Diversity and Compartmentalisation of Monocytes and Macrophages in Patients with Liver Cirrhosis

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Dedicated to my parents

Liane und Karlheinz Geng

“Science and everyday life cannot and should not be separated.”

Rosalind Franklin

Summary

Liver cirrhosis is a widespread systemic condition with increasing global prevalence. Individuals with cirrhosis are particularly vulnerable to bacterial infections, which can trigger acute decompensation (AD) and acute-on-chronic liver failure (ACLF), both of which are linked to high rates of morbidity and mortality, with limited treatment options beyond transplantation. Mononuclear phagocytes are crucial to innate immune responses and serve as a primary defense mechanism against pathogens. The study investigated the heterogeneity of macrophages and monocytes subsets and their compartment-specific variations related to disease progression in liver cirrhosis. Distinct subsets identified here included AXL-expressing and CD52-expressing cells, which play critical roles in regulating innate immune responses and contribute to the complex pathogenesis of immuneparesis in patients with cirrhosis. Strong correlations with clinical parameters, cirrhosis severity scores, infectious complications, and mortality highlight their clinical significance, and suggest their potential as biomarkers for innate immune function integrity on the one hand and as immunotherapeutic targets on the other.

Initial investigations focused on monocyte and macrophage dysfunction in AD/ACLF stages of cirrhosis, where the risk of infection and subsequent morbidity and mortality is highest. Previous studies by the research group showed an increase in monocytic subsets, such as M-MDSC, occurring in compensated cirrhosis with increasing prevalence along with disease progression and highet proportions in AD/ACLF patients, and MERTK-expressing monocytes occurring in patients with AD/ACLF. AXL-expressing monocytes were found to increase with cirrhosis progession from mild to more severe stage, but disappear again in AD/ACLF stage of cirrhosis. With disease progression, these dysfunctional monocytic subsets prevailed over regular monocytes in the circulation of cirrhotic patients in relation to certain stages of disease. These distinct subsets exhibited

unique immune-regulatory functions and immune-suppressive capabilities, compared to regular monocytes. AXL-expressing monocytes demonstrated attenuated inflammatory cytokine production and T-cell activation and enhanced efferocytosis and maintained phagocytosis of *E.coli*. MERTK+ monocytes have immune-regulatory function and dampen the immune response. Futhermore, those monocytes showed enhanced efferocytosis capacity. M-MDSC exhibit immune-suppressive properties, significantly decreasing T cell proliferation, low cytokine responses and reduced bacterial uptake.
AXL, MERTK, and Tyro-3, members of the TAM receptor tyrosine kinases (RTK) family, inhibit TLR-signaling, cytokine receptor-mediated activation, and apoptotic cell removal in monocytes/macrophages. Loss of these RTK leads to liver disease with characteristic apoptotic cell accumulation. In this study, over 90% of liver macrophages expressed AXL in homeostasis, with a reduction in AXL-expressing resident liver macrophages paralleling cirrhosis progression. Interestingly, this process was reversible, as shown by changes in AXL expression in patients transitioning between Child B and Child C stages.
Immunohistochemistry indicated AXL-expressing macrophages were abundant in hepatic plates, while MERTK-expressing macrophages were enriched in both hepatic plates and areas of fibrosis. Phenotypically, AXL-expressing hepatic macrophages under physiological conditions were mature, indicating a need for tolerance at the portal-systemic circulation barrier. The proportion of macrophages phagocytosing gram-negative *E.coli* decreased in cirrhosis, with AXL-expressing monocytes showing increased migratory potential.
Compartment-specific AXL-expression was also assessed in other tissues, including the gut, peritoneum, lymph nodes, and bone marrow. Intestinal macrophages exhibited reduced AXL-expression with cirrhosis progression, similar to hepatic macrophages. Peritoneal macrophages showed lower AXL-expression but higher MERTK-expression compared to monocyte-derived macrophages (MDM). Notably, AXL+ macrophages were absent in the bone marrow but

accumulated in the circulation and lymph nodes of advanced cirrhosis patients, supporting the hypothesis of enhanced migration potential.

These findings, which suggested a stage-specific heterogeneity in monocytes and macrophages during cirrhosis evolution, and prompted an unbiased approach to investigate the underlying pathophysiology of immune. The study employed single-cell RNA sequencing (scRNA-Seq) to systematically decode the stage-specific heterogeneity of circulating monocytes in cirrhosis patients. Seven monocytic clusters were identified, representing classical, non-classical, intermediate monocytes, and M-MDSC, with varying prevalences among different cirrhosis stages. These clusters exhibited distinct marker gene expressions and functions, with inflammatory responses, phagocytosis, and complement system upregulated in compensated cirrhosis but downregulated in decompensated stages, indicating immuneparesis onset.

CD52 expression on monocytes emerged as a significant finding, with enhanced expression in compensated and NAD cirrhosis compared to healthy controls. $CD52^{high}$ monocytes displayed an activated state, with increased phagocytosis capacity, cytokine production potential, and adhesion/migration behavior, and presence of CD52 expressing cells was associated with survival. Interestingly, CD52 expression was downregulated or absent in AD/ACLF stages. Plasma components influenced CD52-expression levels, with bacterial particles elevating CD52 expression, while AD/ACLF plasma dampened phagocytosis capacity.

The study also explored the clinical relevance of CD52-expression on monocytes and soluble CD52 (sCD52) in the bloodstream as non-invasive biomarkers for cirrhosis severity and innate immune function. Elevated phospholipase C (PLC) plasma levels correlated with reduced CD52 surface expression and increased sCD52 plasma levels, suggesting PLC as a potential target for stabilizing CD52 on monocytes. The findings highlight the significance of the CD52 pathway as a clinically relevant biomarker and potential immunotherapeutic target in cirrhosis management.

In conclusion, the study demonstrates that with cirrhosis progression, $CD52^+$ and AXL^+ hepatic macrophages decrease while $CD52^+$ and AXL^+ circulating monocytes increase, reflecting changes in innate immune function. The study provides insights into the potential therapeutic modulation of the AXL-GAS6 and CD52-PLC pathways to enhance innate immune responses, reduce infection susceptibility, and improve survival in cirrhosis patients.

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Abbreviations

ACLF	Acute-on-chronic liver failure
AD	Acute decompensation
AHR	Airway hyperreactivity
AIH	Autoimmune hepatitis
ALT	Alanine-aminotransferase
AP	Alkaline phosphatase
APCs	Antigen-presenting cells
ARLD	Alcohol-related liver disease
AST	Aspartate-aminotransferase
BCR	B cell receptor
BT	Bacterial translocation
CLIF-score	Chronic liver failure score
CLIF-SOFA-score	CLIF- sequential organ failure score
CLL	Chronic lymphocytic leukemia
DALYs	Disability-adjusted life years
DAMP	Damage-associated molecular pattern
DCs	Dendritic cells
GGT	Gamma-glutamyl transferase
GPI-anchored	Glycosylphosphatidylinositol-anchored
GVHD	Graft versus host disease
hASCs	Human adipose stem cells
HBV/HCV/HAV/HDV/HEV	Hepatitis-B/C/A/D/E-virus
HCC	Hepatocellular carcinoma
HE	Hepatic encephalopathy
Hh signalling pathway	Hedgehog signalling pathway
HMGB1	High mobility group box 1
HSCs	Hepatic stellate cells
HVPG	Hepatic venous pressure gradient
IFN-α/-β/-γ	Interferon-α/-β/-γ
IL-4/-13	Interleukin-4/-13

ILC2s	Innate lymphoid cells group 2
LSECs	Liver sinusoidal endothelial cells
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAFLD	Metabolic-associated fatty liver disease
MELD	Model of end-stage liver disease
mDCs	Myeloid DCs
NAD	Not-acute decompensation
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
pDCs	plasmacytoid DCs
PH	Portal hypertension
PBC	Primary biliary cholangitis
PSC	Primary sclerosing cholangitis
PLC	Phospholipase C
RMS	Relapsing remitting multiple sclerosis
RTKs	Receptor tyrosine kinase
SBP	Spontaneous bacterial peritonitis
sCD52	Soluble CD52
Siglec	Sialic acid-binding immunoglobulin-like lectins
SLE	Systemic lupus erythematosus
SSc	Systemic sclerosis
TCR	T cell receptor
TH2 cells	T helper 2 cells
TIPS	Transjugular intrahepatic portosystemic shunt
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
WHO	World Health Organizations

1 Background

1.1 Liver Cirrhosis

1.1.1 Epidemiology

Cirrhosis of the liver represents a global health problem with increasing prevalence and high morbidity and mortality causing more than one million deaths per year worldwide [1, 2]. Cirrhosis is the 10th leading cause of death in Africa, 9th in South East Asia and Europe and 5th in Eastern Mediterranean [3]. Cirrhosis significantly impacts global health and individual quality of life due to its high amount of disability-adjusted life years (DALYs), the potential years of life with full health lost [4]. In 2016 the expenses for liver-related diseases were $32.5 billion [4]. Interestingly two out of 3 liver-related deaths occur in male [2, 5-7].
Alcohol use causing alcohol-related liver disease (ARLD), high caloric intake causing non-alcoholic fatty liver disease (NAFLD) – recently referred to as metabolic-associated fatty liver disease (MASLD) [8, 9] – and hepatitis virus infections e.g. Hepatitis-B-Virus/Hepatitis-C-Virus (HBV/HCV) [5]. The per capita consumption of alcohol was 6.4L worldwide in 2016 [10]. Alcohol use disorders make up to 5.1% globally [11]. Alcohol abuse is the leading cause for cirrhosis worldwide and is responsible for ~60% of cirrhosis cases in Europe, North America and Latin America [12, 13]. The worldwide prevalence of NAFLD is 30.5% with an upward trend [14]. NAFLD is the 2nd leading cause for liver transplantation in the US and the leading cause among females [15, 16]. It is hypothesized that NAFLD will become leading aetiology for liver transplantation in end stage cirrhosis patients in the future [17, 18]. HBV and HCV related diseases accounted for 1.1 million deaths in 2020 [3]. The World Health Organizations (WHO) has estimated that African and the South East Asia regions account for a high proportion of the global HBV burden, but the western pacific region account for 57% of global mortality due to HBV [19, 20]. Approximately 12 million people are co-infected with

HDV worldwide. It is estimated that HDV contributes in 18% of cirrhosis cases in patients with HBV. HDV is dependent on a simultaneous infection with HBV for its life cycle and has a worldwide prevalence of 12 million with Mongolia at top of the list, followed by Guinea-Bissau, Gabon and Mauritania [21-23]. Other aetiologies causing cirrhosis are Primary sclerosing cholangitis (PSC) and Primary biliary cholangitis (PBC), which are more common in Western populations (0.1 – 1.58 and 0.33 – 5.8 per 100,000 people/year) [24-26]. Furthermore, autoimmune hepatitis (AIH) can cause cirrhosis and occurs in all races, ethnic groups and ages with a prevalence of 4.8 – 42.9 per 100,000 people/year [27, 28]. Less prevalent aetiologies are Wilson's disease, haemochromatosis, drug-induced liver injury (DILI) [5] and Alpha-1 antitrypsin (A1AT) deficiency [29].

1.1.2 Pathogenesis of Cirrhosis

Cirrhosis of the liver has been defined as parenchymal loss caused by necrosis, inflammation and fibrogenesis as result of hepatocyte damage through harmful underlying aetiologies. Chronic liver injury is associated with activation and alteration of liver residend and infiltrating immune cells [30]. The loss of hepatocytes results in impaired liver function and causes excessive repair processes, causing formation of nodules of hepatocytes with surrounding fibrous septa (Figure 1.1-1) [2].

Progressive fibrosis can lead to aberrant regeneration, inflammation and vascular changes [2, 31]. Due to the changes in the vascular architecture resistance to portal blood flow increases, called portal hypertension [31]. Liver sinusoidal endothelial cells (LSECs) and activated hepatic stellate cells (HSCs) are involved in the vascular remodelling, formation of intrahepatic shunts and hepatic endothelial dysfunction [2]. The increased pressure on the portal vein, carrying blood from the digestive organs to the liver and the porto-systemic shunting results in pathological bacterial translocation (BT) [32, 33]. Portal hypertension (PH) can cause

complications such as portal-hypertensive gastropathy, variceal bleeding, ascites, portosystemic shunts creating encephalopathy [2].

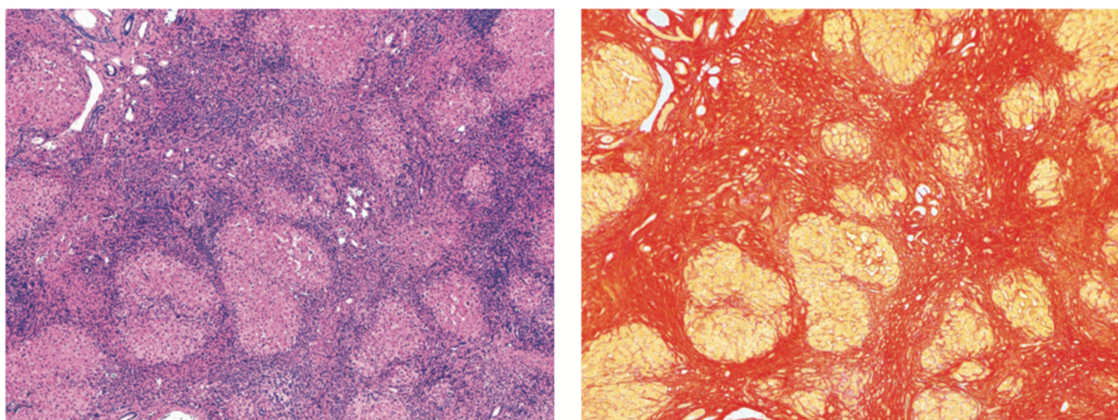

Figure 1.1-1: Histology of Liver Cirrhosis
Liver biopsy of a 53-year-old man with ARLD showing advanced cirrhosis with small cirrhotic nodules, thick internodular septa and fibrous tissue. Left: Haematoxylin and eosin stain. Acidic structures (basophil) appear blue, alkaline structures (eosinophil) appear red. Right: Sirius red stain. Collagen fibres stan red. (adapted from Tsochatzis et al., Lancet, 2014)

1.1.3 Diagnosis

The diagnosis of liver cirrhosis involves the combination of clinical, laboratory, radiology and histological factors.
One tool for the diagnosis are imaging methods such as ultrasonography, where clinicians look for irregular and nodular liver structure, splenomegaly and portosystemic shunts. The grade of liver stiffness can be assessed by performing non-invasive transient elastography (e.g. FibroScan®). This examination gives indirect indication of the degree of fibrosis [34]. There are possible misdiagnoses indicating high liver stiffness suggesting advanced fibrosis grade, due to false high values. This can be caused by multiple factors like operators experience, operating and disease specific conditions [35].
In the medical laboratory blood values like bilirubin, albumin, alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), gamma-glutamyl transferase (GGT) and alkaline phosphatase (AP) are routine laboratory test and easily assessable. Additionally, tests verifying the protein synthesis function of the liver

(albumin, coagulation factor V, International Normalized Ratio (INR)) are in use to investigate the severity of the disease. The gold standard for diagnosis of cirrhosis is liver biopsy and can be performed percutaneous or transjugular. The latter provides additional information about hepatic venous pressure gradient (HVPG) and is favourable in patients with acquired coagulopathy [36]. Histological staining of the biopsy provide information about aetiology of the cirrhosis, accompanying liver diseases, grade of inflammation and fibrosis stage [37]. Biopsies are also advantageous to discriminate between late fibrosis stage and cirrhosis [38]. Liver biopsy is associated with a rather low bleeding risk of 0.6% [39].

In early compensated stages of cirrhosis, symptoms are rare. In advanced cirrhosis symptoms include ascites, jaundice, hepatic encephalopathy (HE), infections, sepsis and variceal bleeding and may facilitate to make a diagnosis even without histological confirmation. The grade of ascites is assessed by ultrasonography and to diagnose oesophageal varices and/or bleeding esophagogastroduodenoscopy is performed.

For the diagnosis of cirrhosis and to distinguish between fibrosis and cirrhosis, especially in compensated patients, non-invasive biomarkers can be helpful tools [40, 41].

1.1.4 Clinical Presentation

Cirrhosis of the liver is a dynamic condition succeeding diverse aetiologies [42]. Over the previous decade it has been observed that fibrosis can be reduced and early forms of cirrhosis can regress when the underlying liver disease is treated successfully [42, 43]. Nevertheless, the treatment of the underlying liver disease does not always lead to regression of cirrhosis or prevention of complications. Such complications include oesophageal varices, ascites, decompensation and development of hepatocellular carcinoma (HCC) [44].

Depending on the circumstances and the underlying liver disease, cirrhosis may remain compensated for an unpredictable period of time, in which fibrosis and PH progress, remain static or regress [44]. The risk for patients with cirrhosis to progress from compensated to decompensation is around 5% per year and is typically characterised by manifestation of peritoneal fluid, so called ascites, oesophageal varices with or without bleeding [44]. Acute decompensation (AD) may be triggered by precipitating events such as the development of infection, variceal bleeding, excessive alcohol consumption, portal-vein thrombosis or surgery [2, 44]. Variceal bleeding is a major emergency with a mortality rate of 10-20% [44]. A major characteristic of decompensation is the manifestation of ascites, with a 5-year mortality rate of 30% [45]. Refractory ascites has a 2-year mortality rate of 65% [46]. Furthermore, HE and jaundice are also signs for decompensation in advanced cirrhosis, with a 5-year survival rate of 20% [47]. The onset of renal dysfunction can also occur in advanced cirrhosis and manifest as kidney injury and hepatorenal syndrome [48]. Precipitating events for decompensation are the development of infections and associated with organ failure and adverse outcome [44]. Acute-on-chronic liver failure (ACLF) is defined based on AD with the development of organ failure [45]. Patients with ACLF have a 28-day mortality ranging from 22-77% compared to 5% in AD patients without ACLF [45].
The phenomenon underlying the increased susceptibility to infection in patients with cirrhosis due to immune dysfunction is called immune paresis and detailed in **chapter 1.2**.
In order to categorise patients with liver cirrhosis according to their outcome, several cirrhosis severity scores have been developed [45, 47, 49, 50]. The Child Pugh classification was established in 1964 and uses points form 5 (early stage cirrhosis) to 15 (end stage cirrhosis) to categorise into Child A, B or C (Table 1) [49].

Table 1: Child Pugh Score

	1 Point	2 Points	3 Points
Bilirubin (µmol/L)	< 34	34 – 51	> 51
Albumin (g/dl)	> 3.5	2.8 – 3.5	< 2.8
INR	< 1.7	1.7 – 2.3	> 2.3
Ascites	none	mild	severe
Hepatic Encephalopathy (grade)	none	I – II	III – IV

	Child A	Child B	Child C
Total points	5 – 6	7 – 9	10 – 15
1-year survival	100 %	81 %	45 %

The model of end-stage liver disease (MELD) score has been established to predict 3-monthy mortality and assess the need for transplantation [50]. MELD score can range from 6 points representing an early stage, to 40 points, with a 3-months survival probability without transplantation of almost 0% [50].

MELD score calculation formular:

$$MELD = 3.8 \times \log_e(\text{bilirubin mg/dL}) + 11.2 \times \log_e(\text{INR}) + 9.6 \times \log_e(\text{creatinine mg/dL}) + 6.4$$

D'Amico and colleagues developed a classification into 4 stages by using the natural course of cirrhosis, reaching from stage 1, compensated to stage 4 decompensated cirrhosis (Table 2) [47].

Table 2: D'Amico Classification

Stage 1	Stage 2	Stage 3	Stage 4
Compensated	Compensated	Decompensated	Decompensated
No varices	Varices	Varices	Variceal Bleeding +/-
No ascites	No ascites	Ascites +/-	Ascites

1-year mortality			
1 %	3.4 %	20 %	57 %

Lately D'Amico et al. developed a new definition for decompensated cirrhosis where they discriminate between an acute onset, into which account previously described AD/ACLF and progressive onset, so called not-acute decompensation (NAD) [45]. This definition is further described and detailed in **chapter 1.2**. The

chronic liver failure (CLIF) score was developed to predict mortality in hospitalized cirrhosis patients by using the CLIF-AD [51], CLIF-ACLF [52] or CLIF- sequential organ failure (SOFA) score [53].

1.1.5 Treatment Options

The treatment options for patients with cirrhosis aim to treat the underlying liver disease and prevent complications but may reverse cirrhosis in a minority of cases only. Eliminating of the underlying trigger and management of complications remain the standard of care treatment options. In cases of NAFLD/MASLD changes in lifestyle and caloric restriction are approached. In ARLD a strict abstinence from alcohol is recommended, while in viral related hepatitis vaccination or viral drugs are used for treatment. Complications related to portal hypertension and the consequent development of varices and variceal bleeding are treated with non-selective betablockers and endoscopic variceal ligation. Furthermore, transjugular intrahepatic portosystemic shunt (TIPSS) can reduce the risk of recurrent bleeding [54]. Antibiotics are used to tread infections, which are triggered due to increased infection susceptibility. Patients with ascites are treated with diuretics and additionally with antibiotics when it comes to spontaneous bacterial peritonitis (SBP). Vasopressors, like terlipressing or vasopressin in combination with albumin are used to treat hepatorenal syndrome (HRS), while HE is being treated with lactulose [2]. Refractory ascites can further be an indication for TIPSS [46].

In end stage liver cirrhosis transplantation may be the last option to cure the disease, but it comes with its pitfalls. The procedure of transplantation is very complex and only applicable to a limited number of patients. Another problem facing when it comes to transplantation is the donor shortage. It was thought that cirrhosis is a non-reversible disease, but it has been shown that it can be reversible under certain circumstances [31, 55, 56]. These conditions require effective and

sustained treatment of the underlying cause of liver injury, e.g. successful treatment of chronic viral hepatitis [43, 57].

Immunomodulatory treatment strategies have been proposed as they may enhance immune defence in decompensated cirrhosis patients and prevent infection as a bridge to liver regeneration or translpantation. Some targets that have been proposed relate to monocyte function and may help to improve innate immune responses in patients with AD/ACLF. Immunomodulatory treatment strategies are further detailed in **chapter 1.2, Table 1**. Compounds like granulocyte-colony stimulating factor (G-CSF) to promote release of $CD34^+$ hematopoietic stem cells from the bone marrow have been extensively studied in the past [58-61]. Other components like UNC569 and BGB324 inhibit specific receptors (MERTK/AXL) on dysfunctional monocyte subsets to restore pro-inflammatory cytokine response *ex vivo* [62] and may be subject to clinical investigation in the future. Also, TLR-4 inhibitor TAK-242 [63-65] and CCR2/CCR5 inhibitor cenicriviroc [66] were used in rodent models to reduce liver injury mediated by macrophages and the recruitment of hepatic monocyte-derived macrophages (MoMF). Another experimental model found a reduction in liver inflammation and fibrosis by using NLRP3 inhibitor MCC950 [67]. *In vitro* poly(I:C), a TLR-3 angonist was found to reverse M-MDSCs expansion [68], methionine sulfoyimine, a glutamin synthetase inhibitor restored phagocytosis and inflammatory response of monocytes [69] and albumin, lowered circulating prostaglandin E2 (PGE2) levels and restored TNF-α production [70, 71].

1.2 Plasticity of Monocytes and Macropahges in Cirrhosis of the Liver

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Plasticity of monocytes and macrophages in cirrhosis of the liver

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Cirrhosis of the liver is a systemic condition with raising prevalence worldwide. Patients with cirrhosis are highly susceptible to develop bacterial infections leading to acute decompensation and acute-on-chronic liver failure both associated with a high morbidity and mortality and sparse therapeutic options other than transplantation. Mononuclear phagocytes play a central role in innate immune responses and represent a first line of defence against pathogens. Their function includes phagocytosis, killing of bacteria, antigen presentation, cytokine production as well as recruitment and activation of immune effector cells. Liver injury and development of cirrhosis induces activation of liver resident Kupffer cells and recruitment of monocytes to the liver. Damage- and pathogen-associated molecular patterns promote systemic inflammation which involves multiple compartments besides the liver, such as the circulation, gut, peritoneal cavity and others. The function of circulating monocytes and tissue macrophages is severely impaired and worsens along with cirrhosis progression. The underlying mechanisms are complex and incompletely understood. Recent 'omics' technologies help to transform our understanding of cellular diversity and function in health and disease. In this review we point out the current state of knowledge on phenotypical and functional changes of monocytes and macrophages during cirrhosis evolution in different compartments and their role in disease progression. We also discuss the value of potential prognostic markers for cirrhosis-associated immuneparesis, and future immunotherapeutic strategies that may reduce the need for transplantation and death.

KEYWORDS
cirrhosis, monocytes, macrophages, liver injury, inflammation, ACLF, immuneparesis, immunotherapy

1 Introduction

Cirrhosis of the liver is a global health burden with increasing prevalence and a high morbidity and mortality causing more than one million deaths per year worldwide (Mokdad et al., 2014; Tsochatzis et al., 2014). Cirrhosis represents a multisystemic disease, that not only involves the liver, but affects multiple organs, diverse immune cells and soluble factors (Ber nsmeier et al., 2020). Chronic liver injury is most frequently caused by alcohol use, nutritive fat accumulation and viral infections and associated with the activation and alteration of liver resident and infiltrating immune cells. It leads to replacement of healthy parenchyma by fibrotic tissue. This results in progressive fibrosis, disrupted liver architecture, aberrant regeneration, inflammation and vascular changes (Marcellin and Kutala, 2018). These processes moreover lead to portal hypertension (PH), an increase in the pressure within the portal vein, which carries blood from the digestive organs to the liver, and porto-systemic shunting, and results in pathological bacterial translocation (BT) (D'Amico et al., 2018; Mandorfer et al., 2020). The clinical course of cirrhosis when patients progress from compensated to decompensated cirrhosis relates to adverse prognosis. In compensated mostly asymptomatic cirrhosis mortality remains low, but dramatically increases in decompensated cirrhosis when ascites, variceal bleeding, bacterial infections, and organ failure develop (Arvaniti et al., 2010; D'Amico et al., 2006). Therapeutic options are limited to the treatment of the underlying cause of cirrhosis and in end stage liver disease transplantation remains the last option (Asrani et al., 2019). Donor organs are limited and don't meet the demand, therefore the development of new effective therapies for cirrhosis patients preventing disease progression could improve clinical outcomes. Immune dysfunction seen in cirrhosis starts with the onset of chronic inflammation originating from damage associated molecular patters (DAMPs) released from the injured liver, and worsens with systemic pathogen associated molecular pattern (PAMP) exposure due to pathological BT. The underlying mechanisms remain incompletely understood, severe immune exhaustion develops following factors precipitating acute decompensation (AD) or acute-on-chronic liver failures (ACLF), mainly infection (Moreau et al., 2013). The liver is a tolerogenic organ with antimicrobial surveillance function between the portal and systemic circulation, governed by innate, and amplified by adaptive immunity. A better understanding of monocyte and macrophages dysfunction at a molecular level is required in order to evaluate potential immunotherapeutic approaches for the future treatment of cirrhosis.

In this article we aim to point out the importance of monocytes and macrophages in the context of cirrhosis. We review the current state of knowledge which mechanisms contribute to the progressing immune dysfunction from early asymptomatic stages to end-stage liver disease by dissecting the phenotypical and functional changes of monocytes and macrophages from patients with cirrhosis in different compartments. In the following we 1) summarise the current concept of the pathophysiology of cirrhosis as systemic inflammatory condition, 2) dissect the differentiation of monocytes and macrophages in context of cirrhosis and 3) discuss potential biomarkers and potential future immunotherapeutic targets modulating monocyte and macrophage function.

2 Cirrhosis

The main underlying aetiologies for cirrhosis in developed countries are alcohol use causing alcohol-related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD)—recently also referred to as metabolic-associated fatty liver disease (MAFLD) (Eslam et al., 2020)—and viral infections such as chronic hepatitis B and C (HBV/HCV) (Asrani et al., 2019). In Europe the aetiologies causing cirrhosis are changing due to the therapeutic cure of HCV and the control of chronic HBV, the increasingly widespread unhealthy use of alcohol, the growing epidemic of obesity, and undiagnosed or untreated liver disease (Karlsen et al., 2022). It is believed that NAFLD will become the most common aetiology for liver transplantation in end stage cirrhotic patients (Haldar et al., 2019; Younossi et al., 2019). Cirrhosis has been defined by severe parenchymal loss due to necrosis, inflammation and fibrogenesis as a consequence of diverse underlying aetiologies. It is histopathologically characterised by diffuse nodular regeneration fibrous septa, parenchymal loss and vascular remodelling (Tsochatzis et al., 2014). Pathophysiologically, it involves the accumulation of fibrous tissue due to the activation of heaptic stellate cells (HSCs), as well as changes in the vascular architecture of the liver, leading to increased resistance to portal blood flow and progressive PH (Wanless, 2020). Over the previous 2 decades, it has been shown that under certain circumstances fibrosis and even cirrhosis may be reversible. These conditions must involve an effective and sustainable treatment of the underlying cause of liver injury (Wanless et al., 2000; Lee et al., 2015a; Hytiroglou and Theise, 2018) e.g., successful treatment of chronic viral hepatitis (Chang et al., 2010; Marcellin et al., 2013).

2.1 Disease stages of cirrhosis

Liver cirrhosis can roughly be categorized into a typically asymptomatic compensated stage and a decompensated stage, marked by the occurrence of complications and reduced survival (D'Amico et al., 2006; Moreau et al., 2013; Planas et al., 2006;

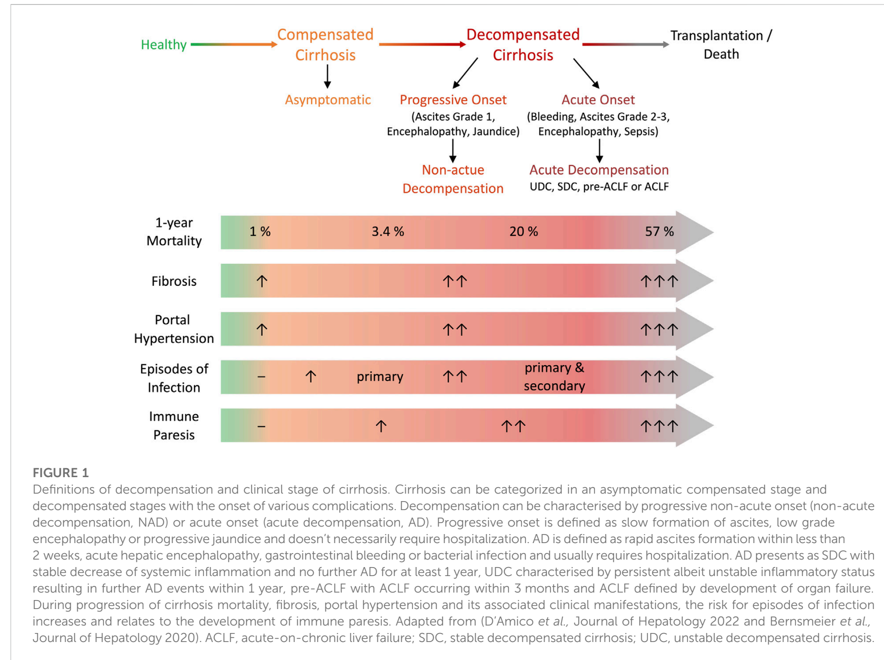

FIGURE 1
Definitions of decompensation and clinical stage of cirrhosis. Cirrhosis can be categorized in an asymptomatic compensated stage and decompensated stages with the onset of various complications. Decompensation can be characterised by progressive non-acute onset (non-acute decompensation, NAD) or acute onset (acute decompensation, AD). Progressive onset is defined as slow formation of ascites, low grade encephalopathy or progressive jaundice and doesn't necessarily require hospitalization. AD is defined as rapid ascites formation within less than 2 weeks, acute hepatic encephalopathy, gastrointestinal bleeding or bacterial infection and usually requires hospitalization. AD presents as SDC with stable decrease of systemic inflammation and no further AD for at least 1 year, UDC characterised by persistent albeit unstable inflammatory status resulting in further AD events within 1 year, pre-ACLF with ACLF occurring within 3 months and ACLF defined by development of organ failure. During progression of cirrhosis mortality, fibrosis, portal hypertension and its associated clinical manifestations, the risk for episodes of infection increases and relates to the development of immune paresis. Adapted from (D'Amico *et al.*, Journal of Hepatology 2022 and Bernsmeier *et al.*, Journal of Hepatology 2020). ACLF, acute-on-chronic liver failure; SDC, stable decompensated cirrhosis; UDC, unstable decompensated cirrhosis.

Arroyo et al., 2020). However, this classification does not discriminate between the prognostic subgroups that characterise the course of decompensation, which depends on the type and number of decompensating events (D'Amico et al., 2022) (Figure 1). Decompensation of cirrhosis was recently defined by the presence or history of any complication of ascites, bleeding, hepatic encephalopathy (HE) or jaundice (D'Amico et al., 2022), and may be characterised by an acute onset (AD) (Moreau et al., 2013) or by a progressive, non-acute onset (NAD) (D'Amico et al., 2022) (Figure 1). NAD involves slow ascites formation, mild HE, or progressive jaundice in non-cholestatic cirrhosis free of other complications and is mostly represented by first decompensation events that do not require hospitalisation (D'Amico et al., 2022) (Figure 1). In contrast, AD is defined as any first or recurrent moderate or severe ascites within less than 2 weeks, first or recurrent acute HE in patients with previous normal consciousness, acute gastrointestinal bleeding, and any type of acute bacterial infection (D'Amico et al., 2022; Trebicka et al., 2021; Arroyo et al., 2021). AD may manifest itself as unstable decompensated cirrhosis (UDC), stable decompensated cirrhosis (SDC), pre-ACLF or ACLF (D'Amico et al., 2022). UDC involves a 3-months mortality of 21–35,6%, while SDC occurs with a 3-months mortality of 0–9.5% (D'Amico et al., 2022). In a pre-ACLF state ACLF develops within a 3-months period and shows a 3-months mortality of 53,7–67,4% (D'Amico et al., 2022) (Figure 1). ACLF, the most severe stage of cirrhosis with a 28-days mortality of up to 77% (D'Amico et al., 2022), is defined by the development of organ failures (liver, kidney, brain, circulation, coagulation and lung) (Moreau et al., 2013; Gustot et al., 2015; Arroyo et al., 2020; Bajaj et al., 2021; Trebicka et al., 2021). Overall, the classification of cirrhosis stages has recently developed and remains debated. Consequently, not all publications refer to the same classification. The exact assignment to the stages of cirrhosis studied is highly relevant regarding the interpretation of experimental research data in the field.

2.2 Pathophysiology of cirrhosis—the systemic inflammation hypothesis

The most important factors in the pathophysiology of immuneparesis in cirrhosis are DAMPs, PAMPs and the systemic inflammatory response (SIRS): Chronic liver injury results in release and exposure to DAMPs, with cirrhosis

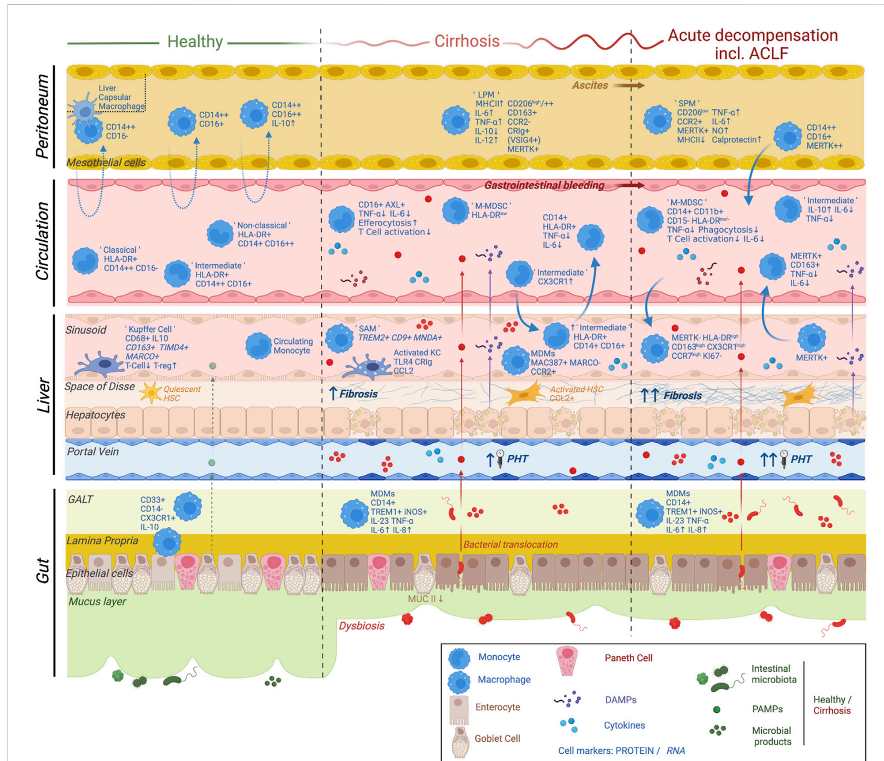

FIGURE 2
Current knowledge on monocyte and macrophage plasticity in relation to the multi-compartmental pathophysiological mechanisms underlying cirrhosis progression and decompensation. The main pathophysiological mechanisms underlying cirrhosis progression and acute decompensation are shown. These include liver fibrosis, hepatic endothelial dysfunction, parenchymal damage, portal hypertension, dysbiosis, bacterial translocation as well as activation of both local and systemic inflammatory responses. A variety of cellular and non-cellular players involved in these mechanisms are depicted (see symbol legend). In summary, homeostatic monocyte and macrophage populations display a distinct phenotype and function in a compartment-specific manner. Changes in monocyte/macrophage differentiation and migration patterns with progression of cirrhosis and acute decompensation are due to multiple factors involving chronic inflammation, exposure to DAMPs (released by injured hepatocytes) as well as exposure to PAMPs/microbial products due to dysbiosis and pathological bacterial translocation. Distinct cell subsets are shown in gut, liver, circulation and peritoneum, underlining the multi-compartmental nature of the disease. Adapted from Bernsmeier *et al.*, Journal of hepatology 2020. ACLF, acute-on-chronic liver failure; LPM, large peritoneal macrophage; SPM, small peritoneal macrophage; M-MDSC, monocytic myeloid-derived suppressor cell; SAM, scar-associated macrophage; MDM, monocyte-derived macrophage; HSC, hepatic stellate cell; PHT, portal hypertension; GALT, gut-associated lymphoid tissue; DAMP; damage-associated molecular patterns; PAMP, pathogen-associated molecular pattern. Created with BioRender.com.

progression pathologic BT develops and can lead to infections subsequently resulting in SIRS.

