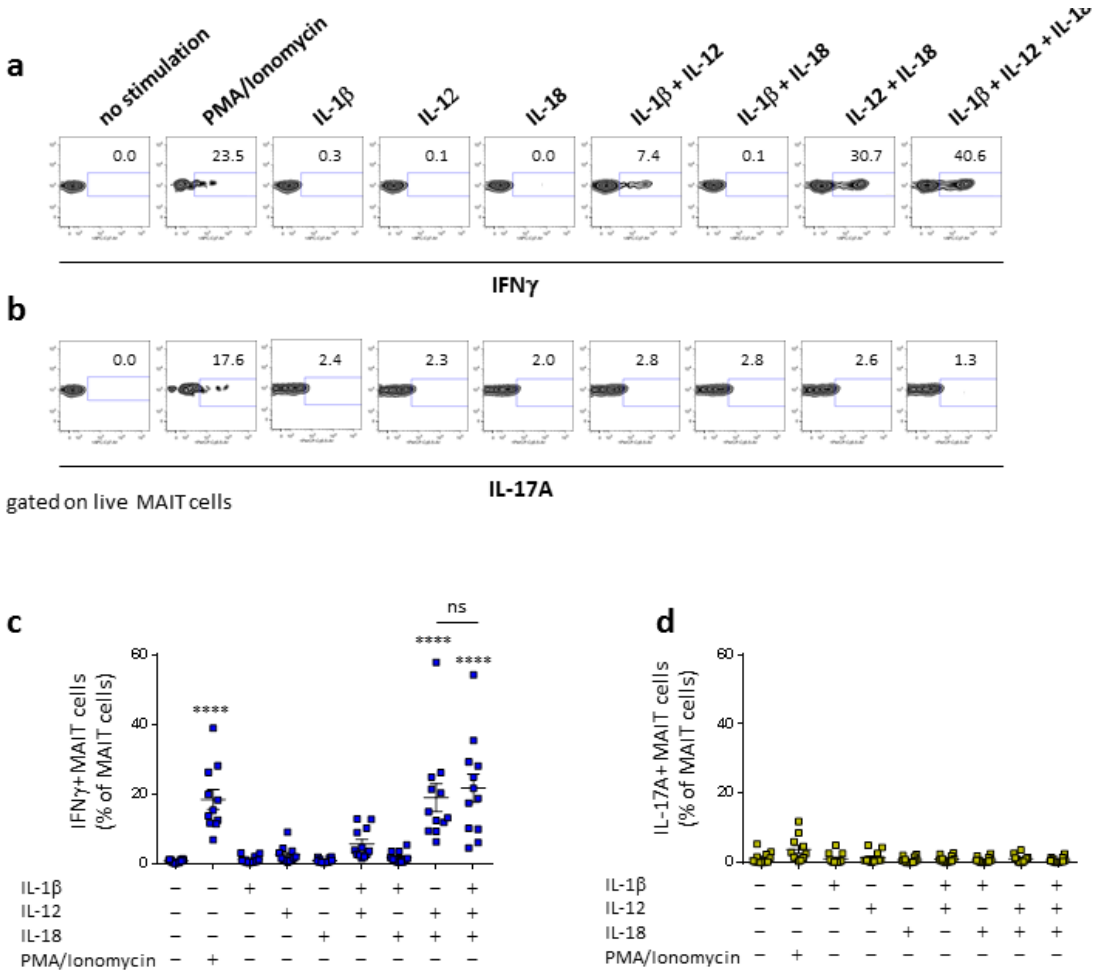


Figure 3.10: IL-12 + IL-18 +/- IL-1β specifically induces IFNγ but not IL-17 expression in MAIT cells.
PBMCs were isolated from peripheral blood of healthy controls and stimulated with PMA (50ng/ml) and Ionomycin (1μM) or IL-1β, IL-12, and IL-18 (50ng/ml each) for 24 hours, intracellular cytokine expression was analysed by flow cytometry. (a) IFNγ and IL-17A expression in MAIT cells, representative flow cytometry plots (b) IFNγ and IL-17A expression in MAIT cells, summary data, each symbol represents data from individual. Data represent mean +/- SEM ****p<0.001 vs. no stimulation, ns = not significant. Pooled data from 4 independent experiments.

To exclude bystander effects from other immune cells in the stimulated PBMC pool on the induction of the IFNγ response in MAIT cells, magnetic cell separation employing a Vα7.2 antibody was used to purify MAIT cells from PBMCs [453]. Using this method, a purity of >80% could be achieved (figure 3.11a). Of note, this technique does not result in MAIT cell activation, permitting the use of MAIT cells

purified with magnetic beads in functional *in vitro* assays [453]. As shown in figure 3.11b, purified MAIT cells likewise responded with IFNγ production to IL-12+IL-18 +/- IL-1β, whereas no IL-17A production was detected after 24h of stimulation (figure 3.11c), consistent with the results observed for activation of MAIT cells in total PBMC cultures. Notably, to our knowledge, this is the first demonstration that purified MAIT cells respond to cytokine mediated stimulation with IL-12, IL-18 and IL-1β, as published data shows MAIT cell activation in total PBMC cultures or within the total $CD8^+$ T cell pool [373].

As shown in figure 3.11a, a contaminating cell population of approximately 20% was found in the purified MAIT cell pool. To further reduce contaminating cells, fluorescence activated cell sorting could be employed to purify cells in future experiments. Moreover, the contaminating cell pool could be analysed in order to gain insight into its composition.

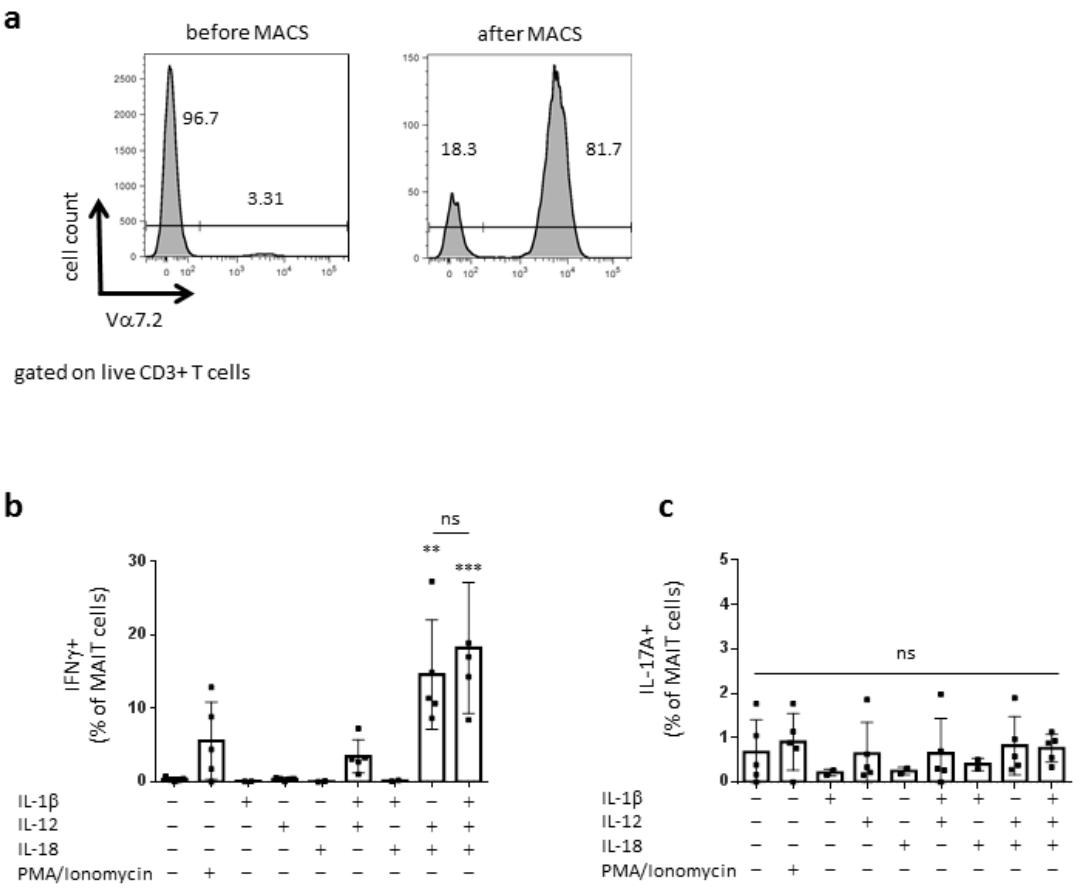


Figure 3.11: IL-12 + IL-18 +/- IL-1β specifically induces IFNγ but not IL-17 expression in purified MAIT cells.

PBMCs were isolated from peripheral blood of healthy controls and purified using magnetic bead based cell separation with a Vα7.2 antibody (see chapter 3.2.4). (a) Representative flow cytometry plot gated on live $CD3^+$ cells. (b)-(c) Purified MAIT cells were stimulated with PMA (50ng/ml) and Ionomycin (1μM) or IL-1β, IL-12 and IL-18 (50ng/ml each) for 24 hours and intracellular expression of IFNγ (b) and (c) IL-17A was analysed by flow cytometry, summary data, n = 5. (b)-(c) Data represent mean +/- SD *p< 0.05, **p<0.01, ***p<0.001 vs. no stimulation, ns = not significant. Pooled data from 2 independent experiments. Each symbol represents data from one individual.

AILD are chronic diseases characterized by persistent liver inflammation [13, 14]. Thus, it is likely that MAIT cells in these patients are repetitively exposed to inflammatory cytokines over longer periods of time.

To mimic such a chronic inflammatory setting more closely, a protocol for repetitive stimulation of MAIT cells was employed to investigate MAIT cell activation. PBMCs

isolated from healthy controls were stimulated repetitively with different combinations of IL-1β, IL-12 and IL-18 for up to 72 hours (50ng/ml each), and IFNγ and IL-17A production by MAIT cells was assessed by intracellular staining and flow cytometric analysis after 24 hours, 48 hours, and 72 hours.

Interestingly, repetitive stimulation with IL-12+IL-18 +/- IL-1β resulted in a significant decline of $IFN\gamma^+$ MAIT cells after 72 hours stimulation, compared to 24 hours stimulation (figure 3.12a). In contrast, IL-17A production was induced after 72 hours stimulation with IL-12 alone, or in combination with IL-1β and/or IL-18 (figure 3.12b).

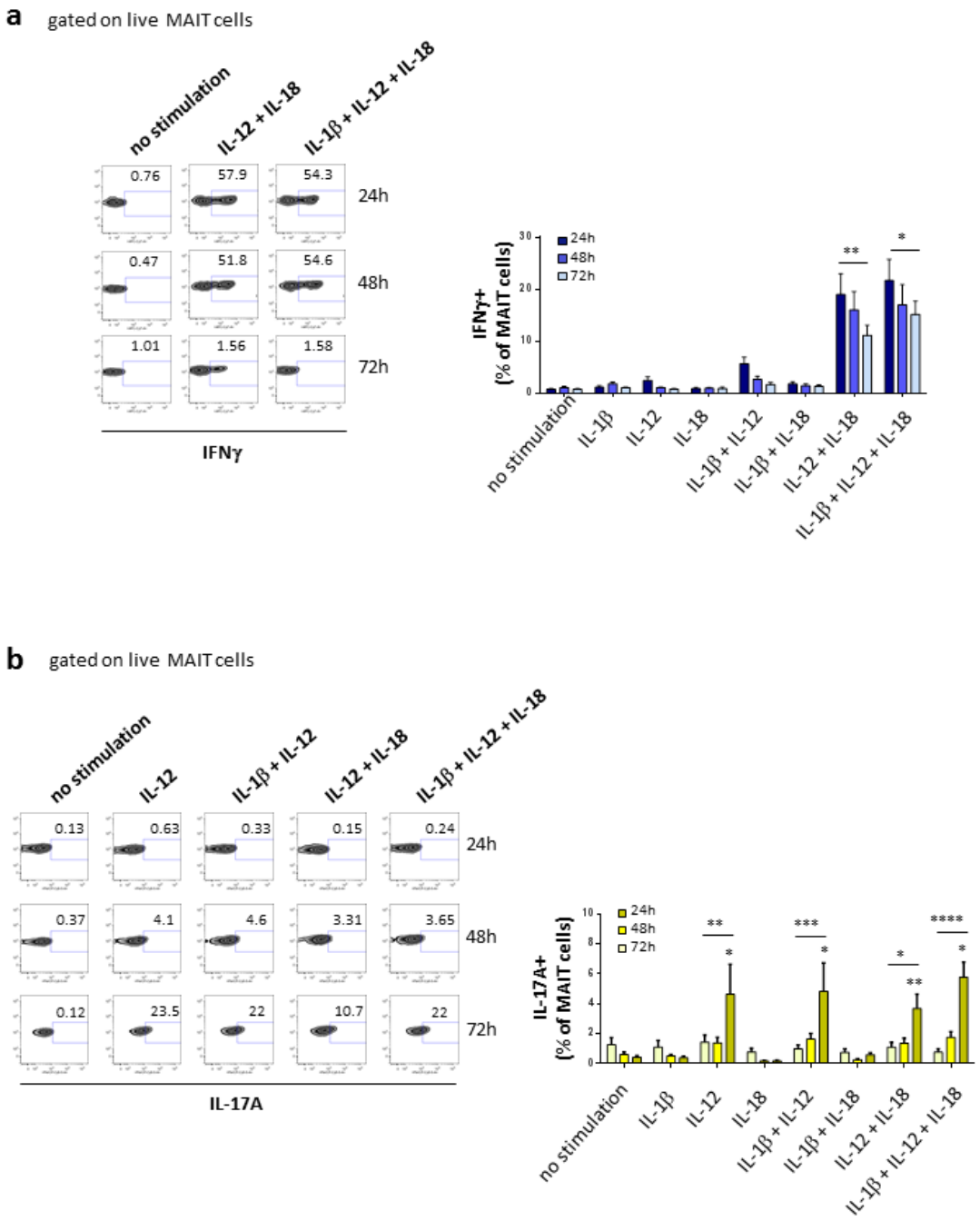


Figure 3.12: Repetitive cytokine stimulation changes cytokine expression pattern of MAIT cells.

PBMCs were isolated from peripheral blood and stimulated with 50ng/ml of IL-1β, IL-12 and IL-18 for 24 -72 hours. Intracellular cytokine expression of (a) IFNγ and (b) IL-17 A were analysed after 24 hours, 48 hours and 72 hours of stimulation. Representative flow cytometry plots and summary data (n = 12). Data represent mean +/- SEM, SD *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. no stimulation or as indicated, pooled data from 4 independent experiments.

To exclude effects of bystander immune cells in the PBMC pool, MAIT cells purified by magnetic bead separation and repetitively stimulated with different combinations of IL-1β, IL-12 and IL-18. Consistent with the results from total stimulation, purified MAIT cells were expressing IL-17A after 72 hours repetitive stimulation with IL-12 alone, or in combination with IL-1β and/or IL-18 (figure 3.13a). Moreover, IFNγ production likewise significantly declined after repetitive stimulation of MAIT cells for 72h with IL-12+IL-18 +/- IL-1β (figure 3.13b).

To our knowledge, this is the first demonstration that MAIT cells express IL-17 upon repetitive stimulation with IL-12.

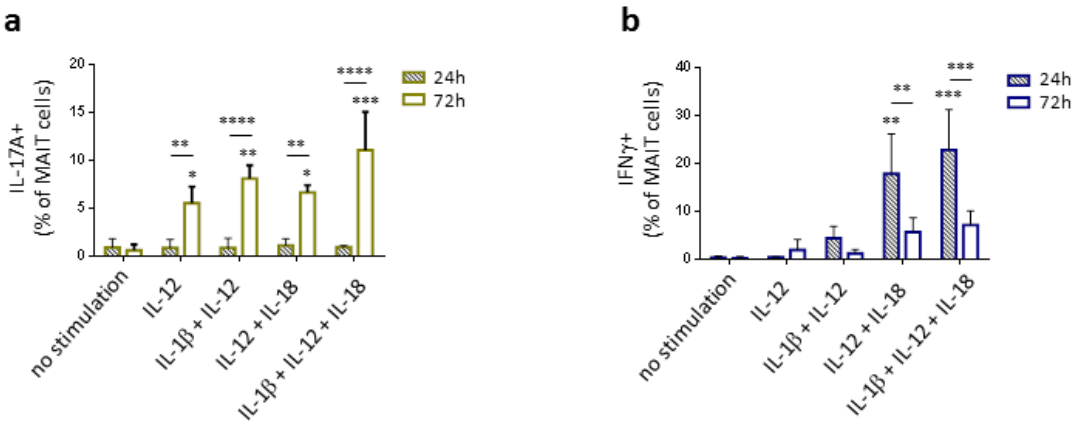


Figure 3.13: Repetitive cytokine stimulation changes cytokine expression pattern of purified MAIT cells.