2.2.1 Chronic liver injury and DAMPs

Importantly, the pathophysiology of cirrhosis is not restricted to the liver, rather it represents a systemic condition involving various compartments, immune cells and soluble factors (Albillos et al., 2014; Ber nsmeier et al., 2020). Upon chronic liver injury fibrosis occurs and its formation involves activated resident or recruited macrophages, hepatocytes and HSCs. HSCs are key players in fibrosis development as they remain quiescent under homeostatic conditions but become

activated following hepatic injury and transform towards myofibroblasts which secrete extracellular matrix proteins (Tsuchida and Friedman, 2017; Kisseleva and Brenner, 2021). Immune dysfunction in cirrhosis initiates with the onset of chronic hepatic inflammation and worsens along with the progression of cirrhosis and the development of PH (Figure 1). It involves components of the innate immune system, which constitutes the first line of defence against pathogens as it plays a key role in maintaining homeostasis by phagocytosis and killing of bacteria, antigen presentation, inflammatory cytokine production, activation of adaptive immune cells and providing physical and chemical barriers. In cirrhosis chronic inflammation, parenchymal damage and scarring of the liver activate local tissue inflammation and contribute to a systemic inflammatory response mediated by DAMPs, cytokines and migration of immunoregulatory innate immune cells (Ber nsmeier et al., 2020). The liver sinusoidal endothelium is highly affected in cirrhosis due to its capillarization and angioarchitectural change (DeLeve, 2015; Su et al., 2021).

2.2.2 Bacterial translocation and PAMPs

The gut-liver axis is an operative unit protecting the body against harmful substances and microorganisms, maintaining homeostasis of the immune system. Liver cirrhosis profoundly alters this complex system and the intestine becomes more permeable allowing the translocation of bacteria and bacterial products (PAMPs) into the portal circulation (Ponziani et al., 2018). In the gut, dysbiosis, i.e. the reduction of beneficial taxa and increase in pathogenic bacteria, and reduction of the mucus layer is observed (Bonnel et al., 2011; Albillos et al., 2014; Bajaj et al., 2014; Wiest et al., 2017; Fernández et al., 2018; Fernández et al., 2019; Piano et al., 2019; Albillos et al., 2020; Bajaj et al., 2021; Van der Merwe et al., 2021). Gut endothelial and vascular barrier dysfunction results in BT, endotoxemia as well as mucosal immune cell alteration (Zapater et al., 2008; Ber nsmeier et al., 2020; Haderer et al., 2022). Pathological BT, present in advanced cirrhosis, is defined by the increased rate of translocation of bacteria or bacterial products from the gut to mesenteric lymph nodes, the portal and systemic circulation as well as other tissues. Gut derived PAMPs further drive hepatic inflammation and immune cell differentiation in the gut, liver, circulation and other organs (Zapater et al., 2008; Ber nsmeier et al., 2020; Stengel et al., 2020; Haderer et al., 2022).

2.2.3 Onset of infections and acute decompensation

Patients with cirrhosis are at high risk of bacterial infections, which are associated with the development of AD, hospitalisation, sepsis and the need for intensive care and organ support involving a substantial mortality (Arvaniti et al., 2010; Moreau et al., 2013; Jalan et al., 2014; Van der Merwe et al., 2021; Triantos et al., 2022) (Figure 1). The most common infections include spontaneous bacterial peritonitis (SBP), urinary tract infection, pneumonia, spontaneous bacteraemia, skin, and soft tissue infections (Arvaniti et al., 2010; Bajaj et al., 2012; Fernández et al., 2018; Piano et al., 2019; Arroyo et al., 2021; Trebicka et al., 2021). Bacterial infections in hospitalised patients are much more frequent in patients with cirrhosis (32–34%) compared to those without (5–7%). Remarkably, around 45% of hospitalised cirrhosis patients with gastrointestinal bleeding develop infections (Borzio et al., 2001; Tandon and Garcia-Tsao, 2008; Arvaniti et al., 2010). Of note, it has been observed that a dual infection (bacterial and fungal) increases the mortality of cirrhosis patients compared to patients with a single infection (Bajaj et al., 2018; Bajaj et al., 2021). As in the general population, infections caused by multi-drug-resistant organisms are progressively increasing in cirrhotic patients and represent up to 30–35% of all infections (Fernández et al., 2012; Fernández et al., 2019; Piano et al., 2019; Onorato et al., 2022).

Pathological BT in the context of immuneparesis can lead to SBP (Assimakopoulos et al., 2012; Wiest et al., 2014). SBP is diagnosed in patients with an elevated neutrophil count in the ascitic fluid (Runyon and Aasld, 2013), but no differences in mortality were observed independently of whether the bacterial culture of ascites was positive or negative (Runyon and Aasld, 2013). Moreover, BT and gut barrier impairment are directly related to the risk of variceal bleeding (Triantos et al., 2022). The plasticity of this process is accompanied by SIRS activation in ACLF and implicates changes in neural regulation, circulating inflammatory mediators and hormones (Bosmann and Ward, 2013; Bernsmeier et al., 2015a). Sepsis and SIRS are associated with hyperinflammation, impaired innate immune function, immunosuppression and complement activation, collectively leading to septic shock and organ failure (Bosmann and Ward, 2013).

Infection susceptibility in cirrhosis has been attributed to a state of immuneparesis, defined by inadequate immune responses to microbial challenge (Bonnel et al., 2011; Bajaj et al., 2012; Albillos et al., 2014). The pathophysiology of immuneparesis is very complex and incompletely understood, involving diverse defects in immune cell function, including monocytes and macrophages in multiple compartments (Bonnel et al., 2011). This suggests that the disease specific differentiation and function of monocytes and macrophages actively contribute to inflammation and infection susceptibility in cirrhosis patients. The following sections focus on the plasticity of mononuclear phagocytes in the context of cirrhosis.

3 Immunepathophysiology of mononuclear phagocytes in cirrhosis

Mononuclear phagocytes (monocytes and macrophages) play a crucial role in initiating immune responses. Their

functions include phagocytosis, killing of bacteria, antigen presentation, cytokine production, immune cell recruitment to sites of infection, and inflammation and activation of immune effector cells. Phagocytes regulate the antimicrobial defence, inflammation in tissue injury, fibrogenesis and tumourigenesis (Yona et al., 2013; Ziegler-Heitbrock and Hofer, 2013). Related to these functions, monocytes and macrophages are essential in the pathophysiology of immune dysfunction in patients with cirrhosis of the liver as a systemic inflammatory condition (Ber nsmeier et al., 2020; Singanayagam and Triantafyllou, 2021) (Figure 2).

3.1 Circulating monocytes

Principally, monocytes can be divided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) subsets (Passlick et al., 1989; Jakubzick et al., 2017; Patel et al., 2017; Guilliams et al., 2018; Kapellos et al., 2019) and further identified by their human leukocyte antigen DR (HLA-DR) expression (Guilliams et al., 2018). The intermediate and non-classical monocyte subsets emerge sequentially from the pool of classical monocytes (Patel et al., 2017). It has been found in mice and confirmed in human that $CD14^{+}CD16^{++}$ non-classical monocytes fulfil a continuous monitoring of the vasculature by a crawling mechanism on endothelial cells (Cros et al., 2010). This behaviour permits the efficient scavenging of luminal microparticles under homeostatic conditions. While this established classification was based on protein assays which involved mainly flow cytometry-based techniques, recent unbiased single-cell RNA sequencing (scRNA-Seq) studies have suggested broader heterogeneity within the intermediate population of monocytes, through the identification of two distinct intermediate monocyte subsets, one expressing classical monocyte and cytotoxic genes, while the other one with so far unknown functions (Villani et al., 2017).

3.1.1 Monocyte development

The development of classical monocytes was firstly discovered with the use of mouse models and some of these findings could subsequently be transferred to humans. In mice, haematopoietic stem cells in the bone marrow give rise to a monocyte/dendritic cell progenitor (MDP) (Guilliams et al., 2018). This MDP population was proposed to give rise to monocytes and classical dendtritic cells (DCs) in both mice (Olsson et al., 2016; Guilliams et al., 2018) and humans (Lee et al., 2015b; Guilliams et al., 2018), by following a binary trajectory towards either dedicated common DC precursors (CDP) or into unipotent common monocyte progenitors (cMoP) (Lee et al., 2015b; Guilliams et al., 2018). Accordingly, the cMoP population has also been identified in human bone marrow (BM) (Kawamura et al., 2017). An important transcription factor maintaining commitment towards the monocyte lineage in humans is interferon regulatory factor 8 (IRF8) (Hambleton et al., 2011). An autosomal recessive IRF8 deficiency in humans leads to reduced numbers of circulating monocytes (Hambleton et al., 2011). Interestingly, investigations into cMoP biology have uncovered that this population possesses a high proliferative capacity and is characterized by CD14 expression in humans (Chong et al., 2016). Further differentiation of cMoP into mature monocytes involves a transient pre-monocyte stage, discriminated by the expression of CXC-motiv chemokinereceptor 4 (CXCR4) in mice and humans (Chong et al., 2016). In mice the downregulation of Cxcr4 expression is accompanied by the up-regulation of C-C chemokine receptor 2 (Ccr2), indicating that Ccr2 regulates the BM exit of murine monocytes (Serbina and Pamer, 2006; Chong et al., 2016). While it remains unclear whether CCR2 also plays a role in BM exit of human monocytes to the circulation (Guilliams et al., 2018), it was shown that CXCR4 participates in the homing of classical and non-classical monocytes to central (BM) and peripheral (spleen) monocyte reservoirs (Chong et al., 2016). In mice, following release of classical monocytes into the circulation from the BM under healthy homeostasis, these cells remain in the circulation and may subsequently either traffic to repopulate a proportion of tissue-resident macrophages (Tamoutounour et al., 2012; Bain et al., 2013; Jakubzick et al., 2013; Bain et al., 2014; Epelman et al., 2014; Calderon et al., 2015; Guilliams et al., 2018) or alternatively convert into non-classical monocytes (Yona et al., 2013; Patel et al., 2017). In an analogous manner, it was observed in humans that the vast majority of classical monocytes are recruited to peripheral tissues where they potentially differentiate into monocyte-derived cells or enter reservoirs of undifferentiated monocytes (Tak et al., 2017a; Patel et al., 2017). The transition of classical monocytes to non-classical monocytes has been observed in different species including mouse (Sunderkötter et al., 2004; Tacke et al., 2006; Varol et al., 2007; Yona et al., 2013), rat (Yrlid et al., 2006), macaque (Sugimoto et al., 2015) as well as in humans (Patel et al., 2017) and therefore represents an evolutionarily conserved program (Guilliams et al., 2018).

3.1.2 Monocyte differentiation in the context of inflammation and cirrhosis

Proportions of circulating monocytes vary depending on monopoiesis, tissue infiltration and their release from central (BM) or peripheral reservoirs. In humans, endotoxin induces a rapid yet transient monocytopenia during the first 2 h after lipopolysaccharide (LPS) injection followed by the sequential reappearance of $CD14^{++}CD16^{-}$ classical monocytes, followed by $CD14^{+}CD16^{+}$ intermediate cells and finally $CD14^{+}$ $CD16^{++}$ non-classical monocytes (Thaler et al., 2016; Tak et al., 2017b; Patel et al., 2017). The number of non-classical monocytes is thus strongly linked to the physiological status of the organism and therefore represents a potential diagnostic tool (Selimoglu-Buet

et al., 2015). Not only do cytokines directly provoke functional changes in monocytes, but they also influence the cellular outcome of haematopoietic stem cells, a phenomenon called "trained immunity" (Quintin et al., 2012; Netea et al., 2016). A number of cytokines have been proposed to play a key role in trained immunity, including interferon-ɣ (INF-ɣ) and interleukine-1β (IL-1β), suggesting that activation of haematopoietic stem cells by cytokines produced by immune or non-immune cells in the BM is crucial for these long-lasting training effects (Boettcher and Manz, 2017). Inflammation-induced emergency haematopoiesis can result in trained immunity characterized by long-term epigenetic effects on haematopoietic stem cells. The epigenetic changes associated with monocyte training involve histone modification of genes encoding pro-inflammatory cytokines such as IL-6, tumor necrosis factor-α (TNF-α) but also genes of the mammalian target of rapamycin (mTOR) pathway (Quintin et al., 2012; Netea et al., 2016). Under pathological conditions, monocytes gain distinct non-redundant functions that often cannot be fulfilled by resident macrophages and DCs. These non-exclusive and sometimes overlapping effector functions comprise pro-inflammatory activities, antigen-presentation, tissue remodelling, or anti-inflammatory abilities (Patel et al., 2017; Guilliams et al., 2018). Furthermore, in mouse models effector monocytes actively participate in fibrosis development (Satoh et al., 2017) and can also differentiate into pathogenic foam cells (Guilliams et al., 2018). This highlights that monocytes are not simply precursors that get recruited to temporarily increase the number of tissue resident macrophages but in fact give rise to functionally distinct monocyte-derived cells (Guilliams et al., 2018).

In patients with cirrhosis of the liver the number of circulating monocytes increases gradually with the progression of disease (Hassner et al., 1979; Zimmermann et al., 2010; Korf et al., 2019a; Brenig et al., 2020). An expansion of the intermediate monocyte population was observed in the circulation and the liver in the context of cirrhosis progression, as a consequence of enhanced recruitment from blood and local differentiation from classical monocytes (Zimmermann et al., 2010; Liaskou et al., 2013; Korf et al., 2019a). This indicates the pathological condition present in patients with cirrhosis is reflected by monocyte differentiation. Intermediate $CD14^{++}CD16^{+}$ monocytes accumulated in chronically inflamed and cirrhotic human liver as a consequence of enhanced recruitment from the blood (Zimmermann et al., 2010; Liaskou et al., 2013; Zimmermann et al., 2016). These monocytes displayed a high level of phagocytosis, antigen presentation, T cell proliferation and secretion of cytokines (TNF-a, IL-6, IL-8, and IL-1b) as well as profibrogenic cytokines (IL-13) and growth factors (granulocyte-colony stimulating factor G-CSF, granulocyte-macrophage-colony stimulating factor GM-CSF) (Liaskou et al., 2013).

3.1.3 Expansion of M-MDSC in cirrhosis

One of the first discoveries of monocyte dysfunction in patients with cirrhosis was their impaired phagocytosis capacity (Sanchez-Tapias et al., 1977; Sherlock, 1977; Hassner et al., 1979; Holdstock et al., 1982). In line with this finding, a reduced expression of major histocompatibility complex class II (MHC II) molecules was observed on monocytes from patients with ACLF and was associated with adverse prognosis (Hassner et al., 1979; Wasmuth et al., 2005). Confirming these findings, it was shown that MHC II molecule expression gradually decreased with cirrhosis progression (Lin et al., 2007; Xing et al., 2007; Bernsmeier et al., 2018; Brenig et al., 2020). HLA-DR, a MHC II family member, can be considered as a monocyte activation marker, due to the increased expression upon immune stimulation and its function in inducing T cell responses (Gordon and Taylor, 2005). On the contrary, the downregulation of HLA-DR appears in acquired immunosuppressive monocyte differentiation (Gordon and Taylor, 2005).

The reduced HLA-DR expression on monocytes in cirrhosis was associated with the expansion of mononuclear myeloid-derived suppressor cells (M-MDSC, $CD11b^{+}CD14^{+}HLA\text{-}DR^{lo/-}CD15^{-}$) (Bronte et al., 2016) in the circulation of patients with cirrhosis (Bernsmeier et al., 2018). The term MDSCs was first introduced in the year 2007 in a cancer-related context (Gabrilovich et al., 2007). MDSCs develop from myeloid cell expansion and conditioning in the BM and spleen with subsequent conversion of monocytes into pathologically activated MDSCs (Condamine et al., 2015; Wang et al., 2019; Ostrand-Rosenberg et al., 2020). Human M-MDSCs are distinct from monocytes by their low expression of MHC II (Bronte et al., 2016). The main characteristic of MDSCs is the inhibition of immune responses, including those mediated by T cells, B cells and NK cells (Gabrilovich, 2017). The suppression of immune responses is mediated by upregulation of signal transducer and activator of transcription 3 (STAT3) expression, induction of endoplasmic reticulum (ER) stress, expression of arginase 1 (Arg1) and expression of S100A8/A9 (Veglia et al., 2021). To fulfil their immunosuppressive function M-MDSCs use nitric oxide (NO), immunosuppressive cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β) and the expression of immune regulatory molecules like programmed death-ligand 1 (PD-L1) (Gabrilovich, 2017). Recently, trajectory analysis showed that M-MDSCs are also molecularly distinct from M1-like and M2-like macrophages (Song et al., 2019), indicating that M-MDSCs represent a subset distinct from monocytes and macrophages.

In the context of cirrhosis, endotoxemia (the presence of bacteria-derived endotoxins in the blood stream) is observed in advanced stages of disease and is associated with the loss of HLA-DR (Zapater et al., 2008; Bernsmeier et al., 2018). Moreover, loss of HLA-DR expression affects the antigen presentation capacity

of monocytes and their ability to induce activation of adaptive immune responses and is associated with adverse clinical outcomes in patients with cirrhosis (Bernsmeier et al., 2018; Brenig et al., 2020). M-MDSCs in AD/ACLF patients displayed immunosuppressive properties in regard to their pro-inflammatory cytokine production (TNF-α, IL-6) (Wasmuth et al., 2005; Lin et al., 2007; Berres et al., 2009; Berry et al., 2011; Bernsmeier et al., 2018; Korf et al., 2019a; Brenig et al., 2020) as well as their reduced T cell activation and decreased phagocytosis capacity, contributing to the dampened innate immune responses of circulating monocytes (Bernsmeier et al., 2018). Sequentially increasing numbers of M-MDSC with disease progression from compensated cirrhosis to AD/ACLF (Bernsmeier et al., 2018) underlie the low TNF-α/IL-6 responses in AD/ACLF patients. Furthermore CD8^{+} T cells from cirrhotic patients displayed high HLA-DR, T cell immunoglobulin and mucin-domain containing-3 (TIM-3), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) expression associated with concomitant infections and disease severity (Lebossé et al., 2019).

In vitro exposure of monocytes from healthy controls to plasma isolated from AD/ACLF patients reduced monocytic HLA-DR expression as well as suppressed TNF-α/IL-6 responses. (Bernsmeier et al., 2018; O'Brien et al., 2014; Bernsmeier et al., 2015b). In a similar fashion healthy human monocytes exposed to activated primary human HSCs, converted mature CD14^{+} monocytes into M-MDSCs in a contact-dependent manner (Höchst et al., 2013; Resheq et al., 2015). Recently it has been shown that MDMCs from patients with non-cirrhotic chronic HBV infection had profound suppressive ability, expressing Arg1/iNOS/PD-L1/CTLA-4/CD40 at significantly greater levels relative to healthy controls (Pal et al., 2022).

3.1.4 TAM receptor expression on monocytes in cirrhosis

Lately, TAM receptors (TYRO3, AXL, and MERTK), which are tyrosine kinase receptors expressed on monocytes and macrophages and essential for the regulation of immune homeostasis, have been shown to play a central role in cirrhosis and contribute to the systemic immune paresis in patients with cirrhosis and liver failure (Roth lin et al., 2007; Bernsmeier et al., 2015b; Triantafyllou et al., 2018; Brenig et al., 2020; Flint et al., 2022). They maintain immune homeostasis by regulating innate immune responses via suppression of inflammatory toll-like receptor (TLR) signalling cascades and promotion of tissue resolution thorough the clearance of apoptotic cells (efferocytosis) (Flint et al., 2022). Immuneregulatory AXL-expressing monocytes expanded along with the progression of cirrhosis, representing <5% in early stages and up to 40% of monocytes in later stages, but were scarce following an event triggering AD (<5%) (Brenig et al., 2020). In compensated cirrhosis and NAD, the expansion of AXL-expressing circulating monocytes correlated with the occurrence of infectious episodes, onset of subsequent AD within 4 months and 1-year mortality (Brenig et al., 2020). On a functional level, AXL-expressing monocytes exhibited a reduced T cell activation potential and dampened TNF-α and IL-6 responses upon TLR4 stimulation through LPS treatment. However, AXL[+] monocytes showed preserved capacity to phagocytose pathogens and enhanced capacity to phagocyte apoptotic cells, i.e. efferocytosis, when compared to AXL[−] monocytes (Brenig et al., 2020). Another TAM receptor family member, MERTK was found to be up-regulated on circulating monocytes in the stage of AD/ACLF in cirrhosis patients (Bernsmeier et al., 2015b; Brenig et al., 2020). MERTK-expressing monocytes represented 14.4% of all circulating monocytes in patients with AD and even 35.7% in ACLF, respectively, and showed dampened innate immune responses to LPS in close association with clinical disease severity scores and the need for transplantation or death (Bernsmeier et al., 2015b).

3.1.5 Functional consequences of monocyte plasticity following acute decompensation

Monocytes from patients with ACLF showed polarisation towards an immunotolerant state by reduced HLA-DR expression, reduced TNF-α/IL-6 production but elevated IL-10 production (Wasmuth et al., 2005; Lin et al., 2007; Berres et al., 2009; Berry et al., 2011; Bernsmeier et al., 2018; Korf et al., 2019a; Brenig et al., 2020), and impaired phagocytic capacity. As indicated, the phagocytic capacity of monocytes was maintained during disease progression, but diminished following insults leading to AD/ACLF stages (Gadd et al., 2016; Bernsmeier et al., 2018).

Under pathological conditions the number, phenotype and function of circulating monocytes changes, indicating that monocytes are not only macrophage precursors, but in fact form a separate cell population giving rise to functionally distinct cells. Immunosuppressive M-MDSCs increase in late stages of cirrhosis either developing earlier in the BM and spleen or originating from mature circulating monocytes in the disease-specific milieu. The appearance of distinct monocyte subsets at early and late stages of disease indicates that monocyte subsets are susceptible to phenotypic and functional changes not only on a mature circulating monocyte level, but already on a developmental level. Thus, monocyte differentiation is continually adapting to the inflammatory milieu in response to PAMPs, DAMPs and SIRS. In NAD, AD, and ACLF stages of disease, immunoregulatory monocytic populations expanding and replace substantial proportions of functionally regular counterparts. In order to prevent infection, decompensation and death from cirrhosis, it is desirable to further explore therapeutic options aimed at restoring systemic innate immune homeostasis by reconstituting monocyte function,

while also assessing the risk of therapy-related side effects (see Section 5).

3.2 Macrophages in the liver

Monocytes are considered to be precursors of macrophages polarizing and generating subpopulations traditionally called activated, pro-inflammatory macrophages "M1" and alternatively activated, anti-inflammatory macrophages "M2" (Nathan et al., 1983; Mosmann et al., 1986; Murray et al., 2014; Chávez-Galán et al., 2015). This categorization of macrophages cannot be applied to hepatic macrophages since they simultaneously express M1 and M2 markers (Gao et al., 2019) and recent evidence using mouse models suggested that liver resident Kupffer cells (KCs) are self-renewing and originate from the yolk sack (Li et al., 2017). These cells exhibit great plasticity which is dependent on their ontogeny, local and systemic microenvironmental mediators and epigenetic programming (Guillot and Tacke, 2019; Wen et al., 2021). To date, markers that distinctly identify KCs in humans and allow to distinguish these cells from monocyte-derived macrophages (MDMs) have been reviewed. Undoubtedly, new technical opportunities provided by 'omics' technologies (genomics, transcriptomics, proteomics, and metabolomics) will allow for the re-evaluation and further exploration of distinct KC and MDM markers (Guilliams et al., 2022). The liver drains blood from abdominal organs through the portal vein and receives systemic blood via the hepatic artery (Jenne and Kubes, 2013). It is a highly organised tolerogenic organ with a large sinusoidal network. These so-called sinusoids are lined by permeable fenestrated liver sinusoidal endothelial cells (LSECs) enabling the exposure of plasma content such as gut-derived bacterial products to hepatocytes and non-parenchymal cells of the liver (Su et al., 2021) and harbour KCs (Figure 2).

3.2.1 Kupffer cells

In homeostatic conditions immune activation is prevented by KCs, LSEC, and DCs, which together promote immune tolerance. KCs are the most abundant hepatic immune cells maintaining liver homeostasis and immune tolerance, due to their continuous exposure to harmless gut-derived antigens and their ability to rapidly identify and eliminate pathogens (Wen et al., 2021). The key role of liver resident KCs is to phagocytose and scavenge DAMPs, recognize PAMPs and remove circulating bacteria to prevent systemic immune activation by secreting IL-10 (Kubes and Jenne, 2018). Furthermore, KCs are capable of self-renewal and show a specific transcriptional program defined by their unique niche (Zigmond et al., 2014; Beattie et al., 2016; Krenkel and Tacke, 2017). KCs are located within the sinusoidal lumen, in continuous contact with LSECs and can also interact with HSCs and hepatocytes across the space of Disse (Jenne and Kubes, 2013; Bonnardel et al., 2019; Sutti et al., 2019). Liver resident KCs are capable of removing bacterial products and secreting tolerogenic factors, regulating this microenvironment (Sutti et al., 2019). KCs have the ability to inhibit T cell expansion and induce regulatory T cells, that may reprogram liver infiltrating monocytes to regulatory IL-10^{+} DCs that further promote tolerance (Heymann et al., 2015; Heymann and Tacke, 2016). Furthermore, hepatocytes, HSCs and endothelial cells shift infiltrating monocytes towards a more tolerogenic phenotype (Blériot and Ginhoux, 2019; Bonnardel et al., 2019).

The balance between immunity and tolerance is essential to liver function (Kubes and Jenne, 2018) and in cirrhosis the mechanisms to maintain tolerance fail (Wu et al., 2010). Chronic liver injury and inflammation lead to progressive liver fibrosis and ultimately to the development of cirrhosis with formation of fibrous septa, regenerative nodules, sinusoidal resistance, intrahepatic shunting and PH (Koyama and Brenner, 2017; Laleman et al., 2018). The liver, which drains the intestinal venous blood, forms a vascular "firewall" that captures gut commensal bacteria entering the blood stream during intestinal pathology (Balmer et al., 2014). The compartmentalization of intestinal microbes was found to be defective in animal models of liver disease due to failure of the hepatic vascular "firewall" that is required to clear microbes from the mesenteric and systemic vasculature efficiently (Balmer et al., 2014). Mononuclear phagocytic cells in the liver can be categorized into liver resident KCs (Kubes and Jenne, 2018), circulating monocytes trafficking through the sinusoids or migrating monocytes, which may persist as monocytes in the tissue, acquire antigen-presenting capability or mature into macrophages (Strauss et al., 2015; Jakubzick et al., 2017; Weston et al., 2019). During the progression from inflammation with fibrosis to cirrhosis, KCs are continuously activated by DAMPs released by dying hepatocytes. Activated TLR4/complement receptor of the immunoglobulin superfamily (CRIg)-expressing KCs lose their tolerogenic phenotype and secrete pro-inflammatory cytokines, amplifying immune responses and recruiting immune cells to the liver (Kolios, 2006; Weston et al., 2019). Experimental models of acute liver injury in rodents have been used to better understand macrophage function during liver disease (Weston et al., 2019). These models showed that extensive hepatocyte damage releases DAMPs which are sensed by KCs leading to the release of cytokines, chemokines and pro-inflammatory macrophage recruitment (Krenkel and Tacke, 2017). During acetaminophen-induced acute liver injury in humans a phenotypic switch in macrophage phenotype is observed, where MAC387 (S100A9) serves as a marker to identify MDMs in contrast to CD68^{+} resident KCs in the liver (Antoniades et al., 2012). Recently, using scRNA-Seq KCs were phenotypically described as CD68^{+}CD163^{+}MARCO^{+}TIMD4^{+} (MacParland et al., 2018; Aizarani et al., 2019; Ramachandran et al., 2019).

3.2.2 Capsular macrophages

Furthermore, a distinct population of MHC II-expressing cells was observed in the hepatic capsule (PRICKE TT et al., 1988). In mice it has been shown that this population represented a macrophage population (liver capsular macrophages LCMs) that is distinct from KCs (Sierro et al., 2017). LCMs in mice are CX3CR1^{hi}TIM4^{-} and replenished in the steady state from blood-borne monocytes (Sierro et al., 2017). Recent spatial transcriptomic experiments in mice have shown that these capsule macrophages can be further characterised by the expression of CD207 and CX3CR1 (Guilliams et al., 2022), but could not be identified in human liver biopsies due to the lack of capsule tissue (Guilliams et al., 2022). In mice, LCMs form a cellular network in the hepatic capsule and potentially sense the blood and the peritoneal cavity due to their extended dendrites (Sierro et al., 2017). It has been suggested that LCMs sense peritoneal bacteria accessing the liver capsule and prevent them from breaching the liver capsule through the recruitment of neutrophils (Sierro et al., 2017).

3.2.3 Monocyte-derived macrophages

In cirrhotic livers the number of intermediate CD14^{++}CD16^{+} monocytes increases, these cells accumulate at sites of inflammation and fibrosis and are characterised by enhanced phagocytosis capacity, antigen presentation capacity, activation potential for T cell proliferation and secretion of numerous soluble factors like chemokines, growth factors, pro-inflammatory, and pro-fibrogenic mediators (Zimmermann et al., 2010; Liaskou et al., 2013; Tsuchida and Friedman, 2017). Intermediate monocytes contribute to the perpetuation of hepatic inflammation and fibrogenesis and show an increased CX3CR1-dependent migratory potential towards liver tissue, while also displaying the ability to transmigrate back to the circulation and contribute to SIRS (Aspinall et al., 2010; Zimmermann et al., 2010). In the context of cirrhosis capillarization of LSECs, formation of basement membranes and activation of HSCs contributes to increased migration of immune cells across the sinusoidal endothelial barrier (Bataller and Brenner, 2005; Henderson et al., 2006; Li et al., 2012; Ramachandran et al., 2012; Shetty et al., 2018; Su et al., 2021). Both, activated KCs and HSCs produce CCL2 and CCL5 promoting the recruitment of MDMs to the liver through CCR2/CCL2 or CCR5/CCL5 signalling (Heymann and Tacke, 2016; Ju and Tacke, 2016; Krenkel and Tacke, 2017; Sasaki et al., 2017; Ramachandran et al., 2020). In addition, activated HSCs can also recruit monocytes through the CCR8/CCL1 and CXCR3/CXCL10 axes (Heymann et al., 2012; Tomita et al., 2016; Zhang et al., 2016). However CCR2 expression on KCs is absent under healthy conditions, but increases in parallel to disease evolution, serving as diagnostic marker in the context of NAFLD (Krenkel et al., 2018). CCR2^{+} macrophages are also seen in patients with acetaminophen (APAP)-induced liver failure (Mossanen et al., 2016) and increased serum CCL2 levels are associated with adverse prognosis (Antoniades et al., 2012) serving as a potential biomarker also for ALF, mitigating its specificity. MDMs have been shown to regulate a number of aspects of liver injury, including perpetuation of inflammation (Wu et al., 2016; Krenkel and Tacke, 2017), but also promotion of fibrosis (Karlmark et al., 2009; Krenkel et al., 2018; Weston et al., 2019; Ramachandran et al., 2020). Both KCs and MDMs can adopt context-dependent fibrogenic and fibrolytic roles due to their heterogeneity and plasticity (Weston et al., 2019).

ScRNA-Seq revealed MDMs as CD68^{+}MARCO^{-} in comparison to CD68^{+}CD163^{+}MARCO^{+}TIMD4^{+} KCs (MacParland et al., 2018; Aizarani et al., 2019; Ramachandran et al., 2019). A cross-species comparison suggested a conserved transcriptional gene signature among human and mouse KCs (Ramachandran et al., 2019; Ramachandran et al., 2020). Recent publications revealed that expression of TREM-1, an amplifier of inflammation, on macrophages promoted hepatic inflammation and fibrosis in mice and humans (Nguye n-Lefebvre et al., 2018; Perugorria et al., 2019). Moreover, TREM-1 promoted pro-inflammatory cytokine production and mobilization of inflammatory cells to the site of injury (Nguye n-Lefebvre et al., 2018). In human liver samples from patients with severe fibrosis an increase in the number of TREM-1^{+} cells in fibrotic areas was observed, compared to only slight TREM-1 expression in and around the hepatic sinusoid in normal liver tissue (Nguye n-Lefebvre et al., 2018). Interestingly, TREM-2 expression on monocytes and macrophages was located around fibrous septa, sinusoids and areas of inflammation in human cirrhotic liver (Perugorria et al., 2019). In a recent study, an unbiased scRNA-Seq approach helped to further characterize the heterogenous population of MDMs in humans. A new pro-fibrogenic scar-associated TREM2^{+}CD9^{+}MNDA^{+} subpopulation of macrophages (scar-associated macrophages, SAMs) was identified, which expanded in the liver in the context of fibrosis, originated from differentiating circulating monocytes (Ramachandran et al., 2019) and was distinct from KCs (Ramachandran et al., 2020). Spatial analysis located SAMs at fibrotic niches in livers of cirrhosis patients and showed expansion of SAMs (Ramachandran et al., 2012; Ramachandran et al., 2019). SAMs also promoted HSC collagen production and proliferation (Ramachandran et al., 2019; Ramachandran et al., 2020). Strikingly, mouse injury-associated macrophages show overlap in distinct marker genes observed in human SAMs, such as TREM2 and CD9, which are conserved across species (Ramachandran et al., 2019; Xiong et al., 2019). Recent scRNA-Seq combined with cellular indexing of transcriptomes and epitopes by sequencing (CITE-Seq) and spatial proteogenomics experiments on NAFLD mouse models showed the expansion of lipid-associated macrophages (LAMs) in the liver, originating from the BM prior to the development of fibrosis and cirrhosis (Remmerie et al., 2020a; Guilliams et al., 2022). The hepatic LAMs also showed some overlap with the SAMs in expression of Trem-2 and Cd9, but could be

distinctively characterised by their expression of Spp1 (Remmerie et al., 2020a).

3.2.4 TAM receptor expression on macrophages

Moreover, macrophages expressing MERTK accumulated in the liver, but also the peritoneal cavity and mesenteric lymph nodes, in patients with decompensated cirrhosis and ACLF (Bernsmeier et al., 2015b). MERTK-expressing monocytes were characterised by increased expression of pro-resolution/anti-inflammatory and tissue- and lymph node-homing markers: HLA-DR^{hi}CD163^{hi}CX3CR1^{hi}CCR7^{hi} and Ki-67 negative, which suggests an origin from recruited monocytes (Bernsmeier et al., 2015b). MERTK-expressing monocytes showed attenuated cytokine responses and an enhanced migratory potential across endothelial layers, suggesting their capacity to infiltrate the liver and reverse migrate back to the circulation where they aggravate the systemic immuneparesis (Bernsmeier et al., 2015b; Triantafyllou et al., 2018). In the context of ALF, it has been shown that these MERTK-expressing macrophages in the liver exhibited enhanced efferocytosis and played a role in resolution of inflammation and tissue restoration (Bernsmeier et al., 2015b; Triantafyllou et al., 2018). Yet, MERTK expressing cells were essential to maintaining homeostasis in the cirrhotic liver, but aggravated systemic immuneparesis (Bernsmeier et al., 2015b; Triantafyllou et al., 2018). In a mouse model for non-alcoholic steatohepatitis (NASH) it was shown that AXL signalling in primary fibroblasts, hepatocytes and liver macrophages promoted fibrosis, while GAS6 or MERTK activation protected primary hepatocytes against lipid toxicity, and the AXL inhibitor bemcentinib diminished liver inflammation and fibrosis in mice (Tutusaus et al., 2020).