PBMCs were isolated from peripheral blood and purified using magnetic bead based cell separation with a Vα7.2 antibody. Purified MAIT cells were stimulated with IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours and intracellular expression of (a) IFNγ and (b) IL-17 was analysed by flow cytometry, n = 3. Data represent mean +/- SD, *p< 0.05, **p<0.01, ***p<0.001 ****p<0.0001 vs. no stimulation or as indicated, data from 1 experiment.

Next, the response to cytokine stimulation by MAIT cells from patients with AILD was tested. PBMCs isolated from peripheral blood of patients with AILD were stimulated with different combinations of IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours, and cytokine production was measured by intracellular cytokine staining and flow cytometric analysis. As shown in figure 3.14a, MAIT cells from patients

with PSC, PBC and AIH similarly responded specifically to IL-12+IL-18 with IFNγ production after 24 hours of stimulation, with no enhancing effect of IL-1β on IFNγ secretion. Similar to MAIT cells from healthy controls, IFNγ production by MAIT cells from AILD patients showed a non-significant trend to decline after repetitive stimulation (figure 3.14a). Moreover, significant IL-17A production could be detected in MAIT cells from patients with PSC, PBC and AIH after repetitive simulation for 72 hours with IL-1β+IL-12 + IL-18 (figure 3.14b).

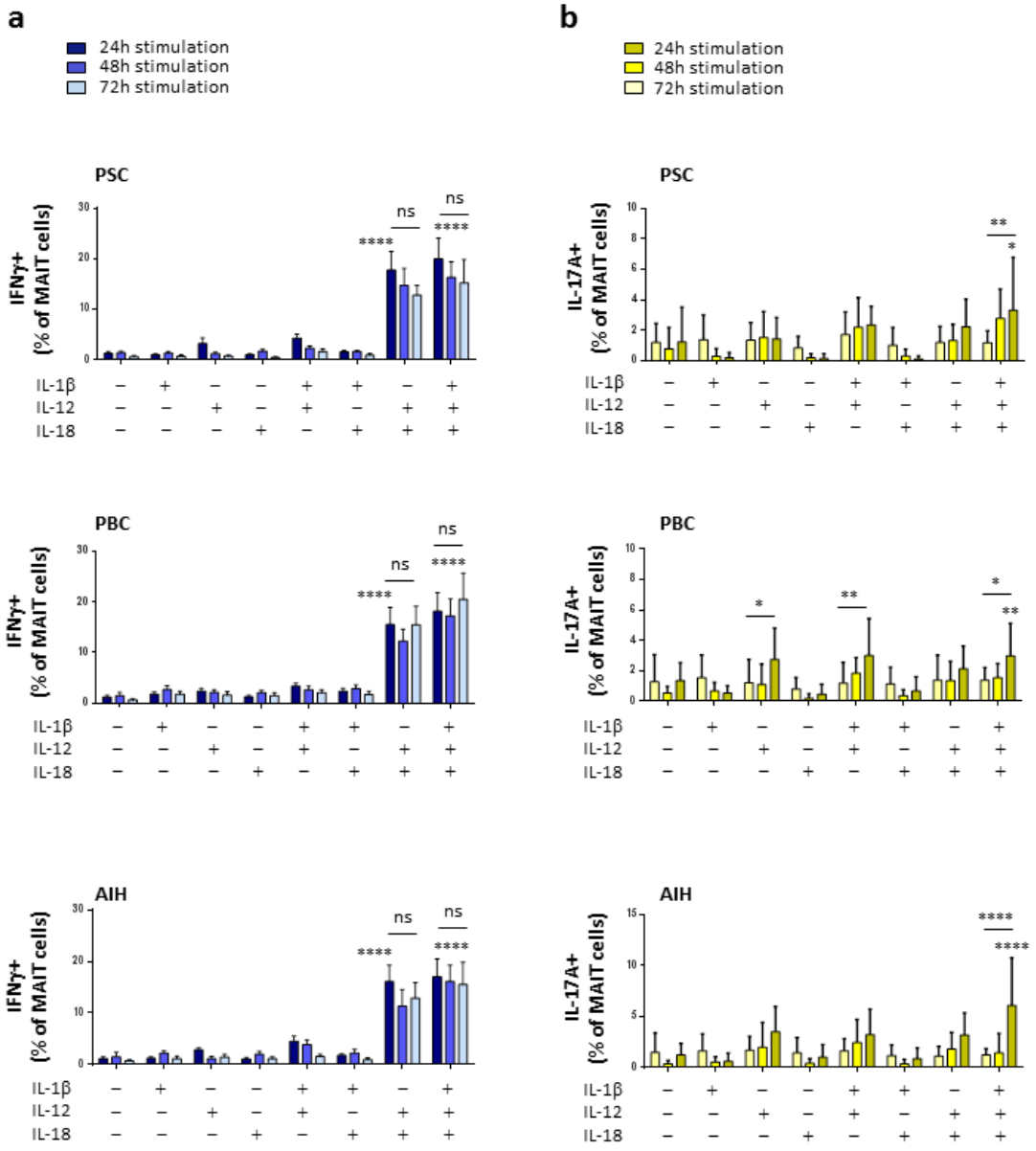


Figure 3.14: MAIT cells from peripheral blood of patients with AILD respond to repetitive cytokine stimulation with decreasing IFNγ and increasing IL-17A expression.

PBMCs were isolated from peripheral blood of patients with PSC, PBC and AIH and stimulated with IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours. (a) IFNγ and (b) IL-17A expression in MAIT cells was analysed by intracellular staining and flow cytometry, n = 9. Data represent mean +/- SEM *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. no stimulation or as indicated, ns = not significant, pooled data from 3 independent experiments.

Interestingly, no significant difference was observed between the percentage of $IFN\gamma^+$ (figure 3.15a), as well as $IL\text{-}17A^+$ (figure 3.15b) MAIT cells after cytokine

stimulation in patients with AILD compared to healthy controls. This indicates that the ability to produce IFNγ and IL-17A in response to cytokine mediated stimulation is maintained in MAIT cells from patients with AILD, in contrast to IFNγ production in response to TCR bypassing stimulation by PMA/Ionomycin (figure 3.9b).

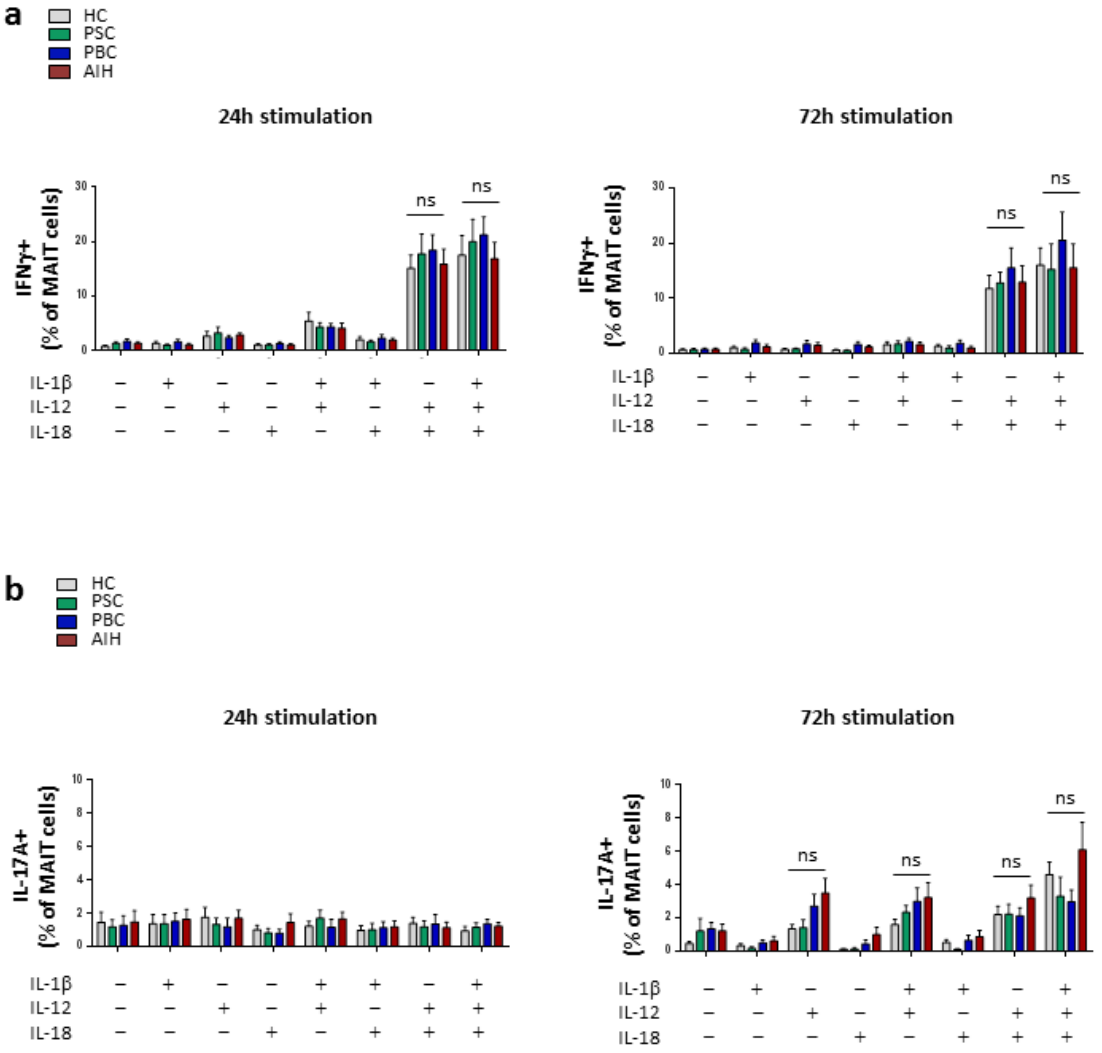


Figure 3.15: Response to cytokine stimulation is maintained in MAIT cells from peripheral blood of patients with AILD.

PBMCs were isolated from peripheral blood of healthy controls and patients with PSC, PBC and AIH and stimulated with IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours, (a) IFNγ and (b) IL-17A expression in MAIT cells was analysed by intracellular staining and flow cytometry, n = 9, data represent mean +/- SEM ns = not significant, pooled data from 3 independent experiments. HC = healthy controls.

These data suggest that circulating MAIT cells are directly stimulated by the pro-inflammatory cytokines IL-12 and IL-18. Of note, repetitive cytokine-mediated stimulation changes the cytokine secretion pattern of MAIT cells towards a declining IFNγ response and increasing IL-17 production, especially in response to IL-12, which has been described for the first time in this thesis. Moreover, the data indicate that, despite showing signs of exhaustion, MAIT cells from AILD patients can respond to cytokine mediated stimulation.

Taken together, the data presented in this chapter provide new insight into the biology of MAIT cells, especially into the previously unknown response to repetitive cytokine stimulation.

3.3.4 Repetitive inflammatory stimuli drive MAIT cell exhaustion and cell death

As shown in figures 3.12 and 3.14, repetitive stimulation with cytokines, such as IL-1β, IL-12 and IL-18, resulted in a decline of IFNγ expression in MAIT cells from both healthy controls and patients with AILD. This could be a result of cytokine receptor downregulation, a mechanism to regulate cytokine response to persisting inflammatory stimuli [462]. Moreover, this could be in line with MAIT cell exhaustion, as T cell exhaustion results from chronic exposure to antigen or inflammatory cytokines [451] and is characterized by a dampened T cell response mediated by inhibitory receptors [463, 464], which results in decreasing cytokine production and associated loss of effector function [465].

To investigate whether surface expression of the IL-12R was down-regulated in MAIT cells in response to repetitive exposure to IL-12, MAIT cells from healthy controls and patients with AILD were stimulated with IL-12 alone or in combination with IL-1β and/or IL-18 for 24-72 hours, and IL-12R expression was measured by flow cytometry. Neither MAIT cells from healthy controls (figure 3.16a), nor MAIT cells from AILD patients (figure 3.16b) showed down-regulation of IL-12R expression following repetitive stimulation with IL-12. Of note, MAIT cells from patients with AILD showed reduced levels of IL-12R expression in culture, compared

to *ex vivo* measurements, however, expression of IL-12R did not change significantly over time (figure 3.16b). These data indicate that downregulation of IL12R cannot account for the observed reduction in IFNγ expression after IL-12 stimulation.

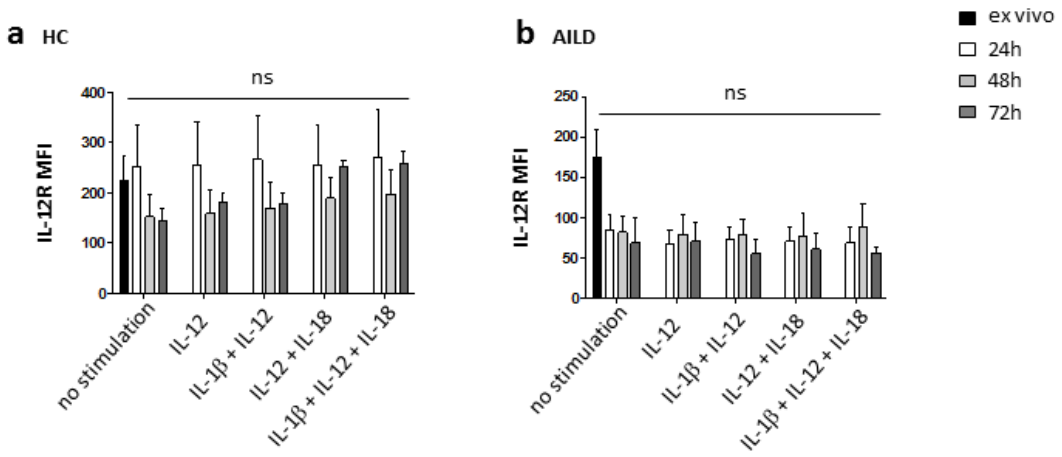


Figure 3.16: IL-12R expression in MAIT cells is unchanged after repetitive cytokine stimulation.
PBMCs were isolated from peripheral blood and stimulated with IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours. IL-12R expression in MAIT cells from (a) healthy controls (HC) and (b) AILD patients analysed by flow cytometry after 24 hours, 48 hours and 72 hours stimulation, summary data, n = 5. Data represent mean +/- SD, ns = not significant. Pooled data from 2 independent experiments. HC = healthy control, MFI = mean fluorescence intensity.

Exhausted T cells show a reduced ability to produce IFNγ, resulting from increased signalling via inhibitory receptors such as PD-1, TIM-3 and CD39 [452, 463, 464] expressed by exhausted T cells.

To investigate whether such inhibitory receptors might be responsible for the reduced ability to produce IFNγ in response to PMA/Ionomycin stimulation, expression of inhibitory receptors, such as PD-1, TIM-3 and CD39, was analysed on MAIT cells after repetitive stimulation with different combinations of IL-1β, IL-12 and IL-18. As shown in figures 3.17a and 3.17b, MAIT cells from healthy controls showed marked up-regulation of PD-1 and TIM-3 after repetitive stimulation and significant up-regulation of CD39 after 72 hours stimulation, compared to 24 hours stimulation. Of

note, significant up-regulation of CD39 was specifically induced by repetitive stimulation with IL-12+IL-18 +/- IL-1β, which also had resulted in IFNγ and IL-17A expression in MAIT cells (figures 3.17a and 3.17b). In MAIT cells isolated from patients with AILD, PD-1 was mildly up-regulated after repetitive stimulation (figure 3.17c). In contrast, TIM-3 was significantly upregulated, both after 72 hours in culture without stimulation and after repetitive stimulation with IL-1β+IL-12 and IL1β+IL-12 + IL-18 (figure 3.17c). CD39 was markedly, but not significantly, up-regulated after repetitive stimulation, which was, however, not restricted to cytokine combinations that provoked IFNγ and IL-17A expression (figure 3.17c).