3.2.5 Function of macrophages in cirrhosis

The impact of aetiologies on the function of monocytes and macrophages at the stage of cirrhosis has not been extensively studied. NAFLD- and ARLD-related cirrhosis display a similar histopathology, pathophysiology, shared genetic and epigenetic factors and frequently coexist (Idalsoaga et al., 2020). Prior to the presence of cirrhosis, in ARLD and NAFLD, hepatic macrophages become activated by LPS, IFN-ɣ, and GM-CSF signalling and show pro-inflammatory cytokine/chemokine secretion (IL-6, IL-1β, and CCL2) (Mandrekar and Szabo, 2009; Gao et al., 2019; Remmerie et al., 2020b). In NAFLD, free fatty acids derived from white adipose tissue promoted hepatocyte triglyceride synthesis and storage as well as lipotoxicity with increased production of both pro-inflammatory cytokines such as TNF-α and IL-6 and macrophage recruiting chemokines such as CCL2, CCL5, and CXCL10 (Kazankov et al., 2019). NAFLD- and ARLD-related cirrhosis was associated with steatosis and hepatocyte cell death (apoptosis, necrosis and pyroptosis) promoting tissue inflammation and injury (Itoh et al., 2013; Gao et al., 2019; Miyata and Nagy, 2020). Autophagy can protect against ARLD tissue damage (Gao et al., 2019) by degrading interferon regulatory factor 1 (IRF1) and damaged mitochondria in hepatic macrophages (Ilyas et al., 2019; Liang et al., 2019; Williams and Ding, 2020). In non-cirrhotic viral hepatitis, KC activation led to the expression of IL-6, IFN-ɣ, and ROS, which in turn inhibited HCV replication and induced apoptosis of infected hepatocytes (Broering et al., 2008; Boltjes et al., 2014). In the setting of non-cirrhotic chronic HBV infection, an impaired immune response was associated with the release of IL-10 (Liu et al., 2018), reduced IL-12 expression (Wang et al., 2013) or increased expression of PD-L1 (Tian et al., 2016). However, in the context of cirrhosis, HCV also triggered the secretion of CCL5 by human macrophages and induced CCR5-dependent activation of HSCs (Sasaki et al., 2017). Patients with cirrhosis due to viral hepatitis showed increased numbers of hepatic macrophages associated with the infiltration of other pro-inflammatory CD14^{+}HLA-DR^{hi}CD206^{+} myeloid cells (Tan-Garcia et al., 2017), which in turn produced pro-inflammatory cytokines such as IL-1β, IL-18, and TNF-α (Weston et al., 2019).

Hepatic macrophages show tolerance during liver homeostasis and protect the liver and subsequently the systemic circulation from bacteria and bacterial products. In chronic liver disease and cirrhosis, KCs become activated and MDMs are recruited to the liver. Recently, due to new 'omics' technologies a list of macrophage markers associated with fibrosis, steatosis and monocyte recruitment could be identified. Hepatic macrophages show a very heterogenous phenotype expressing pro-inflammatory cytokines, but switching to a more immunoregulatory phenotype characterised by pro- and anti-inflammatory cytokines in advanced stages especially AD/ACLF. The further delineation of macrophages in relation to distinct aetiologies underlying cirrhosis and to specific stages of disease is the focus of ongoing research and the basis of potential future therapies.

3.3 Intestinal macrophages

Cirrhosis is a systemic disease that displays immunological alterations in immune cells beyond the liver. The gut connects to the liver by the biliary tract and portal vein, allowing for direct transfer of gut-derived components that impact liver pathophysiology (Hartmann et al., 2015) (Figure 2). In healthy conditions the intestinal mucosal and vascular barrier serve as an intersection for the interactions between the gut and the liver in order to limit systemic dissemination of microbes and toxins as well as to allow nutrients to access the circulation and liver (Albillos et al., 2020). Intestinal macrophages are a subset of CD33^{+}CD14^{-} lamina propria immune cells and are hyporesponsive to LPS (Smythies et al., 2005; Smith et al., 2011). Gut-resident CX3CR1^{+} macrophages contribute to host defence and barrier integrity through their phagocytic capacity (Ber nsmeier et al., 2020). Immunosuppressive cytokine IL-10

plays a key role in the regulatory functions of intestinal macrophages (Scott and Mann, 2020).

In cirrhosis, pathological BT from the gut to mesenteric lymph nodes is driven by a suppressed intestinal immune system with small intestinal bacterial overgrowth and reduced microbial diversity (Albillos et al., 2020; Chopyk and Grakoui, 2020). Following pathological BT in patients with decompensated cirrhosis, $CD14^{+}TREM1^{+}iNOS^{+}$ intestinal monocyte-derived macrophages were recruited from the circulation into the duodenum via MCP-1 secreted by intestinal epithelial cells (Du Plessis et al., 2013). Functionally these activated macrophages were responsive to microbial challenges, which resulted in the production of pro-inflammatory cytokines (IL-23, IL-6, and TNF-α) and contribute to mucosal, epithelial, vascular and immunological barrier dysfunction in cirrhosis (Kamada et al., 2008; Wiest et al., 2017; Sarin et al., 2019; De Muynck et al., 2021). If all defence mechanisms fail, these processes manifest as infection e.g., SBP, bacteraemia and sepsis (Albillos et al., 2020; Ber nsmeier et al., 2020). In murine models of cirrhosis, the mucus layer was reduced in thickness, with a loss of goblet cells, decreased MUC2 expression and bacterial overgrowth. In a NAFLD murine model recent data indicated that a high-fat diet altered the microbiome and impaired the intestinal barrier and the gut vascular barrier, which was confirmed in human gut biopsies (Mouries et al., 2019). Also chronic alcohol intake in humans leads to intestinal inflammation and increased numbers of TNF-α producing monocytes and macrophages in the lamina propria enhancing the intestinal permeability (Chen et al., 2015). It can further lead to intestinal gut microbiota dysbiosis and impaired intestinal barrier integrity (Gao et al., 2019; Sarin et al., 2019). It was shown in humans and mice that chronic alcohol use suppressed intestinal regeneration through reduced antimicrobial Reg3b and Reg3g secretion by Paneth cells (Yan et al., 2011; Hartmann et al., 2013), which led to increased bacteria adhesion to mucosal surfaces, intestinal bacterial overgrowth, enhanced BT of viable bacteria and worsening of liver inflammation (Albillos et al., 2020). Analogously, in cirrhosis patients with chronic HBV and HCV an increased gut permeability and resulting BT was observed (Sandler et al., 2011), indicating shared mechanisms in NAFLD, ARLD and viral-related cirrhosis of the liver. The mechanisms leading to increased gut permeability are not fully understood, but nitration and oxidation of tubulin, damage to microtubule cytoskeleton and activation of iNOS and NF-κB have been shown to impact tight and adherens junctions in the intestinal epithelium (Rao, 2009; Yona et al., 2013; Hartmann et al., 2015; Gao et al., 2019).

In cirrhosis, dysfunctional immune cells are not limited to the liver and the circulation but extend to the innate immune barriers of the intestine. Dysbiosis in the gut and the compromised intestinal barrier led to BT to the liver and circulation, prompting and maintaining systemic inflammation, immuneparesis and bacterial infection susceptibility.

3.4 Peritoneal macrophages

Under healthy conditions peritoneal macrophages (PMs), i.e. macrophages in the peritoneal cavity fluid (PF) are constantly exchanged between blood and PF and clear debris as well as pathogens (Heel and Hall, 2005) (Figure 2). In cirrhosis ascites, the accumulation of fluid in the peritoneal cavity, and SBP may develop. SBP results from intestinal and peritoneal barrier failure, which allows viable bacteria to enter the peritoneal cavity (Van der Merwe et al., 2021) and can induce AD of the liver, ACLF, multiorgan failure and death. Three subsets of PMs have been defined: Classical $CD14^{++}CD16^{-}$, intermediate $CD4^{++}CD16^{+}$ and large granular $CD14^{hi}CD16^{hi}$ (Ruiz-Alcaraz et al., 2016; Ruiz-Alcaraz et al., 2018). PMs highly express pattern recognition receptors (CD14, CD16), as well as phagocytosis receptor CD11b, cytokine receptors (CD116, CD119), T cell receptor ligand (HLA-DR), and co-stimulatory molecules (CD40, CD80) compared to circulating blood monocytes (Ruiz-Alcaraz et al., 2016; Ruiz-Alcaraz et al., 2018). Pathogens and bacterial products originating from BT from the gut may be absorbed by the peritoneal cavity and elicit an inflammatory reaction (Capobianco et al., 2017).

In regard to the aetiology, in ARLD cirrhosis PMs showed a more pro-inflammatory profile, while in hepatitis C higher levels of IL-12 and lower IL-10 levels were found (Tapia-Abellán et al., 2012). The expression of complement receptor of the immunoglobulin superfamily (CRIg) and CCR2 were found to define two phenotypically and functionally distinct peritoneal macrophage subpopulations (Irvine et al., 2016). $CRIg^{hi}$ macrophages showed higher expression of efferocytosis receptors MERTK and TIMD4 and were on a functional level highly phagocytic and displayed enhanced antimicrobial effector activity compared to $CRIg^{lo}$ macrophages (Irvine et al., 2016). Consequently, a high proportion of $CRIg^{hi}$ macrophages was associated with reduced morbidity and mortality (Irvine et al., 2016). Another group defined PM subsets as large PMs (LPMs, $CD206^{+}CD163^{+}$) and small PMs (SPMs, $CD206^{-}$), which differed in granularity and maturation markers in ascites samples from patients with cirrhosis (Stengel et al., 2020). LPMs had an inflammatory phenotype, were less susceptible to tolerance induction and released more TNF-α than SPMs. TLR stimulation and live bacteria altered levels of CD206 on the surface and resulted in release of soluble CD206 (sCD206) (Stengel et al., 2020). In the early phase of SBP, LPMs were lost, but their abundance was reversible and increased after treatment (Stengel et al., 2020). PMs co-expressed classical M1 and M2 markers illustrating their functional plasticity (Ruiz-Alcaraz et al., 2020) and the potential of the ascitic microenvironment to dynamically alter the macrophage phenotype in order to suit the insult. In the event of BT, increased pro-inflammatory cytokine production and increased iNOS/NO production (Van der Merwe et al., 2021) and a reduced HLA-DR expression in $CD14^{+}$ PMs was associated with adverse prognosis (Frances, 2004; Fagan et al., 2015). A counter-regulatory process resulted in ascitic IL-10, resin and reduced expression of CD14, CD16, HLA-DR, CD86, and

CD206 on PMs (Lesińska et al., 2014; Nieto et al., 2015; Nieto et al., 2018; Hadjivasilis et al., 2021). The reduced CD14 expression on PMs in SBP conditions was associated with impaired phagocytosis (Nieto et al., 2015; Nieto et al., 2018). When comparing peripheral circulating monocytes in stable cirrhosis conditions to PMs of patient with ACLF increased immunoregulatory MERTK-expression population was found, indicating a more suppressive phenotype of PMs in ACLF patients (Bernsmeier et al., 2015b; Flint et al., 2022).

Taken together, in the peritoneal fluid from patients with decompensated cirrhosis complicated by ascites, two distinct PM populations have been identified. LPMs have a pro-inflammatory phenotype ($CD14^{+}CD206^{+}CCR2^{-}CRIg^{+}MERTK^{+}$), are less susceptible to tolerance induction, and release more pro-inflammatory cytokines (TNF-α) compared to SPMs. In SBP conditions LPMs release sCD206 and the loss of LPMs occurs, while the number of SPMs ($CD14^{+}CD206^{-}CCR2^{+/-}MERTK^{-}$) increases. Due to the technical challenge to date, PM from patients with compensated cirrhosis without ascitic fluid have not been characterised phenotypically and functionally. This comparison may help to identify immunological and barrier switches in the progression from compensated to decompensated stages of cirrhosis, since ascites is considered as the hallmark of decompensation (D'Amico et al., 2022).

3.5 Macrophages in other tissues

As a systemic disorder, cirrhosis can involve a number of other organs beyond the circulation, liver, gut and peritoneum.

Lymphoid organs like the spleen and lymph nodes harbour mononuclear phagocytes among other immune cells, which are also be affected in cirrhosis patients. In cirrhosis defective immune cells in mesenteric lymph nodes facilitate BT in decompensation stage. A study showed the accumulation of immune-suppressive MERTK-expressing macrophages in the subcapsular sinus and medullary cord in the lymph nodes of patients with AD/ACLF (Bernsmeier et al., 2015b). In the spleen of patients with cirrhosis and PH phagocytosis activity of macrophages in the red pulp and marginal zone was enhanced and related to hypersplenism and cytopenia (Yongxiang et al., 2002). There is evidence also for a spleen-liver crosstalk, given a study showed reduced fibrosis, monocyte infiltration within the injured liver and CCL2 secretion by hepatic macrophages in cirrhosis patients that had undergone splenectomy (Li et al., 2018).

Adipose tissue macrophages (ATMs) have been intensively investigated over the last years in mouse models or in patients with obesity and NAFLD, however not in the context of cirrhosis. In obesity, the adipose tissue mechanism for lipid containment fails leading to dyslipidemia and insulin resistance (Korf et al., 2019b; Remmerie et al., 2020a). Dying adipocytes release DAMPs and cause adipose tissue macrophage (ATM) activation, which form crown like structures around necrotic adipocytes to engulf cell debris (Korf et al., 2019b; Remmerie et al., 2020a). ATMs secrete pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β), promote monocyte recruitment via MCP-1, increase insulin resistance, hepatic lipid flux and lipid accumulation and are associated with disease progression of NAFLD (Du Plessis et al., 2015; Lefere and Tacke, 2019). The CCL2-CCR2 and CCL5-CCR5 chemokine signalling axes stimulates monocyte recruitment to insulted adipose tissue sites and has been suggested as therapeutic target to resolve inflammation in NAFLD (Kazankov et al., 2019).

Overall, the study of macrophages in the context of cirrhosis in organs other than the liver, circulation or peritoneum remain underrepresented. This may be a result of the difficulty to obtain these biological materials such as lymph nodes, spleen, adipose and other tissue from patients. There is a substantial need to investigate immune processes systematically in diverse tissues in order to understand the plasticity of monocytes and macrophages in the human body in the context of cirrhosis.

4 Potential monocyte and macrophage derived markers of immuneparesis in cirrhosis

Immuneparesis in patients with cirrhosis develops as a consequence of persisting systemic inflammation, which attenuates the responses of immune cells required to combat infections (Bonnel et al., 2011; Bajaj et al., 2012; Albillos et al., 2014). Monocyte and macrophage plasticity in tissues and the systemic circulation play a key role in the regulation of tissue homeostasis and defence against infection. Therefore, future prognostic markers indicating the state of immuneparesis would be helpful in clinical decision making.

The soluble form of two scavenger receptors CD163 and CD206 (sCD163 and sMR/sCD206), expressed by circulating blood monocytes and macrophages, were present in plasma, ascites and other body fluids (Grønbæk et al., 2020; Nielsen et al., 2020) and associated with severity and prognosis of chronic liver diseases (Grønbæk et al., 2016; Kazankov et al., 2016; Rainer et al., 2018; Grønbæk et al., 2020; Nielsen et al., 2020; Stengel et al., 2020) and cirrhosis (Holland-Fischer et al., 2011; Grønbaek et al., 2012; Rode et al., 2013; Waidmann et al., 2013). High sCD163 levels were additionally associated with variceal bleeding, but also predicted mortality in alcoholic hepatitis (Waidmann et al., 2013; Saha et al., 2019). However, sCD163 and sMR plasma/serum levels have been associated with a variety of other diseases and infection conditions (Andersen et al., 2018; Suzuki et al., 2018; Gong et al., 2021; Arneth and Kraus, 2022; Davidsson et al., 2022) as shedding of CD163 and CD206 is associated with macrophage activation (Grønbæk et al., 2020; Nielsen et al., 2020) in general and does not specifically indicate immuneparesis in patients with cirrhosis. Moreover, sCD163 plasma levels differed in relation to the

underlying aetiologies (Wang et al., 2015) further mitigating their clinical significance. Given their secretion by LPM, sCD163, and sMR may potentially enhance the diagnostic criteria for SBP in ascitic fluid of cirrhosis patients (Stengel et al., 2020).

AXL expressing immune-regulatory monocytes have been shown to increase in patients during progression of cirrhosis independent of the aetiology and prior to AD and related infectious complications, as well as the future onset of AD and mortality (Brenig et al., 2020). Therefore, the flow cytometry-based analysis of AXL expression on monocytes in patients with cirrhosis might serve as a marker of immuneparesis and identify patients at risk for infection and deterioration. Also its soluble form sAXL was increased in the plasma of cirrhosis patients correlating with cirrhosis progression (Brenig et al., 2020). However, sAXL has been suggested not only as a marker for advanced fibrosis/cirrhosis but also HCC development (Dengler et al., 2017; Staufer et al., 2017). Elevated sAXL levels were not detected in patients with chronic liver diseases of diverse underlying aetiologies, yet patients with advanced fibrosis (F3), cirrhosis (F4) (54.67–94.74 ng/ml) and HCC (82.7–114.5 ng/ml) displayed higher levels compared to healthy controls (40.15 ng/ml) (Dengler et al., 2017). By contrast, MERTK expression has been identified as a marker both for immunosuppressive circulating monocytes and for macrophages in the liver, peritoneum and mesenteric lymph nodes in acute decompensation of cirrhosis (AD/ACLF) (Bernsmeier et al., 2015b). Thus, in addition to AXL expression, flow cytometry-based MERTK expression on monocytes may represent a valuable marker of immuneparesis and indicating patients with AD/ACLF.

The progression from compensated to decompensated stages of cirrhosis is associated with a poor prognosis and a high mortality (D'Amico et al., 2022). Given the increase in circulating neutrophils and concomitant decrease in lymphocyte numbers is a response to stress including sepsis (de Jager et al., 2010), the neutrophil-lymphocyte ratio (NLR) and monocyte-lymphocyte ratio (MLR) have been developed as prognostic markers for patient stratification. Patients with AD and ACLF who died during hospitalisation displayed elevated NLR and MLR (Bernsmeier et al., 2020). In ACLF patients, an NLR >30 was associated with an 80% 3-months mortality risk (Bernsmeier et al., 2020). Thus, NLR and MLR may be used as rapidly available robust initial diagnostic tools for patient stratification in AD/ACLF.

5 Therapeutic modulation of monocyte and macrophage function in immuneparesis

Along cirrhosis progression patients develop multiple complications (PH, inflammation, BT, gut dysbiosis) that predispose them to decompensation and subsequent precipitating events (infection, variceal haemorrhage) lead to AD (Gustot et al., 2021).

The association of immuneparesis with infectious complications, decompensation and mortality has evoked the concept of immunomodulatory therapies as an adjuvant concept in order to prevent decompensation, save organs and reduce mortality in patients with cirrhosis. Over the previous decade, several potential targets for immunotherapy have been defined (Table 1).

Firstly, therapies with G-CSF have gained interest. These therapies aim to improve liver regeneration and innate immune responses in cirrhosis through the release of bone marrow-derived $CD34^+$ haematopoietic stem cells in order to replace the dysfunctional circulating monocytes and have been studied intensively in ACLF patients (Kedarisetty et al., 2015; Newsome et al., 2018; Verma et al., 2018). However, distinct clinical studies showed differing outcomes. On one hand G-CSF improved the survival of patients (Garg et al., 2012; Verma et al., 2018), while on the other hand G-CSF with haemopoietic stem-cell infusion did not improve liver dysfunction and was associated with increased frequency of adverse events (Newsome et al., 2018). Most recently, a large prospective multicentre trial failed to show beneficial effects of G-CSF over standard of care alone (Engelmann et al., 2021). Thus, G-CSF treatment cannot be recommended at any stage of cirrhosis to date and may require further detailed investigation.

Another therapeutic approach may involve inhibition of macrophage activation via pattern recognition receptors by TLR4 inhibition using TAK-242 or SERELAXIN. Proof of concept studies of TLR4 inhibition previously revealed reduced liver injury and inflammation in rodent models (Seki et al., 2007; Shah et al., 2013; Bennett et al., 2017; Engelmann et al., 2020). In a similar fashion the NLRP3 inflammasome inhibitor MCC950 reduced liver inflammation and fibrosis in experimental NAFLD models (Mridha et al., 2017). These concepts await further evaluation in human *in vitro* models and clinical studies.

Ex vivo data obtained from patients at different stages of cirrhosis support the potential strategy of targeting TAM receptors AXL and MERTK (Bernsmeier et al., 2015b; Brenig et al., 2020). The TAM receptor inhibitors UNC569 (MERTK) and BGB324 (AXL) augmented pro-inflammatory cytokine response in *ex vivo* models (Bernsmeier et al., 2015b; Brenig et al., 2020) and therefore deserve evaluation in *vivo* models of cirrhosis and ACLF. TAM receptor inhibitors are in clinical evaluation (phase 2 studies) for different malignant diseases and may eventually be translated to liver disease given their known safety profiles. Another candidate is the established diabetes drug metformin, which led to reduced AXL expression on monocytes and enhanced inflammatory cytokine responses *in vitro* (Brenig et al., 2020). Moreover, the use of metformin in patients with cirrhosis and diabetes appeared safe and was associated with reduced mortality, HCC or decompensation (Kaplan et al., 2021).

TABLE 1 Potential immunomodulatory therapies targeting monocyte/macrophages in cirrhosis.

Target	Compound	Mechanism	References
CD34+ hematopoietic stem cells	G-CSF	G-CSF promotes release of CD34+ hematopoietic stem cells from the bone marrow which may differentiate into monocytes and replace dysfunctional subsets	Kedarisetty et al. (2015) Verma et al. (2018) Newsome et al. (2018) Engelmann et al. (2021)
TLR-4	TAK-242 or SERELAXIN	Inhibition of macrophage TLR-4 by TAK-242 or SERELAXIN reduces liver injury and inflammation in rodent models	Shah et al. (2013) Engelmann et al. (2020) Bennet et al. (2017)
NLRP3	MCC950	NLRP3 inflammasome inhibition by MCC950 reduces liver inflammation and fibrosis in experimental models of NASH	Mridha et al. (2017)
MERTK	UNC569	MERTK inhibition restored LPS-induced pro-inflammatory cytokine response in monocytes from patients with ACLF *ex vivo*	Bernsmeier *et al.* (2015b)
AXL	BGB324	AXL inhibition restored LPS-induced pro-inflammatory cytokine response in monocytes from patients with cirrhosis *ex vivo*	Brenig et al. (2020)
M-MDSC	Poly(I:C)	TLR-3 agonism by Poly(I:C) reversed M-MDSC expansion and increased the antimicrobial function of these cells in patients with ACLF *in vitro*	Bernsmeier et al. (2018)
Glutamine Synthetase	Methionine sulfoximine	Inhibition of glutamine synthetase partially restored both phagocytosis and inflammatory response of monocytes conditioned with plasma from patients with ACLF *in vitro*	Korf et al. (2019a)
Prostaglandin E2	Albumin	Albumin administration in patients with ESLD lowered circulating PGE2 levels and restored TNF-α production by plasma-conditioned MoMF *in vitro*	O'Brien et al. (2014) China et al. (2018)
CCR2/CCR5	Cenicriviroc	Therapeutic treatment with Cenicriviroc reduced recruitment of hepatic Ly-6C+ MoMF in experimental mouse models of NASH	Krenkel et al. (2018)

G-CSF; granulocyte colony-stimulating factor; TLR-4; Toll-like receptor 4; NLRP3; NOD-, LRR- and pyrin domain-containing protein 3; NASH; Non-alcoholic steatohepatitis; MERTK; myeloid-epithelial-reproductive tyrosine kinase; LPS; Lipopolysaccharide; ACLF; Acute-on-chronic liver failure; AXL; Anexelekto; M-MDSC; Monocytic myeloid-derived suppressor cells; Poly(I:C); Polyinosinic-polycytidylic acid; TLR-3; Toll-like receptor 3; ESLD; End-stage liver disease; TNF-α; Tumor necrosis factor α; PGE2; Prostaglandin E2; MoMF; Monocyte-derived macrophages; CCR2/CCR5; C-C chemokine receptor type 2/5.

Another candidate for immunotherapy may be the TLR3 agonist poly (I:C), which reduced proportions of M-MDSCs and improved their anti-microbial function *in vitro* (Bernsmeier et al., 2018). Poly (I:C) has been used as an adjuvant for vaccination and thus *in vivo* studies for the treatment of cirrhosis may be considered.

In addition, feeding of glutamine into the tricarboxylic acid cycle by using a pharmacological inhibitor of glutamine synthetase (GLUL) restored innate immune responses, in particular the phagocytic and inflammatory capacities of monocytes from patients with ACLF (Korf et al., 2019a).

The therapeutic effects of inhibiting monocyte infiltration by using cenicriviroc (CVC), an oral dual chemokine receptor CCR2/CCR5 antagonist, were assessed using mouse models (Krenkel et al., 2018). In all murine models for steatohepatitis, as well as liver fibrosis progression and fibrosis regression, CVC treatment reduced the recruitment of MDMs (Krenkel et al., 2018). Furthermore, CVC did not affect macrophage polarization, hepatocyte fatty acid metabolism or HSC activation (Krenkel et al., 2018). The inhibition of $CCR2^+$ monocyte recruitment shows potential to improve fibrosis in patients with NASH (Krenkel et al., 2018), but needs further evaluation for the potential of cirrhosis reversibility and its effect on cirrhosis due to other underlying liver diseases.

PAMP-mediated macrophage activation may also be prevented by the restoration of a normal gut microbiome using antibiotics and probiotics (Mazagova et al., 2015; Irvine et al., 2019; Wen et al., 2021). Novel approaches to antibiotic prophylaxis have been explored, including strategies targeting intestinal dysbiosis, including non-selective decontamination (rifaximin), probiotics and faecal microbiota transplantation. Moreover, intestinal motility and barrier function may be improved by the use of prokinetics, beta-blockers, and bile acids (Yan and Garcia-Tsao, 2016). Treatments improving intestinal homeostasis could beneficially impact systemic immune function by reducing exposure to pathological taxa and chronic immune stimulation. Many antibiotics, including those prophylactically used in patients with decompensated cirrhosis, have direct impact on the immune system, although the underlying mechanisms are not well-defined (Zapater et al., 2015).

In addition, the cyclooxygenase (COX)-derived eicosanoid prostaglandin E_2 (PGE_2) was elevated in the plasma of

decompensated cirrhosis patients in association with suppressed cytokine responses and bacterial killing by macrophages *in vitro* (O'Brien et al., 2014). Albumin and COX inhibitors modulated endosomal TLR signalling and improved pro-inflammatory cytokine production (O'Brien et al., 2014; China et al., 2018; Casulleras et al., 2020). An *in vitro* study revealed that the augmentation of serum albumin above 30 g/L was associated with the reversibility of plasma-mediated immune dysfunction by binding and inactivation PGE_2 (China et al., 2018). Intravenous albumin had however no effect on systemic inflammation, albumin function, cardiovascular mediators and biomarkers compared with standard of care in hospitalized decompensated cirrhosis (China et al., 2022). To date, the standard of care remains the treatment with albumin in addition to appropriate antibiotics in SBP (Bajaj et al., 2021) as well as in acute kidney injury. Further studies may evaluate the additive use of COX inhibitors and their potential to improve immuneparesis and restore cytokine production in cirrhosis patients.

In summary, there are a variety of immunomodulatory strategies in clinical evaluation which may shape the future therapy of patients with cirrhosis by improving their antimicrobial defence, and hereby hopefully save organs and reduce mortality.

6 Conclusion

In conclusion, research over the last decade has significantly improved our understanding of the plasticity of monocyte and macrophage differentiation in relation to different compartments and clinical conditions. Their relevance in immuneparesis development in patients with cirrhosis as well as their prognostic value is substantial, and thus supports the concept of adjuvant immune-modulatory treatments. This review specifically summarizes findings originating from patient data or distinctly describing the translation from mouse to human findings. To date immune-pathophysiological findings in humans with cirrhosis are little due to the limitation in assessing samples. Furthermore, translation of mouse related findings to human have to be drawn carefully due to the lack of a proper cirrhosis mouse model. Methodological advances such as single-cell based technologies have enabled the identification of tissue specific mononuclear phagocytic subsets in the context of their functions in limited samples from patients with liver cirrhosis. However, further studies are needed to clarify monocyte and macrophage function also in relation to the diverse underlying aetiologies and in diverse compartments other than the liver and the circulation. Decompensation of cirrhosis is accompanied with the development of diverse complications, such as ascites and infections. Infections, which are due to a dysfunctional immune response, in turn represent a great risk to patients with decompensated cirrhosis. Most of these infections originate from barrier dysfunction in the gut and peritoneum, which result in BT to the circulation. In addition to the inflamed liver, BT has the potential to change the milieu in the gut, liver, circulation and peritoneum and affects tissue specific differentiation of monocytes and macrophages. Treatment with antibiotics and albumin have reduced the mortality in case of infection, but antibiotics should be prescribed with caution given the increasing prevalence of MDRO in cirrhosis patients. Research over the past years has identified not only potential prognostic molecular markers to better predict cirrhosis progression and immuneparesis development in patients, but also molecules on immune cells which may be targeted in order to restore immune function and prevent infectious episodes. The development of immune-modulatory treatments however is challenging given the tissue-specific plasticity of immune cells and requires careful evaluation *in vivo*. At present, experimental studies and clinical trials aimed at either identifying novel immunotherapeutic targets or delineating beneficial effects of distinct immune-modulatory treatments from their off-target effects are ongoing.

Author contributions

AG and CB contributed to the conception of the review. EF designed Figure 2 and Table 1. AG wrote the first draft of the manuscript under supervision of CB. All authors read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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1.3 TAM Receptors: Tyro3, AXL and MERTK

The TAM receptors Tyro3, AXL and MERTK are a family of receptor tyrosine kinases [72]. The three receptors share similarity in structure and are expressed in a variety of cells and tissues [72]. Tyro3 is mostly expressed in the central nervous system, cerebral cortex and cerebellum [73-75]. AXL on the other hand is expressed in cell populations in the liver, kidney, heart, sceletal muscle, testis, but also blood monocytes and platelets [73]. MERTK is expressed in cell populations in ovaries, testis, liver, lung kidney cerebral cortex, retina and blood cells such as NK cells, monocytes and platelets [73, 76]. The structure of TAM receptors comprises an extracellular domain, a transmembrane domain and and intracellular part [77]. Ligand binding TAM receptors are GAS6, Protein S and Galecin-3 [78-81].

1.3.1 TAM Receptor Expression Regulation

In vitro AXL is upregulated on human monocyte derived macrophages (MDM) upon treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) [82]. Monocytes also upregulate AXL following TLR stimulation by LPS treatment [83]. MERTK on the other hand is getting upregulated by IL-17A and IL-10, which are both, pro- and anti-inflammatory cytokines [84]. Furthermore, glucocorticosteroid treatment and stimulation of the liver X receptor also results in MERTK upregulation [85, 86]. Tyro3 is the least studied receptor of the family and mechanisms of its upregulation are not very well established [72]. TAM receptors were found in the tumour microenvironment. They can be be expressed on tumour cells as well as pro- and anti-tumour macrophages indicating a dual mechanism of anti-TAM receptor strategy in oncology. Interestingly, monocytes upregulate MERTK when differentiating into macrophages [85, 86]. There are contrasting results for the AXL expression pattern and further research needs to be conducted [72]. While MERTK is rather attributed to an immunosuppressive

and pro-restorative phenotype [87, 88], AXL is observed in pro- and anti-inflammatory contidions. Macrophages stimulated by TLR and INF-γ (pro-inflammatory), but also cells stimulated with IL-4 or IL-13 (anti-inflammatory) show AXL expression [89, 90].

1.3.2 Signalling Pathway of TAM Receptors

TAM receptors bind the ligands GAS6 and Proteins S [79, 80]. The ligands' structure consists of an N-terminal gamma-carboxyglutamic acid (Gla), four epidermal growth factor (EGF)-like repeates and a C terminal domain consisting of two lamining G (LG) domains [91]. GAS6 has the potential to bind all the the three TAM receptors, but showing highest affinity to AXL, while Protein S binds only to Tyro-3 and MERTK [92]. Galectin-3 was lately shown to bind MERTK and Tyro-3, but not AXL [93-95]. Mechanistically, the LG domain of the ligand binds to the TAM receptor, which in turn causes dimerization of the receptor and subsequent phosphorylation and activation of intracellular signalling [96, 97]. To less extend, Tubby, binding MERTK, tubby-like protein 1 (Tulp1), binding all TAM receptors and Galectin3, binding MERTK have also been described [98].
TAM receptors play an important role in the regulation of the innate immune response. These receptors regulate efferocytosis, the phagocytosis of apoptotic cells. This process is important for restoration of the immune- and tissue-homeostasis. Phosphatidyl-serine (PtdSer), which is exposed on the surface of apoptotic cells can bind to TAM receptors and initiate their uptake. When Ca^{2+} is present, the PtdSer can bind to the Gla domain of the TAM ligands GAS6 or Proteins S and uptake of apoptotoic bodies by macrophages or other phagocytes gets increased [96, 97, 99].
Furthermore, TAM receptors serve as negative feedback loop for the innate immune response through suppression of TLR signalling [100]. The ligand activated dimerized TAM receptor interacts with IFNAR/STAT complex which in first place amplifies the inflammatory response. This interaction also causes functional

changes of IFNAR/STAT1 towards an anti-inflammatory molecule and suppressors of cytokine signaling 1 and 3 (SOCS1/3) are transcribed. This in turn inhibits TLR signalling and pro-inflammatory cytokine signalling (Figure 1.3-1) [100]. Shedding of the ectodomain of the TAM receptors also plays a role in regulation of these processes. ADAM10 and ADAM17 cleave TAM receptors at the transmembrane domain and solube forms of TAM receptors Tyro3, sAXL and sMER are released [101, 102]. The ligands GAS6 and Protein S still bind the soluble TAMs, this subsequently dampens the ligand-induced signalling (Figure 1.3-1) [103].

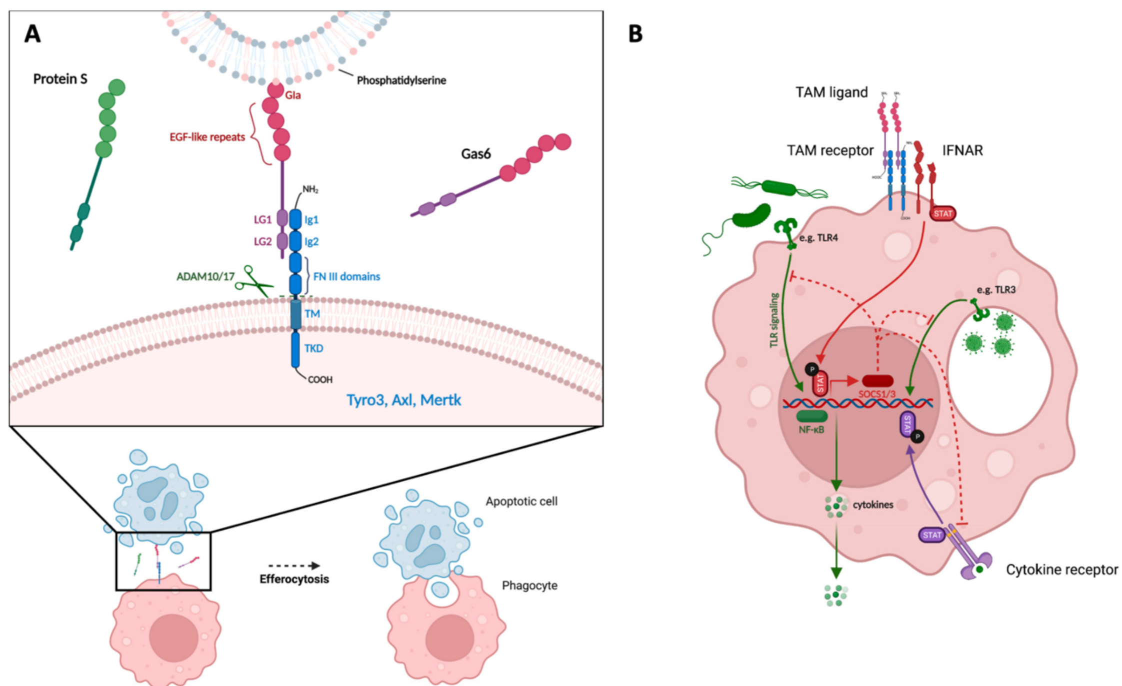

Figure 1.3-1:TAM receptors promote efferocytosis and inhibit TLR signaling
(A) TAM receptors promote efferocytosis by GAS6/Protein S binding both, PtdSer (apoptotic cells) and TAM receptors (phagocyte). ADAM10/17 cleave TAM receptor within TM domain. (B) TLR signaling inhibition by TAM ligand binding and TAM activation with subsequent cytokine release via NF-κB. Interaction with IFNAR/STAT complex and transcription of SOCS1/3 inhibits TLR- and cytokine receptor signaling and reduces NF-κB induced cytokine production. (adapted from Flint et al., Livers, 2022)

1.3.3 TAM Receptors in Disease

Triple KO mice (*Tyro3-/-, Axl-/-, Mertk-/-*) develop inflammation, which continues and leads to chronic inflammation [104]. AXL and MERTK are barely co-expressed in mice, with the exception of murine liver-resident macrophages. These cells were shown to co-express AXL and MERTK, while Tyro3 was absent [89]. Knocking out AXL or MERTK in mice resulted in the accumulation of apoptotic cells in the liver due to impaired efferocytosis. These mice also show inflammatory infiltrates in the liver, inflammatory cytokines and chemokines in the liver and high serum levels of liver damage markers [105].