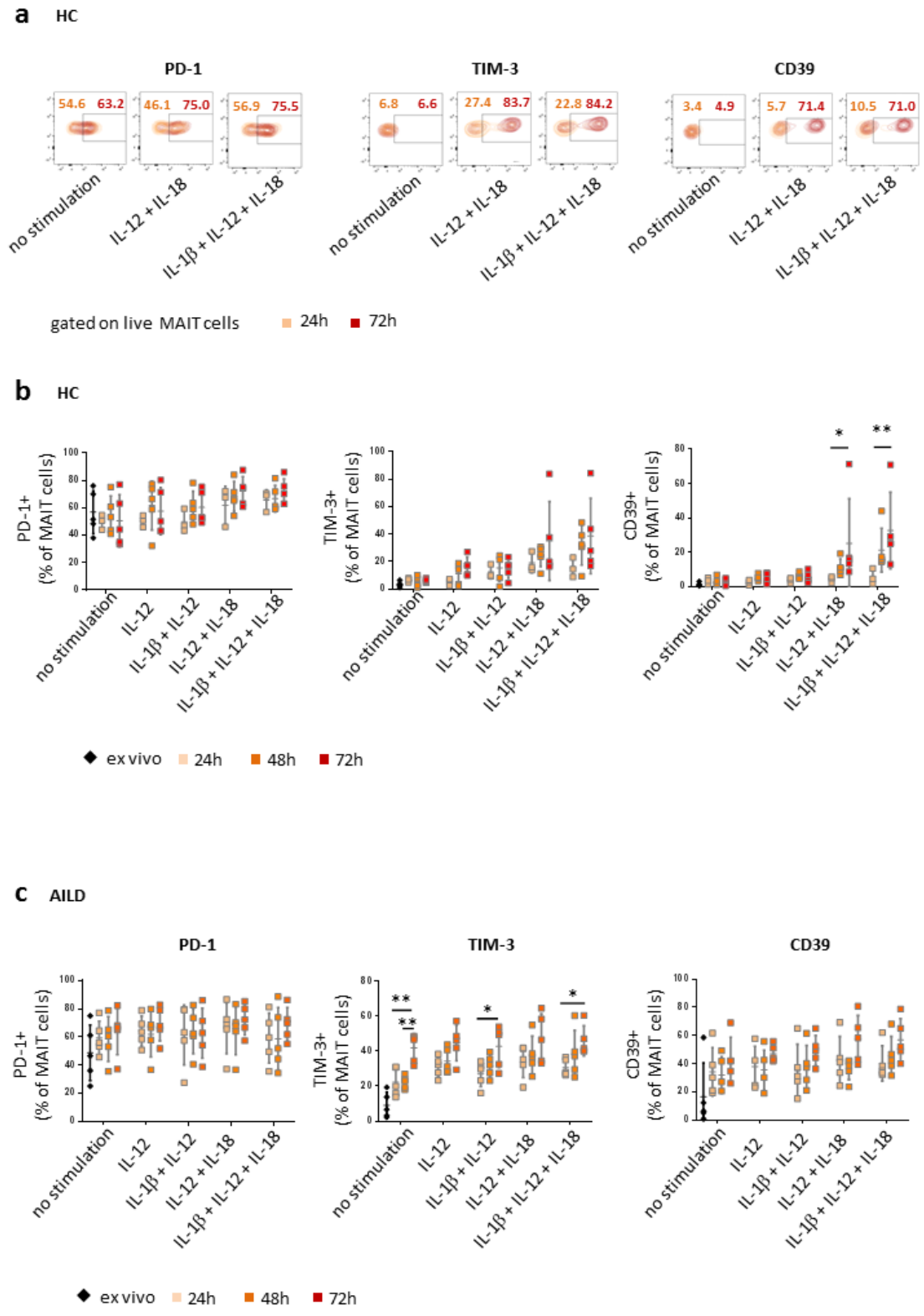


Figure 3.17: Repetitive cytokine stimulation results in upregulation of inhibitory receptors in MAIT cells.

PBMCs were isolated from peripheral blood and stimulated with IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours. Expression of PD-1, TIM-3 and CD39 in MAIT

cells from (a)-(b) healthy controls and (c) patients with AILD analysed by flow cytometry after 24, 48 and 72 hours stimulation. (a) Representative flow cytometry plots and (b)-(c) summary data, each symbol represents data from one individual. Data represent mean +/- SD, *p< 0.05, **p<0.01. Pooled data from 2 independent experiments is shown. HC = healthy control.

Furthermore, a non-significant decline of live MAIT cell frequency was observed in MAIT cells from AILD patients over time in culture, most evident after repetitive stimulation with IL-12+IL-18 +/- IL-1β (figure 3.18a). Of note, the frequency of live MAIT cells derived from healthy controls only declined after repetitive stimulation with IL-12+IL-18 +/- IL-1β (figure 3.18a). In contrast, live $CD3^+$ T cell frequency remained constant over time, suggesting that MAIT cell loss was specific and not accompanied by general T cell death in culture (figure 3.18b).

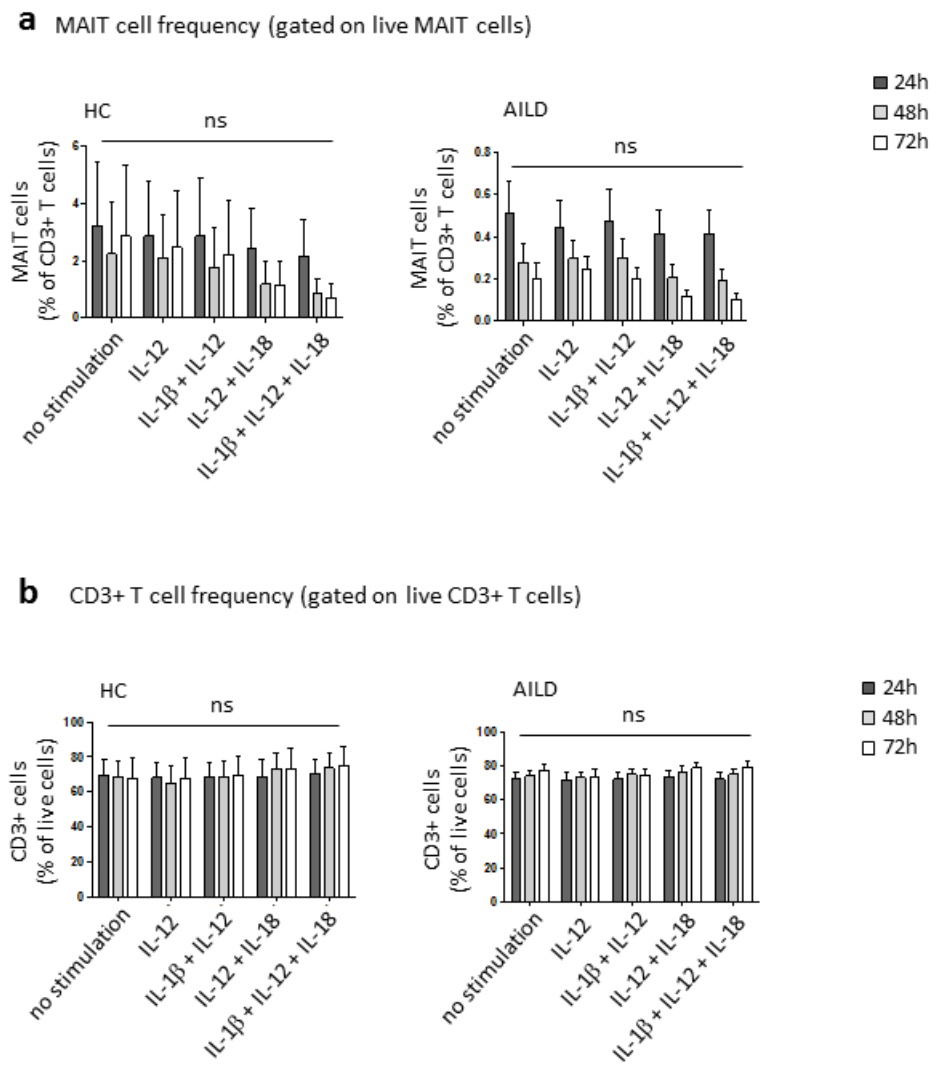


Figure 3.18: Repetitive cytokine stimulation results in MAIT cell death in patients with AILD.

PBMCs were isolated from peripheral blood and stimulated with IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours. (a) MAIT cell and (b) CD3+ frequency in healthy controls and AILD patients after 24, 48 and 72h stimulation *in vitro*, analysed by flow cytometry, summary data, HC n = 5, AILD n = 11 (4 PSC, 4 PBC, 3 AIH). Data represent mean +/- SD, ns = not significant. Pooled data from 2 independent experiments. HC = healthy controls

It has been described that MAIT cells in HIV infected individuals are exhausted following chronic activation, which is associated with a persistent decline of MAIT cells *in vivo* [390]. In AILD patients, similarly, MAIT cell frequency inversely correlated with the expression of exhaustion markers (figure 3.8), supporting the

conclusion that chronic exposure to pro-inflammatory cytokines is able to drive MAIT cell exhaustion, which is characterized by a decline of effector function (figure 3.9), up-regulation of inhibitory receptors (figure 3.7 and figure 3.17) and cell death (figure 3.18a).

3.3.5 Liver MAIT cells produce large amounts of IL-17A in response to repetitive cytokine stimulation

Despite being abundant in peripheral blood, MAIT cells are particularly enriched in the liver where they comprise up to 20-40% of $CD3^+$ lymphocytes [335, 356, 372] and preferentially locate around portal tracts [356]. This high abundance within the liver is thought to be due to chemokine dependent recruitment of MAIT cells to the liver by liver-specific homing receptors, such as CCR6 and CXCR6 which are highly expressed by MAIT cells [335, 356].

In order to characterize liver MAIT cells (liMAITS) in healthy human livers, liver-associated lymphocytes were isolated from non-diseased liver tissue derived from liver surgery for benign liver lesions or liver metastasis, or from explanted healthy livers that were unsuitable for transplantation due to long ischemic time.

Indeed, liMAITs accounted for up to 38% of $CD3^+$ lymphocytes in the liver and were significantly more frequent among $CD3^+$ cells, compared to blood-derived MAIT cells (bMAITs) (figure 3.19a). Similar to bMAITs (figures 3.3a and 3.5), the majority of liMAITs expressed the TCR co-receptor CD8, followed by $CD4^-/CD8^-$ liMAITs (figure 3.19b). Moreover, there was no significant difference in IL-12R and IL-18R expression on liMAITs compared to bMAITs (figure 3.19c, see also [356]).

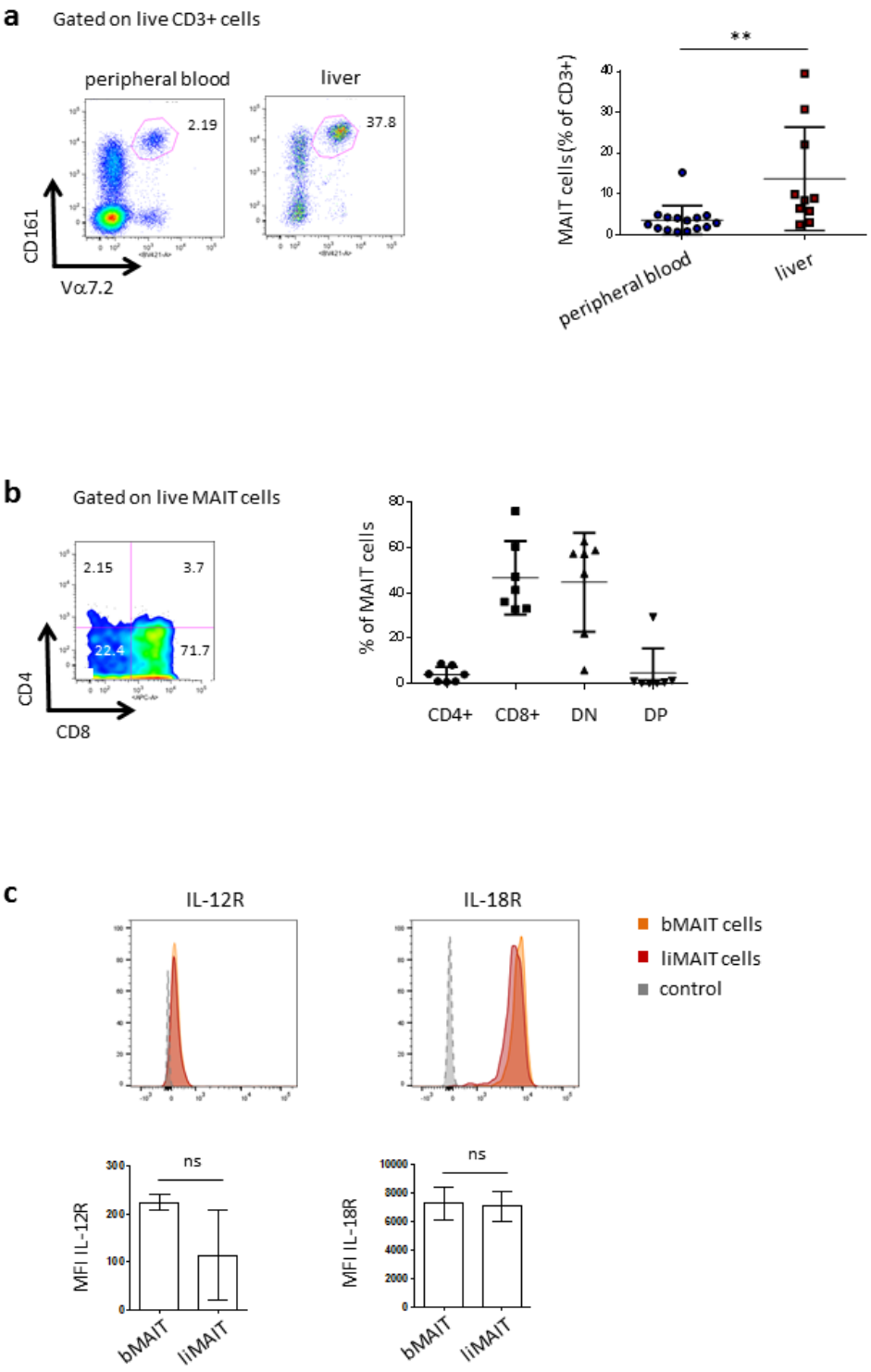


Figure 3.19: Frequency of MAIT cells is significantly higher in the liver than in peripheral blood.

Liver associated lymphocytes and PBMCs were isolated from control livers and peripheral blood of healthy individuals as described in chapter 3.2.2.1 and 3.2.2.2. (a) MAIT cell frequency in peripheral blood and liver determined by flow cytometry. MAIT cells were defined as $CD3^{+}$, $CD161^{++}$, $V\alpha7.2^{+}$, representative flow cytometry plot and summary data. (b) CD4 and CD8 expression in liver MAIT cells, DN = $CD4^{-}$

/CD8$^-$, DP = CD4$^+$/CD8$^+$, representative flow cytometry plot and summary data. (c) IL-12R and IL-18R expression in liver MAIT cells, representative flow cytometry plot and summary data of mean fluorescence intensity (MFI), n = 3. (a)- (b) Each symbol represents data from one individual. Data represent mean +/- SD, pooled data from 3 experiments. (c) Data represent mean +/- SEM, Data from 1 experiment. bMAIT = MAIT cells isolated from peripheral blood, liMAIT = MAIT cells isolated from liver tissue.

Is has been described that liMAITs show a partially activated phenotype, characterized by higher expression levels of the activation markers CD38, CD69 and HLA-DR, but low expression of the proliferation marker Ki67 [372]. Indeed, besides expressing significantly higher level of CD38, liMAITs tended to express higher levels of CD69 and HLA-DR compared to bMAITs (figure 3.20a). Furthermore, liMAITs expressed non-significantly higher levels of the inhibitory receptors PD-1, TIM-3 and CTLA-4, as well as CD39, a marker for terminal exhaustion (figure 3.20b). These data should be interpreted with care, since only three livers have been available for analysis. Moreover, the analysis of liMAITs isolated from healthy liver tissue will be expanded in the future.

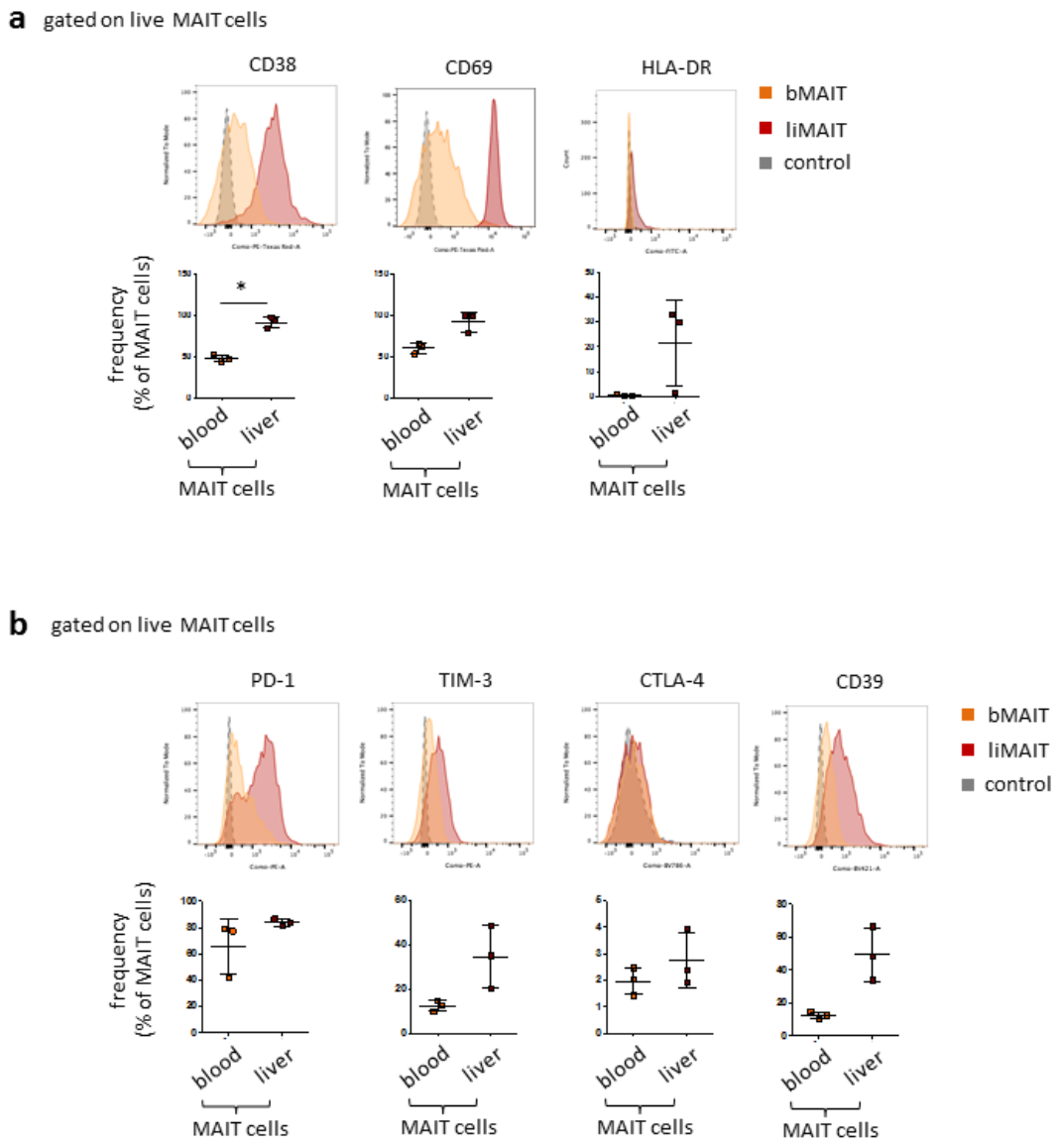


Figure 3.20: Activation markers and inhibitory receptors are highly expressed in liMAIT cells.
PBMCs and liver-associated lymphocytes were isolated from peripheral blood and liver tissue of healthy controls. Expression of (a) CD38, CD69 and HLA-DR and (b) PD-1, TIM-3, CTLA-4 and CD39 in MAIT cells was analysed by flow cytometry *ex vivo*. Representative flow cytometry plots and summary data, each symbol represents data from one individual. Data represent mean +/- SD, *p< 0.05, data from one experiment. bMAIT = MAIT cells isolated from peripheral blood, liMAIT = MAIT cells isolated from liver tissue.

In order to determine the response of liMAITs to cytokine stimulation, liver-associated lymphocytes were stimulated with different combinations of IL-1β, IL-12

and IL-18 (50ng/ml each) for 24 hours, and IFNγ and IL-17A expression in liMAITs was analysed by intracellular cytokine staining and flow cytometry. Analogous to bMAITs, liMAITs significantly expressed IFNγ in response to stimulation with IL-12+IL-18 +/- IL-1β (figure 3.21a). Similar to bMAITs, IL-1β+ L-12+IL-18 did not enhance IFNγ production of liMAITs compared to IL-12+IL-18 (figure 3.21a). Of note, stimulation with PMA/Ionomycin, IL-1β, IL-1β+ IL-12 also induced IFNγ production in liMAITs, however this was not significant compared to no stimulation (figure 3.21a). Interestingly, there was no significant difference between liMAIT and bMAIT IFNγ response to cytokine stimulation (figure 3.21a).

In line with the previous findings in bMAITs, no IL-17A expression was observed in liMAITs after 24h stimulation with cytokines or PMA/Ionomycin (figure 3.21b). However, liMAITs showed a markedly, but non-significantly, higher basal IL-17A expression compared to bMAITs (figure 3.21b).

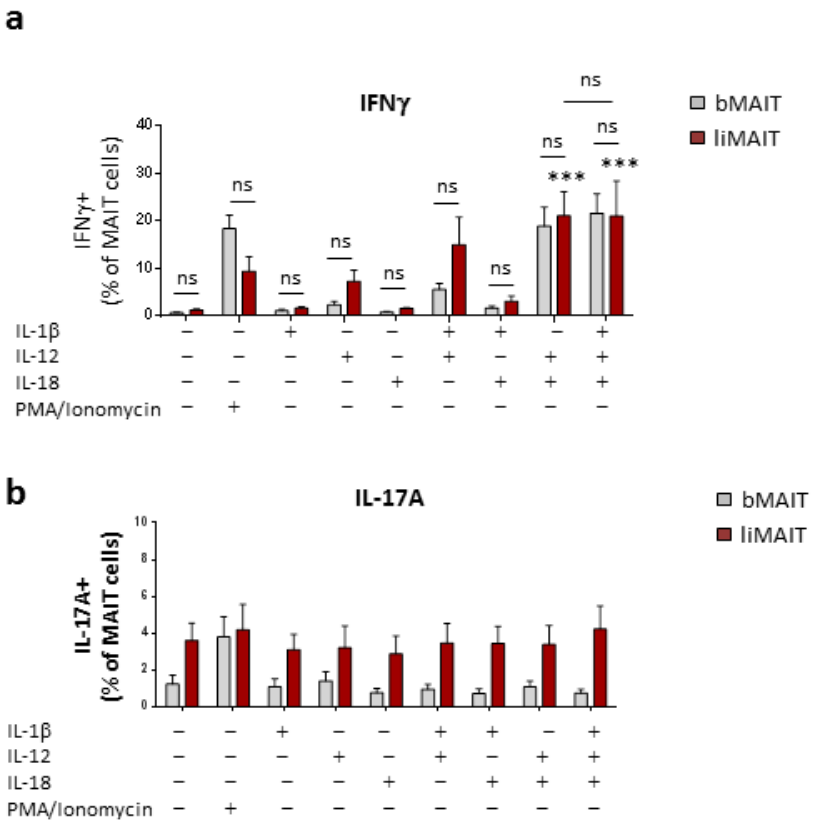


Figure 3.21: LiMAITs express IFNγ, but no IL-17 following stimulation with IL-12+IL-18+/-IL-1β.
PBMCs and liver-associated lymphocytes were isolated from peripheral blood and liver tissue of healthy controls and stimulated with PMA (50ng/ml) and Ionomycin (1uM) or IL-1β, IL-12 and IL-18 (50ng/ml each) for 24 hours. (a) IFNγ and (b) IL-17A expression in MAIT cells was analysed by intracellular staining and flow cytometry. Summary data, bMAITs n = 12, liMAITs n = 7, data represent mean +/- SEM. ***p<0.001 vs. no stimulation or as indicated, pooled data from 3 independent experiments, bMAIT = MAIT cells isolated from peripheral blood, liMAIT = MAIT cells isolated from liver tissue.

As described in figure 3.12, IFNγ expression decreased in bMAITs after repetitive cytokine stimulation (figure 3.12a), while IL-17A production was induced by repetitive stimulation with IL-12 alone or in combination with IL-1β and/or IL-18 (figure 3.12b). To test the effect of repetitive cytokine stimulation on liMAITs, liver-associated lymphocytes were stimulated with different combinations of IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours, and IFNγ and IL-17A expression was

analysed by intracellular cytokine staining and flow cytometry. Interestingly, in contrast to bMAITs, IFNγ production in liMAITs initially increased after stimulation with IL-12+IL-18 +/- IL-1β (48 hours stimulation compared to 24 hours) and then declined significantly after 72 hours stimulation (figure 3.22a). Similar to bMAITs, significant IL-17A expression was observed in liMAITs after 72h stimulation with IL-12 and IL-1β+IL-12 (figure 3.22b). Moreover, IL-17A expression significantly increased in liMAITs following repetitive stimulation with IL-1β, IL-12, IL-1β+IL-12 and IL-1β+IL-18 (figure 3.22b). Of note, as opposed to bMAITs, liMAITs did not show induction of IL-17A expression after repetitive stimulation with IL-12+IL-18 or IL-12+IL-18+IL-1β (figure 3.22b).

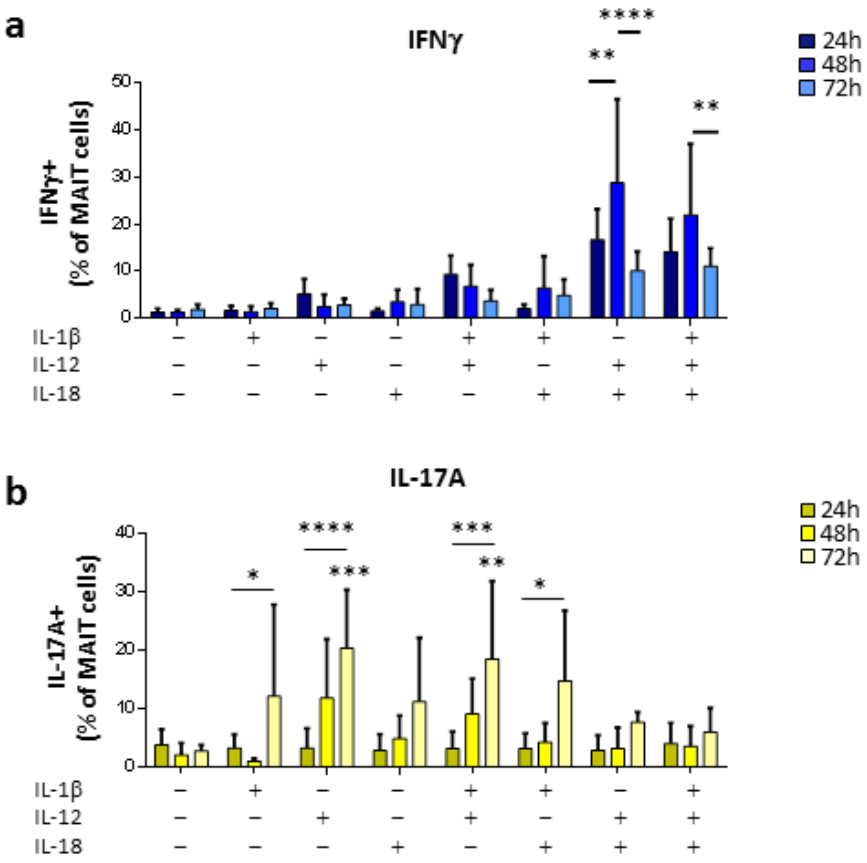


Figure 3.22: LiMAITs express large amounts of IL-17A in response to repetitive stimulation with IL-12 + IL-1β.
Liver-associated lymphocytes were isolated from control liver tissue and stimulated with IL-1β, IL-12 and IL-18 (50ng/ml each) for 24-72 hours, (a) IFNγ and (b) IL-17A expression in MAIT cells was analysed by intracellular staining and flow cytometry. Summary data, n = 6, data represent mean +/- SD, *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. no stimulation or as indicated, data from 1 experiment. bMAIT = MAIT cells isolated from peripheral blood, liMAIT = MAIT cells isolated from liver tissue.

When comparing cytokine expression of bMAITs and liMAITs following 72 hours cytokine stimulation, liMAITs expressed significantly more IL-17A in response to IL-12 and IL-1β+IL-12 stimulation compared to bMAITs (figure 3.23a). In contrast, no difference was observed in IFNγ expression following 72 hours stimulation with IL-12+IL-18 +/- IL-1β between liMAITs and bMAITs (figure 3.23b).

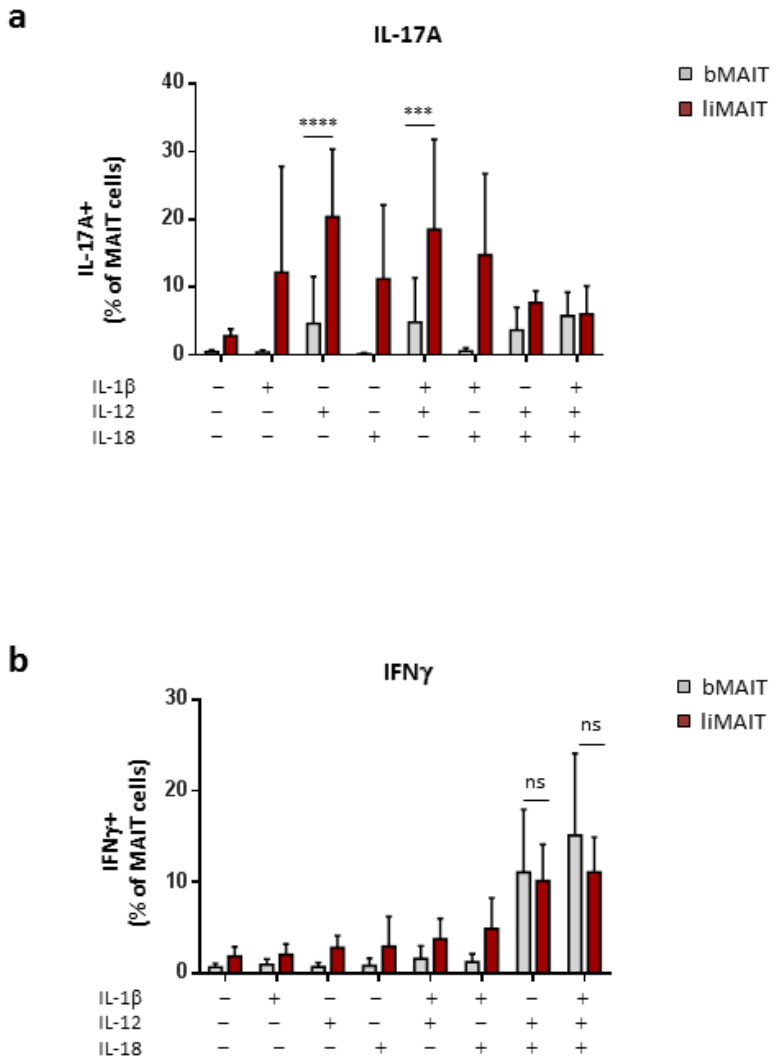


Figure 3.23: LiMAITs express significantly more IL-17 in response to 72 hour stimulation than bMAITs.