In humans, findings of TAM receptors need to be put in context of clinical presentations. In the context of cirrhosis, cirrhosis-associated immune dysfunction (CAID) occurs, where immunodeficiency and systemic inflammation are present in cirrhosis. Bacterial translocation occurs from the gut to mesenteric lymph nodes in healthy conditions and is important to maintain host immunity. This process is increased in patients with liver cirrhosis and goes beyond hosts ability to maintain tolerance [32]. The progression of cirrhosis is associated with systemic inflammation and increased serum levels of pro-inflammatory cytokines [106]. Circulating immune cells are activated and display changes in phenotype and function [107-110]. Furthermore, the hepatic protein synthesis is impaired and subsequently the complement system, which regults in a reduced pathogen revognition [111, 112]. This goes hand in hand with reduced numbers of Kupffer cells (KC) [113] and expansion of immunosuppressive cells [83, 114]. Taken together these mechanisms increase susceptibility to infections and are associated with increased mortality in paitents with cirrhosis [115-117].

TAM receptors seem to play an important role in CAID. For example, the AXL/GAS6 signaling pathway induces autophagy in liver macrophages, which prevents NLRP3 inflammasome activation, and thereby dampens hepatic inflammatory response [118]. Interestingly, when hepatic injury is induced by LPS or CCl_4, *Axl-/-* mice are protected compated to wild type controls [118]. It was shown that $MERTK^+$ monocytes increased in circulation of ACLF patients, compared to

helathy and compensated cirrhosis and have immunoregulatory function [114]. MERTK-expressing macrophages were increased also in liver, peritoneum and mesenteric lymph nodes of these patients and the elevated expression level correlated with disease severity scores like Child Pugh, MELD, CLIF-SOFA and NACSELD scores as well as systemic inflammation score SIRS [114]. Functionally, MERTK$^+$ monocytes dampened pro-inflammatory cytokine production (IL-6, TNF) upon bacterial stimulation and show an anti-inflammatory phenotype. Increased migration behaviour into inflamed tissues in cirrhosis-associated endothelial dysfunction has been attributed to MERTK$^+$ monocytes. It was suggested that repeated migration cycles may contribute for MERTK upregulation in ACLF [114].

In regard to AXL, a population of AXL$^+$ monocytes already expanded in compensated cirrhosis and increases with disease progression in the circulation of patients, but was found to be absent in stage of AD/ACLF [83]. Functionally, AXL-expressing monocytes produced pro-inflammatory cytokines (IL-6, TNF) upon TLR-4 stimulation with LPS, dampened T cell activation and showed increased phagocytosis and efferocytosis capacity [83]. Moreover, levels of AXL$^+$ monocytes in the circulation correlated, similar to MERTK, with Child Pugh and MELD score as well as D'Amico classification and the development of ascites, varices and HE [83]. The frequency of AXL-expressing monocytes can also be seen as predictor for the development of immune dysfunction or infection susceptibility, the onset of AD and increased mortality. From a regulatory perspective, AXL gets upregulated on monocytes *in vitro* by exposure to PAMPS, uptake of apoptotic cells and bacteria. Inhibition of AXL has the potential to restore inflammatory cytokine production, which make AXL a potential target in CAID [83].

It is suggested that plasma components of TAM signalling pathway may serve as non-invasive markers in liver cirrhosis. Interestingly, GAS6 appears to be a promising plasma marker for varices detection [119]. An accurate biomarker for fibrosis/cirrhosis in NAFLD, HCC and viral hepatitis appeared to be sAXL plasma levels in patients [120]. The accuracy can be further improved by including albumin

levels and assessing sAXL/albumin ratio [120]. In addition, GAS6 levels are elevated in plasma of patients with cirrhosis as well as sAXL, sMERTK and Protein S and Galectin3 is increased in pateints with ACLF [83, 114, 121].

1.3.4 TAM Receptor Inhibitors

Given the context deduced above, modulation of TAM receptors in the context of liver cirrhosis appeared to be a promising future therapeutic target. The inhibition of AXL and MERTK may improve anti-bacterial responses without having adverse effect on tissue homeostasis and repair processes. The small molecule AXL inhibitor bemzentinib (BGB324) was used in a CCl_4 mouse model and reduced collagen deposition in the liver [122]. Metformin, an anti-diabetic drug, was described to reduce HCC [123] and portal hypertension in cirrhosis models [124]. Metformin targets and and down-regualtes AXL and Tyro3 [125]. In *ex vivo* experimental setup, metformin enhanced innate immune response in monocytes, making it an interesting immune-modulatory treatment option. The MERTK inhibitor UNC1062 was described to reduce activation of MERTK downstream signaling, although in the background of metastatic melanoma [126]. Another MERTK inhibitor UNC569 reduced proliferation and survival *in vitro* and increased sensiticity to cytotoxic chemotherapies in background of acute lymphoblastic leukemia (ALL) [127]. In cirrhosis it was shown *ex vivo* that UNC569 and bemzentinib restored pro-inflammatory cytokine response [83, 114]. The two TAM receptor inhibitors are also described in **chapter 1.2**.

1.4 CD52: Glycosylphosphatidylinositol-anchored Glycoprotein

CD52 is a 21 to 28 kDa non-modulating membrane glycosylphosphatidylinositol-anchored (GPI-anchored) glycoprotein [128]. CD52 has no intracellular domains [129] and is expressed on mature lymphocytes, eosinophils, dendritic cells (DCs) and monocytes [130]. It is also found to be expressed in tissues like the male genital tract and mature sperm cells [130, 131], but not on erythrocytes, platelets and haematopoietic progenitor cells [132]. CD52 expression on immune cells is only poorly characterized and its role is barely understood.

1.4.1 Expression of CD52 in Immune Cells

CD52 is relatively high expressed on all lymphocytes, especially on CD4 T cells [131, 133]. It was reported that a costimulatory signal to human CD4 T cells induces regulatory T (Treg) cells. Tregs then execute a suppressing response effect to adjacent CD4 T cells in a contact dependent manner [129]. CD52 expressing Treg cells have the potential to suppress the proliferation not only of CD4 but also CD8 T cells [129]. This suppressive action is dependent on cell-to-cell contact [134].

Interestingly CD52 expression was also found on monocytes, DCs, eosinophils, but not on neutrophils [133]. Memory B cells, myeloid DCs (mDCs) show relatively high CD52 expression levels, while plasmacytoid DCs (pDCs) and natural killer (NK) cells show rather low expression [135]. Furthermore CD16high non-classical monocytes were found to express higher levels of CD52 compared to their CD16low classical and intermediate monocyte counter parts [135]. IL-4 and IL-13 can upretgulate CD52 on monocytes via STAT6 pathway, while LPS, IFN-α, -β and -γ cause downregulation of CD52 on moncoytes [136].

1.4.2 CD52 Signalling Pathway

It is known that GPI anchored proteins are cleaved by phospholipases, while the effect of phospholipase C (PLC) was investigated in activated T cells [134, 137]. It was found that surface CD52 is cleaved by PLC and its soluble form is released [134]. Sialoside structures, such as CD52, are recognized by sialic acid-binding immunoglobulin-like lectins (Siglec), which are inhibitory receptors of the Ig superfamily [138]. Soluble CD52 (sCD52) directly binds to the inhibitory Siglec-10 and mediates immune regulation [134]. Therefore, the immunosuppressive characteristics of CD52high CD4 T cells is mediated by PLC-mediated release of sCD52, which binds to Siglec-10 and inhibits the phosphorylation of T cell receptor (TCR)-associated kinases Lck and Zap70 and following inhibits T cell activation [134]. CD52 can therefore act as a costimulatory molecule by activating effector and regulatory T cells, but on the other hand, sCD52 binds to Siglec-10 and inhibits T cell activation [132]. The release of sCD52 could be inhibited by the use of PLC inhibitor [139]. Also blocking of Siglec-10 reversed the inhibitory effect of CD52high CD4 T cells [138].

There are two mechanisms on CD52high CD4 T cells (Tregs) to execute their inhibitory effects. One is the release of sCD52 and the subsequent binding of Siglec-10 impairing Lck and Zap70 phosphorylation, the other one is the cross-linkage of CD52 surface molecules by undefined endogenous ligands, which results in the expansion of CD52high CD4 T cells (Figure 1.4-1) [133]. Siglec-10 has been described to bind vascular adhesion protein-1 and to mediate lymphocyte binding to the endothelium [140]. The binding of sCD52 to Siglec-10 can therefore modify the inflammatory microenvironments [133]. CD52 knockout mice showed increased cytokine production and hypothermic responses after low dose lipopolysaccharide (LPS) injections. In contrast, injection of CD52-Fc to wild type mice suppressed responses to LPS [141, 142]. It was reported that CD24, another GPI-anchored protein, first associates with damage-associated molecular pattern (DAMP) proteins like high mobility group box 1 (HMGB1) before binding to Siglec-10. It was found that CD52-Fc binding to Siglec-10 suppressed T cells

depending on the DAMP protein HMGB1 [141]. Soluble CD52 first sequesters HMGB1 to cancel out its proinflammatory Box B, which is then followed by Siglec-10 binding, triggering SHP1 recruitment and Lck/Zap70 phosphorylation [141]. Furthermore, sCD52 inhibited Toll-like receptor (TLR) and tumour necrosis factor (TNF) receptor signalling. This limited the activation of nuclear factor kappa B (NF-κB) and suppressed the production of inflammatory cytokines by macrophages, monocytes and subset of cells that were also high for CD52 expression, which is consistent with the homeostatic role for Siglec-10 in human CD4 T cells [143]. In systemic lupus erythematosus (SLE) patients it was found, that that sCD52, cleaved by PLC, inhibited B cell receptor (BCR) signalling in a Siglec-10 dependent manner. On B cells CD52 functions also as a homeostatic protein by inhibiting response to BCR signalling [144].

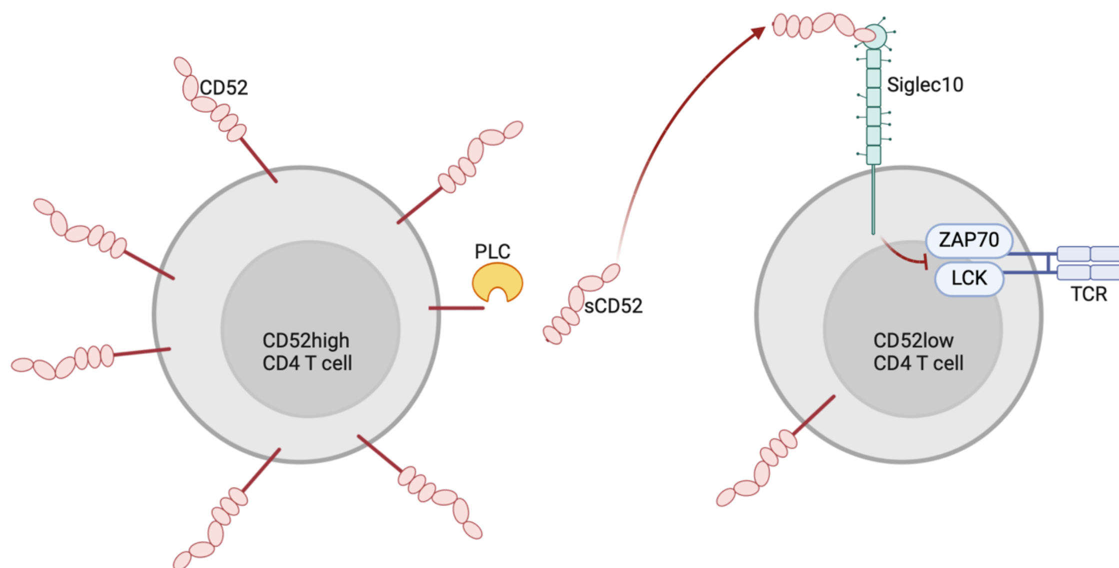

Figure 1.4-1: T cell regulation by sCD52
CD52 is cleaved by cleaved by phospholipase C (PLC) and soluble CD52 (sCD52) is released. Siglec10 binds sCD52. Activation of Siglec10 impairs phosphorylation of T cell receptor (TCR) associated Lck and ZAP70 suppressing T cell activation. Created with biorender.

Little however is known about how CD52 is upregulated, but it was found that the membrane receptor CD24, which belongs to the same GPI-anchored protein family, is expressed under direct control of the Wnt/β-catenin signalling pathway [145]. Human adipose stem cells (hASCs) start to express CD24 *in vitro* when

incubated with hedgehog (Hh) signalling pathway activating molecules [146]. In acute promyelocytic leukemia CD52 expression can be induced by treatment with all-*trans*-retinoic acid [128]. On monocytes CD52 expression is upregulated by interleukin-4 (IL-4)/IL-13 through the STAT6 pathway, while it was found to be downregulated by LPS and IFN-α, -β and -γ [136].

1.4.3 CD52 in Disease

It is reported that in human sperm cells *N*-glycans of CD52 isoforms are associated with human infertility [147]. In a T cell transfer model, the depletion of CD52high cells was related to the onset of autoimmune diabetes [148]. But CD52 knockout mice show no concerning phenotype regarding their immune or reproductive system [149].

There are several immunologically related diseases where CD52 is described to play an important role in disease development.

An upregulation of CD52 was found in SLE patients [144]. These patients showed increased CD52-expression levels on B cells and also increased sCD52 plasma levels. Furthermore, sCD52 levels correlated with SLE disease activity [144].

It was suggested that innate lymphoid cells (ILC2s) and T helper 2 (TH2) cells produce type 2 cytokines which contribute to development of airway hyperreactivity (AHR). CD52 was found to be upregulated in IL-33 induced AHR in mice. CD52-targeted depletion of of type 2 immune cells (IL2s and TH2s) could reverse the establishment of IL-33 induced AHR in mice by reducing airway resistance and easing lung inflammation [150]. Importantly CD52-depletion could be successfully used to treat allergic AHR induced by *Alternaria alternata* and house dust mite in mice [150]. The anti-CD52 antibody alemtuzumab could be used to reduce pulmonary inflammation, abrogate eosinophilia, improve lung function and treat allergic AHR [150].

In cases of breast cancer, high levels of CD52 expression on tumor infiltrating cells were detected in early stages and associated with favourable prognosis

[151]. A higher infiltration rate of M1 macrophages, monocytes, T follicular helper cells and resting memory CD4 T cells was observed after CD52 overexpression. On the contrary, a downregulation of CD52 resulted in M2 macrophage infiltration [151]. In the context of breast cancer, high expression of CD52 may negatively regulate M2 macrophage infiltration, but promote infiltration of anti-cancer immune cells, resulting in protective effect and improving prognosis of patients [151].

In sepsis CD52 was described as a prognostic biomarker and therapeutic target. The increased expression of CD52 on lymphocytes correlated with improved sepsis outcomes [152]. Patients with systemic sclerosis (SSc) showed dysregulation of type I interferon (IFN) signalling [136]. In monocytes of these patients reduced CD52 expression was found and enhanced adhesion to endothelial cells. The overexpression of CD52 resulted in decreased CD18 expression and reduced monocyte adhesion, while the knockout of CD52 was shown to increase adhesion potential of monocytes [136].

Taken together sCD52 is associated with immune suppressive function, making it a potential candidate for immunotherapeutic agent.

1.4.4 Alemtuzumab, a CD52 Inhibitor

The anti-CD52 monoclonal antibody (mAb) alemtuzumab is clinically used for the treatment of chronic lymphocytic leukemia (CLL) and relapsing remitting multiple sclerosis (RMS). Furthermore, prior to transplantation immunosuppression is induced by alemtuzumab to prevent steroid-refractory acute graft versus host disease (GVHD) [128, 129, 132, 153]. It is also known under the tradename Campath-1H and is a humanized monoclonal antibody binding the C-terminal part of the peptide to an epitope that includes part of the GPI anchor [133, 135]. Alemtuzumab inhibits trans endothelial migration of T cells and depletes lymphocytes by cytolytic effects *in vivo* [129, 135]. The CD52 inhibitor negatively modulates CD4 and CD8 expression on T cells through apoptosis-independent

pathways [154]. Furthermore alemtuzumab can kill T cells via complement- and non-complement mediated mechanisms [132]. Therefore, alemtuzumab may cause cell death by multiple mechanisms, e.g. complement-mediated, antibody-mediated and apoptosis enhancing [155-158].

The cytolytic effects of alemtuzumab are related to the density of CD52 expression on cells and it was found that monocytes are less susceptible to alemtuzumab cytolysis, which leads to the suggestion that density is not the only relevant factor for cytolysis [135]. The elimination half-life of alemtuzumab is 12 days, but its clinical effects are more persistent and a prolonged lymphocyte depletion is observed [132, 159, 160].

Although alemtuzumab was FDA approved in 2001 for CLL and in 2014 for RMS, its risk of autoimmune disorders has led to the recommendation to restrict the use of alemtuzumab to patients who failed at least two other treatments approved for RMS [161]. *In vitro* the use of alemtuzumab on human blood samples increased the adhesion potential of monocytes and enhanced type I IFN responses [136]. In autoimmune encephalomyelitis mice anti-CD52 treatment resulted in increased expression of MHC II and costimulatory molecules on blood and splenic innate immune cells. This was also associated with increased potential of activate antigen-specific T cells [162]. The use of alemtuzumab comes with its advantages and disadvantages and needs to be considered disease specific.

2 Aim of the Thesis

While patients with compensated cirrhosis may be asymptomatic and may have a normal life expectancy, the prognosis of patients with decompensated cirrhosis is adverse.
When cirrhosis progresses to a certain stage, a state of immuneparesis has been observed, and infection can trigger decompensation as a precipitating factor. The origins and cause of immuneparesis are unknown and may potentially preceed infection. Additionally, immuneparesis can occur after an episode of infection, making patients more susceptible to secondary infections.
We aimed to examine the dynamic phenotypical and functional changes in circulating monocytes throughout different stages of cirrhosis (stages Child-Pugh A, B, C and AD/ACLF or compensated cirrhosis, NAD, AD/ACLF).

In a first part, we investigated differential expression of TAM receptors AXL and MERTK on tissue specific macrophages using a cohort of patients with compensated and decompensated cirrhosis. We identified AXL-expressing $CD68^+$ resident liver macrophages decreasing with cirrhosis progression. The same pattern was observed on gut and peritoneal macrophages, while regional lymph nodes showed an increase in AXL-expressing macrophages, as previously observed in the blood compartment. Mechanistically we showed that GAS6, secreted by HSC, was enriched in cirrhotic livers and appeared to down-regulate AXL *in vitro*.

In a second part, we sought to systemically dissect the stage-specific heterogeneity of monocyte differentiation in the circulation of liver cirrhosis patients using scRNA-Seq. We identified seven monocyte clusters with varying frequency between cirrhosis stages. Phenotypically and functionally activated CD52-expressing monocytes were found to be enhanced in compensated and not-acutely decompensated (NAD) cirrhosis. In patients presenting with acute decompensation

CD52 was cleaved by elevated PLC levels, resulting in immunosuppressive CD52^{low} circulating monocytes in patients with AD and high levels of plasma sCD52.

To this day liver transplantation is the only curative therapy for advanced cirrhosis. Understanding and deciphering the pathophysiology of monocyte and macrophage dysfunction and immuneparesis might promote the development of future immunotherapeutic treatments for patients with stable and advanced cirrhosis in order to enhance immune responses, prevent infectious complications, decompensation events, need for transplantation and death.

3 Material and Methods

Material and methods used for the experiments are listed in the material and methods section of the respective publications and manuscript in **chapter 4.1** and **chapter 4.2**.

4 Results

4.1 AXL Expression of Homeostatic Resident Liver Macrophages is Reduced in Cirrhosis following GAS6 Production by Hepatic Stellate Cells

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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

AXL Expression on Homeostatic Resident Liver Macrophages Is Reduced in Cirrhosis Following GAS6 Production by Hepatic Stellate Cells

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SUMMARY

Enhanced AXL expression on circulating monocytes attenuated immune responses in cirrhosis patients. Resident liver macrophages highly express AXL physiologically. GAS6, secreted by activated hepatic stellate cells, decreased AXL on liver macrophages in relation to cirrhosis progression. This suggests a role for AXL in the regulation of hepatic immune homeostasis.

BACKGROUND & AIMS: AXL and MERTK expression on circulating monocytes modulated immune responses in patients with cirrhosis ($CD14^+HLA\text{-}DR^+AXL^+$) and acute-on-chronic liver failure ($CD14^+MERTK^+$). AXL expression involved enhanced efferocytosis, sustained phagocytosis, but reduced tumor necrosis factor-α/interleukin-6 production and T-cell activation, suggesting a homeostatic function. Axl was expressed on murine airway in tissues contacting the external environment, but not interstitial lung- and tissue-resident synovial lining macrophages. Here, we assessed AXL expression on tissue macrophages in patients with cirrhosis.

METHODS: Using multiplexed immunofluorescence we compared AXL expression in liver biopsies in cirrhosis (n = 22), chronic liver disease (n = 8), non-cirrhotic portal hypertension (n = 4), and healthy controls (n = 4). Phenotype and function of isolated primary human liver macrophages were characterized by flow cytometry (cirrhosis, n = 11; control, n = 14) *ex vivo*. Also, AXL expression was assessed on peritoneal (n = 29) and gut macrophages (n = 16) from cirrhotic patients. Regulation of AXL expression was analyzed *in vitro* and *ex vivo* using primary hepatic stellate cells (HSCs), LX-2 cells, and GAS6 in co-culture experiments.

RESULTS: AXL was expressed on resident (CD68^{+}) but not tissue-infiltrating (MAC387^{+}) liver macrophages, hepatocytes, HSCs, or sinusoidal endothelial cells. Prevalence of hepatic CD68^{+}AXL^{+} cells significantly decreased with cirrhosis progression: (healthy, 90.2%; Child-Pugh A, 76.1%; Child-Pugh B, 64.5%; and Child-Pugh C, 18.7%; all $P < .05$) and negatively correlated with Model for End-Stage Liver Disease and C-reactive protein (all $P < .05$). AXL-expressing hepatic macrophages were CD68^{high}HLA-DR^{high}CD16^{high}CD206^{high}. AXL expression also decreased on gut and peritoneal macrophages from cirrhotic patients but increased in regional lymph nodes. GAS6, enriched in the cirrhotic liver, appeared to be secreted by HSCs and down-regulate AXL *in vitro*.

CONCLUSIONS: Decreased AXL expression on resident liver macrophages in advanced cirrhosis, potentially in response to activated HSC-secreted GAS6, suggests a role for AXL in the regulation of hepatic immune homeostasis. *(Cell Mol Gastroenterol Hepatol 2023;16:17–37; https://doi.org/10.1016/j.jcmgh.2023.03.007)*

Keywords: TAM Receptors; Innate Immunity; Cirrhosis; Resident Liver Macrophages.

Under homeostatic conditions, the liver is a tolerogenic organ acting as a firewall mediating mutualism between the host and its gut commensal microbiota.[1] Hepatic non-parenchymal cells including resident liver macrophages, dendritic cells (DCs), infiltrating monocytes, hepatic stellate cells (HSCs), and liver sinusoidal endothelial cells actively promote tolerance by removing bacterial products, secreting tolerogenic factors, and modulating immune cell phenotype and function.[1,2] During the progression of cirrhosis and evolution of portal hypertension, these mechanisms fail to maintain tolerance, which has pivotal consequences for the hepatic and systemic milieu.[3] Also, a dysfunctional intestinal barrier resulting in pathologic bacterial translocation[4] and altered peritoneal macrophages (pMACs), which are critical for regulating inflammation and controlling bacterial infections,[5] contribute to the loss of systemic immune surveillance during development of cirrhosis. The current pathophysiological concept assumes that the breakdown of these "immunologic firewalls" in tissues in contact with the external environment predisposes to the development of bacterial infections, sepsis, organ dysfunction, progressive hepatocellular failure, and accelerates morbidity and mortality.[6–8]

TYRO-3, AXL, and MERTK belong to the TAM receptor tyrosine kinases family and are expressed on monocytes and macrophages. They are important regulators of innate immune homeostasis[9,10] and operate by inhibiting toll-like receptor (TLR) signaling pathways and promoting phagocytic removal of apoptotic cells.[9,11]

The function of AXL-expressing innate immune cells in tissues acting as immunologic barriers has been widely addressed in distinct compartments such as the lung,[12] central nervous system,[13,14] joints,[15] and skin[16] and associated with prevention and resolution of inflammation. In mouse livers, recent analyses indicated that Mertk and Axl were predominantly expressed on Kupffer cells (KCs) and endothelial cells and critical regulators to maintain liver homeostasis.[17] In patients with acutely decompensated cirrhosis and especially those with acute-on-chronic liver failure, MERTK-expressing macrophages were detected in the liver and other tissue sites such as the peritoneum and regional lymph nodes.[18] MERTK-expressing monocytes and macrophages represented an immune-modulatory pro-restorative cell population, characterized by enhanced efferocytosis of apoptotic neutrophils while expressing attenuated cytokine responses.[18,19] Recently, we observed circulating AXL-expressing monocytes in patients with cirrhosis of the liver in parallel with disease progression and portal hypertension characterized by preserved phagocytosis capacity but reduced T-cell activation and inflammatory cytokines suggesting a homeostatic function.[20]

Considering cirrhosis as a systemic disease condition, in this study we sought to assess the plasticity of AXL expression on tissue macrophages in different compartments of patients with advanced cirrhosis and to investigate its potential role in immune homeostasis regulation.

Results

Patient Characteristics

The 2 cohorts comprising patients with cirrhosis distinguished between Child-Pugh A, B, and C and compared with healthy controls and chronic liver disease without cirrhosis, and patients with cirrhosis undergoing liver resection compared with histologically normal controls are summarized in Tables 1 and 2. Both cohorts were characterized by etiology, disease severity scores, and extensive clinical parameters. In the first cohort in patients with cirrhosis, 1-year mortality rate was 13.6% (n = 3)

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Abbreviations used in this paper: aHSC, activated hepatic stellate cell; α-SMA, alpha smooth muscle actin; CCl_4, carbon tetrachloride; DC, dendritic cell; FFPE, formalin-fixed and paraffin-embedded; HPF, high-power field; HSC, hepatic stellate cell; Ig, immunoglobulin; NCPH, non-cirrhotic portal hypertension; KC, Kupffer cell; MELD, Model for End-Stage Liver Disease; MoMF, monocyte-derived macrophages; PBS, phosphate-buffered saline; pMAC, peritoneal macrophage; TLR, toll-like receptor.

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Table 1. Clinical Characteristics of Patients With Cirrhosis Compared with Chronic Liver Disease Without Cirrhosis and Healthy Controls

Variables, median (range)	Cirrhosis (n = 22)	CLD without cirrhosis (n = 8)	Healthy controls (n = 4)
Age (y)	59 (34–79)	38 (24–66)[a]	55 (47–69)
Sex (m:f)	14:8	5:3	—
Underlying liver disease(s) [%]	ALD 11 [50] HCV 4 [18] NAFLD 2 [9] PBC 2 [9] Wilson's disease 1 [5] Hemochromatosis 1 [5] Unknown 1 [5]	HBV 4 [50] NAFLD 3 [37.5] AIH 1 [12.5]	NA
Death (1 year)	3 [14]	—	—
Child-Pugh	8 (5–11)	5 (5–5)	NA
MELD	14 (6–29)	6 (6–6)	NA
BMI (kg/m^2)	27 (17–32)	—	—
Na$^+$ (mmol/L)	138 (120–144)	139 (137–142)	140 (138–142)
K$^+$ (mmol/L)	3.9 (3.0–5.1)	3.8 (3.7–4.0)	3.95 (3.40–4.20)
Creatinin (μmol/L)	84 (47–171)	70.5 (61.0–83.0)	83.5 (73.0–93.0)
Urea (mmol/L)	5.9 (3.1–14.7)	3.7 (3.5–3.9)	6.75 (4.8–7.6)
Bilirubin (μmol/L)	34 (8–147)	16 (5–32)	9.5 (6–12)[b]
AST (U/L)	49 (16–168)	28 (17–133)	23 (22–27)[b]
ALT (U/L)	29.5 (7.0–114.0)	27 (11–207)	30.5 (15.0–35.0)
GGT (U/L)	85 (26–1013)	26 (10–272)	119 (20–133)
AP (U/L)	117 (49–483)	76 (35–106)	56 (33–93)[c]
INR	1.45 (0.90–2.30)	1 (1–1)[a]	—
Albumin (g/L)	26.5 (19.0–39.0)	39.8 (37.0–44.4)[d]	39 (38–40)[e]
Hemoglobin (g/L)	99.5 (7.2–152.0)	142 (113–164)[d]	—
Hematocrit (%)	0.314 (0.242–0.440)	0.42 (0.35–0.5)[a]	—
Leukocytes (G/L)	5.05 (2.40–70.0)	5.4 (4.3–6)	—
Neutrophils (G/L)	3.6 (1.6–41.3)	48.9 (48.6–55.1)[f]	—
Eosinophils (G/L)	0.29 (0.017–0.91)	3.9 (2.2–5.7)	—
Basophils (G/L)	0.37 (0.001–0.28)	0.7 (0.6–1.3)	—
Lymphocytes (G/L)	1.55 (0.4–2.5)	2.3 (1.6–2.5)	—
Monocytes (G/L)	0.62 (0.23–7.49)	5.8 (5.7–7.3)	—
Platelets (G/L)	100.5 (8.5–341)	218 (172–235)[a]	—
CRP (mg/L)	9 (0.4–67)	2 (0.6–5.4)[f]	1.35 (0.70–2.40)[b]

NOTE. Data indicated as median with (minimum and maximum) and [percentage].
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; HBV, hepatitis B virus; HCV, hepatitis C virus; INR, international normalized ratio; MELD, Model for End-Stage Liver Disease; NAFLD, nonalcoholic fatty liver disease; PBC, primary biliary cirrhosis.
[a]*P* = .01.
[b]*P* = .01.
[c]*P* = .05.
[d]*P* = .001 indicate cirrhosis vs CLD without cirrhosis.
[e]*P* = .001 indicate cirrhosis vs healthy controls; comparisons by Mann-Whitney *U* tests.
[f]*P* = .05.

(hepatocellular carcinoma 33% [n = 1], sepsis 33% [n = 1], and acute respiratory distress syndrome 33% [n = 1]), and liver transplantation occurred in 13.6% (n = 3). Infections at time of inclusion were seen in 9% of patients (urinary tract [n = 2]), whereas 18.2% (n = 4) (spontaneous bacterial peritonitis 50% [n = 2], hospital-acquired pneumonia 25% [n = 1], sepsis of unknown origin 25% [n = 1]) of patients with cirrhosis developed infections within 15 months after inclusion. Of note, one patient presented with infection both at time of inclusion and after inclusion. In the second cohort, 1-year mortality in patients with cirrhosis was 27.2% (hepatocellular carcinoma [n = 3]).

Table 2. Clinical Characteristics of Patients With Cirrhosis, Pathologic Controls, and Histologically Normal Controls

Variables, median (range)	Cirrhosis (n = 11)		Pathologic controls (n = 3)		Histologically normal controls (n = 12)	
Age (y)	67 (48–84)		71 (64–78)		61 (28–78)	
Sex (m:f)	6:5		2:1		5:10	
Underlying liver disease(s) [%]	ALD HCV AIH Unknown	6 [54] 3 [27] 1 [9] 1 [9]	NA Echinococcosis	2 [66] 1 [33]	NA Echinococcosis	11 [79] 3 [21]
Death (1 year)	3 [27]		0 [0]		0 [0]	
Child-Pugh	A (n = 9), B (n = 2)		NA		NA	
MELD	8 (6–15)		NA		NA	
BMI (kg/m^2)	27 (22–34)		23 (21–25)		23 (20–27)	
Na^+ (mmol/L)	139 (132–143)		140 (140–141)		140 (136–141)	
K^+ (mmol/L)	4 (3.7–4.7)		4.1 (3.8–4.4)		4.3 (3.8–4.5)	
Ca^{2+} (mmol/L)	2.4 (2.1–2.6)		2.4 (2.3–2.5)		2.4 (2.2–2.5)	
Creatinin (μmol/L)	63 (48–132)		79 (61–100)		58 (42–89)	
GFR	92 (51–109)		80 (62–92)		97 (66–127)	
Urea (mmol/L)	5.7 (2.6–10.7)		6.5 (4.6–8.6)		4.9 (3.8–8.4)	
Bilirubin (μmol/L)	10.5 (4.6–19.6)[a]		5.8 (4.1–8.8)		5.1 (2.5–14.2)	
AST (U/L)	33 (20–83)		30 (24–36)		31 (18–52)	
ALT (U/L)	26 (13–48)		24 (14–29)		30 (16–56)	
GGT (U/L)	70 (18–796)		316 (31–858)		57 (16–576)	
AP (U/L)	80 (51–557)		221 (67–487)		95 (44–354)	
INR	1.1 (1.0–1.9)[a]		1 (0.9–1.0)		1 (0.9–1.1)	
Albumin (g/L)	38 (25–43)		37 (35–42)		36 (29–41)	
Hemoglobin (g/L)	134 (88–143)		126 (107–155)		119 (104–152)	
Hematocrit (%)	40 (28–43)		36 (31–43)		36 (31–43)	
Leukocytes (G/L)	5.65 (2.74–8.67)		6.00 (4.98–7.05)		5.4 (3.1–12.0)	
Erythrocytes (G/L)	4.3 (3.03–5.17)		4.14 (3.67–5.01)		4.12 (3.64–4.98)	
Neutrophils (G/L)	2.66 (1.97–6.23)		3.94 (2.91–4.91)		3.26 (2.02–4.85)	
Eosinophils (G/L)	0.07 (0.05–0.16)		0.14 (0.03–0.32)		0.12 (0.04–0.59)	
Basophils (G/L)	0.02 (0.01–0.03)		0.03 (0.02–0.04)		0.02 (0.01–0.08)	
Lymphocytes (G/L)	1.02 (0.48–1.85)		1.42 (1.2–1.65)		1.46 (1.05–2.37)	
Monocytes (G/L)	0.31 (0.19–0.61)		0.40 (0.30–0.46)		0.33 (0.22–1)	
Platelets (G/L)	137 (99–279)[a]		238 (211–264)		242 (100–363)	
CRP (mg/L)	3.4 (0.5–74.2)		7.3 (0.5–10.8)		2.35 (0.7–13.2)	

NOTE. Data indicated as median with (minimum and maximum) and [percentage].
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GFR, glomerular filtration rate; GGT, gamma-glutamyl transferase; HCV, hepatitis C virus; INR, international normalized ratio; MELD, Model for End-Stage Liver Disease.
[a]P = .05 indicates comparison between cirrhosis and histologically normal controls by Mann-Whitney U test.

AXL Is Expressed on the Majority of Resident Human Liver Macrophages

Using immunofluorescence microscopy, we investigated AXL expression on hepatocytes, resident macrophages ($CD68^+$, Figure 1*A*), recently infiltrated macrophages ($MAC387^+$,[21] Figure 1*B*), endothelial cells ($CD31/CD34/vWF^+$, Figure 1*C*), and activated HSCs (aHSCs) (alpha smooth muscle actin [α-SMA^+],[22] Figure 1*D*). We hereby identified AXL expression on the majority of resident macrophages in normal liver tissue but not on hepatocytes or other non-parenchymal cells. Recently infiltrated macrophages were sparse in healthy liver tissue and barely expressed AXL (Figure 1). Co-expression of the TAM receptors Mertk and Axl had been described on freshly isolated murine KCs.[17] In our study on human liver tissue, we did not observe AXL and MERTK co-localization (Figure 2*A*).

AXL Expression on Macrophages Is Reduced in Patients With Advanced Cirrhosis and Portal Hypertension

Having observed AXL expression on most resident macrophages in healthy livers, we assessed AXL expression

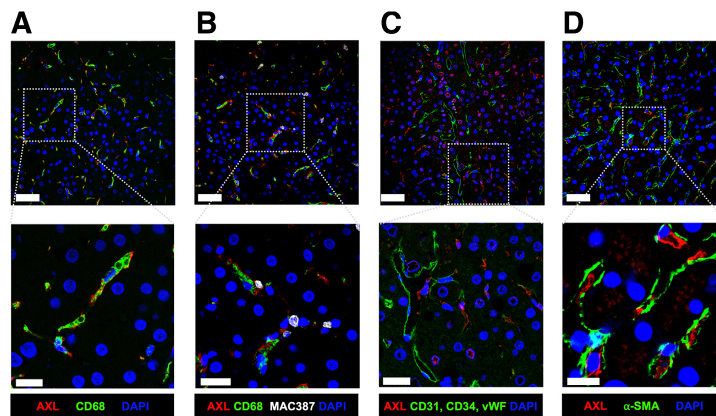


Figure 1. AXL is expressed on resident liver macrophages. Representative micrographs from immunofluorescence stains of liver biopsies from the control group (n = 4). (*A*) AXL/CD68/DAPI stain showing AXL expression on resident liver macrophages. (*B*) AXL/CD68/MAC387/DAPI stain showing absence of AXL expression on liver-infiltrating macrophages. (*C*) AXL/CD31+CD34+vWF/DAPI stain showing absence of AXL expression on LSECs. (*D*) AXL/α-SMA/DAPI stain of a control liver biopsy showing absence of AXL expression on aHSCs. Upper panels: original magnification, 400×; scale bar = 50 μm; lower panels: details, scale bar = 20 μm. AF594, *red*; AF488, *green*; AF647, *white*; DAPI, *blue*.

at different disease stages of cirrhosis (Figure 2*A*). Although $CD68^+$ macrophages did not differ significantly across different stages of cirrhosis, the number of AXL-expressing $CD68^+$ resident liver macrophages per high-power field (HPF) was significantly reduced in advanced stages of cirrhosis, as was the percentage of AXL^+ cells relative to the $CD68^+$ macrophage population (Figure 2*B*). Similarly, AXL-expressing macrophages were reduced in non-cirrhotic portal hypertension (NCPH) (Figure 2*A* and *B*). As shown previously,[18,19] MERTK-expressing cells were increased in patients with advanced cirrhosis compared with controls (Figure 2*A*).