PBMCs and liver associated lymphocytes from healthy controls were stimulated with IL-1β, IL-12 and IL-18 (50ng/ml each) for 72 hours. (a) IL-17A and (b) IFNγ expression in MAIT cells was analysed by intracellular staining and flow cytometry, summary data, bMAIT n = 12, liMAIT n = 7. Data represent mean +/- SD, ***p<0.001, ****p<0.0001, ns = not significant. Pooled data from 6 independent experiments. bMAIT = MAIT cells isolated from peripheral blood, liMAIT = MAIT cells isolated from liver tissue

Taken together, liMAITs show an activated phenotype compared to bMAITs as shown by increased expression of activation and exhaustion markers (figure 3.20). Moreover, the cytokine expression pattern changes in liMAITs in response to repetitive cytokine

stimulation similar to bMAITs, resulting in decreasing IFNγ and increasing IL-17A expression following 72 hours repetitive exposure to cytokines (figure 3.22). Interestingly, liMAITs not only showed high basal expression of IL-17A *ex vivo* (3.21), but also expressed significantly more IL-17A than bMAITs after repetitive stimulation with IL-12+/- IL-1β (figure 3.23). As IL-17A is a mediator of tissue inflammation [466] and HSC activation [436], two processes that promote tissue damage and the development of liver fibrosis and cirrhosis, these data suggest that liMAITs might contribute to the maintenance of chronic inflammation and the development of fibrosis in the liver by IL-17A production.

3.3.6 Liver MAIT cells from patients with AILD show an activated phenotype

As discussed before, MAIT cells circulating in human blood show a significantly reduced frequency (figure 3.3) and an exhausted phenotype (figure 3.7) in patients with AILD, but no defect in their response to cytokine-mediated stimulation (figure 3.15). Whether MAIT cells in the liver of AILD patients show similar alterations in phenotype and function has not been investigated so far. Thus, phenotypic and functional characteristics of liMAITs isolated from patients with AILD were determined. Due to restricted access to liver tissue from AILD patients, only liver tissue from PSC patients has been analysed up until now, but analysis will be expanded towards liver tissue from PBC and AIH patients in the future.

MAIT cell frequency was significantly reduced in PSC livers compared to healthy controls (figure 3.24a), in line with a recent report on decreased frequencies of liMAITs in patients with chronic liver disease, such as alcoholic liver disease, non-alcoholic steatohepatitis, PBC and PSC [356]. Similar to bMAITs from AILD patients (figure 3.5b), the proportion of $CD4^+$ MAIT cells was significantly increased among liMAITs from PSC patients, compared to healthy controls (figure 3.24b). In addition, analysis of cytokine receptor expression revealed no difference between IL-12R and IL-18R expression in liMAITs from patients with PSC and healthy controls (figure 3.24c, see also [356]), similar to bMAITs.

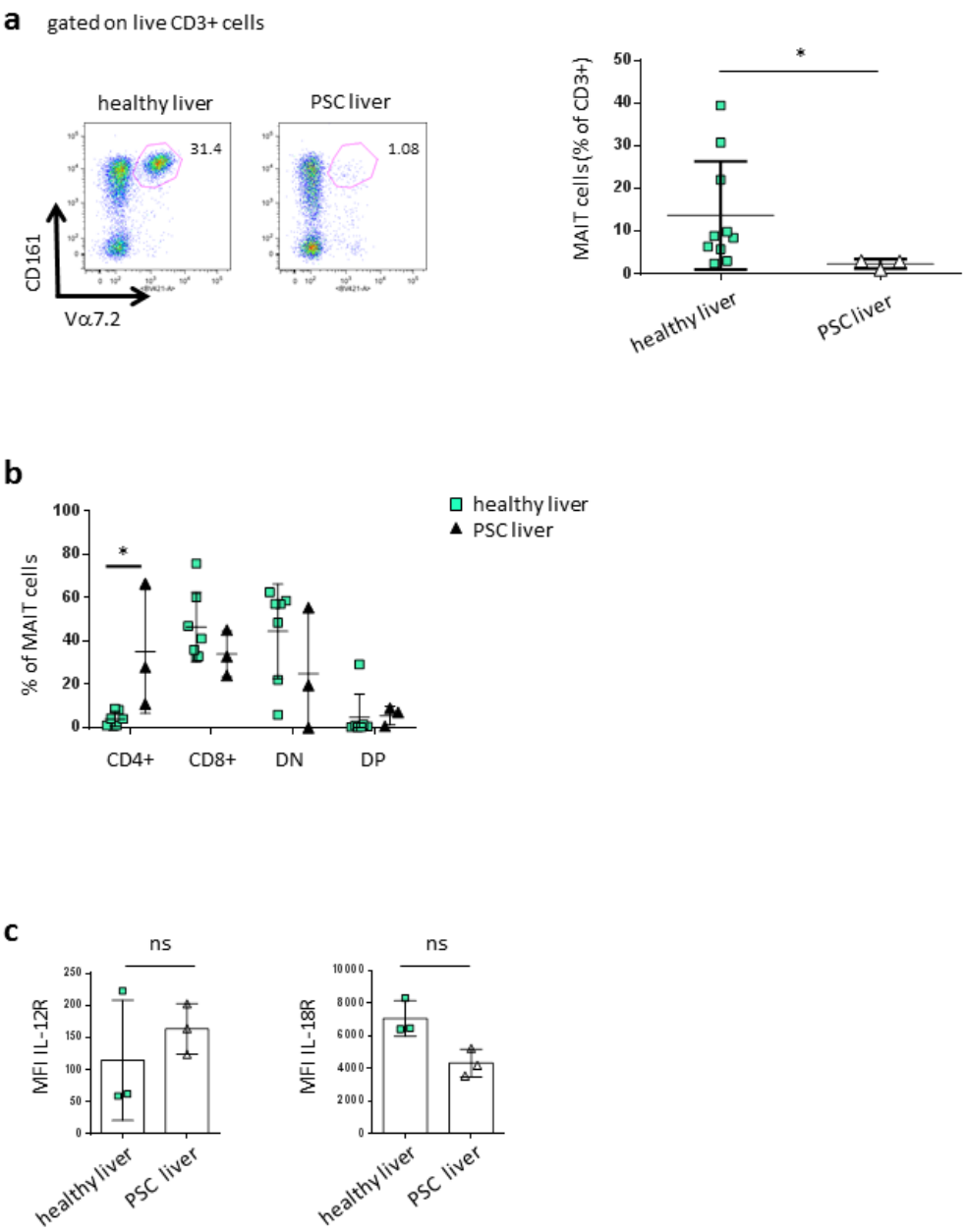


Figure 3.24: MAIT cells are significantly reduced in livers from PSC patients.
Liver associated lymphocytes were isolated from liver tissue from PSC explants and healthy controls and analysed by flow cytometry *ex vivo*. (a) Frequency of MAIT cells in healthy and PSC livers, representative flow cytometry plot and summary data. (b) Expression of CD4 and CD8 in MAIT cells, DN = $CD4^-/CD8^-$, DP = $CD4^+/CD8^+$, summary data (c) Mean fluorescence intensity (MFI) of IL-12R and IL-18R in MAIT cells, n = 3. (a) – (b) each symbol represents data from one individual, pooled data from 3 independent experiments, (c) Data from 1 experiment. (a) – (c) Data represent mean +/- SD, *p< 0.05, ns = not significant.

As bMAITs from AILD patients displayed an exhausted phenotype (figure 3.7) which might have resulted from chronic activation within the diseased liver, one would speculate that liMAITs would show similar characteristics. In order to investigate whether this was the case, expression of surface activation markers and inhibitory receptors commonly expressed by exhausted T cells were analysed on liMAITs from patients with PSC and compared to liMAITs from healthy controls.

Interestingly, in patients with PSC, expression of the activation markers CD38, CD69 and HLA-DR was higher in liMAITs than in bMAITs (figure 3.25). However, no significant differences in expression of the activation markers CD38, CD69 and HLA-DR were observed between liMAITs from patients with PSC and liMAITs from healthy controls (figure 3.25).

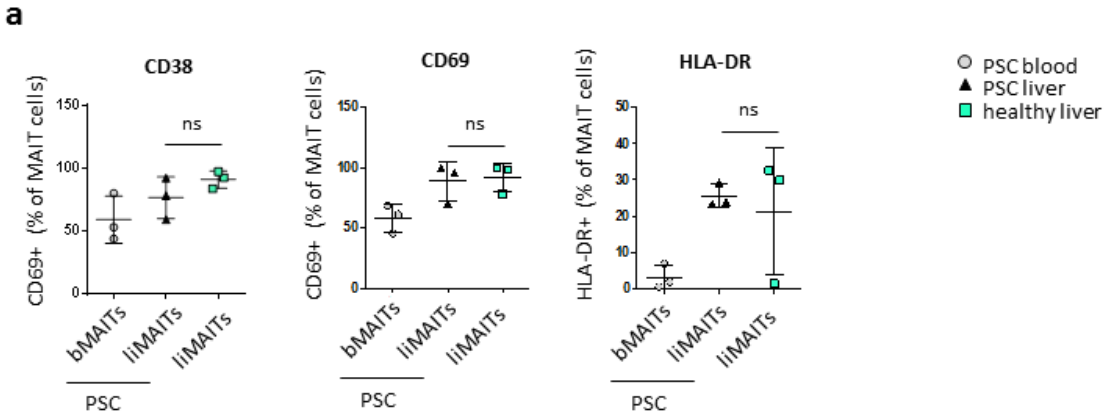


Figure 3.25: LiMAITs from PSC liver tissue highly express activation markers.
Ex vivo expression of (a) CD38, CD69 and HLA-DR in MAIT cells isolated from peripheral blood and liver of patients with PSC and healthy livers, analysed by flow cytometry, summary data. Each symbol represents data from one individual. Data represent mean +/- SD, ns = not significant. Data from one experiment.

Next, the expression of inhibitory receptors on liMAITs was investigated. LiMAITs from PSC patients expressed significantly higher levels of CTLA-4 and higher levels of PD-1, TIM-3 and CD39 compared to bMAITs from patients with PSC (figure 3.26). Expression levels of PD-1 and CD39 in liMAITs from PSC patients were similar to liMAITs isolated from healthy controls (figure 3.26). However, liMAITs

from patients with PSC expressed higher levels of CTLA-4 and markedly lower levels of TIM-3 compared to liMAITs from healthy controls (figure 3.26). These data suggest that liMAITs from both healthy controls and AILD patients show an activated phenotype compared to bMAITs. However, the expression of most activation and exhaustion markers was similar on liMAITs from AILD patients and healthy controls (figures 3.25 and 3.26), although these result have to be interpreted with care due to the low number of analysed samples (n=3).

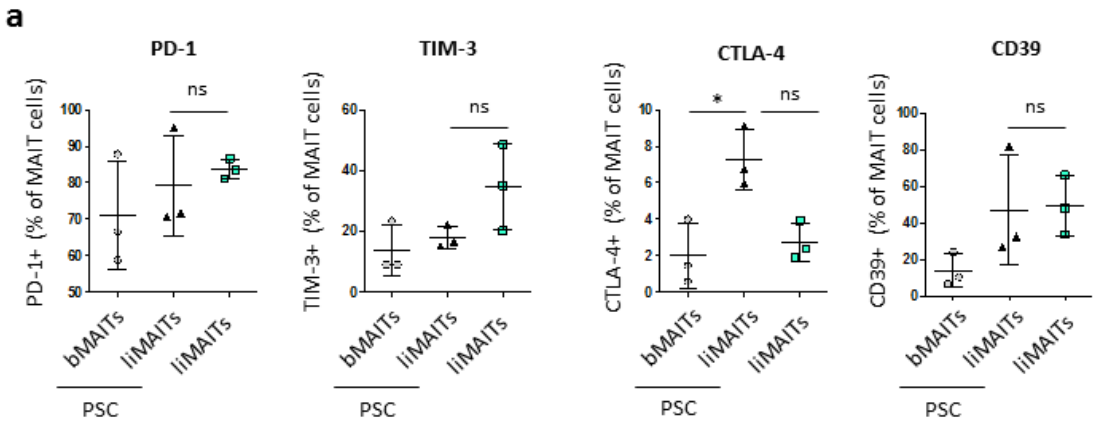


Figure 3.26: LiMAITs from AILD patients express lower levels of TIM-3, but higher levels of CTLA-4, compared to healthy controls.
Ex vivo expression of (a) PD-1, TIM-3, CTLA-4 and CD39 in MAIT cells isolated from peripheral blood and liver of patients with AILD and healthy livers, analysed by flow cytometry, summary data. Each symbol represents data from one individual. Data represent mean +/- SD, *p< 0.05, ns = not significant. Data from one experiment.

Taken together, liver MAIT cells are highly activated and produce large amounts of IL-17A upon repetitive cytokine-mediated stimulation (figures 3.22 and 3.23). Both circulating and liver-resident MAIT cells are depleted in patients with AILD (figures 3.3b-c and 3.24a), accompanied by a relative increase in $CD4^+$ MAIT cells (figures 3.5b and 3.24b). Moreover, liMAITs from AILD patients are highly activated (figures 3.25 and 3.26).

3.3.7 MAIT cells stimulate HSC proliferation

IL-17 plays an important role in fibrosis development [435, 436, 467], which is most likely due to its ability to induce HSC activation and collagen deposition [436], two important features of fibrosis development [6, 24]. As MAIT cells show an activated phenotype in AILD patients (figures 3.7, 3.25 and 3.26) and expressed IL-17A in response to stimulation by PMA/Ionomycin (figure 3.9, see also [335]) or repetitive cytokine stimulation (figure 3.14), it was investigated whether MAIT cells are able to induce HSC proliferation, which is considered a feature of HSC activation (see chapter 2.1.1.2 and [6, 24].

PBMCs were isolated from peripheral blood of healthy controls and MAIT cells were purified by magnetic bead-based cell separation using a Vα7.2 antibody. MAIT cells were then co-cultured with primary human HSCs in a ratio of 2:1 in presence of CD3/CD28 beads, which are expected to activate MAIT cells [335, 372]. After 48h co-culture, proliferation of HSCs was assessed by BrdU incorporation assay.

Co-culturing MAIT cells with HSCs led to significant increase of BrdU incorporation by HSCs, suggesting increased HSC proliferation (figure 3.27). This was only slightly increased upon additional activation of MAIT cells by CD3/28 beads (figure 3.27a).

Moreover, increasing the ratio of MAIT cells:HSCs did not result in increased HSC proliferation (figure 3.27b), suggesting that a ratio of 1:1 is sufficient for induction of HSC proliferation.

These data indicate that MAIT cells are able to induce features of HSC activation, a process that might contribute to fibrosis development in the liver *in vivo*.

Nevertheless, further measures of HSC activation, e.g. expression of pro-fibrogenic and pro-inflammatory genes or collagen secretion, need to be tested in order to confirm the hypothesis that MAIT cells can induce HSC activation. Moreover, this study aims to determine a mechanism for such HSC activation by MAIT cells in the future. In this context, it will be determined whether stimulation of HSCs by MAIT cells requires direct cell contact and if HSC activation is dependent on TCR-MR1 interaction. Furthermore, the role of IL-17 for HSC activation by MAIT cells will be investigated and IL-17 blocking antibodies, as well as IL-17 receptor antagonists will be employed to test this hypothesis.