AXL expression on liver macrophages correlated negatively with disease severity scores (Child-Pugh and Model of End-Stage Liver Disease [MELD] scores) and a marker of inflammation (C-reactive protein) (Figure 2*C*). Furthermore, presence of ascites, hepatic encephalopathy, and infectious complications were associated with low numbers of $CD68^+AXL^+$ cells (Figure 2*C*).

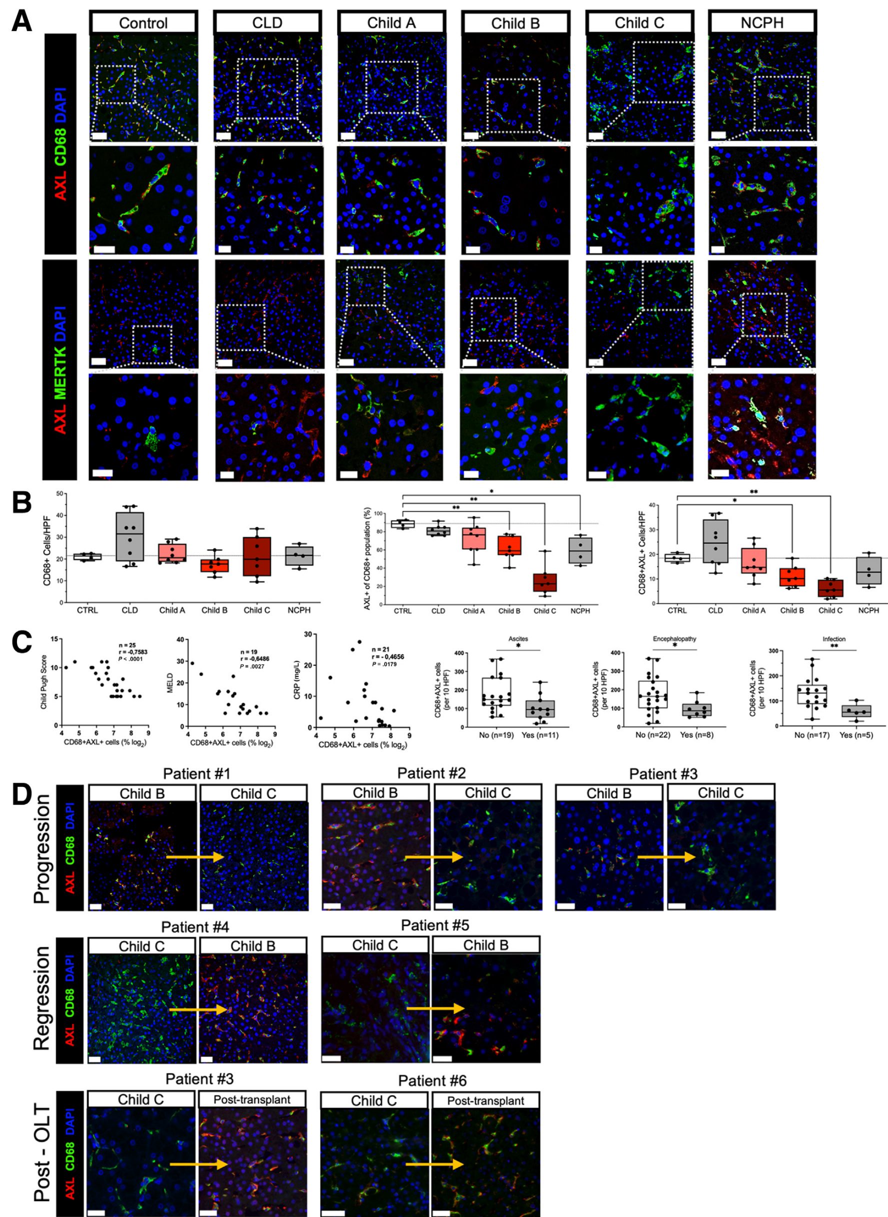
In 6 patients we assessed AXL expression longitudinally. These patients clinically showed either progression from early to advanced stages of cirrhosis and underwent follow-up biopsies or transplantation, respectively, or regression of their initially advanced cirrhosis stage. A different condition studied was resolution of cirrhosis in those patients who underwent transplantation. We found a decrease in AXL^+ liver macrophages in parallel with progression of cirrhosis stage (Figure 2*D*, patients 1–3). Inversely, we observed an increase of AXL^+ macrophages along with cirrhosis regression (Figure 2*D*, patients 4 and 5) or after liver transplantation (Figure 2*D*, patients 3 and 6). Altogether, the data suggest dynamic changes of resident macrophage states during disease evolution.

AXL and MERTK Expression in Areas of Fibrosis

Although we performed quantification of the nonparenchymal cells in hepatic panels, this was challenging to describe in areas of fibrosis because of their significant anatomic variability. Immunofluorescent staining for AXL/MERTK/collagen I/DAPI revealed no expression of AXL in the areas of fibrosis, whereas MERTK-expressing cells seem to approximate collagen I deposition in stages Child-Pugh B and C (Figure 3).

Phenotypic Characterization of the AXL-Expressing Hepatic Macrophages

In addition to immunofluorescence, we used flow cytometry to characterize the phenotype and function of primary human macrophages obtained from liver resections (Figure 4*A*). In line with our microscopic findings, we found significantly fewer AXL-expressing macrophages in cirrhotic livers when compared with controls (Figure 4*B* and *C*). Using FlowSOM, liver macrophages were split into 5 main clusters and annotated on the tree map (Figure 4*D*). High CD68 expression in clusters 2, 3, and 4 indicated resident liver macrophages (Figure 4*D*), whereas low CD68 in clusters 0 and 1, mainly present in cirrhosis samples, indicated infiltrating macrophages (Figure 4*E*). AXL-expressing macrophages were found in most of the clusters, with AXL^{hi} in clusters 2, 3, and 4 (Figure 4*D*). In contrast, MERTK expression was low in all clusters and allocated to cluster 1, confirming that AXL and MERTK were not co-expressed (Figure 4*D*). Also, a decrease of AXL expression was seen in all clusters, whereas MERTK-expressing macrophages increased slightly in cirrhosis cluster 1 (Figure 4*E*).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A
Control
CLD
Child A
Child B
Child C
NCPH
AXL CD68 DAPI
AXL MERTK DAPI
B
CD68+ Cells/HPF
CTRL
CLD
Child A
Child B
Child C
NCPH
AXL+ of CD68+ population (%)
CD68+AXL+ Cells/HPF
C
Child Pugh Score
MELD
CRP (mg/L)
CD68+AXL+ cells (% log2)
Ascites
Encephalopathy
Infection
No (n=19)
Yes (n=11)
No (n=22)
Yes (n=8)
No (n=17)
Yes (n=5)
D
Progression
Regression
Post - OLT
AXL CD68 DAPI
Patient #1
Patient #2
Patient #3
Patient #4
Patient #5
Patient #6
Child B
Child C
Post-transplant

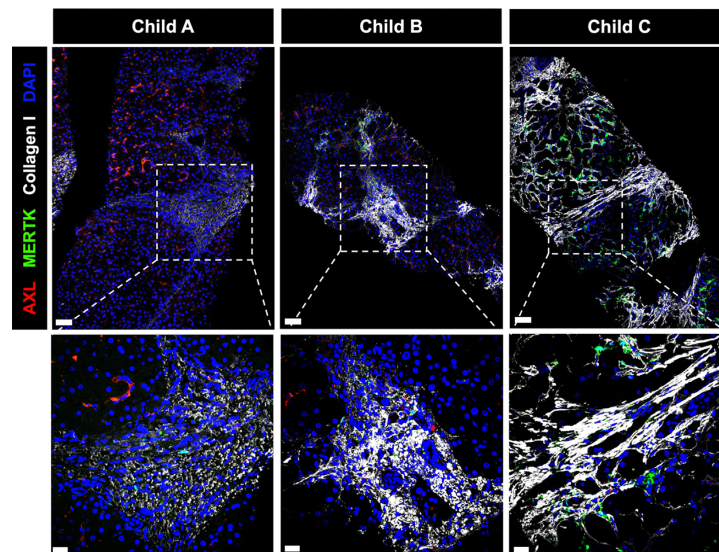

Figure 3. Reduced AXL expression in portal-septal areas. Representative micrographs from immunofluorescence stains of liver biopsies from patients with Child-Pugh A (*left*), Child-Pugh B (*middle*), and Child-Pugh C (*right*) cirrhosis. AXL/MERTK/collagen I/DAPI stain showing AXL and MERTK expression in relation to fibrotic areas; FITC, *green*; AF594, *red*; AF647, *white*; DAPI, *blue*; scale bar in upper panels = 50 μm, scale bar in lower panels = 20 μm.

We reported higher proportions of CXCR4-expressing and lower proportions of CD64- and CCR5-expressing macrophages within the entire resident macrophage population in cirrhosis (Figure 4*F*, upper panel). The expression of CD32, TLR4, and CXCR4 was increased in cirrhosis, whereas the expression of IFNAR, CD206, CCR5, and CCR7 was reduced (Figure 4*F*, lower panel). AXL-expressing macrophages were identified as $CD16^{high}HLA\text{-}DR^{high}$, indicating a mature macrophage population (Figure 4*G*). In addition, AXL-expressing macrophages expressed higher levels of mannose receptor CD206 and TAM co-receptor interferon α/beta-receptor (IFNAR, Figure 4*G*).

AXL-Expressing Hepatic Macrophages Reveal Unaltered Phagocytosis Capacity

Phagocytosis of resident liver macrophages is crucial to maintain tolerance at the barrier between the portal and the systemic circulation. We thus investigated *ex vivo* phagocytosis capacity of resident macrophages from compensated cirrhotic compared with control livers for Gram-negative *Escherichia coli* (*E coli*) and Gram-positive *Staphylococcus aureus* (*S aureus*) particles, respectively (Figure 5*A* and *B*), and revealed a reduced percentage of macrophages able to take up *E coli* in cirrhosis, and it was similar for *S aureus* (Figure 5*B*). AXL expression on macrophages was unaltered on incubation with bacterial bioparticles (Figure 5*C*). Phagocytosis capacity of bacterial bioparticles of $CD68^{+}AXL^{+}$ cells did not differ from $CD68^{+}AXL^{-}$ cells (Figure 5*D*).

Because of the accumulation of AXL-expressing cells in the circulation and lymph nodes in cirrhosis, we hypothesized these cells may have migratory properties. Indeed, cells that underwent either transendothelial or reverse migration displayed higher AXL expression than non-migrated cells (Figure 5*E*).

AXL-Expressing Homeostatic Macrophages Are Reduced in the Gut Mucosa and Peritoneum but Increased in Regional Lymph Nodes in Advanced Cirrhosis

Having described a reduction of AXL expression on liver macrophages and a concurrent accumulation of AXL-expressing circulating monocytes in advanced cirrhosis,[20] we assessed compartment-specific

Figure 2. (***See previous page***). **Loss of AXL-expressing resident liver macrophages with the progression of cirrhosis.** (*A*) Representative immunofluorescence micrographs from AXL/CD68/DAPI and AXL/MERTK/DAPI stains of liver biopsies from the control group (n = 4), chronic liver disease (CLD) (n = 8), Child-Pugh A (n = 8), Child-Pugh B (n = 7), Child-Pugh C (n = 7), and NCPH (n = 4). Upper panels: original magnification, 400×; scale bar = 50 μm; lower panels: details, scale bar = 20 μm. (*B*) Cell count of $CD68^{+}$ resident liver macrophages per HPF, AXL^{+} macrophages per HPF, and percentage of AXL^{+} cells of $CD68^{+}$ population. (*C*) Correlations of AXL^{+} macrophages with Child-Pugh and MELD scores, C-reactive protein, encephalopathy, ascites, and infections. $^{*}P \leq .05$/$^{**}P \leq .01$ (Mann-Whitney tests, Spearman correlation coefficients). (*D*) AXL/CD68/DAPI stain displaying longitudinal AXL expression on resident liver macrophages from patients undergoing either progression or regression or resolution of cirrhosis post transplantation (OLT). AF488, *green*; AF647, *red*; DAPI, *blue*; scale bar = 50 μm.

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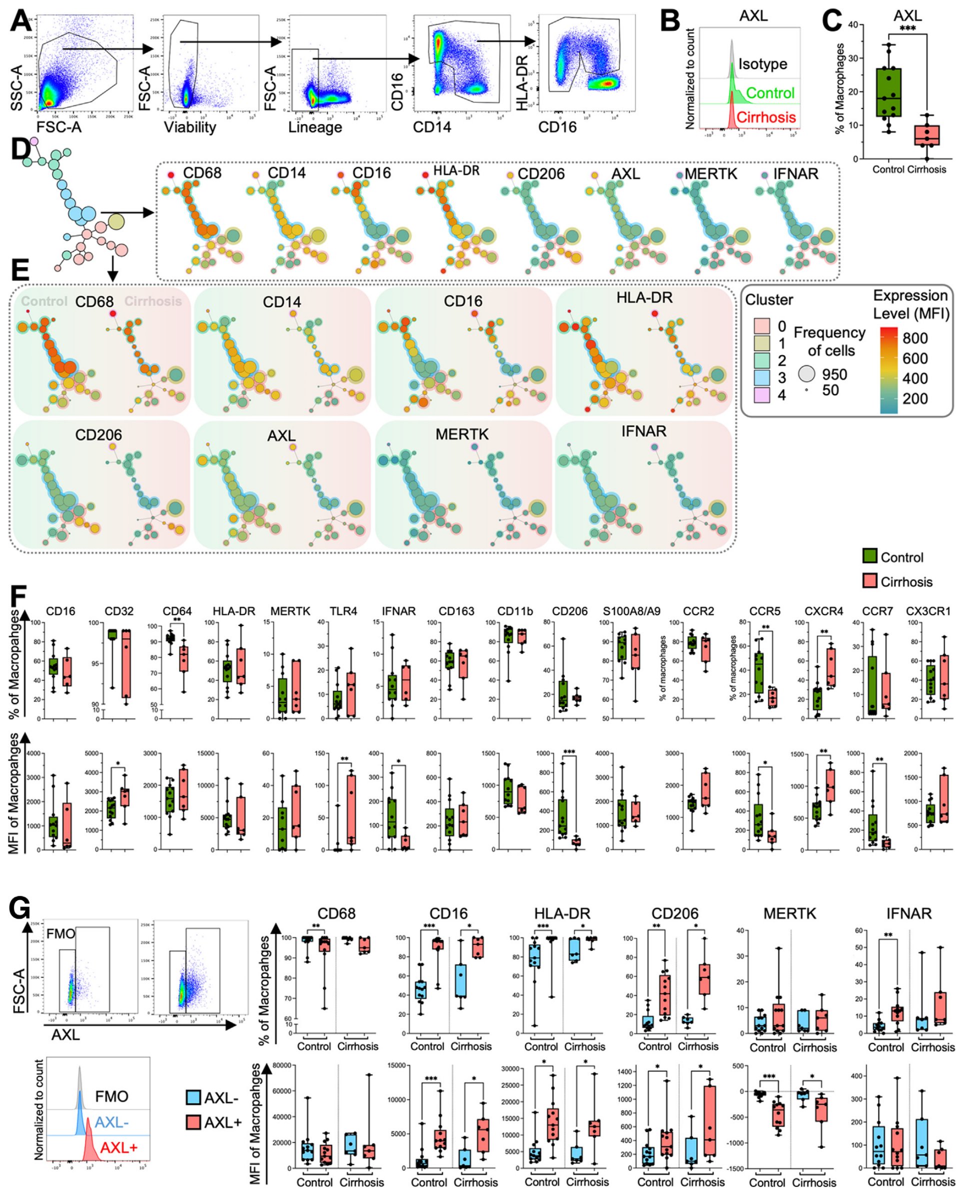


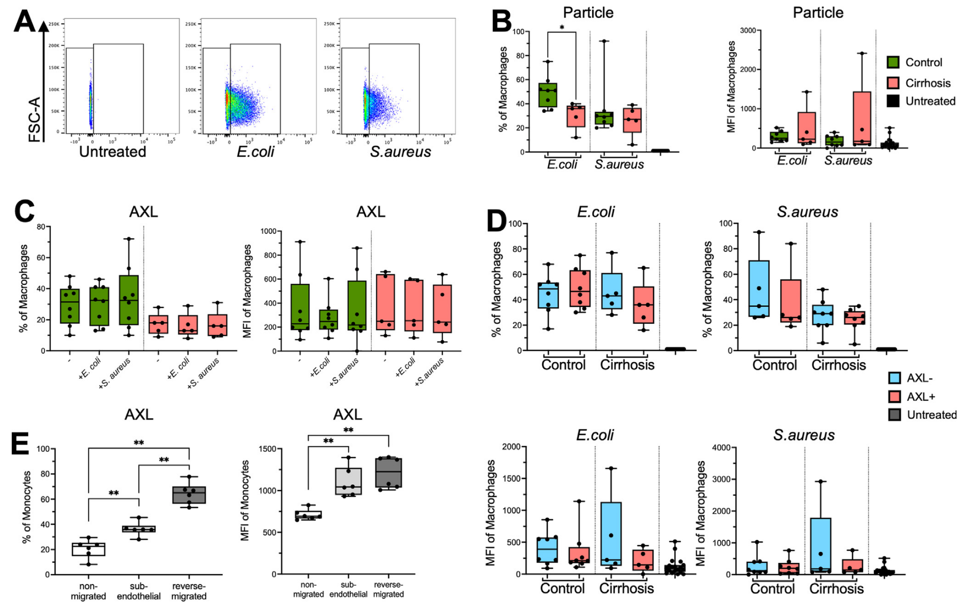


Figure 5. Phagocytosis of bacteria by primary liver macrophages and AXL on migrating monocyte. (*A*) Gating strategy for determination of bioparticle (*E coli*, *S aureus*) positive macrophages from liver resections based on 1% border of untreated control. FSC-A (forward scatter area). (*B*) *E coli* and *S aureus* bioparticle uptake by primary liver macrophages from controls (n = 8) and compensated cirrhosis samples (n = 5); untreated control (n = 8). In percentage and median fluorescence intensity (MFI) of macrophages. (*C*) AXL expression in percentage and MFI on *E coli* and *S aureus* bioparticle uptake on macrophages from controls (n = 8), compensated cirrhosis (n = 5), and untreated controls (n = 8). (*D*) Phagocytosis capacity for *E coli* and *S aureus* bioparticles of AXL^{+}/AXL^{-} liver macrophages in percentage and MFI of macrophages from control (n = 8) and compensated cirrhosis samples (n = 5) and untreated controls (n = 8). (*E*) AXL expression levels as percentage and MFI on magnetically sorted $CD14^{+}$ monocytes from healthy donors in an established migration assay (n = 6). *Box plots* showing median with 10–90 percentile and all points min-max. **P* ≤ .05, ***P* ≤ .01, Wilcoxon, Kruskal-Wallis, and Mann-Whitney test.

occurrence and proportion of AXL-expressing macrophages in the gut, the peritoneum, lymph nodes, and the bone marrow.

On intestinal macrophages from colon biopsies we identified AXL expression on the majority of resident macrophages in healthy gut mucosa, whereas a significant decrease of $CD68^{+}AXL^{+}$ macrophages was found in patients with advanced cirrhosis and similarly in conditions of chronic inflammation unrelated to cirrhosis such as in patients with ulcerative colitis (Figure 6*A* and *B*). Plasma levels

Figure 4. (***See previous page***). **Immunophenotyping by flow cytometry of liver macrophages.** (*A*) Gating strategy with representative flow cytometry scatter plots. Side scatter area (SSC-A), forward scatter area (FSC-A). (*B*) Representative histograms for AXL expression on liver macrophages in control and compensated cirrhosis. (*C*) AXL expression on liver macrophages (%) in controls (n = 13) and compensated cirrhosis (n = 7). *Box plots* showing median with 10–90 percentile and all points min-max. ****P* ≤ .001 (Mann-Whitney test). (*D*) Scaffold reference map of the mixed control (n = 5, 2550 cells/sample) and compensated cirrhosis (n = 5, 2550 cells/sample) macrophages landscape constructed from fluorescence cytometry data displaying 25 unsupervised FlowSOM nodes in 5 clusters with representative fluorescent marker expression in the scaffold map. (*E*) Comparison of control (n = 5, 2550 cells/sample) and compensated cirrhosis (n = 5, 2550 cells/sample) macrophages representative fluorescent marker expression mapped in the scaffold map. (*F*) Immunophenotyping of control and compensated cirrhosis liver macrophages. Expression level as percentage of all macrophages and as median fluorescence intensity (MFI) of all macrophages. (*G*) Gating strategy and histograms for AXL expression used to distinguish AXL-expressing from AXL-negative liver macrophages. Immunophenotyping of AXL^{+} and AXL^{-} liver macrophages in controls (n = 13) and compensated cirrhosis (n = 7). Expression level as percentage (%) and MFI of all macrophages. *Box plots* showing median with 10–90 percentile and all points min-max. **P* ≤ .05/***P* ≤ .01/****P* ≤ .001, Mann-Whitney and Wilcoxon test.

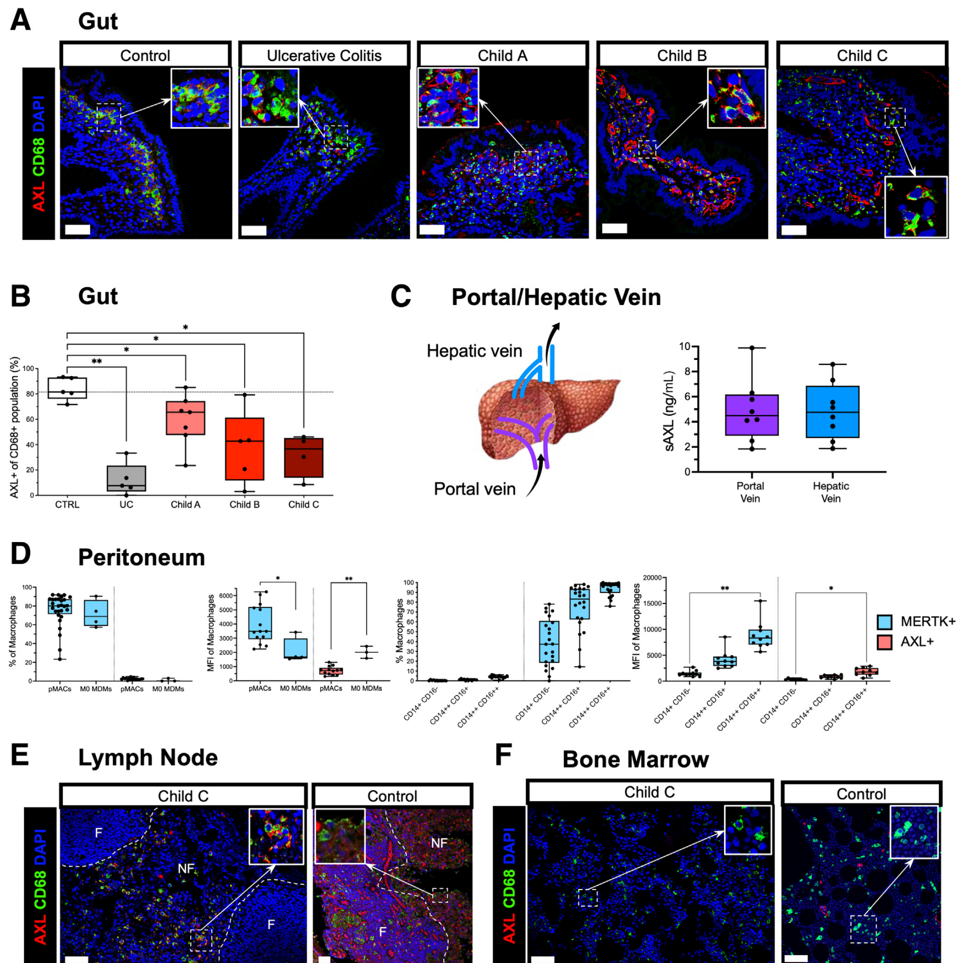


Figure 6. Prevalence of AXL expression on macrophages in gut, peritoneum, bone marrow, and lymph node. (*A*) Representative immunofluorescence micrographs from AXL/CD68/DAPI stains of gut biopsies from control group (n = 4), ulcerative colitis (UC) (n = 5), Child-Pugh A (n = 7), Child-Pugh B (n = 5), and Child-Pugh C (n = 4). (*B*) Percentage of AXL^{+} macrophages lining the epithelial barrier relative to total population per HPF. (*C*) Schematic drawing of liver vessels with blood flow indicated and soluble AXL (sAXL) levels (pg/mL) in blood from portal and hepatic veins (n = 8). (*D*) AXL and MERTK expression of peritoneal macrophages from cirrhosis patients (pMACs, n = 23) in classical ($CD14^{+}CD16^{-}$, class), intermediate ($CD14^{++}CD16^{+}$, inter), and non-classical ($CD14^{++}CD16^{++}$, non-class) subsets and monocyte-derived macrophages (M0-MDMs). (*E*) Representative immunofluorescence micrographs from AXL/CD68/DAPI stains of a lymph node from explant patient (Child-Pugh C) and control in follicular (F) and non-follicular (NF) regions. (*F*) Representative micrograph from AXL/CD68/DAPI stains of bone marrow from explant patient (Child-Pugh C) and control. *Box plots* showing median with 10–90 percentile and all points min-max. *$P \leq .05$/**$P \leq .01$, Mann-Whitney and Kruskal-Wallis test. AF594, *red*; AF488, *green*; AF647, *white*; DAPI, *blue*; scale bar = 50 μm.

of soluble AXL from portal venous and hepatic venous blood in patients undergoing transplantation may indicate a slight tendency of higher soluble AXL levels in the hepatic venous blood, albeit no significant difference (Figure 6*C*).

The pMACs isolated from the ascitic fluid of decompensated cirrhotic patients expressed significantly lower levels of AXL but higher levels of MERTK when compared with monocyte-derived M0 macrophages. MERTK expression by pMACs was lowest in the classical and increased in the intermediate and non-classical subsets, whereas AXL expression was low in all subsets but showed slightly elevated levels in the non-classical subset (Figure 6*D*).

From one patient with advanced cirrhosis (Child-Pugh C) who underwent transplantation, we investigated macrophages in mesenteric lymph nodes and observed the presence of $CD68^+AXL^+$ macrophages accumulating in the non-follicular regions. In control lymph nodes, $CD68^+$ macrophages did not express AXL; however, AXL was detectable on endothelial cells of vessels (Figure 6*E*). Neither bone marrow from the Child-Pugh C patient nor from control displayed AXL expression (Figure 6*F*).

Mechanisms Down-Regulating AXL Expression on Liver Macrophages

Next, we aimed to understand the underlying mechanisms as to how AXL expression on liver macrophages was reduced on the evolution of cirrhosis. It has been shown that GAS6, a ligand with high affinity for AXL, down-regulated AXL expression *in vitro*.[23] Moreover, we previously showed that patients with cirrhosis had elevated plasma GAS6 levels increasing with severity of the disease.[20] Although the cellular origin of GAS6 in the blood of cirrhotic patients is unknown, a rat model of carbon tetrachloride (CCl_4)-induced liver injury showed increased expression of GAS6 by aHSCs.[24] Thus, we hypothesized that GAS6 released by aHSCs in the context of cirrhosis may lead to AXL down-regulation in resident liver macrophages.

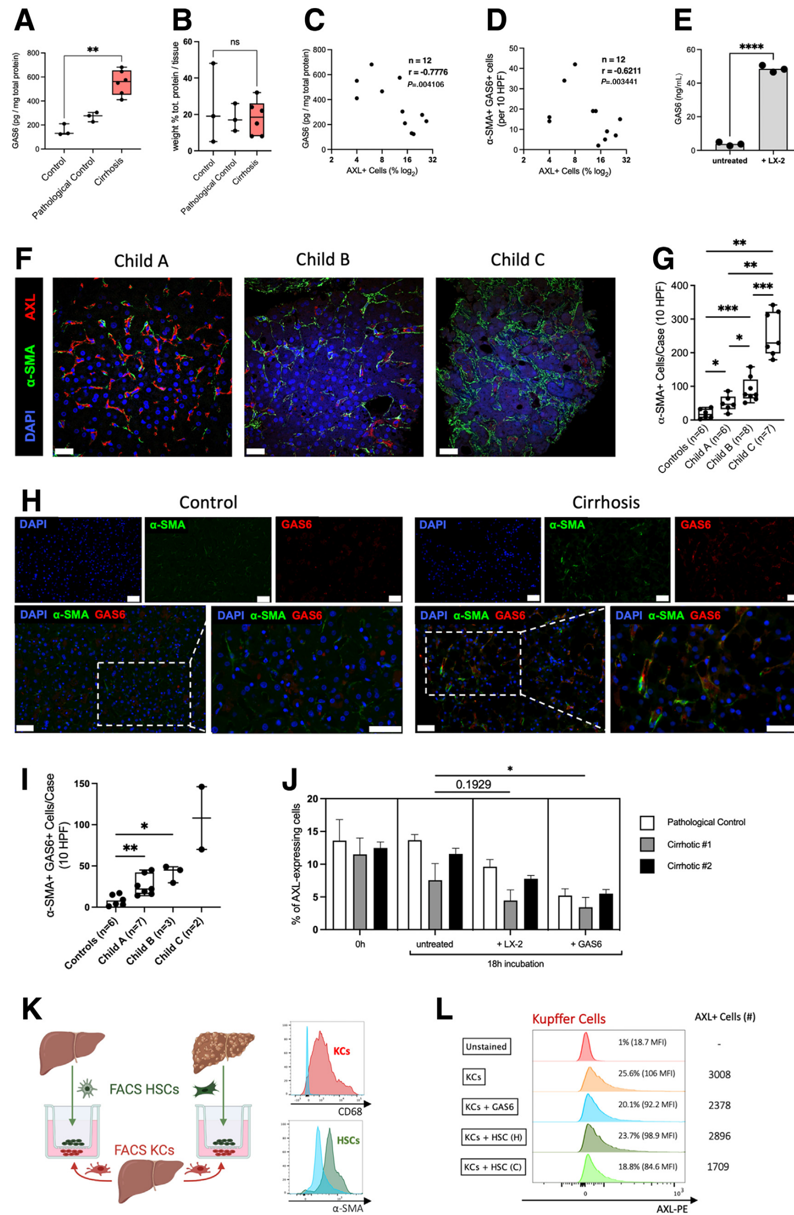
Supporting this hypothesis, we measured elevated GAS6 levels in liver tissue lysates from cirrhotic patients compared with controls (Figure 7*A*), and the percentage of total protein was comparable between control and cirrhotic tissue (Figure 7*B*). Both GAS6 levels in liver tissue and α-SMA^+GAS6^+ cells inversely correlated with AXL expression on liver macrophages isolated from the same tissue (Figure 7*C* and *D*). Moreover, GAS6 levels were higher in the supernatants of macrophages co-cultured with LX-2 cells, a cell line for aHSCs, compared with liver macrophages cultured *ex vivo* alone (Figure 7*E*). We also performed immunofluorescent staining on liver biopsies and liver resection tissue from patients with cirrhosis and controls showing AXL, α-SMA, and GAS6 expression (Figure 7*F* and *H*). We observed significantly increased numbers of α-SMA^+ as well as α-SMA^+GAS6^+ cells across Child-Pugh stages of cirrhosis, suggesting that GAS6 was produced by aHSCs in the context of disease (Figure 7*G* and *I*). This is in line with recently published data showing that the α-SMA^+ area significantly increased in patients with nonalcoholic fatty liver disease and advanced fibrosis (F3) or cirrhosis (F4).[25] For further endorsement, we performed 2 proof-of-principle experiments. Stimulation with GAS6 significantly decreased AXL expression on primary liver macrophages isolated from either 2 patients with cirrhosis or a pathologic control (NCPH) *ex vivo*, and co-culturing with LX-2 cells displayed a trend for reduced AXL expression (Figure 7*J*). To further mimic *in vivo* settings, we co-cultured sorted liver macrophages from a histologically normal liver with sorted HSCs from either a cirrhotic liver or a histologically normal liver. This revealed lower AXL expression and numerically decreased AXL^+ KCs in cirrhotic conditions and on GAS6 stimulation alone (Figure 7*K* and *L*).

Axl-Expressing Macrophages Are Reduced in a Murine CCl_4 Model of Fibrosis

Recurrent CCl_4 treatment is used as a murine model of liver fibrosis.[26] Thus, we aimed to assess Axl expression levels in liver monocytes and macrophages in a CCl_4 model using wild-type mice. Subsequently, we assessed the efficacy and safety of Axl inhibition *in vivo* by administering bemcentinib (a small molecule inhibitor of Axl) in CCl_4-treated mice (Figure 8*A*).

First, to investigate Axl expression in the liver, we isolated hepatic nonparenchymal cells and performed flow cytometry analysis (Figure 8*B*). Although KCs vastly outnumbered other liver monocyte/macrophage subsets in healthy controls, both infiltrating monocytes and monocyte-derived macrophages (MoMF) expanded in the CCl_4 model (Figure 8*C*). In line with our findings in human livers, Axl expression levels on total liver monocytes and macrophages were reduced in the CCl_4 model compared with healthy controls in terms of both percentage and expression values. Interestingly, KCs displayed higher Axl expression compared with both infiltrating monocytes and MoMF (Figure 8*D*). In accordance with our flow cytometry data, immunofluorescence staining showed that more than 90% of the $F4/80^+$ resident macrophages in control murine liver tissue expressed Axl, whereas $F4/80^+Axl^+$ macrophages were significantly decreased in livers from CCl_4-treated mice. Moreover, $F4/80^+Mertk^+$ macrophages increased compared with healthy controls (Figure 8*E* and *F*). Using flow cytometry analysis of blood (Figure 8*G*), we did not observe differences in phenotype of blood monocytes between the CCl_4 model and controls (Figure 8*H*).

Next, we assessed the potential efficacy and safety of bemcentinib administration in our CCl_4 model and performed histopathology analyses on both hematoxylin-eosin and Sirius Red stainings of formalin-fixed and paraffin-embedded (FFPE) liver sections from bemcentinib-treated CCl_4 mice (Figure 8*I*). Although Ishak grade, a scoring system for liver necroinflammation, indicated no change after bemcentinib administration compared with CCl_4 controls, Ishak stage, a scoring system for liver fibrosis, was significantly reduced after bemcentinib administration (Figure 8*J*), which is in accordance with findings in murine nonalcoholic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A
B
C
D
E
F
Child A
Child B
Child C
AXL
α-SMA
DAPI
G
H
Control
Cirrhosis
DAPI
α-SMA
GAS6
I
J
Pathological Control
Cirrhotic #1
Cirrhotic #2
18h incubation
K
FACS HSCs
FACS KCs
KCs
HSCs
CD68
α-SMA
L
Kupffer Cells
AXL+ Cells (#)
Unstained
KCs
KCs + GAS6
KCs + HSC (H)
KCs + HSC (C)
AXL-PE

steatohepatitis.[27] Importantly, plasma levels of bilirubin and albumin as well as plasma levels of alanine transaminase were unvaried after administration of bemcentinib in the CCl_4 model, underlining its safety relating to the liver for therapeutic use (Figure 8*K*). Bemcentinib administration had no effect on frequency of liver monocyte/macrophage subsets compared with CCl_4 controls (Figure 8*L*). Moreover, Axl and Mertk on both liver monocytes/macrophages and blood monocytes were unvaried after therapeutic immunomodulation with bemcentinib (Figure 8*M*).

Discussion

Previously we identified the emergence of circulating immune-modulatory monocytes expressing AXL in parallel with disease progression of cirrhosis that dampened immune responses to microbial challenge.[20,27] The role of AXL expression on tissue macrophages in the liver and associated compartments such as gut and peritoneum in the context of immune-homeostasis regulation in health and cirrhosis as a multisystem disorder remained unknown.

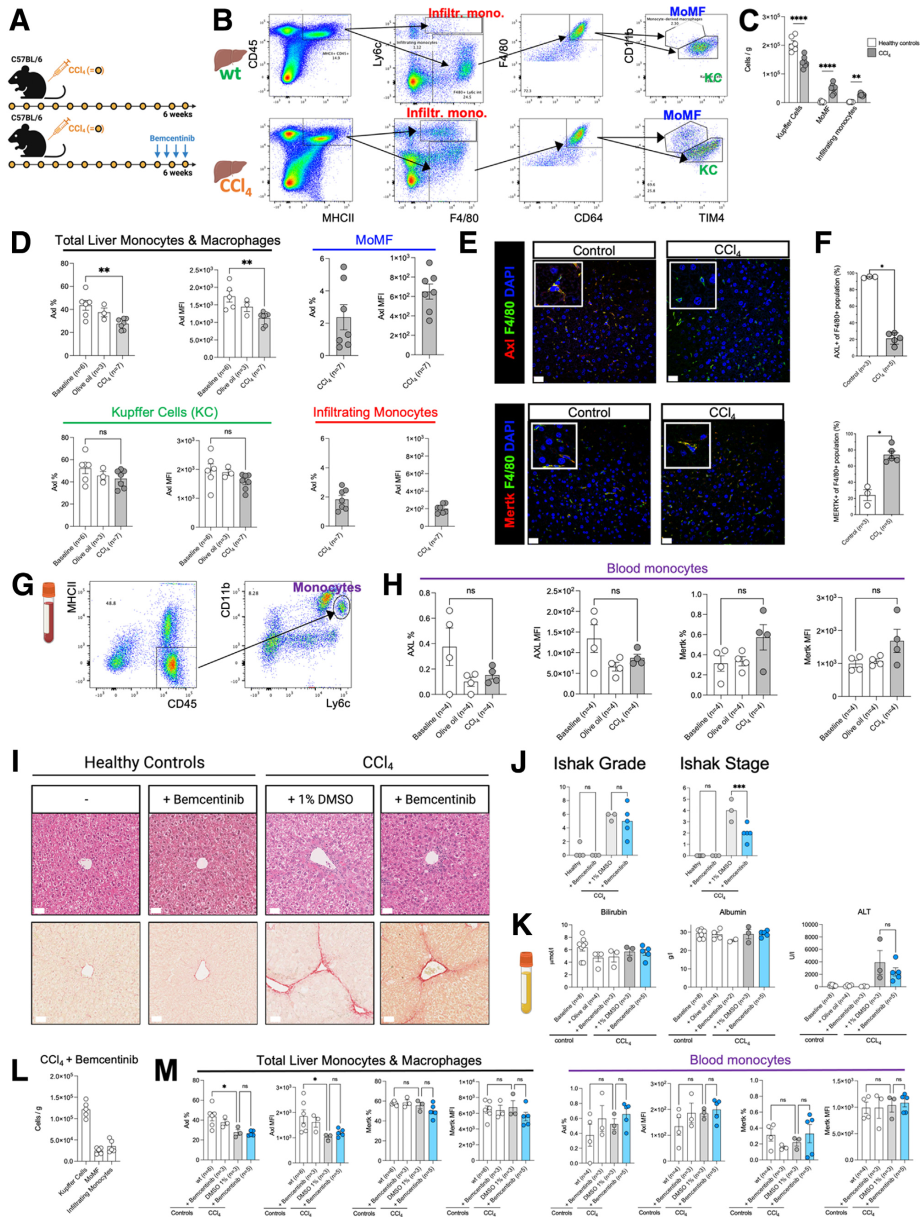
In this study we newly identified the ubiquitous expression of AXL on resident liver macrophages in healthy livers, whereas endothelial cells, HSCs, and hepatocytes did not express AXL. In cirrhosis, AXL expression on KCs was significantly reduced in association with disease severity and infectious complications. In patients with cirrhosis, we also observed a reduced expression of AXL on gut and peritoneal macrophages, whereas AXL expression was elevated on macrophages in mesenteric lymph nodes. Because of these distinct expression patterns, we hypothesize that regulatory mechanisms of AXL expression are presumably disease- and compartment-specific. As a mechanism, we showed previously that AXL expression on monocytes was enhanced in response to microbial products and efferocytosis,[20] which may explain their abundance in the circulation in the context of cirrhosis. Data from this study hint at a mechanism involving GAS6, produced by aHSCs in the cirrhotic liver, potentially down-regulating AXL on human liver macrophages.

Phenotypically, in line with our previous findings on monocytes,[20] AXL-expressing macrophages were characterized $CD16^{high}HLA\text{-}DR^{high}$, indicating a mature macrophage. In addition, higher expression levels of the mannose receptor CD206 were detected, which is known to identify tissue macrophages and for its essential function in phagocytosis and immune homeostasis by scavenging adverse mannoglycoproteins.[28]

Functionally, phagocytosis capacity for pathogens was unchanged between AXL^+ and AXL^- macrophages, similarly to our recently published data, where we observed AXL-expressing circulating monocytes characterized by preserved phagocytosis of pathogens and enhanced efferocytosis capacity.[20] Nevertheless, liver macrophages in cirrhosis patients presented reduced phagocytosis capacity of Gram-negative bacteria unrelated to AXL. Because of the heterogeneity, there may be other macrophage subsets responsible for this observation. Also in the literature, TAM receptors have been described to be dispensable in the phagocytosis and killing of bacteria.[29]

In cirrhosis, we observed a significant decrease in AXL-expressing resident liver macrophages in parallel with disease severity. AXL levels on resident liver macrophages were similarly decreased in patients with NCPH, suggesting potential involvement of mechanisms eliciting portal hypertension. In line with these findings on protein level, within open available databases of a recently published study using unbiased large-scale techniques, transcriptomes of single liver cells revealed higher AXL expression on KCs of uninjured controls compared with cirrhotic human livers,[26] which is similar to the results from a dataset of single-cell transcriptomes of KCs from a murine nonalcoholic fatty liver disease/nonalcoholic steatohepatitis model.[30] Sequential assessment of individual patients, in whom the clinical cirrhosis stage progressed or regressed, demonstrated the relevance of our trans-sectional data. We revealed in exemplary cases that AXL-expressing macrophages undergo a dynamic process depending on disease evolution. Because it is rarely clinically indicated to take a biopsy in patients with biopsy-proven cirrhosis who clinically improved while cirrhosis persisted, trans-sectional biopsy data for cirrhosis regression were highly limited. Hence, further studies are necessary to investigate the dynamics of AXL expression on

Figure 7. (***See previous page***). **Mechanism of AXL loss on resident liver macrophages.** (*A*) GAS6 levels in liver tissue lysates and (*B*) protein percentage of liver tissue lysates assessed by bicinchoninic acid assay (BCA) from controls (n = 3), pathologic control (n = 3), and compensated cirrhosis liver resections (n = 6). (*C*) GAS6 levels in liver tissue in correlation with AXL expression on liver macrophages determined by flow cytometry (n = 12, Spearman r correlation). (*D*) α-SMA^+GAS6^+ cell numbers (immunofluorescence) in correlation with AXL expression on liver macrophages (flow cytometry) (n = 12, Spearman r correlation). (*E*) GAS6 levels in supernatants after 18 hours of liver macrophage cultures or co-cultures of liver macrophages and LX-2 cells (n = 3). (*F*) Representative immunofluorescence micrographs from α-SMA/AXL/DAPI stains of either liver resections from histologically normal livers (n = 6) or liver biopsies from patients with cirrhosis (Child-Pugh A, n = 6; Child-Pugh B, n = 8; Child-Pugh C, n = 7). AF594, *red*; AF488, *green*; DAPI, *blue*; original magnification, 400×; scale bar = 20 μm. (*G*) Cell count of α-SMA^+ cells per 10 HPF in healthy controls and patients with cirrhosis. (*H*) Representative immunofluorescence micrographs from α-SMA/GAS6/DAPI stains of liver resections from histologically normal livers (n = 6), as well as liver biopsies and liver resections from patients with cirrhosis (Child-Pugh A, n = 7; Child-Pugh B, n = 3; Child-Pugh C, n = 2). AF647, *red*; AF488, *green*; DAPI, *blue*. Upper panels: original magnification, 400×; scale bar = 50 μm; lower panels: details, scale bar = 50 μm. (*I*) Cell count of α-SMA^+ $GAS6^+$ cells per 10 HPF in healthy controls and patients with cirrhosis. (*J*) AXL expression change in liver macrophages on either co-culture with LX-2 cells or GAS6 treatment (n = 3, technical triplicates); bar plots with standard deviation error, nested *t* tests. (*K*) Experimental setup for co-culture of healthy KCs with either healthy HSCs or cirrhotic HSCs in a transwell system and confirmatory flow cytometry analysis of sorted cells. (*L*) Flow cytometry analysis of either $CD68^+$ KCs or α-SMA^+ HSCs after co-culture experiment depicted in *G* (n = 1). *Box plots* showing median with 10–90 percentile and all points min-max. $^*P \leq .05$/$^{**}P \leq .01$, Mann-Whitney and Kruskal-Wallis test.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A
B
C
D
Total Liver Monocytes & Macrophages
MoMF
E
F
Kupffer Cells (KC)
Infiltrating Monocytes
G
Monocytes
H
Blood monocytes
I
Healthy Controls
CCl4
+ Bemcentinib
+ 1% DMSO
J
Ishak Grade
Ishak Stage
K
L
CCl4 + Bemcentinib
M
Total Liver Monocytes & Macrophages
Blood monocytes

KCs during cirrhosis evolution. We also studied AXL on KCs of patients with advanced cirrhosis who underwent transplantation and observed an increase of AXL expression after transplantation. Even though liver transplantation does not represent regression of the disease, interestingly it has been shown that recipient leukocytes rapidly repopulated the transplanted liver and were reprogrammed toward $CD68^+$/ $CD206^+$ liver macrophages, whereas only a small residual population of donor KCs persisted.[31,32]

In line with previous data from our group,[18] we observed few MERTK-expressing macrophages in healthy liver tissue. Instead, their accumulation occurred in cirrhotic livers in relation to disease severity and inversely to the reduction of AXL expression. Moreover, AXL and MERTK did not co-localize on human macrophages. We therefore hypothesize a distinct regulation of AXL and MERTK on liver macrophages as suggested in previous studies on bone marrow–derived macrophages and DCs: MERTK was expressed in response to tolerogenic and AXL in response to inflammatory stimuli.[11] Under homeostatic conditions in mice in turn, Axl and Mertk co-expression has previously been observed on murine KCs[17] and on murine lung airway macrophages.[12]

KCs represent ∼20% of all liver cells and ∼80% of body's tissue macrophages.[33] They are pivotal sinusoidal cells maintaining hepatic immune homeostasis and tolerance due to their ability to clear pathogens and their products derived from the intestine.[34] Balmer et al[1] proposed that the liver may act as a vascular firewall of the systemic circulation facilitating phagocytosis of commensal organisms derived from the intestine. TAM receptors have been generally described to maintain homeostasis and promote phagocytic removal of apoptotic cells (efferocytosis) during resolution phases of inflammation.[9,10] In the liver, TAM receptor triple knockout mice developed spontaneous chronic hepatic inflammation.[35] DCs of triple knockout mice showed a hyper-responsiveness to endotoxins and impaired efferocytosis.[9] In acute liver injury models, more severe parenchymal damage was observed in $Axl^{-/-}$ compared with $Mertk^{-/-}$ mice; nonetheless, Mertk was required for efferocytosis of apoptotic cells.[17] Besides being involved in immune homeostasis, MERTK and AXL were also associated with deleterious processes in some liver diseases.[36] In mouse models of nonalcoholic steatohepatitis[27] and fibrosis,[17,37] Axl signaling was associated with increased inflammation[27] and fibrogenesis[17,27,37], whereas Mertk signaling was involved in protection of hepatocytes against lipotoxicity.[27] The respective effects of AXL and MERTK thus depend on expressing cell types and compartments, disease stage, and the degree of expression. Axl-expressing macrophages have been particularly implicated in immunologic barrier functions in various compartments. Axl was expressed under homeostatic conditions on murine airway but not interstitial lung macrophages. Axl-expressing airway macrophages expanded after influenza infection, thereby preventing excessive tissue inflammation through efferocytosis.[12] In the central nervous system, Axl was highly expressed on microglia in the context of neurodegeneration[13] and autoimmune encephalomyelitis[14] and associated with efferocytosis of myelin debris. In the joint, murine tissue-resident synovial lining CX_3CR1^+ macrophages expressed a high level of Axl in their transcriptional profiling and formed an internal immunologic barrier as a protective shield for intra-articular structures.[15] Of note, we did not detect AXL expression on bone marrow macrophages, highlighting their suggested role in microbial defense in physiological conditions.

In cirrhosis with emerging portal hypertension, the load of translocated bacteria and bacterial products from the gut to the systemic circulation is enhanced, which is termed as pathologic bacterial translocation.[38,39] Here, we question whether decreased AXL expression on macrophages in the cirrhotic liver may relate to reduced immune tolerance and barrier protection in liver tissue and enhanced liver inflammation in the context of elevated load of translocated microbes and pathogen-associated molecular patterns in advanced cirrhosis. On the other hand, the concurrent accumulation of AXL-expressing monocytes in mesenteric lymph nodes and in the circulation[20] may possibly favor a state of systemic tolerance, i.e.e, immuneparesis in cirrhosis patients. The following arguments may support this hypothesis. AXL expression on innate immune cells has been allocated to barrier compartments as described above. Moreover, the function of AXL-expressing cells (including

Figure 8. (***See previous page***). **Efficacy and safety of Axl inhibition in a carbon tetrachloride-induced liver fibrosis model.** (*A*) Overview of carbon tetrachloride (CCl_4) model involving biweekly administration of CCl_4 (intraperitoneal 0.4 mL/kg) for 6 weeks and therapeutic immunomodulation with bemcentinib for 1 week every other day (intraperitoneal 100 mg/kg). (*B*) Flow cytometry gating strategies for liver cell subsets including infiltrating monocytes, monocyte-derived macrophages (MoMF), and Kupffer cells (KCs) in a wild-type (wt) and CCl_4 mouse model. (*C*) Monocyte/macrophage cell numbers per gram of liver tissue in healthy controls and CCl_4 model. (*D*) Flow cytometry analysis of Axl expression levels displayed as either % or median fluorescence intensity (MFI) on liver cell subsets identified in *B*. (*E*) Representative micrographs from either Axl/F4/80/ DAPI stains or Mertk/F4/80/DAPI stains of mouse livers from healthy controls and CCl_4-treated animals. (*F*) Percentage of either Axl-expressing macrophages or Mertk-expressing macrophages relative to total population (mean per mouse). (*G*) Flow cytometry gating strategy for blood monocytes. (*H*) Flow cytometry analysis of either Axl or Mertk expression levels displayed as % or MFI on blood monocytes identified in G. (*I*) Representative images of hematoxylin-eosin stainings (above) and Sirius Red stainings (below) of FFPE liver sections from either healthy controls or CCl_4-treated mice with or without bemcentinib treatment. (*J*) Liver necroinflammation score (Ishak grade) and liver fibrosis score (Ishak stage) based on histopathology analysis of H&E and Sirius Red stainings. (*K*) Plasma levels of bilirubin, albumin, and alanine aminotransferase (ALT) assessed with a clinical chemistry analyzer. (*L*) Cells per gram of liver tissue from CCl_4-treated mice after administration of bemcentinib. (*M*) Axl and Mertk (% and MFI values) on either liver monocytes/macrophages or blood monocytes assessed with flow cytometry analysis. ns = not significant/*$P < .05$/**$P < .01$, unpaired *t* tests and one-way ordinary analysis of variance. AF594, *red*; AF488, *green*; DAPI, *blue*; scale bar = 50 μm.

enhanced efferocytosis, preserved phagocytosis, reduced cytokine production, and reduced T-cell activation as reported previously[20]) reflects an innate immune state at the interface of exposure to potential pathogens and tolerance to commensal microbes. Finally, there are the mechanisms up-regulating AXL expression upon efferocytosis, phagocytosis, and stimulation with microbial products (TLR agonists).[20]

We also confirmed that murine resident liver macrophages showed reduced Axl expression on CCl_4-induced fibrosis. However, it must be considered that although CCl_4 induces fibrosis and models using CCl_4 + TLR ligands such as lipopolysaccharide may mimic inflammatory events occurring on top of fibrosis, murine models reflecting the advanced stage of cirrhosis as a multisystemic disease do not exist. Reduction in Axl in CCl_4-induced fibrosis was mainly caused by reduction of KCs and substitution by Axl^{low} MoMF and Axl^{low} infiltrating monocytes. As expected, Axl on circulating mouse monocytes in this stage of fibrosis did not differ from controls, considering our previous data[20] describing an expansion of AXL-expressing circulating monocytes in patients with cirrhosis. Our finding partially corroborates previously published murine data (C57/BL6 background), where Axl was expressed on hepatic KCs but also on endothelial cells.[17] In their CCl_4 model, hepatic Axl up-regulation was observed, whereby Axl-expressing cell types have been detailed by immunofluorescence without quantification only, whereas we used immunofluorescence and flow cytometry to quantify AXL on KCs.[17]

The idea of using a selective Axl inhibitor (bemcentinib) in a CCl_4 model was to evaluate the hepatic and systemic effects of AXL inhibition in the context of fibrosis. We recently described the occurrence of AXL-expressing circulating monocytes in cirrhosis characterized by impaired inflammatory responses, thereby favoring immuneparesis, which were reversed by bemcentinib *ex vivo*.[20] At certain stages of cirrhosis, the effect of AXL inhibition might be desirable in the systemic circulation but not necessarily in the liver, gut, and presumably other tissues because liver and gut macrophages revealed reduced AXL expression in advanced cirrhosis. Nevertheless, in our CCl_4 model, bemcentinib did not enhance hepatic inflammation and also reduced fibrosis, as previously shown by other groups through its inactivating effect on HSCs[37] and prevention of liver fibrosis/inflammation in early nonalcoholic steatohepatitis.[27] This may imply first evidence that AXL inhibitors may be safely used and further evaluated in patients with cirrhosis. How AXL inhibition might eventually find therapeutic application in humans with regard to its particular compartmental expression profiles in health and disease conditions needs to be carefully dissected in future murine *in vivo* studies to investigate distinct target and off-target effects.

In extrahepatic sites, we discovered AXL expression on resident macrophages in healthy gut tissue, whereas it was lower in patients with cirrhosis and ulcerative colitis, supporting our hypothesis of AXL being a crucial player in maintaining immune homeostasis by providing defense against microbes not only in the liver. In agreement with this, high expression of genes associated with phagocytosis, such as *Mertk, Gas6,* and *Axl,* has been described in murine intestinal macrophages.[40] On the other hand, activated intestinal macrophages in patients with cirrhosis have been associated with an increased intestinal permeability enhancing the translocation of pathogens by releasing interleukin-6 and nitric oxide.[41] In summary, in chronic inflammatory conditions, the invasion of pathogens from the intestine might be favored by reduced AXL expression on macrophages, thereby promoting disease progression and inflammation.

Similarly, we observed low AXL but increased MERTK expression on peritoneal macrophages of decompensated cirrhosis patients with ascites. Generally, little is known about healthy human pMACs because of limited sample availability. Murine pMACs have been shown to strongly express Mertk[12,42] (and no Axl[12]), which mediated apoptotic cell engulfment.[42] It remains unknown whether pMACs express AXL under physiological or disease conditions other than cirrhosis.

Although translocated bacterial products as well as pathogen and apoptotic cell uptake lead to an increase of AXL expression on circulating monocytes in patients with advanced cirrhosis,[20] it remained unknown which mechanisms underlie the down-regulation on macrophages. Because of the enhanced migratory potential of AXL-expressing monocytic cells and their enhancement in other compartments in the condition of advanced cirrhosis, it is conceivable that AXL-expressing cells may egress the liver and migrate towards regional lymph nodes and/or the systemic circulation. Our data do not support the assumption that in cirrhosis AXL^+ macrophages may be transformed towards $MERTK^+$ macrophages, because we did not see that loss of AXL would lead to an up-regulation of MERTK on primary human macrophages *ex vivo* (data not shown). Moreover, a transformation of AXL- into MERTK-expressing cells has to our knowledge not yet been described in the literature.[9–11] Rather, shown by proof-of-principle experiments here, we think that enhanced GAS6 production by aHSCs in the cirrhotic liver may down-regulate AXL expression on primary liver macrophages. As a limiting factor, however, it must be considered that the relevant experiments could only be conducted with small sample sizes because of the requirement of highly limited resources such as primary HSCs and KCs from patients with and without cirrhosis, respectively, undergoing liver resections. GAS6 has been shown to down-regulate AXL in prostate cancer cells,[23] GAS6 production by aHSCs has previously been described in the context of fibrosis,[24] and its deficiency reduced fibrosis in mice.[37] GAS6 is also known to be expressed in chronic inflammatory conditions,[43] and GAS6 deficiency has been shown to prevent liver inflammation and fibrosis, potentially by restoring AXL on KCs.[44] Because of the dynamic changes of AXL expression demonstrated by our longitudinal data, inactivation, senescence, and apoptosis of aHSCs[45] might lower levels of GAS6, thereby permitting AXL re-expression on macrophages. In summary, the reduction of AXL expression on liver macrophages on the evolution of cirrhosis presumably involves

different mechanisms, potentially including GAS6-mediated down-regulation of AXL and migration of AXL-expressing monocytic cells to extrahepatic compartments. To what extent these mechanisms interact in reducing AXL expression requires further investigation such as genetic manipulation of these pathways in animal models or the use of high-dimensional analyses. Moreover, we did not investigate the mechanism underlying AXL down-regulation on intestinal macrophages. The role of AXL expression on gut macrophages in intestinal inflammation and cirrhosis is subject of ongoing studies.

In conclusion, we identified AXL as a constitutively expressed tyrosine kinase on resident liver macrophages known to maintain liver tolerance. AXL expression on macrophages was lost in the process of fibrosis progression and portal hypertension in cirrhosis and implicated infectious complications. The mechanism may involve GAS6 production by aHSCs, a process deserving future evaluation in relation to potential immune-modulatory and anti-fibrotic therapies involving the GAS6-AXL axis.

Materials and Methods

Patients and Sampling

A cohort of 33 patients with cirrhosis was identified at the University Hospital Basel and the Cantonal Hospital St. Gallen, Switzerland between January 2016 and April 2021 and 29 patients with cirrhosis and ascites at King's College and St. Mary's Hospitals, London, United Kingdom, between January 2014 and August 2018. Patients were recruited and categorized according to Child-Pugh scores (Child-Pugh A [biopsy n = 8, resection n = 9], B [biopsy n = 7, resection n = 2], and C [biopsy n = 7]). We further included control subjects such as healthy (biopsy n = 4), patients with NCPH due to nodular regenerative hyperplasia (biopsy n = 4) or chronic liver disease without cirrhosis (biopsy n = 8). All subjects provided written informed consent. Histologically normal non-lesional, cirrhotic, and NCPH liver tissues for immunohistochemistry were obtained from liver biopsy or resection. Histologically normal non-lesional and cirrhotic liver tissues for macrophage isolation were obtained from patients undergoing surgical liver resection for solitary colorectal metastasis and echinococcosis (control n = 14) or hepatocellular carcinoma (cirrhosis n = 11). Exclusion criteria were age younger than 18 years, immunosuppressive therapy, and human immunodeficiency virus infection. The study had been approved by the local ethics committees (EKSG 15/074/EKNZ 2015-308, BASEC-ID 2019-00816, BASEC-ID 2019-02118; UK: 12/LO/0167) and recorded in the clinical trial register ClinicalTrials.gov (identifier: NCT04116242) and Swiss National Clinical Trials Portal (SNCTP000003482).

Clinical, Hematologic, and Biochemical Parameters

Full blood count, liver and renal function tests, and clinical variables were entered prospectively into a database. The following disease severity scores were calculated, Child–Pugh and MELD, and infections were documented.

Fluorescent Immunohistochemistry and Confocal Microscopy

Tissue sections, fluorescent immunohistochemistry staining, and confocal microscopy were undertaken as previously described.[18] Tissue samples were FFPE. The 4-μm serial sections were cut and placed on slides. Single and multiplexed immunofluorescence on FFPE tissue was performed on serial sections to assess CD68, AXL, MERTK, MAC387, CD31, CD34, vWF, α-SMA, GAS6, collagen I, and F4/80 expression.

For CD68 and AXL, sections were incubated for 18 hours at 4°C with a goat anti-human AXL antibody (R&D Systems, catalogue number AF154, lot number DMG0618111, dilution 1:200) in 1× phosphate-buffered saline (PBS), followed by 1-hour incubation at room temperature with an Alexa Fluor 594 donkey anti-goat immunoglobulin (Ig) G (H+L) antibody (Invitrogen, catalogue number A11058, lot number 1842799, dilution 1:400) in 1× PBS. Slides were then incubated again for 18 hours at 4°C with a monoclonal mouse anti-human CD68 antibody (Agilent, clone PG-M1, catalogue number M086, lot number 20029531, dilution 1:100), followed by 1-hour incubation at room temperature with an Alexa Fluor 488 donkey anti-mouse Ig G (H+L) antibody (Jackson Immunoresearch, catalogue number 715-545-150, dilution1:400) in 1× PBS. Slides were then counterstained with DAPI and mounted using fluorescence mounting medium (Dako, catalogue number S3023).

For AXL, MERTK, and collagen I, sections were incubated for 18 hours at 4°C with a goat anti-human AXL antibody (R&D Systems, catalogue number AF154, lot number DMG0618111, dilution 1:250) in 1× PBS, followed by 1-hour incubation at room temperature with Alexa Fluor 594 donkey anti-goat Ig G (H+L) antibody (Invitrogen, catalogue number A11058, lot number 1842799, dilution 1:400) in 1× PBS. This was followed by another incubation for 18 hours at 4°C with a monoclonal rabbit anti-human MERTK antibody (Abcam, clone Y323, catalogue number ab52968, lot number 1829926, dilution 1:200) and 1-hour incubation at room temperature with fluorescein goat anti-rabbit Ig G (H+L) antibody (Invitrogen, catalogue number F2765, lot number 1829926, dilution1:400) in 1× PBS. A third overnight incubation with a monoclonal mouse anti-human collagen I antibody (Abcam, catalogue number ab88147, clone 3G3, dilution 1:300) labeled with Alexa Fluor 647 using a labeling kit from Invitrogen (catalogue number A20186) was followed by counterstaining with DAPI.

For CD68, AXL, and MAC387, sections were incubated for 18 hours at 4°C with goat anti-human AXL antibody (R&D Systems, catalogue number AF154, lot number DMG0618111, dilution 1:200) in 1× PBS, followed by 1-hour incubation at room temperature with Alexa Fluor 594 donkey anti-goat Ig G (H+L) antibody (Invitrogen, catalogue number A11058, lot number 1842799, dilution 1:400) in 1× PBS. Slides were then incubated again for 18 hours at 4°C with monoclonal mouse anti-human CD68 antibody (Agilent, clone PG-M1, catalogue number M086, lot number 20029531, dilution 1:100), followed by 1-hour incubation at room temperature with Alexa Fluor 488 donkey

anti-mouse Ig G (H+L) antibody (Jackson Immunoresearch, catalogue number 715-545-150, dilution1:400) in 1× PBS. A third overnight incubation with monoclonal mouse anti-human S100A9 + calprotectin (S100A8/A9 complex) antibody (Abcam, catalogue number ab22506, lot number GR309896-4, clone MAC387, dilution 1:300) labeled with Alexa Fluor 647 using a labeling kit from Invitrogen (catalogue number A20186) was followed by counterstaining with DAPI.

For AXL and CD31/CD34/vWF, sections were incubated for 18 hours at 4°C with a cocktail of primary antibodies: goat anti-human AXL antibody (R&D Systems, catalogue number AF154, lot number DMG0618111, dilution 1:200), monoclonal mouse anti-human CD31 (Agilent, catalogue number M0823, clone JC70A, lot number 20049471, dilution 1:50), monoclonal mouse anti-human CD34 Class II (Agilent, catalogue number M7165, clone QBEnd 10, lot number 20055214, dilution 1:50), and mouse anti-human vWF (Invitrogen, catalogue number MA5-14029, clone F8/86, lot number TF2582851, dilution 1:20) in 1× PBS, followed by 1-hour incubation at room temperature with a cocktail of secondary antibodies: Alexa Fluor 594 donkey anti-goat IgG (H+L) (Invitrogen, catalogue number A11058, lot number 1842799, dilution 1:400) and Alexa Fluor 488 donkey anti-mouse IgG (H+L) antibody (Jackson Immunoresearch, catalogue number 715-545-150, dilution 1:400) in 1× PBS. Slides were counterstained with DAPI.

For AXL and α-SMA, sections were incubated for 18 hours at 4°C with goat anti-human AXL antibody (R&D Systems, catalogue number AF154, lot number DMG0618111, dilution 1:200) in 1× PBS, followed by 1-hour incubation at room temperature with Alexa Fluor 594 donkey anti-goat IgG (H+L) antibody (Invitrogen, catalogue number A11058, lot number 1842799, dilution 1:400) in 1× PBS. Slides were then incubated again for 18 hours at 4°C with monoclonal mouse anti-human smooth muscle actin antibody (Agilent, catalogue number M0851, clone 1A4, lot number 20049711, dilution 1:150), followed by 1-hour incubation at room temperature with a fluorescein goat anti-mouse IgG (H+L) antibody (Invitrogen, catalogue number F2761, lot number 1820001, dilution 1:400) in 1× PBS. Slides were counterstained with DAPI.

For AXL and F4/80, sections were incubated for 18 hours at 4°C with a cocktail of antibodies: goat anti-mouse AXL antibody (R&D Systems, catalogue number AF854, lot number CTC0218071, dilution 1:20) and monoclonal Alexa Fluor 647 rat anti-mouse F4/80 antibody (BioLegend, catalogue number 123122, clone BM8, dilution 1:50) in 1× PBS. Sections were then incubated for 1 hour at room temperature with Alexa Fluor 594 donkey anti-goat IgG (H+L) antibody (Invitrogen, catalogue number A11058, lot number 1842799, dilution 1:400) in 1× PBS. Slides were counterstained with DAPI.

For Mertk and F4/80, sections were incubated for 18 hours at 4°C with a cocktail of antibodies: goat anti-mouse Mertk antibody (R&D Systems, catalogue number AF591, lot number DGS0517091, dilution 1:20) and monoclonal Alexa Fluor 647 rat anti-mouse F4/80 antibody (BioLegend, catalogue number 123122, clone BM8, dilution 1:50) in 1× PBS. Sections were then incubated for 1 hour at room temperature with Alexa Fluor 594 donkey anti-goat IgG (H+L) antibody (Invitrogen, catalogue number A11058, lot number 1842799, dilution 1:400) in 1× PBS. Slides were counterstained with DAPI.

Image Acquisition and Analysis

All micrographs for fluorescent images were acquired using an LSM980 confocal microscope with Airyscan 2 (Zeiss, DE). For the quantitative morphometry, 10 HPF (400× magnification) were acquired for each slide, and the number of positive cells per HPF was assessed for the following combinations of markers: CD68$^+$, F4/80$^+$, CD68$^+$AXL$^+$, AXL$^+$MERTK$^+$, MAC387$^+$AXL$^+$, CD31$^+$CD34$^+$vWF$^+$AXL$^+$, α-SMA$^+$AXL$^+$, F4/80$^+$Axl$^+$, and F4/80$^+$Mertk$^+$.

Human Macrophage Isolation

Small resection samples (<5 cm × 5 cm × 1 cm) were mechanically disrupted using a filter mesh,[46] whereas large resection samples were processed using a Stomacher 400 circulator (Seward).[19]

Flow Cytometry-Based Phenotyping of Hepatic Macrophages

Phenotyping of hepatic macrophages isolated from liver resection specimen was undertaken using flow cytometry (LSRFortessa Cell Analyzer, BD Biosciences) as previously described.[18] Flow cytometry antibodies were purchased from the indicated companies below. Flow cytometry data were analyzed using FlowJo software (V.10.7.1, Becton Dickinson & Company) including the clustering and visualization technique of FlowSOM.[47]

Antibody information is as follows: BD Biosciences: CD14-PE-Cy7 (557742), CD16-BV650 (563692), CD64-FITC (555527), CD163-PE (556018), CD11b-PE (555388), isotype controls Mouse IgG1 κ BV650 (563231), Mouse IgG2a κ Pe-Cy7 (557907); R&D Systems: AXL-AF488 (FAB154G), MERTK-APC (FAB8912A), IFNAR-PE (FAB245P), S100A8/A9-AF488 (IC9337G); BioLegend: HLA-DR-PerCP-Cy5.5 (307630), TLR4-APC (312816), CD206-BV421 (321126), CCR2-BV510 (357218), CCR5-BV421 (359118), CCR7-BV421 (353208), CX3CR1-BV510 (341621), CXCR4-BV510 (306536), CD68-BV785 (333826), CD3-APC-Fire750 (300470), CD19-APC-Fire750 (302258), CD56-APC-Fire750 (362554), Mouse IgG2b κ BV785 (400355), Mouse IgG2a κ PerCP-Cy5.5 (400252), Mouse IgG1 κ APC-Fire750 (400196); and eBioscience: CD32-FITC (11-0329-42), Fixable Viability Dye eFluor 455UV (65-0868-14) for cell viability, and FOXP3/Transcription Factor Staining Buffer Set (00-5523-00) for intracellular staining.

Flow Cytometry–Based Phagocytosis Assay in Hepatic Macrophages

Cells were incubated with pHrodo *E coli* (P35361) or *S aureus* (A10010) Red BioParticles (Phagocytosis Kit for Flow Cytometry from Invitrogen/Thermo Fisher Scientific) according to the manufacturer's protocol. At least 100,000

cells in 100 μL FACS Buffer supplemented with 10% human AB serum were incubated with 5 μL of BioParticles for 30 minutes at 37°C. Cells were fixed and stained with antibodies.

Flow Cytometry–Based Phenotyping of Peritoneal Macrophages

The pMACs from ascites were obtained from ascitic tap fluid by centrifugation. The pMACs were stained after isolation by centrifugation (500*g*, 5minutes) and stained for AXL, MERTK, CD14, CD16, and HLA-DR for flow cytometry as previously described.[18]

In Vitro Co-Culture Experiments of Hepatic Macrophages With LX-2 Cells, Primary Hepatic Stellate Cells, or Treatment With GAS6

Hepatic macrophages were isolated from liver resection specimens, plated for 18 hours in RPMI 1640 medium (10% fetal bovine serum, 1% Pen/Strep), and either co-cultured with LX-2 cells (an aHSCs cell line[48]; gift of Prof Dr Scott Friedman, Mount Sinai School of Medicine) at a ratio of 1:1 in a transwell system (cellQART, cat. #9320402) or treated with GAS6 (500 ng/mL) (885-GSB R&D Systems). In addition, hepatic macrophages were isolated with fluorescence-activated cell sorting from liver resection specimens and co-cultured in a transwell system with either sorted aHSCs from histologically normal liver tissue or sorted aHSCs from cirrhotic tissue. Subsequently, cells were harvested and phenotypically characterized by surface and intracellular marker expression levels via flow cytometry.

Protein Extraction From Snap-Frozen Liver Tissues

Snap-frozen tissue samples from resection specimens were lysed using metallic beads and radio-immunoprecipitation lysis buffer system (ChemCruz, cat. Sc-24948). Samples were lysed in the tissue lyser (Qiagen) for 5 minutes at 25*g*. Supernatants underwent 3 thawing/freezing cycles before centrifugation (20,800*g*, 20 minutes).

Total Protein, GAS6, Soluble AXL Level Assessment

Total protein levels in liver tissue lysates from resection specimens were assessed with a bicinchoninic acid assay. Briefly, colorimetric detection of bicinchoninic acid-Cu1+ complexes was detected at 562 nm using a microplate reader (BioTek Synergy H1), indicating total protein concentrations. GAS6 levels in liver tissue lysates from resection specimens and in supernatant from *in vitro* experiments were assessed using a GAS6 ELISA kit (BMS2291, ThermoFisher Scientific). Soluble AXL levels in serum samples were measured using a sAXL ELISA kit (ab99976, Abcam).

Migration Assay

Transendothelial migration of monocytes across stimulated endothelium was assessed using an established[49] *in vitro* migration assay and performed according to a previously published protocol.[18]

Carbon Tetrachloride–Induced Liver Fibrosis Model

Wild-type C57BL/6 8- to 10-week-old male mice (Charles River, UK) were administered 0.4 mL/kg CCl_4 (Sigma-Aldrich), diluted in olive oil (Sigma-Aldrich) at a 1:3 ratio. Mice were injected intraperitoneally twice a week for total of 6 weeks (n = 13 intraperitoneal CCl_4 injections). In addition, a group of wild-type mice were administered bemcentinib (Selleckchem, cat. #S2841) intraperitoneally at 100 mg/kg dose 4 times over the last week before death. Twenty-four hours after the last CCl_4 injection, mice were killed, livers were perfused with PBS, and liver tissue was FFPE for fluorescent immunohistochemistry analysis. All animal experimental protocols were approved by Imperial College London in accordance with UK Home Office regulations (PPL numbers: 70/7578 and P8999BD42).

Flow Cytometry–Based Phenotyping of Murine Liver Monocytes/Macrophages and Blood Monocytes

Mouse hepatic non-parenchymal cells were isolated as previously described,[50] and liver monocytes and macrophages were phenotypically characterized by flow cytometry. Cells were incubated for 30 minutes at 4°C with a viability dye (Zombie Live Dead, #423101, BioLegend) and the following flow cytometry antibodies: BioLegend: F4/80-BV421 (cat. #123137), CD45-BV650 (cat. #103151), CD11b-BV711 (cat. #101242), CD64-PerCP-Cy5.5 (cat. #139308), Ly6c-PE-Cy7 (cat. #128018); eBioscience: Tim4-AF488 (cat. #53-5866-82), Mertk-AF700 (cat. #56-5751-82), MHCII-APC-eFluor780 (cat. #47-5321), and Axl-PE (R&D cat. #FAB8541P). Blood monocytes were phenotypically characterized with flow cytometry using the same antibodies.