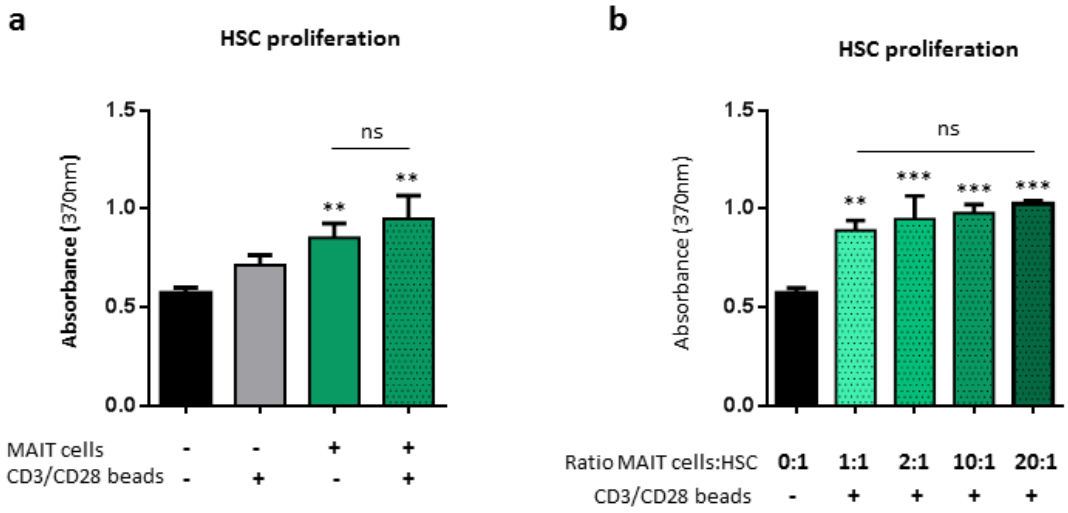


Figure 3.27: MAIT cells are able to stimulate HSC proliferation.
PBMCs were isolated from peripheral blood of healthy controls and purified with magnetic bead separation using a Vα7.2 antibody. Purified MAIT cells were co-cultured with HSCs +/- CD3/CD28 beads in triplicates in a ratio of (a) MAIT cells:HSCs 2:1 or (b) varying ratios. (a)- (b) HSC proliferation was assessed after 48 hours of co-culture by BrdU incorporation assay. Data represent mean +/- SD **p<0.01, ***p<0.001 vs. the unstimulated control group (black bar), ns = not significant. Representative data from 3 experiments.

3.4 Discussion

Autoimmune damage is a central feature of AILD, i.e. PSC, PBC and AIH. Although characterised by different pathophysiological mechanisms, all AILD are marked by chronic tissue inflammation associated with the development of liver fibrosis and eventually cirrhosis [13, 14]. As AILD are strongly associated with IBD, it has been hypothesised that AILD and IBD result from a shared pathogenic mechanism. One potential explanation for the association between AILD and IBD is that lymphocytes which are activated in the inflamed gut enter the portal circulation of the liver, where they get reactivated and cause tissue inflammation and liver injury [370]. In general, chronic autoimmune injury in AILD leads to destruction of the liver parenchyma, thereby compromising the liver's function, but also stimulating the development of liver fibrosis which is characterised by activation of HSCs, which is central for fibrosis development [6, 14, 370]. Once activated, HSCs proliferate, produce large amounts of ECM proteins and inhibit the breakdown of excess ECM, culminating in the accumulation of ECM, i.e. liver fibrosis [6, 24].

MAIT cells are the largest population of innate like T cells in humans and are particularly enriched in the gut and liver, where they account for 20-40% of all T lymphocytes (figure 3.24a, see also [335]). Upon activation by their cognate antigen, bacterial-derived vitamin B metabolites, or by cytokine-mediated, TCR independent stimulation, MAIT cells secrete inflammatory cytokines, such as TNFα, IFNγ and IL-17 [339, 373, 453]. Of note, in IBD highly activated MAIT cells accumulate in the inflamed gut, suggesting that MAIT cells contribute to mucosal inflammation in this clinical condition [361, 401, 468]. Moreover, MAIT cells are decreased in peripheral blood in chronic liver disease, such as chronic HCV infection [402-404], NASH, PSC and PBC [356].

However, it remains unknown if the phenotype and function of MAIT cells is altered in AILD and whether MAIT cells contribute to fibrosis development in AILD.

Therefore, in this thesis the MAIT cell compartment in patients with AILD was characterised, and the interaction between MAIT cells and HSCs was analysed. The most striking observation in the experiments conducted here was the significant decrease in MAIT cells in both peripheral blood and livers of patients with AILD, irrespective of its type. Interestingly, the MAIT cells that could be found in these

patients showed an exhausted phenotype, likely as a result of chronic activation. Moreover, the response of MAIT cells to different types and kinetics of stimulation was altered in AILD patients. Whereas IFNγ production was impaired in circulating MAIT cells from AILD patients in response to PMA and Ionomycin stimulation, response to cytokine-mediated stimulation was maintained. Furthermore, MAIT cells from both peripheral blood and liver expressed large amounts of IL-17A upon repetitive cytokine-mediated stimulation. As IL-17A has been shown to be involved in fibrosis development and HSC activation [372, 467, 469], the interaction between MAIT cells and HSCs was investigated next. Indeed, MAIT cells were able to stimulate HSC proliferation, which is considered as a feature of HSC activation [6, 24].

Although further experiments are necessary to determine the exact contribution of MAIT cells to fibrosis development in AILD, the data presented in this thesis suggest that MAIT cells are highly activated in AILD, eventually resulting in MAIT cell exhaustion. One could hypothesise that this is directly linked to the reduced numbers of MAIT cells observed in AILD patients, which could follow from MAIT cell death resulting from exhaustion. Moreover, the observed production of large amounts of IL-17A by MAIT cells and their ability to stimulate HSC proliferation suggest that MAIT cells play a role for fibrosis development. Taken together, these data provide new insights into both the pathogenesis of AILD and MAIT cell biology, suggesting MAIT cells as a potential target for novel anti-inflammatory and anti-fibrotic therapy.

3.4.1 Circulating MAIT cells are severely reduced and show a chronically activated and exhausted phenotype in AILD

MAIT cells are a subset of innate-like T cells, which is important for anti-bacterial defence [347, 365, 382]. In addition, MAIT cells are altered in several non-infectious diseases, including autoimmune disorders [361, 394, 396]. Whereas the role of MAIT cells in multiple sclerosis remains unclear [396], MAIT cells are decreased in peripheral blood and accumulate in the inflamed intestinal mucosa in IBD patients, suggesting a contribution of MAIT cells to mucosal inflammation in IBD [361, 401].

In this thesis, both circulating and liver-resident MAIT cells were investigated in patients with AILD. Interestingly, in all AILD patients, irrespective of their disease, both MAIT cell frequency and absolute number were significantly reduced in peripheral blood. $CD8^+$ as well as $CD4^-/CD8^-$ MAIT cells, which represent the most frequent subsets of MAIT cells, were significantly reduced.

Decrease of MAIT cells in peripheral blood seems to be a common feature of MAIT cells in chronic infectious and non-infectious disease, as MAIT cell numbers were also significantly reduced in patients with chronic HIV infection [362, 389, 390] and IBD [361, 401]. Moreover, the MAIT cell frequency is altered in chronic liver disease. In peripheral blood of HCV-infected patients, the MAIT cell frequency is severely reduced [388, 402, 403]. Moreover, the frequency of circulating and liver MAIT cells has been described as significantly decreased in chronic non-viral liver disease, such as ALD, NASH and AILD, i.e. PSC and PBC [356]. Here, it has to be noted, that Jeffery et al. [356] have included PSC and PBC patients as an examples of chronic liver disease in their study, however, these patients only represent a small number of total cases that were investigated.

The cause of this decline is unclear, and one could speculate that MAIT cell decrease in peripheral blood is a result of compartmentalization in the liver, as MAIT cells have been shown to rapidly translocate to sites of infection and inflammation. This has been shown in experimental pulmonary infection in mice [364] and in the inflamed mucosa in patients with IBD [361, 401]. In contrast, the results of the present work show that the MAIT cell frequency was significantly reduced in PSC livers, and a similar finding was reported in liver tissue from patients with ALD and NASH [356]. This could be due to several reasons. Firstly, MAIT cell accumulation in inflammatory settings could be specific to tissues that do not include the liver, like the lungs or the gut mucosa. This, however, seems rather unlikely as MAIT cells express high levels of liver-specific homing-markers under steady-state conditions, as well as in chronic liver disease [335, 356]. Secondly, it is possible that MAIT cells initially accumulate in the liver and become activated, but then either become exhausted and eliminated, or undergo activation induced cell death, a phenomenon that limits immune responses by induction of apoptosis [470]. Notably, MAIT cells have been shown to be highly sensitive to activation-induced cell death [471]. As a third possibility, other immune cells might specifically eliminate MAIT cells in the liver in order to reduce inflammation. It has been shown that natural killer (NK) cells kill activated T cells in

different autoimmune diseases. Such elimination of $CD4^+$ T cells has been shown to reduce inflammation in a murine colitis model [472].

As compartmentalization in the liver is not the cause of the decrease of circulating MAIT cells, what are the possible reasons for this observation? It has been reported, that treatment with corticosteroids leads to a decline of MAIT cells in peripheral blood, as well as to reduced IFNγ production by MAIT cells [360, 395]. Of note, in this study only one patient with PSC and one patient with PBC received treatment with corticosteroids at the time of blood sampling. In contrast, 20 out of 21 AIH patients received immunosuppressive treatment, of which 14 were treated with corticosteroids. Therefore, it is unlikely that corticosteroid treatment induced MAIT cell decline in peripheral blood in PSC and PBC patients, although it cannot be excluded in AIH patients. Nevertheless, there was no difference in MAIT cell decrease between PSC, PBC and AIH patients, implicating that corticosteroid treatment is unlikely to be causing the MAIT cell decrease in AIH patients.

Like MAIT cells in HIV infection [390], MAIT cells in AILD showed increased expression of activation and exhaustion markers *ex vivo* and impaired IFNγ production upon PMA/Ionomycin stimulation. Moreover, the frequency of MAIT cells in peripheral blood inversely correlated with the expression of the inhibitory receptors CTLA-4, TIM-3 and CD39, suggesting that the decline of MAIT cells in peripheral blood is associated with MAIT cell exhaustion. Historically, T cell exhaustion has first been described for conventional T cells in chronic viral infection [473]. However, it also occurs in other diseases, e.g. after chronic antigen exposure during bacterial infections and cancer [474]. During viral infection, naïve T cells differentiate into effector T cells that are able to eliminate infected cells, with a small proportion further differentiating into memory T cells after clearance of infection. Persistent infection or chronic exposure to antigen, however, prevents memory T cell formation and leads to a hierarchical loss of T cell function. First, a decline of cytokine production, such as IL-2 production, as well as a decrease of cytotoxicity and proliferative capacity is observed in virus-specific T cells. This is accompanied by up-regulation of inhibitory receptors on the surface of these T cells, which further limits T cell function due to encounter of these inhibitory receptors with their ligands on other cells. Ultimately,

severely exhausted T cells lose their ability to produce IFNγ and are physically deleted [451, 465]. Although not studied in great detail, it seems likely that MAIT cells, in analogy to conventional T cells, are susceptible to similar mechanisms upon chronic infection/liver disease [383, 390]. Hence, the data presented in this thesis suggest, that MAIT cell loss in AILD results from chronic MAIT cell activation, eventually resulting in exhaustion and finally deletion of MAIT cells.

To challenge this hypothesis, and to further stress the importance of inhibitory receptor upregulation for MAIT cell exhaustion, it would be interesting to test whether blocking inhibitory receptors can rescue MAIT cell effector function. It has previously been shown that blocking PD-1 and CTLA-4 can rescue anti-viral [475] effector functions of exhausted T cells and blocking PD-1 and TIM-3 enhances anti-tumour properties of tumour infiltrating $CD8^+$ T cells [476].

The liver is perfused by a unique dual blood supply, with only 30% of hepatic blood flow being derived from the hepatic artery, whereas 70% of blood is supplied by the portal vein [2]. Originating from the gut, portal venous blood contains bacterial metabolites, food-derived antigen and LPS, which is derived from the wall of gram-negative bacteria and a known TLR4 agonist. Moreover, in AILD associated with IBD, the gut mucosa becomes leaky, allowing for translocation of bacteria and increased delivery of bacterial products from the gut to the portal circulation, which could lead to increased exposure to antigen and other MAIT cell stimulating molecules in these patients [370, 477]. For example, LPS levels are increased in portal venous blood and in the systemic circulation in cirrhotic patients [478, 479]. It thus seems plausible that MAIT cells experience chronic exposure to their antigens, bacterial-derived vitamin B metabolites, in AILD patients, which could explain the characteristics of MAIT cell exhaustion observed here. Furthermore, it has been shown that MAIT cells can indirectly be stimulated by TLR4 agonists, such as LPS, through LPS-activated APCs [373], further supporting antigen activation of MAIT cells and thereby contributing to MAIT cell exhaustion.

Moreover, LPS-driven MAIT cell activation is dependent on IL-12 and IL-18 secretion by APCs [373]. In this thesis, it was demonstrated that MAIT cells specifically up-regulated activation markers and inhibitory receptors upon stimulation with IL-12 and IL-18, indicating that exposure to IL-12 and IL-18 can drive MAIT

cell exhaustion. Therefore, in AILD MAIT cell exhaustion could be driven by LPS-induced IL-12 and IL-18 secretion by other immune cells in the liver.

Furthermore, chronic exposure to commensal-derived antigen could contribute to MAIT cell activation in the liver in health and disease. It still remains unclear whether and how MAIT cells can distinguish between vitamin B metabolites derived from commensals or pathogens, and if MAIT cells develop tolerance towards antigen derived from commensals. However, it has been described, that MAIT cell accumulation and proliferation requires co-stimulatory signals provided by bacteria and TLR agonists [364], suggesting that MAIT cell activation is a complex process that requires further research in order to fully understand it.
Recent studies show that AILD are accompanied by changes of the gut microbiota. In PSC, the composition of the microbiota is less diverse and is characterized by overexpression of the species Enterococcus, Fusobacterium and Lactobacillus, independently of concomitant IBD [480]. In AIH, the microbiome composition is altered as well [477] and the microbiome in PBC harbours less beneficial bacterial species and more opportunistic pathogens, compared to healthy controls [481]. Such alterations in microbiome composition could differentially influence MAIT cell activation and may contribute to MAIT cell exhaustion in AILD. As different species display different potential to activate MAIT cells [371, 482], it is indeed likely that alterations in the microbiome influence MAIT cell activation in AILD.

In addition to a reduced frequency, the analyses conducted in this study demonstrated a change in the subset composition of MAIT cells. It was observed that there was a relative increase in $CD4^+$ MAIT cells in both peripheral blood and liver of AILD patients. The reason for this is unknown, although such an increase in $CD4^+$ MAIT cells was also observed in livers of other chronic liver disease, e.g. ALD and NASH [356], and in peripheral blood of patients with inflammatory bowel diseases [361, 401]. The increase in $CD4^+$ MAIT cells is compensated for by the decrease in $CD8^+$ MAIT cells. One could speculate that the relative increase of $CD4^+$ MAIT cells stems from a reduced exhaustion of this subset, or these cells could be less susceptible to activation induced cell death. Alternatively, $CD8^+$ MAIT cells could specifically be targeted and eliminated by other immune cells. However, it remains to be seen whether the changes in subset frequency are dependent on such a mechanism.

Moreover, it remains unclear if the decline of $CD8^+$ MAIT cells has any functional relevance, although it is important to consider that $CD8^+$ MAIT cells are the main cytokine producing MAIT cell subset (data not shown, see also [356]).