Statistical Analysis

Data are expressed as the median/interquartile range unless otherwise specified. For data that did not follow a normal distribution, the significance of differences was tested using the Mann–Whitney or Wilcoxon tests; Spearman correlation coefficients were calculated. Graphs were drawn using Prism 9.3.0 (GraphPad, La Jolla, CA).

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4.2 Circulating Monocytes Upregulate CD52 and Sustain Innate Immune Function in Cirrhosis unless Acute Decompensation Emerges

Circulating monocytes upregulate CD52 and sustain innate immune function in cirrhosis unless acute decompensation emerges

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Data Availability Statement: The single-cell RNA sequencing data generated and analyzed in this study have been submitted to the Gene Expression Omnibus (GEO) under the accession number GSE263100. The data are currently in a "private" status. Raw sequencing data are considered sensitive and thus cannot provided on public data repositories. Upon request to the authors, a data access agreement can be signed between involved institutions to share these raw data on a case-by-case basis.

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61 ## Abstract
62 **Background & Aims:** Infectious complications determine the prognosis of cirrhosis patients.
63 Their infection susceptibility relates to the development of immuneparesis, a complex
64 interplay of different immunosuppressive cells and soluble factors. Mechanisms underlying
65 the dynamics of immuneparesis of innate immunity remain inconclusive. We aimed to dissect
66 the heterogeneity of circulating monocyte states in different cirrhosis stages, and pursued the
67 function of selected differentially expressed genes.
68 **Methods:** We systematically investigated circulating monocytes in health, compensated and
69 not-acutely decompensated (NAD) cirrhosis using single cell RNA sequencing. Selective genes
70 were confirmed by flow cytometry and diverse functional assays on monocytes *ex vivo*.
71 **Results:** We partitioned monocytes into seven clusters. Their abundances varied between
72 cirrhosis stages, confirming previously reported changes i.e. reduction in $CD14^{low}CD16^{++}$ and
73 emergence of M-MDSC in advanced stages. DE genes between health and disease and among
74 stages were detected, including for the first time CD52. CD52-expression on monocytes
75 significantly enhanced throughout compensated and NAD cirrhosis. Heretofore the biological
76 significance of CD52-expression on monocytes remained unknown.
77 $CD52^{high}CD14^{+}CD16^{high}HLA\text{-}DR^{high}$ monocytes in patients with cirrhosis revealed a functional
78 phenotype of active phagocytes with enhanced migratory potential, increased cytokine
79 production, but poor T cell activation. Following acute decompensation (AD), CD52 was
80 cleaved by elevated phospholipase C (PLC), and soluble CD52 (sCD52) was detected in the
81 circulation. Inhibition and cleavage of CD52 significantly suppressed monocyte functions *ex*
82 *vivo* and *in vitro*, and the predominance of immunosuppressive $CD52^{low}$ circulating monocytes
83 in patients with AD was associated with infection and low transplant-free survival.
84 **Conclusion:** CD52 may represent a biologically relevant target for future immunotherapy.
85 Stabilising CD52 may enhance monocyte functions and infection control in the context of
86 cirrhosis, guided by sCD52/PLC as biomarkers indicating immuneparesis.
87
88 **Highlights**:
89 - scRNA-seq partitioned monocytes into 7 distinct clusters, varying between
90 compensated and decompensated cirrhosis stages
91 - Transcriptome analysis revealed changes in inflammatory and immune-related genes
92 and pathways in relation to stages
93 - Activated $CD52^{high}$ circulating monocytes increased in compensated and NAD
94 cirrhosis in correlation with survival
95 - CD52-expression on monocytes and sCD52 may serve as biomarkers for innate
96 immune function in cirrhosis
97 - PLC induces sCD52 cleavage from circulating monocytes in AD/ACLF, deteriorating
98 innate immune function and prognosis
99
100 **Impact and Implications:**
101 Monocyte dysfunction substantially contributes to infection susceptibility which determines
102 the prognosis of cirrhosis patients, and represents a critical condition with unmet therapeutic
103 needs. Its underlying mechanisms remain poorly understood, although among hepatologists
104 a better understanding of the monocyte pathophysiology in relation to cirrhosis stages, and
105 a therapeutic reconstitution of monocyte function are believed to enhance defence against
106 infection and thus reduce morbidity and mortality of patients with cirrhosis in the future. By
107 systematically delineating the heterogeneity and function of circulating monocytes ex vivo,

108 we identified that the absence of CD52 expression on monocytes represented a distinct
109 biomarker of monocyte dysfunction in patients with cirrhosis, discriminating patients at
110 substantial risk for infectious complications. Otherwise, given the beneficial antimicrobial
111 functions of CD52-expressing monocytes, CD52-stablisation may also represent such a target
112 for immunotherapy worth exploring, at it has been desired in clinical practise.
113

114 ## Introduction

115 Liver Cirrhosis is a systemic inflammatory condition initiated by chronic liver injury and
116 scarring, destruction of the distinct liver vasculature, loss of hepatic function and
117 consequently significant systemic metabolic and immunological changes[1, 2]. Onset and
118 recurrence of bacterial infections in patients with cirrhosis relate to significant morbidity and
119 mortality being the most common factors precipitating decompensation, hospitalisation and
120 significantly increase the risk of death[3]. The sequence of pathophysiological events remains
121 incompletely understood. Particularly, understanding the onset and evolution of systemic
122 immuneparesis in relation to different stages, i.e. compensated and not-acutely
123 decompensated cirrhosis (NAD), acute decompensation (AD) and liver failure (ACLF), may
124 enable future immunotherapy to prevent deterioration and bridge to regeneration[4] or
125 transplantation.
126 Immuneparesis in cirrhosis involves an attenuated function of diverse innate and adaptive
127 immune cells, and soluble factors in multiple compartments[5]. Along with disease
128 progression, circulating monocytes from patients with cirrhosis lose their regular
129 differentiation and function. In AD/ACLF stages immunosuppressive states i.e. low HLA-DR-
130 expressing monocytic myeloid derived suppressor cells (M-MDSC) and MERTK-expressing
131 monocytes prevail[6, 7]. Yet, M-MDSC already occur in earlier compensated stages as well as
132 immune-modulatory AXL-expressing monocytes[5, 8].
133 CD52 is a small glycosylphosphatidylinositol-anchored protein, expressed on all leukocytes,
134 but mostly described on T cells, limiting T cell activation [9]. A monoclonal antibody against
135 CD52 (alemtuzumab) is in clinical use for the treatment of haematologic malignancies and
136 multiple sclerosis and depletes lymphocytes by cell-mediated cytotoxicity[10], aiming at
137 beneficial reconstruction of the immune system. The function of CD52-expression on
138 monocytes remains ill-defined. Soluble CD52 (sCD52) suppressed inflammatory cytokine
139 responses by monocytes, CD52 deletion exacerbated inflammation[11]. CD52 upregulation
140 and co-localisation with its receptor SIGLEC-10 was shown on monocytes and quiescent stem-
141 like cells in leukaemia[12]. Of note, CD52-expression on lymphocytes is a prognostic
142 biomarker for sepsis indicating survival[13].
143 In this work, we hypothesised that heretofore unrecognised monocyte states may modulate
144 immune responses in cirrhosis, and thus we pursued a systematic unbiased approach to
145 identify states of monocyte differentiation in relation to disease stages.
146

5

147 ## Material and Methods
148
149 ### Patients and Sampling
150 A cohort of 157 patients was identified at the University Hospital Basel and the Cantonal
151 Hospital St.Gallen, Switzerland, from June 2019 until September 2024. Patients with biopsy-
152 proven cirrhosis were recruited during outpatient consultation or hospitalisation for
153 decompensation (Table S1) or liver-resection (Table S2), respectively. Cirrhosis patients were
154 categorised according to D'Amico[14] into compensated[n=71], NAD[n=43] and
155 AD/ACLF[n=19]. We also included healthy controls (HC,[n=41]). Resection tissue samples
156 were categorised into cirrhosis (n=9) and morphologically-normal controls (n=15). In total 188
157 samples (including follow-ups) from 157 donors were collected (Table S3). Exclusion criteria
158 were age under 18 years and HIV infection. Blood samples were obtained for *ex vivo* analyses
159 of monocyte differentiation and function, plasma/serum, and peripheral blood mononuclear
160 cells (PBMC) were stored. Liver tissue specimens were obtained from liver resection surgery
161 for *ex vivo* analysis of liver macrophage differentiation and function. Patients were followed
162 up for 1 year for adverse events. The study was approved by the local ethics committees
163 (EKSG_15/074/EKNZ_2015-308, BASEC-ID 2019-00816, BASEC-ID 2019-02118, BASEC-ID
164 2022-01370/2023-02318) and recorded at ClinicalTrials.gov (NCT04116242, NCT06092385).
165
166 ### Clinical, Haematologic, and Biochemical Parameters
167 Full blood count, liver and renal function tests, and clinical variables were obtained by the
168 clinician and entered into a database.
169
170 ### Cell isolation
171 Monocytes and T cells were isolated by negative selection[8]. Human hepatic macrophages
172 were isolated as previously described[15].
173
174 ### Single cell RNA sequencing methods
175 14 blood samples from healthy donors (n=5), compensated cirrhosis (n=4) and NAD (n=5)
176 patients were processed for scRNA-seq, across 8 batches from July 2019 to August 2021.
177 Monocytes isolated by magnetic cell separation (MACS) were stained for viability and
178 monocyte markers and sorted using BD FACSMelody Cell Sorter. The dataset was analyzed by
179 the Bioinformatics Core Facility, Department of Biomedicine, University of Basel
180 (Supplementary Methods).
181
182 ### Flow Cytometry-based Phenotyping
183 Phenotyping of peripheral blood monocytes, neutrophils, lymphocytes, PBMC and hepatic
184 macrophages was undertaken using flow cytometry (BD-LSR-Fortessa-Cell-Analyzer) as
185 previously described[6]. Monocytes from HC expressing $CD52^{low}$ (Median: 32.4%/348.5MFI)
186 (<40%/<600MFI CD52-expressing monocytes) and monocytes from patients with cirrhosis
187 expressing $CD52^{high}$ (Median: 75.80%/72.65%, 644MFI/496MFI) (>60%/>600MFI CD52-
188 expressing monocytes) were identified and processed for phagocytosis capacity, mixed-
189 lymphocyte-reaction, adhesion and migration assays, *in vitro* inhibition with alemtuzumab,
190 phospholipase C (PLC) incubation and plasma conditioning.
191

192 *In vitro* assessment of monocyte function
193 Phagocytosis capacity, cytokine responses of monocytes upon Toll-like receptor (TLR)
194 stimulation and T cell proliferation activation was measured using flow cytometry as
195 previously described[7, 8]. Monocyte adhesion and CD52 inhibition was assessed as
196 previously described[16]. For plasma conditioning $CD52^{low}$ and $CD52^{high}$ monocytes were
197 incubated with plasma as previously described[7]. Monocyte incubation with PLC was
198 assessed as previously described[17].
199
200 Migration Assay in Static Conditions through Human Liver-derived Endothelial Cells
201 $1x10^6$ Human liver-derived endothelial cells (HLEC) were plated on 30%-collagen-coated
202 transwell inserts. At confluency $2x10^6$ monocytes were plated and migrated monocytes were
203 assessed after 24h by flow cytometry[18].
204
205 Generation of THP-1 Cell Line Stably Expressing CD52
206 pLV[Exp]-CMV>hCD52[NM_001803.3]-vector, psPAX2 and pMD2.G were used for lentivirus
207 production[19]. After sorting (BD FACSMelody), 60% of THP-1-hCD52 cells expressed hCD52,
208 while the CD52-expression level of non-transduced counterparts was 0%.
209
210 ELISA
211 sCD52 and PLC were measured using ELISA in plasma and supernatants according to the
212 manufacturers protocol.
213
214 Fluorescent Immunohistochemistry (IHC)
215 Tissue sections, fluorescent IHC staining and microscopy were undertaken as previously
216 described[20]. FFPE liver tissue samples were stained to assess CD52 and CD68 expression.
217
218 Statistical Analysis
219 Statistics and graphs were performed in Prism. Data are expressed as median/interquartile
220 range unless otherwise specified. For non-normally distributed data, the significance of
221 differences was tested using the Mann-Whitney-, Wilcoxon-, Kruskal-Wallis- or Friedman-
222 tests; Spearman correlation coefficients were calculated and $p<0.05$ values were considered
223 statistically significant. Data were presented as Column or box plots showing median with 10-
224 90 percentile and all points. *$p<0.05$/**$p<0.01$/***$p<0.001$/****$p<0.0001$
225

7

226 ## Results

227

228 ### scRNA-seq revealed distinct monocyte clusters in compensated and decompensated
229 cirrhosis

230 Our scRNA-seq dataset comprises monocytes from 14 healthy and cirrhotic individuals (Fig.
231 1A; Fig. S1A-C). After quality filtering (Fig. S1D) and correction for patient-specific effects (Fig.
232 S1C), a total of 93,318 monocytes were partitioned into 7 clusters (Fig. 1B), which were
233 annotated using unbiased comparisons to reference atlases (Fig. S1D), and the expression of
234 cluster specific genes (Fig. 1C).
235 Cluster 1 was defined by Fc-gamma receptors (e.g., *FCGR3A*), and represented non-classical
236 monocytes ($CD14^{+}CD16^{++}$). Expression of MHC class II (e.g., *HLA-DRB1, HLA-DRA*) was
237 enriched in cluster 2 and represented intermediate monocytes ($CD14^{++}CD16^{+}$). *CD52* served
238 as a gene defining cluster 1 and 2. Clusters 3 to 6 represented classical monocytes
239 ($CD14^{++}CD16^{-}$). In cluster 3 MHC II and ribosomal protein genes (e.g., *RPL13, RPS18)* were
240 highly expressed. Interferon-induced proteins (e.g., *MX1, IFIT1)* defined cluster 4. Cluster 5
241 and 6 showed similar expression patterns, although cluster 5 expressed genes involved in
242 phagocytic processes (e.g., *MPEG1, CD93*). Cluster 6 displayed slightly elevated expression of
243 ribosomal proteins (e.g., *RPS3A, RPL3*) and genes related to cell degradation and recycling
244 (e.g., *CD99, VIM, MGST1*). Cluster 7 was enriched in S100-protein genes (e.g., *S100A8,*
245 *S100A9*), *CD84, ITGAM, CD33* but low in MHC II, thus likely corresponded to M-MDSC (Fig.
246 1C/D).
247 Overall, the observed continuum included functionally distinct monocytic subsets,
248 recapitulating in particular the well-known distinction between $CD16^{high}$/MHC II^{high}-
249 expressing monocytes and $CD14^{high}$ monocytes.
250 Subsequently, relative frequencies of monocytic clusters were compared between patient
251 groups (Fig. 1E/F), however a large variability was present across patients within each group
252 (Fig. S1), which likely accounted for the absence of significant differences between
253 compensated cirrhosis and healthy. However, direct comparison of NAD cirrhosis to healthy
254 showed significant trends: cells from cluster 1 (non-classical) were more abundant in healthy
255 (FDR=0.00019%), while cells from cluster 7 (M-MDSC) were more abundant in NAD (FDR=7%),
256 as previously described [7, 8] (Fig. 1E/F).

257

258 ### Transcriptome changes of monocytes in cirrhosis

259 Differential expression (DE) analysis between patient subgroups was stratified by cluster to
260 eliminate the potential effects of cluster frequency differences. Notably, significant DE genes
261 were identified in non-classical (cluster 1), intermediate (cluster 2), classical monocytes
262 (cluster 3-6) and M-MDSC (cluster 7) (Fig. 2A/S2; Table S4).

263

264 In cirrhosis, genes related to pro-inflammatory cytokines were downregulated, while genes
265 related to apoptosis, complement and TLR signalling, calprotectin expression, protein C
266 activation and TAM receptor signalling were upregulated. Comparing the different stages of
267 cirrhosis, we found an upregulation of STAT1 inhibitor as well as genes indicating
268 inflammation and migration in NAD (Fig. 2A, Supplementary Results).

269

270 Notably, the analyses suggested differential expression of genes of the phosphatidylinositol
271 signalling pathway, i.e., *PLCG2, PLD4, IMPA2,* on monocytes in cirrhosis (Fig. 2A). Interestingly,

272 *CD52,* a (GPI)-anchored protein, was consistently upregulated in cirrhosis compared to HC
273 (although not significantly after multiple testing correction, e.g., NAD cirrhosis vs. HC in cluster
274 1: $log_2FC=0.66$, p-value=0.038, FDR=1). CD52 not only defined cluster 1 and 2 in health, but
275 was enhanced in most clusters in compensated and NAD cirrhosis (Fig. 1D/E). Other GPI-
276 anchored proteins were upregulated upon WNT-signalling[21], and WNT-signalling genes
277 (*WWC3, RHOU, SRPRB*) were differently expressed in cirrhosis. Furthermore, CD52 is shed by
278 PLC in T cells[22], and its gamma isoform (*PLCG2*) was upregulated in NAD. Moreover, sCD52
279 binds to SIGLEC10 to suppress T cell activation, and DE gene analysis identified *SIGLEC12* (Fig.
280 2B). Thus, we selected *CD52* as a gene of interest with putative central role in the regulation
281 of monocyte function in cirrhosis affecting genes involved in WNT- and SIGLEC-signalling
282 pathways.
283 Firstly, $CD52^{pos}$ monocytes were characterised revealing an association with *S100* proteins in
284 all clusters. $CD52^{pos}$ monocytes showed higher *IFI30* and *MNDA* expression in cluster 1,
285 interferon-induced proteins (e.g., *IFITM2*) and Fc-receptors (e.g., *FCGR3A*) in cluster 2, MCH
286 II (e.g., *HLA-DRA, HLA-DRB1*) in cluster 3-6 and *S100* proteins, *FCER1G* and *HLA-B* in cluster 7
287 (Fig. 2C).
288
289 To identify common up- or downregulated pathways, gene set enrichment analysis (GSEA)
290 was performed using the Hallmark Signatures gene sets.
291 In cirrhosis, monocytic inflammatory responses like interferon-α/-γ, IL-2/IL-6-signalling,
292 phagocytosis and the complement system were upregulated. Interestingly, also pathways
293 involved in coagulation, cholesterol- and lipid-homeostasis were upregulated in cirrhosis,
294 while DNA repair and MYC targets were downregulated compared to HC (Fig. 2D). Comparing
295 pathways differentially regulated in NAD compared to compensated cirrhosis, inflammatory
296 responses like interferon-α/-γ and TNFα-signalling were downregulated (Fig. 2E).
297 Using Gene Ontology categories (data not shown) dataset for GSEA, multiple pathways
298 regulating T cell proliferation/differentiation and cellular adhesion were upregulated, which
299 were affected by CD52 overexpression[22].
300

301 ## Upregulation of CD52 on monocytes indicates low infection and survival

302 We analysed peripheral blood monocytes to confirm *CD52*-upregulation at the protein level
303 using flow cytometry. Monocyte count was unchanged in cirrhosis (Fig. 3A). $CD52^{high}$
304 monocytes expanded in compensated and NAD cirrhosis, compared to HC, but not AD/ACLF
305 (Fig. 3B). Downregulation of CD52 in AD/ACLF compared to compensated/NAD stages, was
306 further observed in neutrophils but no other PBMC (Fig. 3C).
307 CD52-expression on monocytes was independent of the aetiology and associated with
308 transplant-free survival (TFS), development of infectious complications, hepatic
309 encephalopathy (HE) and ascites (Fig. 3D), and predicted 1-year TFS (AUROC 0.9412) and the
310 development of infection (AUROC 0.8363, Fig. 3E). CD52-expressing monocytes negatively
311 correlated with disease severity scores (Child Pugh, MELD, CLIF-AD) and liver function tests
312 (Fig. 3F).
313 To determine whether CD52 was shed from monocytes when patients progress to AD/ACLF,
314 sCD52 in plasma was assessed. Indeed, high sCD52 plasma levels detected in AD/ACLF were
315 associated with low TFS, infection, HE and ascites, independent of the aetiology, and
316 predicted 1-year TFS (AUROC 1, Fig. 3G/H). Plasma sCD52 positively correlated with disease
317 severity scores and liver function parameters (Fig. 3I). Monocytic cells from the hepatic vein
318 showed a numerically higher expression of the hepatic tissue macrophage marker TIM4 and

319 CD52 compared to peripheral monocytes sampled simultaneously in the same patient with
320 AD of cirrhosis (Fig. 3J).
321

322 CD52^{high} monocytes implicate macrophage-like activated phagocytes dampening 323 adaptive immune stimulation

324 CD52-expression was assigned to monocytic subsets: Classical monocytes increased in
325 cirrhosis, while non-classical decreased significantly in AD/ACLF compared to HC. In
326 compensated and NAD cirrhosis CD52-expression increased in classical monocytes, but
327 decreased significantly in AD/ACLF in all subsets (Fig. 4A-C).
328 CD52^{high} monocytes (Fig. 4D) were CD14^{+}CD16^{high}HLA-DR^{high} indicating a mature population
329 expressing higher macrophage markers (CD206, TIM-4[4]), TAM receptors (AXL, MERTK[6, 8]),
330 but lower MNDA[23] (Fig. 4E).
331 The biological function of CD52 on monocytes is not well-known. We detailed functional
332 characteristics of circulating CD52^{high} monocytes in cirrhosis. CD52^{high} cirrhotic monocytes
333 showed increased phagocytosis capacity (Fig. 5A/B), and enhanced cytokine production (TNF-
334 α/IL-6/IL-10) upon TLR stimulation (Fig. 5C/D).
335 In inflammatory conditions, e.g. liver injury, blood monocytes infiltrate tissues, e.g. the liver,
336 and differentiate into monocyte-derived macrophages[24]. We observed enhanced adhesion
337 and migration capacities of CD52^{high} monocytes from cirrhosis patients (Fig. 5E).
338 Furthermore, CD52^{high} cirrhotic monocytes dampened T cell proliferation[8] *ex vivo* (Fig. 5F).
339

340 Modulation of CD52 expression *ex vivo* induces significant functional changes

341 Next, we developed a THP-1 cell line overexpressing human CD52 (hCD52; THP-1-hCD52).
342 Lentiviral transduction of THP-1 cells with hCD52 resulted in a secondary antigen with a
343 continuous expression pattern (Fig. 6A/B). Similar to CD52^{high} monocytes from cirrhosis
344 patients, THP-1-hCD52 cells showed increased expression of CD16, HLA-DR, CD206, TIM-4,
345 AXL, MERTK compared to THP-1, albeit the expression levels of CD16, CD206, TIM-4, AXL were
346 below 5% (Fig. 6C). Similar to patient-derived monocytes, cytokine production was increased
347 in THP-1-hCD52 cells (Fig. 6D).
348 Treatment with the CD52-inhibitor alemtuzumab reduced CD52 expression on isolated
349 cirrhotic (CD52^{high}), but not HC (CD52^{low}) monocytes, viability was unchanged (Fig. 6E/F).
350 Functionally, alemtuzumab treatment dampened phagocytosis of *E.coli*/*S.aureus* in cirrhosis
351 but not HC, TNF-α/IL-6-production, and adhesion and migration capacities of CD52^{high}
352 monocytes from cirrhosis patients (Fig. 6G-K). The suppressive phenotype of cirrhotic CD52^{high}
353 monocytes towards T cell activation was reversed (Fig. 6M).
354

355 Plasma components regulate CD52 expression and phagocytosis capacity

356 We evaluated possible mechanisms modulating CD52-expression on monocytes. In cirrhosis,
357 pathological bacterial translocation (BT) enables microbial products to access the
358 circulation[24], potentially changing immune cell differentiation. Conditioning of healthy
359 monocytes led to CD52-upregulation in plasma from compensated and NAD cirrhosis, but not
360 AD/ACLF; CD52^{high} monocytes from cirrhosis patients downregulated CD52-expression in
361 AD/ACLF or HC plasma (Fig. 7A/B). CD52-expression significantly increased on monocytes
362 after phagocytosis of *E.coli*/*S.aureus* bioparticles, but not upon TLR-stimulation (Fig. 7C/D). In
363 addition, migrated monocytes from cirrhosis patients showed higher CD52-expression

364 compared to non-migrated (Fig. 7E). Furthermore, AD/ACLF plasma dampened phagocytosis
365 capacity of monocytes from HC and cirrhosis (Fig. 7F/G).
366

367 PLC downregulates CD52 on monocytes and indicates infection and low transplant-free 368 survival

369 PLC cleaves GPI-anchored proteins[17], representing a putative candidate downregulating
370 CD52. PLC plasma levels were below detection limit in compensated and NAD cirrhosis, but
371 significantly increased in AD/ACLF (Fig. 7H). PLC levels correlated negatively with CD52-
372 expression on monocytes and positively with sCD52 plasma levels (Fig. 7I). PLC elevation was
373 associated with low TFS, development of infection within 4 weeks, HE and ascites,
374 independent of the underlying aetiology, and predicted 1-year TFS (AUROC 0.7880) and
375 infection development (AUROC 0.7269) (Fig. 7J/K).
376 To investigate whether PLC cleaves CD52-expression on monocytes *ex vivo*, we incubated
377 monocytes from HC and cirrhosis at different stages with PLC, inducing a significant reduction
378 of CD52-expression on monocytes from compensated and NAD cirrhosis patients (Fig 7L).
379 With respect to other immune cells, CD52-downregulation was consistently observed 20min
380 after PLC incubation in monocytes and neutrophiles, however less in lymphocytes. In parallel,
381 sCD52 levels in supernatants increased, but not in AD samples (Fig. 7M).
382

383 Physiological CD52-expression on liver macrophages is reduced in cirrhosis

384 In relation to the activated macrophage-like state of circulating CD52^{high} monocytes, liver
385 macrophages isolated from patients with compensated cirrhosis and histologically-normal
386 liver were investigated. Hepatic macrophages highly expressed CD52 in healthy conditions.
387 However, in cirrhosis, CD52-expression was reduced (Fig. 8A). Using IHC staining, CD52^{+}CD68^{+}
388 cells were fewer in hepatic plates from patients with NAD and AD compared to compensated
389 cirrhosis and controls (Fig. 8B/C). Immunophenotyping of CD52^{high} hepatic macrophages
390 revealed CD68^{+}CD14^{+}CD16^{high}HLA-DR^{high} cells expressing higher levels of CD206 and AXL
391 similar to CD52^{high} monocytes (Fig. 8D/E). Hepatic macrophages showed lower phagocytosis
392 capacity in cirrhotic tissues, while CD52^{high} macrophages showed higher phagocytosis capacity
393 compared to CD52^{low} similar to circulating CD52^{high} monocytes (Fig. 8F/G).
394

395 ## Discussion

396 This work transcriptionally and systematically dissects monocyte states circulating in patients
397 with compensated and decompensated cirrhosis, hereby recapitulating the reduction of non-
398 classical monocytes and the accumulation of M-MDSC with disease severity, and identifying
399 previously unknown distinct changes within the classical monocyte subset, i.e. upregulation
400 of genes involved in resolution of inflammation and macrophage differentiation.
401
402 Cirrhosis-associated immune dysfunction involves changes in the prevalence of certain
403 circulating monocyte states, including a reduction in non-classical monocytes that are
404 recruited to the injured liver[6]. Our scRNA-seq data clearly confirmed this finding, distinctly
405 identifying non-classical, intermediate and classical subsets by their transcriptional profile
406 and showing a reduction of non-classical monocytes in NAD. Additionally, our dataset
407 confirmed the accumulation of M-MDSC in NAD, as previously described[7, 8, 25]. Albeit
408 AD/ACLF samples were not included, which previously showed highest numbers of
409 immunosuppressive M-MDSC[7].
410 Other distinct circulating monocyte states have been reported at certain stages, e.g.
411 monocytes expressing TAM receptors [6, 8, 20]. Given the high monocyte plasticity[8, 20, 26],
412 it is essential to thoroughly dissect clinical conditions and stages of cirrhosis in translational
413 studies. Our scRNA-seq data revealed more monocytic states within the classical subset which
414 differed between HC, compensated and NAD cirrhosis, respectively. While in health cluster 6
415 (cell degradation and recycling) predominated the classical monocytes, we observed a shift
416 towards cluster 5 (phagocytosis) in compensated and cluster 7 (M-MDSC) in NAD. These data
417 imply activation of survival signals in monocytes in cirrhosis, confirming viability was
418 preserved even in AD/ACLF[7]. Also, it highlights the importance of phagocytosis in circulating
419 monocytes in presence of pathological BT[7, 27].
420 *GAS6*, ligand and activator of the TAM receptors MERTK and AXL, was upregulated on
421 monocytes in cirrhosis. This may relate to the upregulation of MERTK or AXL on monocytes[8].
422 Of note, the source of GAS6 is not restricted to monocytic cells, but expressed in several
423 tissues and secreted into the blood. We previously observed a significant upregulation of
424 GAS6 in hepatic stellate cells from cirrhosis patients[20]. *MERTK,* important for the inhibition
425 of TLR signalling and efferocytosis in the context of resolution of inflammation[28, 29], was
426 upregulated on monocytes in cirrhosis, again highlighting the relevance of *MERTK* as a
427 biomarker of immuneparesis, and a potential immunotherapeutic target[6].
428 Upregulation of inflammatory pathways and cytokines in cirrhosis was repeatedly reported
429 and led to the systemic inflammation hypothesis[1, 2]. Due to the transition of stages during
430 cirrhosis progression and inconsistencies in the nomenclature over the years[30], the
431 dynamics of inflammatory responses across stages remain insufficiently understood. The
432 upregulation of genes involved in inflammatory pathways including humoral responses but
433 also interferon signalling in monocytes confirms previous observations, while the lower
434 expression in NAD compared to compensated cirrhosis is novel and confirms the concept that
435 attenuated inflammatory responses may occur in NAD and prevail in AD/ACLF[6, 7].
436 The upregulation of genes involved in phagocytosis, plasma membrane organisation and
437 endocytosis in compensated and NAD stages is intriguing. We previously reported that
438 phagocytosis was preserved in cirrhosis, unless AD/ACLF occurred[7, 8]. Indeed, in this study
439 we revealed that $CD52^{high}$ monocytes, were active phagocytes maintaining the patients'
440 infection defence in compensation and NAD. Similar findings have recently been reported
441 using scRNA-seq/snRNA-seq from cirrhotic liver myeloid cells[31].

442 Deterioration of synthetic liver function in cirrhosis is known, but its onset in relation to the
443 dynamic stages remains unclear. In particular, the presence of coagulopathy and its clinical
444 implications are subject of discussion and research[32]. Here, genes involved in coagulation
445 were upregulated in monocytes in cirrhosis, also in NAD, supporting enhanced rather then
446 failed coagulation. Complement pathways in cirrhosis were less well studied[33], however in
447 this dataset, clearly enhanced in cirrhosis, even NAD, indicating preservation of soluble
448 immune components.
449 The upregulation of CD52, PLC and other phosphatidyl-signalling pathway components on
450 monocytes in compensated and NAD cirrhosis is novel and highly interesting in relation to the
451 poorly understood function of CD52 on monocytes on the one hand, and the beneficial
452 prognostic profile on the other. In health, CD52-expression is highest on B and T cells; among
453 myeloid cells on $CD16^+$ monocytes and mDC[34]. CD52 on T cells limited their activation[9]
454 and sCD52 inhibited TLR activation of NF-kB and suppressed inflammation in THP-1 cells[11].
455 In our cohort, we observed that circulating monocytes upregulated CD52 during early
456 cirrhosis stages with acceptable morbidity, immune control and prognosis. $CD52^{high}$ cells were
457 characterised as $CD14^+CD16^{high}HLA\text{-}DR^{high}$ mature monocytes with high phagocytosis
458 capabilities and cytokine responses, required for pathogen clearance and initiation of
459 immune responses, and high potential to migrate across endothelial barriers, as required for
460 the recruitment of monocytes to inflamed tissues, and back to the circulation, however with
461 low potential to activate T cell proliferation. Enhancing these selective monocyte functions
462 appeared beneficial in the context of pathological BT and sporadic sterile or infectious
463 inflammation of the liver and other tissues[4], and may represent a rescue process enhancing
464 innate immune function in cirrhosis. This may explain the clear association of $CD52^{high}$
465 monocyte prevalence with survival in our cohort, and indicate marker function of the CD52
466 pathway predicting innate immune function.
467 Which signals upregulate CD52 on circulating monocytes, and downregulate CD52 on liver
468 macrophages in cirrhosis, respectively? *In vitro* we revealed that plasma components of
469 cirrhosis patients induced the upregulation of CD52 on healthy monocytes, similarly
470 upregulation was observed after phagocytosis of bacterial particles. Another process
471 upregulating CD52 was migration across liver sinusoidal endothelia, and $CD52^{high}$ cells
472 revealed enhanced migration capacities. These findings implicate that the enhanced CD52-
473 expression on circulating monocytic cells originates from diverse processes including changes
474 in plasma composition, circulating bacterial products, and migration of monocytic cells across
475 endothelia, potentially towards the circulation.
476 By contrast, in AD/ACLF, $CD52^{high}$ monocytes disappeared from the circulation, associated
477 with mortality. $CD52^{low}$ monocytes had reduced phagocytosis potential, low cytokine
478 responses, and low migratory potential. A similar immunosuppressive profile occurred
479 following treatment with alemtuzumab, but also PLC treatment. PLC has been shown to
480 cleave CD52 on T cells, and release sCD52 to the plasma[17]. Interestingly, AD patients
481 displayed $CD52^{low}$ monocytes and high plasma levels of both PLC and sCD52. We
482 demonstrated *ex vivo* that PLC cleaved CD52 from monocytes and neutrophils from patients
483 with cirrhosis, releasing sCD52. Of course, this may not be the only cellular source of sCD52
484 in patients with AD/ACLF as a systemic disease condition. Also, the source of plasma PLC in
485 AD/ACLF remains unknown, various isoforms, splicing variants exist in diverse human
486 tissues[35]. Phospholipase A release from liver and kidney was reported in cirrhosis rat
487 models[36]. PLC may originate from sources of tissue inflammation in AD/ACLF, e.g. the
488 peritoneum or kidney, less likely the inflamed liver itself[37].

13

489 CD52 is a biomarker indicating survival also in sepsis. A scRNA-seq study evaluating dynamic
490 gene expression on PBMC in patients with gram-negative sepsis revealed increased CD52-
491 expression over 6-hours in lymphocytes of survivors[13].
492 In this paper we recapitulate the distinct functional phenotype of CD52^{high} monocytes *in vitro*
493 by CD52 overexpression. The question arises, whether stabilisation of CD52-expression on
494 immune cells could be used for future therapeutic immune-modulation. In the endoplasmic
495 reticulum, GPI-anchored proteins are stabilized by p24-complex during post-translational
496 modification[13]. If stabilisation of CD52 enhanced immune responses in cirrhosis, this may
497 lower susceptibility to infection and prevent infectious episodes, AD and death – allowing the
498 liver to regenerate or bridge to transplantation.
499
500 In summary, we detailed circulating monocytes by transcriptome analysis in cirrhosis patients,
501 recapitulating the loss of CD14^{+}CD16^{++} monocytes and the emergence of M-MDSC in
502 decompensated stages. Moreover, we detect CD14^{++} monocytes with enhanced phagocytosis
503 emerging in adaptation to changes in liver architecture and composition of portal and
504 systemic milieu. We newly identified CD52^{high} functionally-activated circulating monocytes in
505 correlation with survival. In AD/ACLF by contrast, these CD52^{high} monocytes were lost, by PLC-
506 cleavage, potentially released from sites of inflammation, relating to poor prognosis.
507 Regulation of CD52 on monocytes may be a marker of innate immune function and an
508 immunotherapeutic mechanism worth future exploration.
509

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533 Author names in bold designate shared co-first authorship.
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Figure Legends

Figure 1: Single-cell atlas of human circulating monocytes

A: Overview of monocyte isolation from HC (n=5), compensated (n=4) and NAD (n=5) cirrhosis patients. **B:** Heatmap of cluster-defining marker genes. The log-normalized expression levels were averaged across cells from the same cluster and sample, averaged values were centred and scaled per gene. **C:** Final monocyte clustering visualized on a UMAP embedding. **D:** Gene expression of selected cluster-defining markers across cells. **E:** Monocyte densities and *CD52* expression across disease states. **F:** Cluster frequencies across disease state.

Figure 2: Identification of differential Gene expression in monocytes in liver cirrhosis

A: Volcano plots showing DE genes in cirrhosis (n=9) vs. HC (n=5) (top row) and NAD (n=5) vs. compensated (n=4) cirrhosis (bottom row) in different clusters. **B:** Violin plots of DE gene expression. **C:** Heatmap of DE genes comparing $CD52^{high}$ vs. $CD52^{low}$ monocytes. GSEA of **D:** cirrhosis vs. HC and **E:** NAD vs. compensated.