3.4.2 Cytokine-mediated stimulation of MAIT cells

Activation of MAIT cells by bacterial-derived antigens presented on MR1 molecules on other cells results in IFNγ and IL-17A secretion by MAIT cells [348, 356, 371, 453]. This activation can be mimicked by stimulation with the mitogen PMA and the calcium ionophore Ionomycin [335, 483]. In addition to antigen-dependent activation by other immune cells or epithelial cells, MAIT cells within the PBMC pool can be activated in a TCR-independent manner by the pro-inflammatory cytokines IL-12 and IL-18 [373], which are secreted by myeloid immune cells such as DCs, monocytes and macrophages in response to pathogenic stimuli [484, 485]. Of note, IL-12 and IL-18 are thought to play a role for the development of AILD and both IL-12 and IL-18 levels are elevated in serum of AILD patients [486, 487]. The contribution of IL-12 and IL-18 to the pathogenesis of AILD will be discussed in more detail below. IL-1β is thought to be involved in the development of liver fibrosis through HSC activation and recruitment of inflammatory cells [458, 459]. Moreover, IL-1β has been shown to induce IFNγ production in NK cells in combination with IL-12 [460] and IL-1β + IL-23 potently stimulated IFNγ and IL-17 expression in MAIT cells when combined with TCR-mediated stimulation [372].

To investigate the ability of MAIT cells to respond to TCR-independent stimuli in this thesis, MAIT cells were stimulated with different combinations of IL-1β, IL-12 and IL-18. Here, it was demonstrated for the first time that purified MAIT cells are specifically stimulated by IL-12 + IL-18. However, IL-1β showed no enhancing effect on IL-12 + IL-18 induced IFNγ production in MAIT cells in the experiments conducted in this study. This shows that MAIT cells are directly stimulated by IL-12 + IL-18. Furthermore, these findings highlight the importance of IL-12 + IL-18 as a potent way of MAIT cell stimulation, further considering that both long-term (20 hour) stimulation of MAIT cells with bacteria, as well as TLR4 agonist mediated

stimulation are dependent on IL-12 + IL-18 [373]. In contrast, IL-1β does not seem to play a role for induction of IFNγ in MAIT cells.

Furthermore, the response of MAIT cells to different protocols of stimulation with different kinetics was investigated. When a single cytokine stimulus was applied for 24 hours, MAIT cells responded with IFNγ production to stimulation with IL-12+IL-18 +/- IL-1β, whereas no IL-17A production could be detected. Interestingly, repetitive stimulation with IL-12 alone, or in combination with other cytokines, stimulated IL-17A production significantly, showing that MAIT cell response to chronic inflammatory stimuli differs fundamentally from the response to a single stimulus. This is important for the understanding of MAIT cell behaviour in chronic infection and inflammation, as the repetitive stimulation protocol most likely resembles inflammatory settings *in vivo* more closely.

Why does repetitive IL-12 stimulation induce IL-17A production in MAIT cells?

For conventional T cells, it is known that IL-12 induces the differentiation of naïve CD4+ T cells into Th1 cells, and to skew the immune response towards a Th1 cytokine profile with high levels of IFNγ [488, 489].

In contrast, production of IL-17A is observed by Th17 cells, iNKT cells, γδ T cells and conventional T cells. Differentiation of Th17 cells from naïve T cells requires TGF-β, IL-6, IL-21, IL-1β and IL-23 and the function of Th17 cells is mainly regulated by IL-4, IL-10 and IFNγ. IL-17 secretion by iNKT cells is stimulated by anti CD3 and IL-23, IL-1β + IL-23, as well as the synthetic ligand α-galactosylceramide [490-492]. In γδ T cells, IL-1, IL-6, IL-18 and IL-23 are able to induce IL-17 production [493]. Importantly, in conventional, activated T cells IL-18 stimulates IL-17 secretion, whereas IL-12 inhibits IL-17 production [494]. Thus, to our knowledge, there is no data about stimulation of IL-17 secretion by IL-12 in MAIT cells or other immune cells, highlighting the importance of this finding for the understanding of MAIT cell biology.

It is known that IL-12 mediates its effects through activation of the transcription factors signal transducer and activator of transcription (STAT) STAT1, STAT3, STAT5, and in particular STAT4 [495, 496], which then induce IFNγ production [497]. In contrast, IL-17 expression is induced by IL-23, which is structurally closely related to IL-12. Like IL-12, IL-23 activates several members of the STAT family, STAT1, STAT3, STAT4 and STAT5, although IL-23 preferentially signals through

activation of STAT3 [498]. Moreover, the transcription factor retinoic acid-related orphan receptor γT (RORγT), which has been shown to induce IL-17 expression [379], is constitutively highly expressed in MAIT cells [367]. As RORγT expression is regulated by STAT 3 [499], it is possible that induction of IL-17 production in MAIT cells by repetitive and prolonged exposure to IL-12 may be mediated through STAT3-regulated RORγT induction, although this hypothesis requires further investigation.

Whereas a single stimulus with IL-12+IL-18 for 24 hours lead to high IFNγ production, repetitive stimulation over 72 hours resulted in a significant decrease in IFNγ production by MAIT cells. In response to ongoing exposure to their ligand, cytokine receptors can be internalized in order to limit the immune response [462, 500]. Nevertheless, in MAIT cells, the decline of IFNγ production following repetitive stimulation was not due to IL-12 receptor down-regulation.
Instead, declining IFNγ production of MAIT cells could rather be a sign of T cell exhaustion, as inhibitory receptors, such as PD-1 and TIM-3, which were shown to inhibit cytokine production of T cells [451, 501], were significantly up-regulated in response to repetitive stimulation.
This, however, does not seem to be a sign of terminal MAIT cell exhaustion, as IL-17 expression is increasing, and IFNγ expression is not fully abrogated. To further test the extent of MAIT cell exhaustion on a functional level it would be interesting to analyse the ability of MAIT cells to lyse infected target cells in this setting in future experiments.

Furthermore, IFNγ expression was impaired in MAIT cells from patients with AILD after mitogenic stimulation of MAIT cells using PMA and Ionomycin. In contrast, the production of IFNγ as well as IL-17 production - was maintained in response to single and repetitive cytokine stimulation. This suggests that exhausted circulating MAIT cells in AILD patients are still susceptible to cytokine-mediated stimulation.
As described above, PMA and Ionomycin stimulation is a rather artificial way of T cell stimulation, where TCR signalling is mimicked by induction of calcium flux [483]. In order to gain more specific insight into the IFNγ response to TCR mediated

stimulation, MAIT cells from AILD patients will be stimulated by their physiological ligands in future experiments by employing APCs presenting E. coli-derived vitamin B metabolites on MR1 [453]. Notably, it has been shown for MAIT cells from HIV infected patients that the use of such physiological ligands for TCR stimulation resulted in similar or even reduced production of IFNγ compared to PMA and Ionomycin stimulation [390]. Thus, it is likely that the production of cytokines in response to PMA/Ionomycin stimulation observed here reflect the response to physiological ligands adequately.

Before conventional naïve $CD4^+$ and $CD8^+$ T cells can carry out their effector functions, they have to differentiate into effector T cells. This differentiation process requires 3 signals provided by APCs: signal 1 being TCR-mediated antigen stimulation, signal 2 provided by co-stimulatory receptors such as CD28, and signal 3 provided by pro-inflammatory cytokines such as IL-12 and/or type I IFN [302, 303]. Once differentiated into effector T cells, conventional T cells show a reduced threshold for cytokine secretion and cytotoxicity towards infected target cells. MAIT cells belong to the family of innate-like T cells and are not naive but exist in an activated effector T cell state. They can therefore carry out their effector functions immediately, without the need of further differentiation [502]. Furthermore, they do not only produce effector cytokines and are able to carry out cytotoxic effector function upon TCR signalling, but also respond to cytokine mediated stimulation [373], thus showing features of both innate and adaptive immune cells.

The data presented in this thesis show that certain innate stimulation, a specific combination of cytokines is able to evoke IFNγ production MAIT cells from AILD patients. Such induction of IFNγ production was specifically driven by IL-12 + IL-18 with or without IL-1β. In addition, repetitive stimulation with IL-12 was sufficient to induce IL-17A production in MAIT cells from AILD patients. Interestingly, this is in contrast to mitogenic stimulation with PMA/Ionomycin, which seems to be impaired in MAIT cells from AILD patients, compared healthy controls.

Therefore, one could speculate, that interfering with the innate activation of MAIT cells by IL-12+IL-18 might be a promising target for anti-inflammatory and anti-fibrotic therapy, which is potentially superior to inhibition of TCR mediated stimulation.

3.4.3 The potential role of MAIT cell-derived IL-17A in the development of fibrosis in AILD

In this thesis, it was demonstrated that MAIT cells from healthy controls, as well as from patients with AILD, respond with IL-17A production to repetitive stimulation with IL-12.

Notably, IL-12 signalling has been shown to play a role for the development of AILD and is extensively studied for potential exploitation for novel therapeutic approaches to treat AILD patients [405]. For example, genome-wide association studies (GWAS) have identified genetic variants in the IL-12 pathway to be associated with the development of PBC [503]. Moreover, deletion of the IL-12 subunit p40 ameliorates the development of autoimmune cholangitis in a mouse model [455], and overexpression of IL-12 leads to the development of persistent liver inflammation in mice resembling AIH [504]. Interestingly, serum levels of IL-12 were elevated in patients with PSC and significantly elevated in patients with PBS and AIH [487], suggesting that MAIT cells in these AILD patients are chronically exposed to high levels of IL-12. Although the exact contribution of MAIT cells to AILD is not known up to now, the potent effect of IL-12 on MAIT cell activation observed in this study suggests that persistent exposure of MAIT cells to IL-12 in AILD patients most likely results in MAIT cell activation and the secretion of pro-inflammatory cytokines such as IL-17, in the liver *in vivo*, thereby contributing to disease progression.

IL-17 is considered a key cytokine for the development of tissue-specific autoimmune disease. IL-17 can stimulate various cell types, such as endothelial cells, macrophages and fibroblasts, to produce pro-inflammatory cytokines including IL-1, IL-6 and TNFα [505]. Due to this effect, IL-17 is thought to play a role in various autoimmune diseases, e.g. multiple sclerosis, rheumatoid arthritis and IBD [506] but also in the development of autoimmune liver disease [507, 508]. Consistent with this hypothesis, serum levels of IL-17 are elevated in PSC, PBC and AIH [432, 509, 510]. Interestingly, the number of IL-17$^+$ cells infiltrating bile ducts is increased in PBC patients [432] and PSC patients show an increase in IL-17$^+$ cells within the peri-portal tracts of the liver [433]. Furthermore, the number of IL-17$^+$ cells positively correlates with the severity of hepatic inflammation and fibrosis in AIH [421]. Although until now these findings have mostly been attributed to conventional Th17 T cells, it is

likely that MAIT cells contribute to IL-17 production in these patients and that IL-17$^+$ cells identified in these studies include MAIT cells. This assumption is underscored by a recent report suggesting that MAIT cells are the dominating population of IL-17-producing T cells within the liver, representing more than 60% of all IL-17 producing cells [372].

Interestingly, IL-17 also plays a role for the development of non-immune-mediated liver disease, such as ALD, NASH and viral hepatitis [507, 508], implicating that IL-17 secretion by MAIT cells may contribute to the pathogenesis of chronic liver disease and liver fibrosis of various aetiologies.

In this study, MAIT cells were significantly decreased in peripheral blood and liver of AILD patients. This raises the question whether such reduced numbers of MAIT cells are sufficient to contribute to fibrosis development in AILD. As we hypothesised that the decrease of MAIT cells is associated with MAIT cell exhaustion following chronic activation, it is possible that the damage caused by MAIT cells predates the moment of analysis. AILD are notoriously asymptomatic for a long time [13] making it very difficult to analyse MAIT cells at an early disease state. This is further aggravated by the fact that 30% of patients with AIH are cirrhotic at the time of diagnosis, and up to 35.9% of patients with PSC show dominant strictures when first diagnosed [405, 511]. Therefore, it is possible that MAIT cells do accumulate in the inflamed liver in early stage AILD, become activated in the liver and then die due to activation-induced cell death and/or exhaustion.

One potential mechanism of how IL-17 produced by MAIT cells contributes to the development liver fibrosis might be the activation of other liver resident cells, e.g. HSCs. It has been shown that activated HSCs are the main cell type responsible for fibrosis development, as they produce excess extracellular matrix and at the same time inhibit ECM degradation, leading to net accumulation of ECM and eventually liver fibrosis [6, 24, 51]. Interestingly, HSC express the IL-17 receptor [508] and IL-17A is able to activate HSCs and induced the production of collagen I in HSCs via STAT3 signalling [435, 436]. As both circulating MAIT cells and liver resident MAIT cells produced high levels of IL-17A upon repetitive stimulation with IL-12, these data suggest that the IL-12 - IL-17 axis in MAIT cells could contribute to fibrosis development in humans. This hypothesis is further supported by the fact that MAIT cells were able to stimulate HSC proliferation *in vitro*. Interestingly, MAIT cell

mediated HSC proliferation was already observed upon co-culture of HSCs with MAIT cells isolated from healthy volunteers in absence of further stimulation. Furthermore, additional TCR-triggering on MAIT cells with CD3/CD28 beads did not enhance HSC proliferation. This could be due to rather low levels of cytokines, in particular IL-17, secreted by MAIT cells upon CD3/CD28 stimulation without additional cytokine stimulation [335, 372].

These data further suggest that HSC proliferation induced by MAIT cells relies on MAIT cell-derived soluble mediators that are produced due to their recent activation *in vivo*, or that HSCs provide sufficient signals to activate MAIT cells, e.g. by producing MAIT cell activating cytokines. This hypothesis is supported by the fact that human HSCs indeed secrete low levels IL-12 in culture, which is significantly increased by inflammatory signals [512].

Notably, it still needs to be clarified whether MAIT cell HSC interactions are MR-1 and/or cell contact dependent. Moreover, it needs to be tested whether MAIT cells can stimulate other features of HSC activation, such as upregulation of α-SMA or increased collagen deposition. Furthermore, to test the hypothesis, that MAIT cells stimulate HSC proliferation by secretion of IL-17, experiments involving IL-17 blockade need to be conducted.

Of note, biliary epithelial cells also express the IL-17 receptor and produce the pro-inflammatory cytokines IL-1, IL-6 and IL-23 upon IL-17 exposure, which leads to the activation of innate immune cells and MAIT cells, but also induces periductular fibrosis [435, 436]. Moreover, MAIT cells respond with cytokine secretion to bacterially infected biliary epithelial cells [356]. These findings suggest that interactions between MAIT cells and biliary epithelial cells, besides MAIT cell – HSC interactions, might contribute to the perpetuation of liver inflammation and fibrosis development.

3.4.4 Circulating and liver-resident MAIT cells – differences and similarities

In this thesis, circulating as well as liver MAIT cells from patients with AILD have been investigated. MAIT cell frequency was significantly higher in the healthy liver, compared to peripheral blood, which might be due to the high expression of the liver-specific homing receptors CCR6 and CXCR6 on MAIT cells, resulting in their homing to the liver [335, 356, 372]. Whereas the different subsets of MAIT cells were similarly represented in both bMAITs and liMAITs, with $CD8^+$ and $CD4^-/CD8^-$ MAIT cells being the most abundant subsets, liMAITs showed a more activated phenotype than bMAITs, evident by higher expression of activation markers and inhibitory receptors. Moreover, basal IL-17 expression without stimulation was markedly higher in liMAITs compared to bMAITs.

As discussed above, the liver is constantly exposed to antigens derived from food and gut bacteria, even in healthy humans [478, 479]. Because of such continuous antigen exposure, the liver has been described as a tolerogenic environment [3, 4], and antigen-presentation to conventional $CD4^+$ and $CD8^+$ T cells within the liver, e.g. by non-professional APCs such as LSEC seem to result in a tolerant or regulatory phenotype of T cells [324].

Although the response of liMAITs to antigen presentation by non-professional liver-resident APCs such as LSEC or HSCs has not been investigated so far, it has been shown that liMAITs isolated from chronically diseased livers express IFNγ and TNFα in response to E.coli-exposed biliary epithelial cells [356]. This suggests that liMAITs not only express high levels of activation markers, but also produce effector cytokines in chronic liver disease. Nevertheless, it remains unclear if liver-resident non-professional APCs can present antigen to MAIT cells, and if such presentation results in a cytotoxic or rather tolerant phenotype in the steady-state.

Upon stimulation with cytokines, both bMAITs and liMAITs responded to singular stimulation with IL-12+IL-18 +/- IL-1β with IFNγ production that was indistinguishable between the two. Moreover, similar to bMAITs, no IL-17A production could be evoked in liMAITs by singular cytokine stimulation, although liMAITs showed a slightly higher basal expression of IL-17 than bMAITs.

In contrast, liMAITs responded with IL-17A production to repetitive stimulation for 72 hours with IL-12+/-IL-1β, which was significantly higher than in bMAITs. Moreover, repetitive stimulation with IL-1β+/-IL-18 resulted in a significant increase in IL-17A production in liMAITs, but not bMAITs. Thus, despite being able to express the Th1 cytokine IFNγ, liMAITs seem to be skewed towards a Th17 cytokine expression pattern with high IL-17A expression, perhaps owing to their high expression of RORγt [356, 378, 379], suggesting that liMAITs play an important role in tissue inflammation in the liver.

Interestingly, repetitive stimulation with IL-12+IL-18 +/- IL-1β liMAITS did not evoke IL-17A production in liMAITs. It is unlikely, that unresponsiveness to repetitive IL-12+IL-18 +/- IL-1β stimulation is due to receptor down-regulation, as IL-17A production was induced by repetitive stimulation with IL-12 +/- IL-1β. Alternatively, the lack of IL-17A expression after repetitive stimulation with IL-12+IL-18 +/- IL-1β could rather be a sign of overstimulation or regulation of the immune response, respectively.

Moreover, upon repetitive stimulation, IFNγ production first increased in liMAITs and then decreased, whereas IFNγ expression continuously declined in bMAITs. This implies that liMAITs are able to respond with increasing cytokine production to repetitive stimulation, which is then followed by negative regulation of cytokine expression to avoid an excessive immune response.

Despite the differences in the activation status and cytokine production between bMAITs and liMAITs in general, in AILD patients even bMAITs showed an activated and exhausted phenotype, indicating that MAIT cells might circulate between the liver and the peripheral blood in AILD patients. So far, it is unclear whether MAIT cells circulate between organs and peripheral blood, or if there is a resident MAIT cell population that is confined to the liver microenvironment. The high percentage of $CD69^+$ MAIT cells in the liver however suggests that these MAIT cells are resident to the liver, as CD69 has been shown to be a marker for tissue residency in T cells [513-515].

Overall, despite sharing some characteristics, bMAITs and liMAITs show differences in their activation state and response to cytokine stimulation, making it necessary to study MAIT cells from both liver and blood, especially when investigating the role of

MAIT cells in liver disease. However, as MAIT cells from the blood expressed high levels of exhaustion markers, and the frequency of MAIT cells negatively correlates with the fibrosis stage in AILD patients, blood derived MAIT cells might serve as a marker for disease progression, if this correlation is confirmed in studies enrolling a sufficient number of cases.

3.4.5 Alterations in liMAIT phenotype and function in AILD patients

When liMAITs from AILD livers were analysed, their frequency was significantly reduced compared to healthy controls, analogue to the findings for bMAITs in these patients. As discussed above, this was surprising, as MAIT cells have been shown to translocate to sights of infection and inflammation [361, 364, 401], therefore one would expect to observe an enrichment of liMAITs in AILD patients. Of note, liMAITs were still more frequent than bMAITs in AILD, and it has been reported that MAIT cells from healthy and diseased livers show no differences in CCR6 and CXCR6 expression [356], indicating that that preferential homing of MAIT cells to the liver is maintained in AILD livers.

It has been shown that bacterial translocation from the gut to the liver occurs in chronic liver disease, as shown for example for ALD [479, 516], NASH [517] and AILD [477, 518, 519]. Therefore, exposure of MAIT cells to their antigens is most likely higher in AILD livers than in healthy livers, which might result in a more activated or even exhausted phenotype of liMAITs from AILD livers.

Interestingly, although the expression levels of the activation markers CD38, CD69 and HLA-DR were higher in liMAITS than in bMAITs in AILD patients, liMAITS from AILD livers did not express higher levels of these activation markers compared to healthy controls. However, expression levels of inhibitory receptors associated with T cell exhaustion differed between liMAITs from AILD patients and healthy controls. Whereas TIM-3 expression was lower in liMAITS from AILD patients compared to MAIT cells from healthy controls, the expression of CTLA-4 was increased.

The liver is considered to be a tolerogenic organ [3, 4, 314] and it is generally believed that this tolerance has to be overcome in order to establish autoimmune disease in the liver [520, 521]. It has been suggested that a lack of inhibitory receptor signalling contributes to the breakdown of tolerance in AIH. In line with this hypothesis, TIM-3 expression is decreased in $CD4^+$ effector T cells in patients with AIH, rendering them less susceptible to Treg control [522]. Likewise, the lower TIM-3 expression in liMAITs from AILD patients, suggests that they show an effector phenotype and the low expression of TIM-3 might contribute to the ability of these cells to produce inflammatory cytokines. This is supported by the fact that liMAIT cells are able to express effector cytokines in the setting of chronic liver disease, as shown by Jeffery et al. [356].

In contrast, CTLA-4 expression was highly increased in liMAITs from AILD livers, compared to healthy controls. Moreover, CTLA-4 was significantly higher in liMAITs, compared to bMAITs in AILD.

This finding seems particularly interesting, as GWAS have identified single-nucleotide polymorphisms (SNPs) in the CTLA-4 gene as a risk factor for the development of PSC, PBC and AIH [523, 524]. Loss of CTLA-4 function results in defective activation of inhibitory pathways and therefore in autoimmunity [525, 526], supported by reports showing that decreased CTLA-4 expression or CTLA-4 blockade results in an AIH-like liver pathology in mice [527, 528]. In contrast, high expression of CTLA-4 leads to an impaired immune response to various pathogens, including bacteria [524]. Of note, bacterial infections are very common in cirrhotic patients and increase morbidity and mortality in cirrhotic patients [529, 530]. Therefore, it is possible that increased expression of CTLA-4 in liMAITs in AILD limits indeed the immune pathology, but also contributes to increased susceptibility to infection in cirrhotic AILD patients. Overall, these data should however be interpreted with caution, as only three PSC livers have been available for analysis.

In order to gain insight into the functionality of liMAITs isolated from AILD patients, preliminary experiments were performed. LiMAITs from two PSC patients and from healthy controls were stimulated with different combinations of the cytokines IL-1β, IL-12 and IL-18 (50ng/ml each) for 24 hours and cytokine production by MAIT cells was analysed by flow cytometry. As shown in figure 3.28, like liMAITs from healthy livers, liMAITs from AILD livers specifically responded to stimulation with IL-

12+IL-18 +/- IL-1β with IFNγ production. Similarly to healthy controls, IL-17A production of liMAITs from AILD patients was not induced by cytokine stimulation for 24 hours, although there was a greater increase of IL-17A production in cytokine stimulated liMAITs from AILD patients, compared to no stimulation. It is very difficult to draw valid conclusions from these data, as only two PSC livers have been available for experiments. Nevertheless, this could hint towards IL-17A expression being maintained in liMAITS from AILD patients, even with end-stage liver disease, as the liver tissue for analysis originated from liver explants.

This suggests that liMAITs could contribute to liver inflammation and tissue damage in AILD by secretion of IL-17A, although further experiments are required to characterize the functionality of liMAITs from AILD patients in more detail.

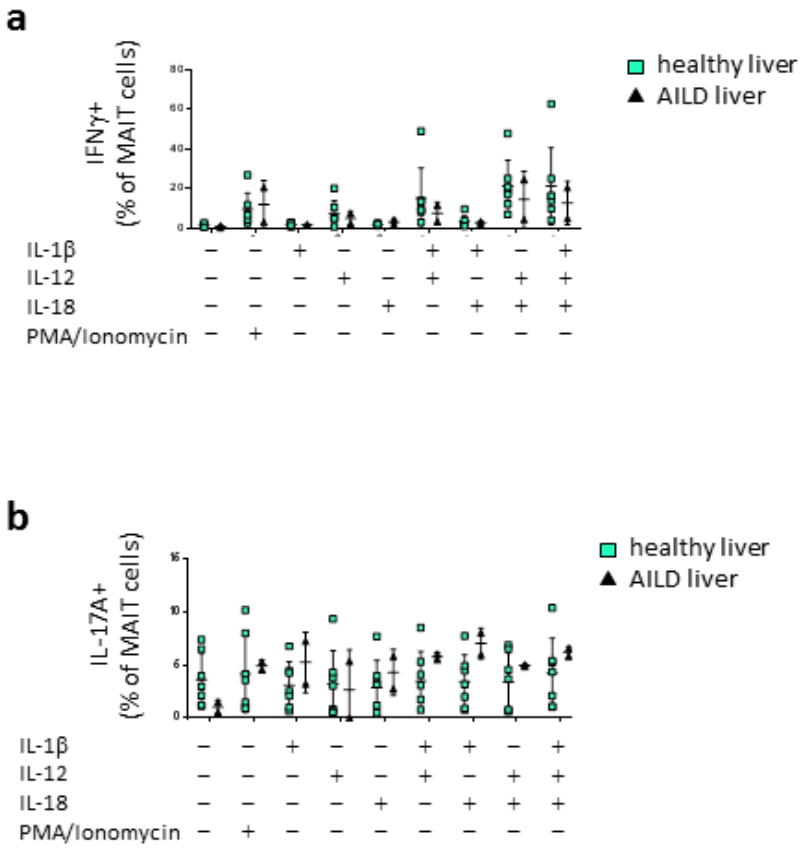


Figure 3.28: IFNγ but not IL-17A response is impaired in liMAITs from PSC livers.
Liver associated-lymphocytes from PSC and control livers were stimulated with PMA (50ng/ml) and Ionomycin (1μM) or IL-1β, IL-12 and IL-18 (50ng/ml) for 24 hours, and (a) IFNγ and (b) IL-17 expression in MAIT cells analysed by intracellular staining and flow cytometry, summary data. Each symbol represents data from one individual. Data represent mean +/- SD, pooled data from 2 independent experiments.

Overview over the potential role and fate of MAIT cells in AILD

Considering the data presented in this thesis as well as the potential mechanisms discussed in chapter 3.4, it can be hypothesised that MAIT cells may contribute to fibrosis development in AILD. The potential role and the fate of MAIT cells in AILD are summarized in figure 3.29.

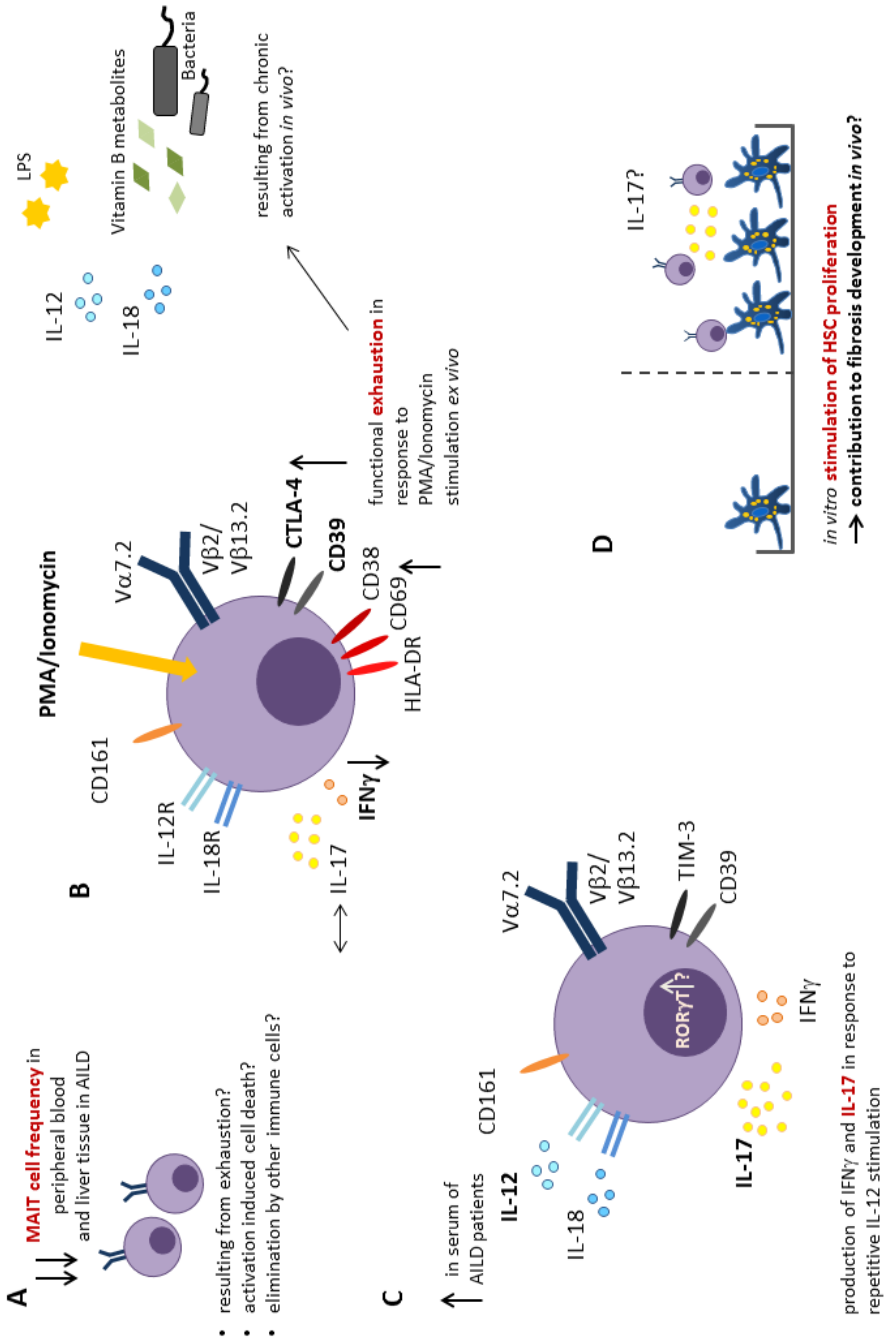


Figure 3.29: Potential role for MAIT cells in AILD

A: The MAIT cell frequency is significantly reduced in peripheral blood and liver tissue of MAIT cells in AILD, eventually due to severe exhaustion as a result from chronic exposure to antigen and/or LPS, IL-12 and IL-18 *in vivo*. **B**: Whereas MAIT cells from AILD patients show signs of exhaustion *ex vivo* in response to PMA/Ionomycin stimulation, the response to cytokine stimulation is maintained. **C** : Repetitive stimulation with IL-12 results in expression of IL-17 by MAIT cells, which might contribute to tissue damage and is able to stimulate HSC proliferation. **D**: The induction of HSC proliferation by MAIT cells *in vitro* suggests that MAIT cells may play a role for fibrosis development *in vivo*.

Appendix chapter 3

The work presented in this chapter has been presented at various national and international meetings in the last three years:

"Mucosa associated T (MAIT) cells in autoimmune liver disease"
Oral presentation, 7th Meeting of the European Club of Liver Cell Biology (ECLCB) 2016, Ascot, UK

"Mucosa associated T (MAIT) cells in autoimmune liver disease"
Oral presentation, Annual Meeting of the Association of Physicians of Great Britain and Northern Ireland 2016, Plymouth, UK

"Mucosa associated invariant T (MAIT) cells are phenotypically altered and functionally impaired in patients with autoimmune liver disease"
Poster presentation, The International Liver Congress 2016, European Association for the Study of the Liver, Barcelona, Spain

"Mucosa associated invariant T (MAIT) cells are phenotypically altered and functionally impaired in patients with autoimmune liver disease"
Poster presentation, Division of Medicine Research Retreat 2016, University College London, London, UK

"Mucosa associated invariant T (MAIT) cells are phenotypically altered and functionally impaired in patients with autoimmune liver disease"
Poster presentation, Institute for Immunity and Transplantation Symposium 2016, University College London, London, UK

Moreover, I have been awarded the **EASL Physician Scientist Fellowship** for12 months in 2017 in order to continue working on the project **"Mucosal-associated invariant T cells (MAIT cells) in autoimmune liver diseases – a potential target for anti-fibrotic therapy"**.

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