Figure 3: Clinical relevance of CD52 expression on monocytes

A: Monocyte counts (box plot, Kruskal-Wallis test). CD52-expression on **B:** circulating monocytes (% and MFI) with representative FACS-histograms (box plots, Kruskal-Wallis test), **C:** Neutrophils (%), T-cells, B-cells and NK cells (%) across cirrhosis stages (box plots, Kruskal-Wallis tests). CD52-expression on monocytes **D:** related to survival, infection, HE, ascites and cirrhosis aetiology (box plots, Mann-Whitney and Kruskal-Wallis tests), **E:** predicts 1-year mortality, infection (ROC Analyses, ****p=0.0001) and **F:** correlates negatively with Child Pugh, MELD, CLIF-C-AD, bilirubin, albumin, INR, CRP (Pearson's r Correlations) Plasma levels of sCD52 **G:** across cirrhosis stages, related to survival, infection, HE ascites, cirrhosis aetiology, (box plots, Mann-Whitney and Kruskal-Wallis test), **H:** predicts survival (ROC Analysis, **p=0.0021) and **I:** correlate with Child Pugh, MELD, CLIF-C-AD, bilirubin, albumin, CRP, AST, ALT (Pearson's r Correlations). J: TIM4 and CD52 expression in hepatic venous blood (% and MFI) (box plots, Wilcoxon test). *p=0.05; **p=0.01; ***p=0.001; ****p=0.0001

Figure 4: Phenotypic characterisation of $CD52^{high}$ monocytes

A: Gating strategy for monocytic subset identification (classical=$CD14^{++}CD16^{-}$, intermediate=$CD14^{++}CD16^{+}$, non-classical=$CD14^{+}CD16^{++}$). **B:** Monocyte count across cirrhosis stages (box plots, Kruskal-Wallis tests). **C:** CD52-expression (% and MFI) of monocytic subsets across cirrhosis stages (box plots, Kruskal-Wallis tests). **D:** Gating strategy and representative histograms for $CD52^{high}$ and $CD52^{low}$ monocytes. Forward scatter (FSC), fluorescence minus one (FMO) **E:** Immunophenotyping of $CD52^{high}$ and $CD52^{low}$ monocytes in cirrhosis (box plots, Wilcoxon tests). *p=0.05; **p=0.01; ***p=0.001; ****p=0.0001

Figure 5: Functional characteristics of $CD52^{high}$ monocytes *ex vivo*

Phagocytosis capacity (% and MFI) of monocyte in **A:** HC vs. cirrhosis (box plots, Mann-Whitney and Kruskal-Wallis tests) and **B:** $CD52^{high}$ vs. $CD52^{low}$ from cirrhosis (box plots, Wilcoxon tests). Cytokine production (% and MFI) upon TLR stimulation of monocytes in **C**: HC vs. cirrhosis (box plots, Friedman tests) and **D:** $CD52^{high}$ vs. $CD52^{low}$ from cirrhosis (box plots, Wilcoxon tests). **E:** Adhesion, migration behaviour (MFI and ratio) (box plots, Mann-Whitney tests) and **F:** T-cell proliferation (MFI^-1) in co-culture with monocytes, of previously

defined CD52^{high} vs. CD52^{low} samples (box plot, Mann-Whitney test). *p=0.05; **p=0.01; ***p=0.001; ****p=0.0001

Figure 6: CD52 overexpression in THP-1 cells and inhibition in monocytes *in vitro*

A: Plasmid used for lentiviral transduction. **B:** Representative FACS plot and histograms to identify THP-1-hCD52 cells. Forward scatter (FSC), side scatter (SSC), fluorescence minus one (FMO). **C:** Immunophenotyping of THP-1-hCD52 and THP-1 cells (bar graphs, median Mann-Whitney tests). **D:** Cytokine production upon TLR stimulation of THP-1-hCD52 and THP-1 cells (bar graphs, median, Mann-Whitney tests). Effect of CD52 inhibition by alemtuzumab treatment on monocytes of previously defined CD52^{high} vs. CD52^{low} samples: **E:** CD52-expression (%) (box plots, Friedman tests), **F:** survival (% and MFI) (box plots, Friedman tests), **G:, H:** phagocytosis capacity (% and MFI) (box plots, Wilcoxon tests), **I:** cytokine production (%) upon TLR stimulation (box plots, Wilcoxon tests), **J:** adhesion (box plots, Wilcoxon tests) and **K:** migration potential (ratio) (box plot, Wilcoxon test). **M:** T-cell proliferation (MFI^-1) in co-culture with monocytes from previously defined CD52^{high} patients and CD52^{low} HC samples (box plots, Wilcoxon tests). *p=0.05; **p=0.01; ***p=0.001; ****p=0.0001

Figure 7: Effect of plasma conditioning on CD52^{high} monocytes *in vitro*

CD52 expression (% and MFI) of **A:** HC monocytes (box plots, Friedman tests) and **B:** previously defined CD52^{high} monocytes from cirrhosis patients upon plasma conditioning from different disease stages (box plots, Friedman tests). CD52 expression (%) on monocytes of HC and cirrhosis upon **C:** bacterial treatment (box plots, Kruskal-Wallis and Mann-Whitney tests) and **D:** TLR stimulation (box plots, Kruskal-Wallis and Mann-Whitney tests). **E:** monocyte CD52-expression (%) of previously defined CD52^{high} and CD52^{low} samples after migration (box plots, Kruskal-Wallis and Wilcoxon tests). Phagocytosis capacity (% and MFI) of **F:** HC monocytes (box plots, Friedman tests) and **G:** previously defined CD52^{high} cirrhotic monocytes upon plasma conditioning from different disease stages (box plots, Friedman tests). PLC plasma levels (ng/ml) **H:** at different stages of cirrhosis (box plots, Kruskal-Wallis tests) and **I:** correlate negatively with monocyte CD52-expression and positively with sCD52 plasma levels in cirrhosis patients (Pearson's Correlations). PLC plasma levels (ng/ml) **J:** related to survival, infection, HE, ascites and cirrhosis aetiology (box plots, Kruskal-Wallis and Mann-Whitney tests) and **K**: predicts survival and infections (ROC Analyses, *p=0.0332). **L:** CD52-expression (% and MFI) of monocytes at different disease stages upon treatment with recombinant PLC (Dot plot, Wilcoxon test *p=0.05). **M:** Time course of CD52 downregulation on monocytes, neutrophiles and lymphocytes upon PLC treatment and corresponding sCD52 levels in the supernatant (Dot plots, Friedman and Wilcoxon tests). *p=0.05; **p=0.01; ***p=0.001; ****p=0.0001

Figure 8: CD52 expression on isolated human hepatic macrophages *ex vivo*

A: CD52 expression (% and MFI) on human hepatic macrophages (box plots, Kruskal-Wallis and Mann-Whitney tests). **B:** Cell count of CD68^{+} cells/HPF and percentage of CD52^{+} cells of CD68^{+} population (box plots, Kruskal-Wallis tests). **C:** Representative IHC micrographs from CD52/CD68/DAPI stains of liver biopsies (NAD, AD) and resections (Control, Compensated). **D:** Gating strategy and representative histograms to identify CD52^{high} and CD52^{low} macrophages. Forward scatter (FSC), fluorescence minus one (FMO). **E:** Immunophenotyping of CD52^{high} and CD52^{low} macrophages in cirrhosis (box plots, Wilcoxon tests). Phagocytosis

717 capacity (% and MFI) of macrophages in **F:** control vs. cirrhosis (box plots, Mann-Whitney
718 tests) and **G:** $CD52^{high}$ vs. $CD52^{low}$ macrophages (box plots, Wilcoxon tests). *p=0.05;
719 **p=0.01; ***p=0.001; ****p=0.0001
720

721 ## Graphical Abstract

722 scRNA-seq allocated seven circulating monocyte states, changing in cirrhosis patients at
723 different stages of disease. Circulating monocytes overexpress CD52 in cirrhosis, but are
724 absent in AD/ACLF due to PLC. CD52-expressing monocytes show high capability for
725 phagocytosis, cytokine production, adhesion and migration potential and T cell suppression.
726 Created with BioRender.com
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Figure 1

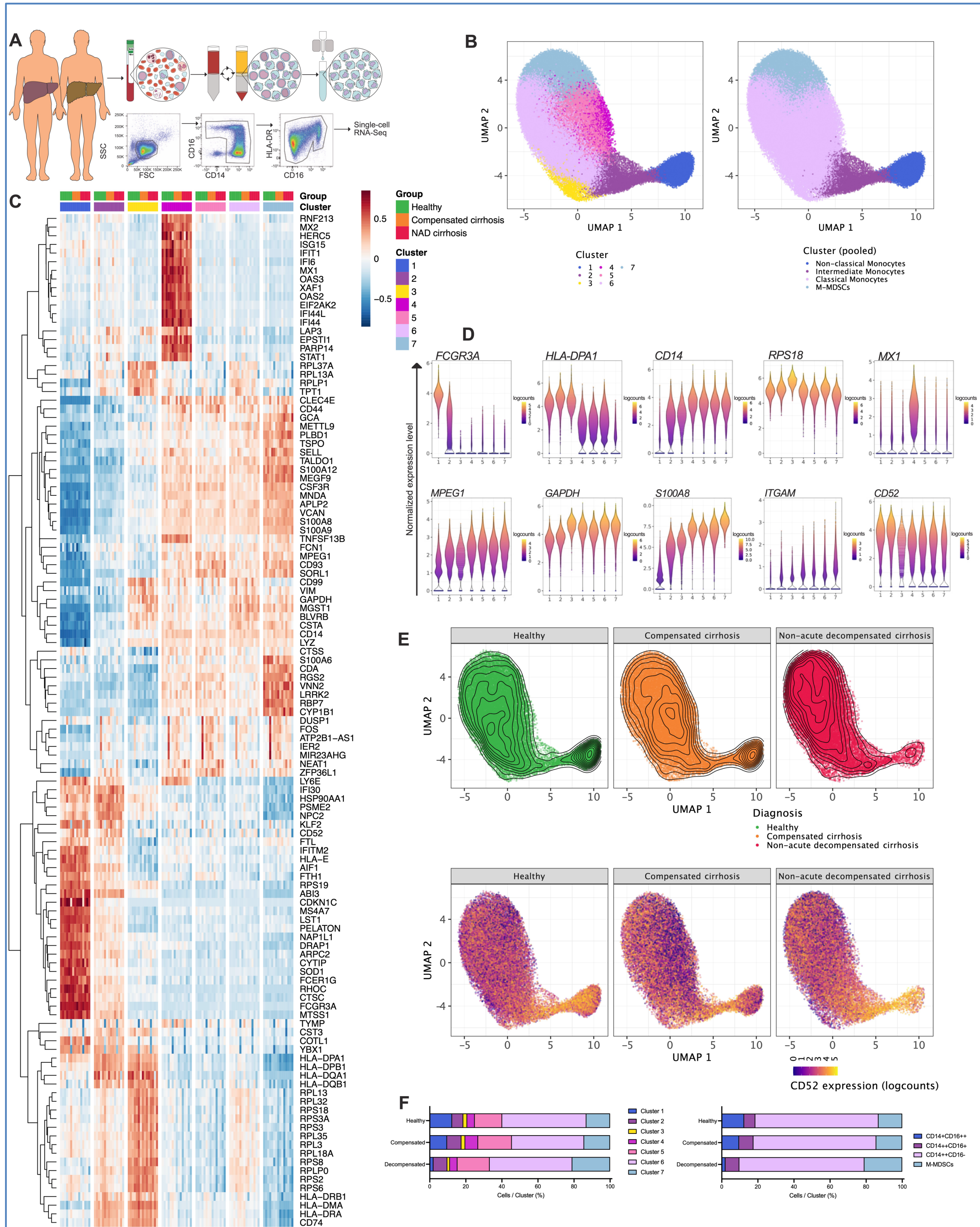
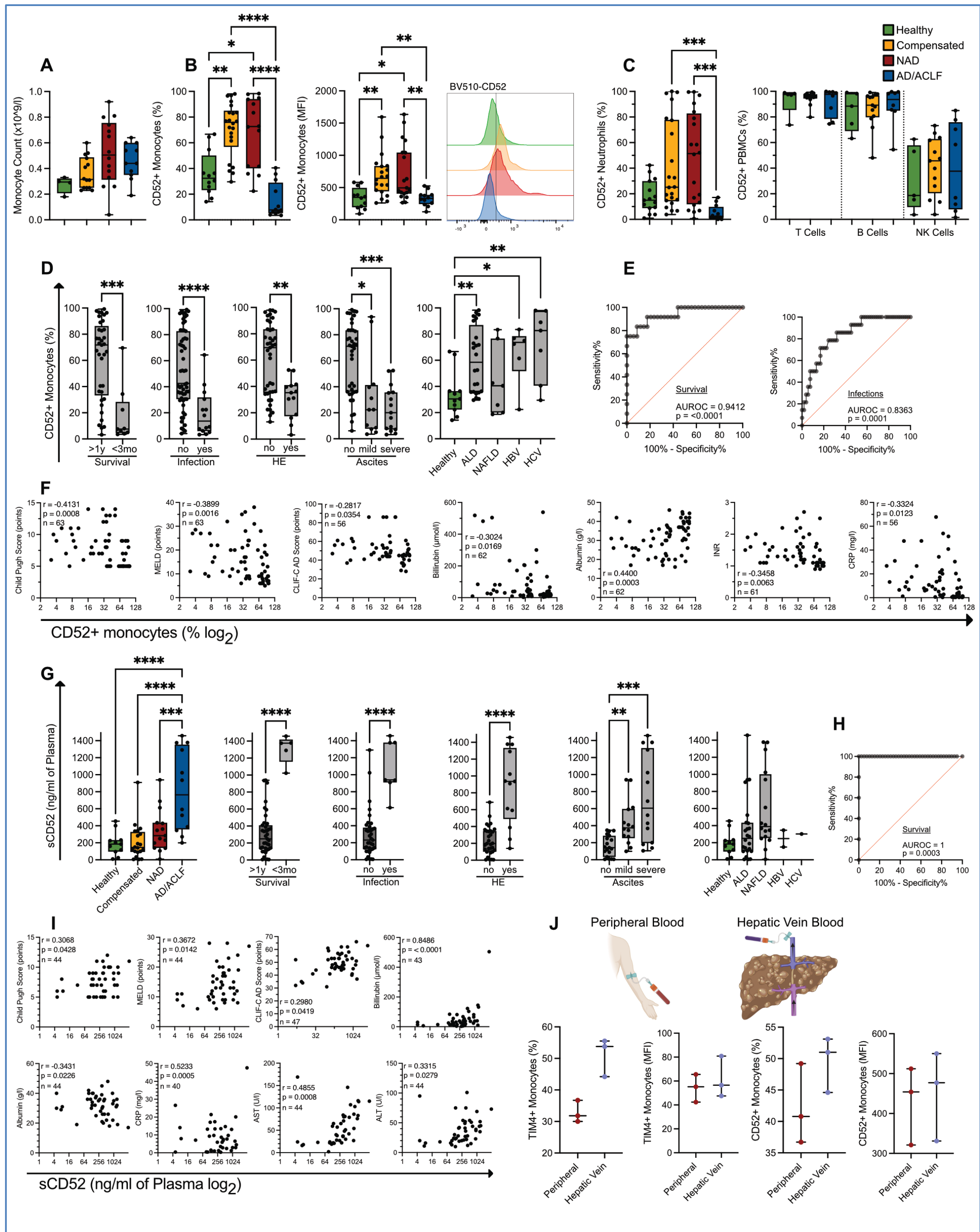


Figure 2

Figure 3



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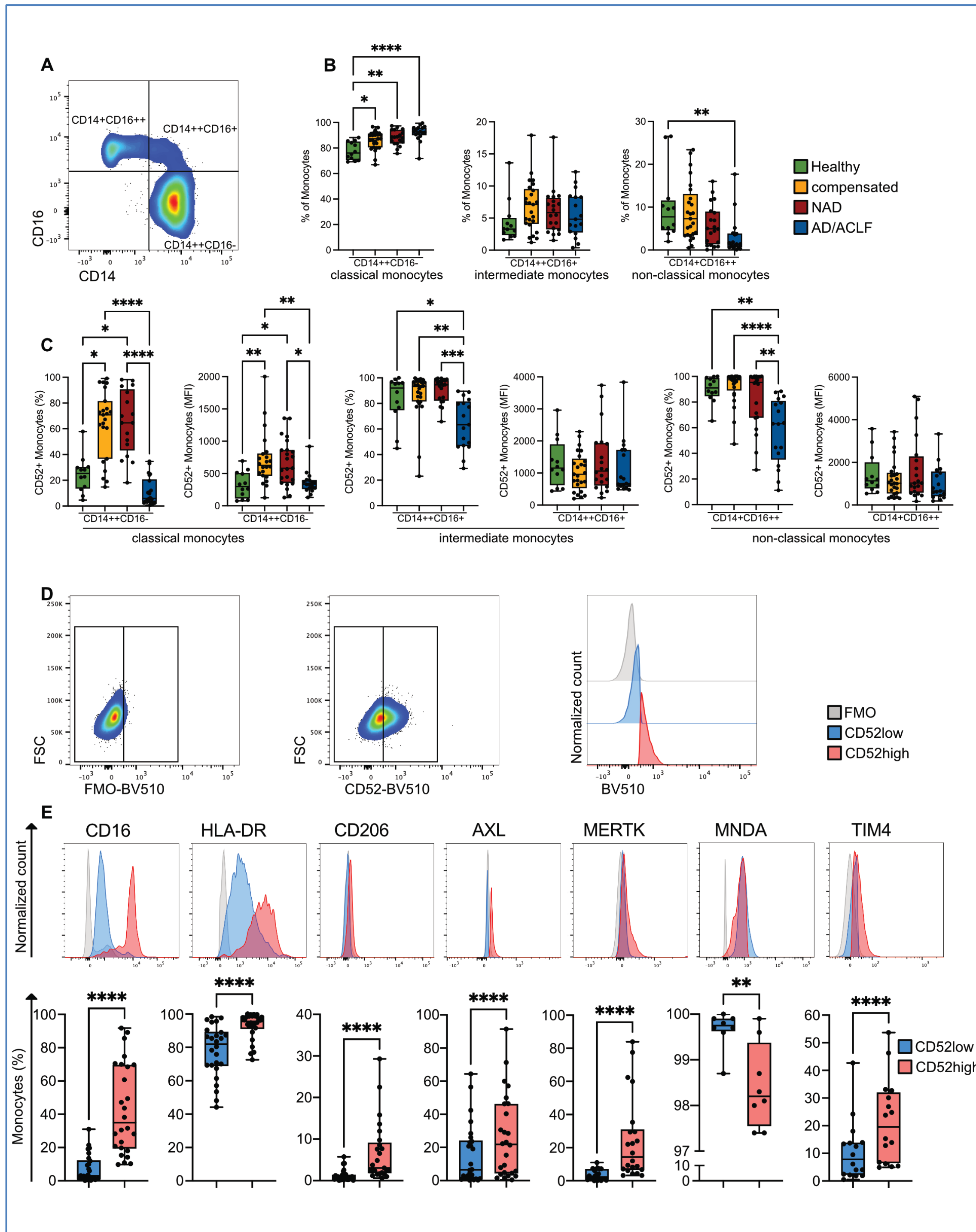
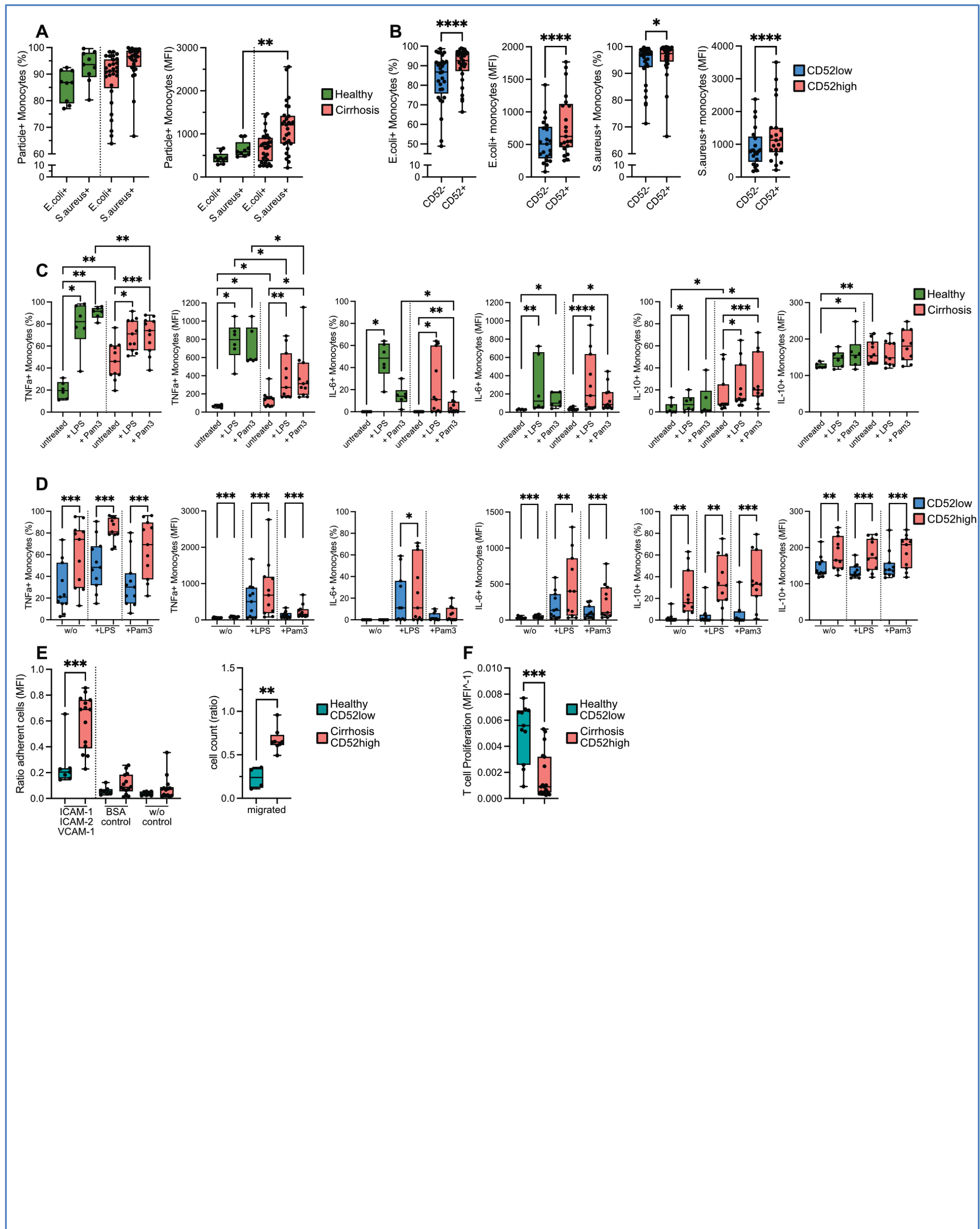


Figure 5



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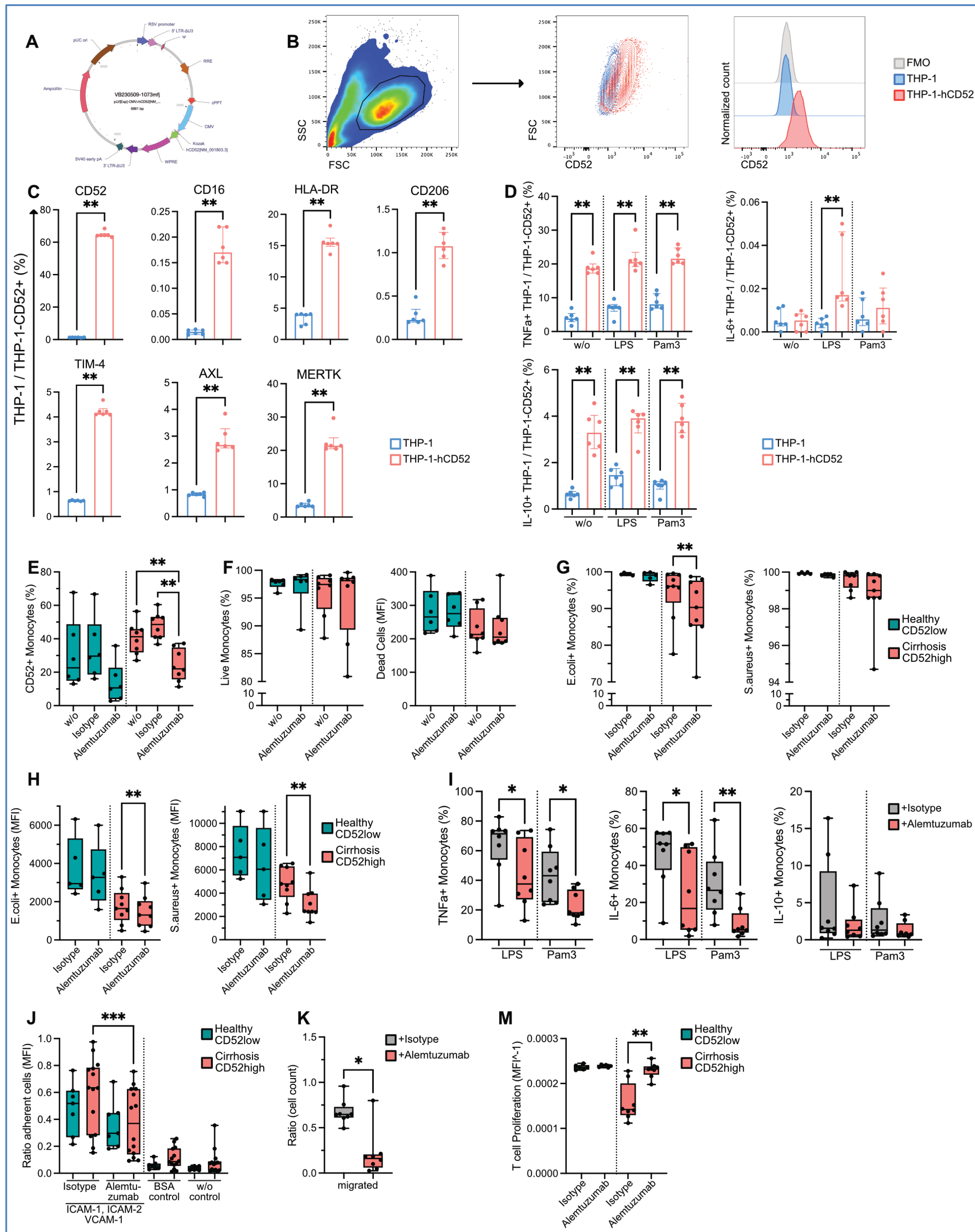


Figure 7

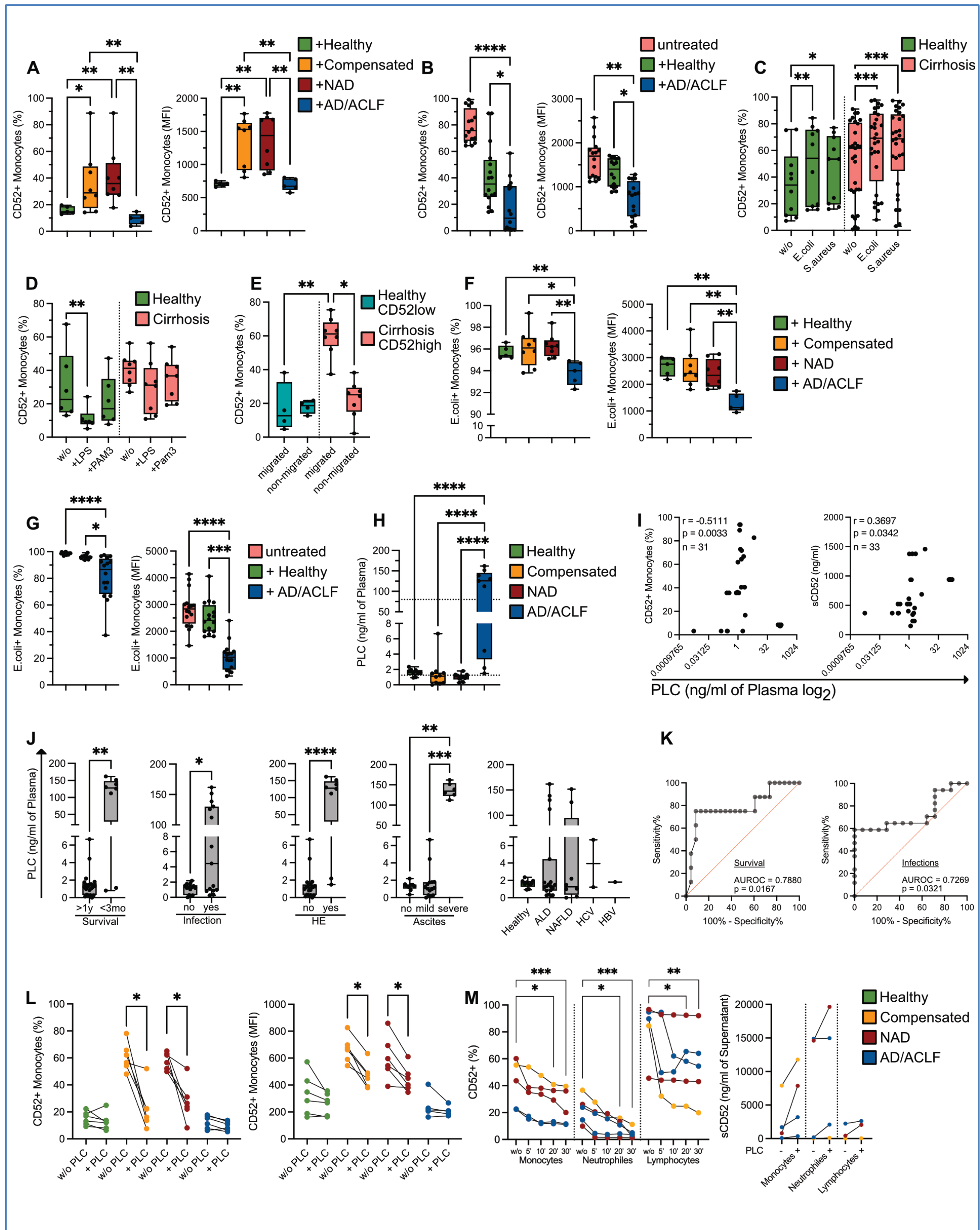
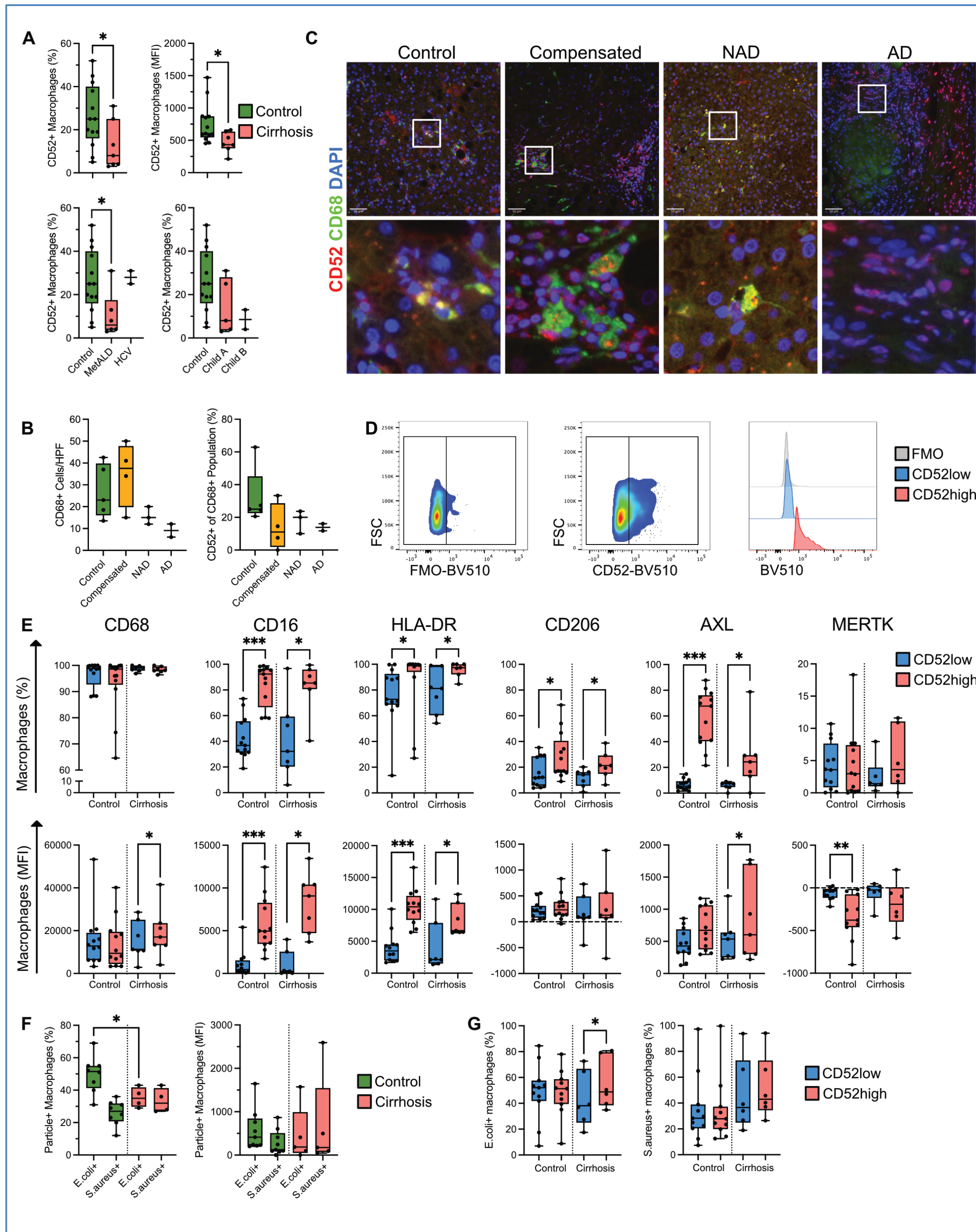
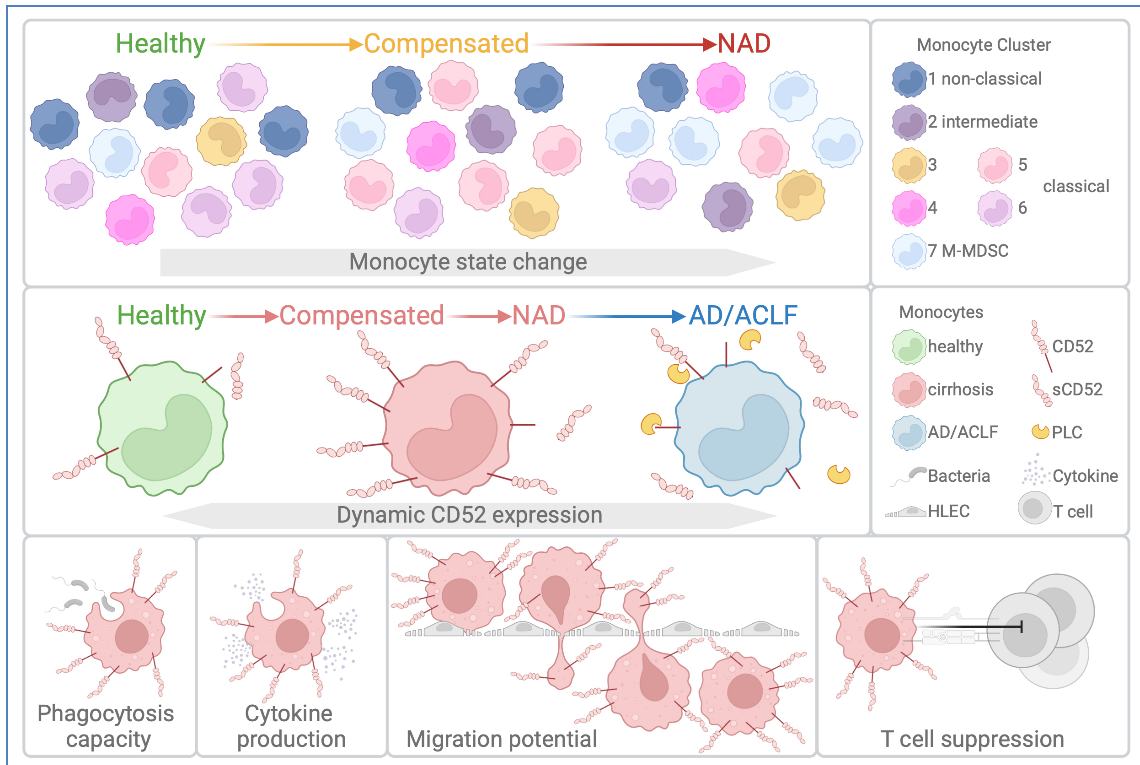


Figure 8



Graphical Abstract



5 Synthesis

We detailed distinct macrophage and monocyte subsets including AXL-expressing and CD52-expressing cells, occuring in a compartment-specific manner in relation to disease progression which regulate innate immune responses, hereby contributing to the complex pathogenesis of immuneparesis in patients with cirrhosis. Strong correlations with clinical parameters, cirrhosis severity scores, development of infections and mortality emphasize their clinical significance. The findings may suggest their role as biomarkers for the integrity of innate immune functions in patients with cirrhosis and potentially as immunotherapeutic targets.

Initial studies investigated monocyte and macrophage dysfunction in AD or ACLF stages of cirrhosis, when the probability to develop infection and the subsequent morbidity and mortality is highest [114, 163-166]. In our group we previously showed that M-MDSC, distinct AXL-expressing and MERTK-expressing monocytic subsets increased with disease progression and prevailed over regular monocytes in the circulation of patients with cirrhosis (Figure 5-1) [68, 83, 114]. Furthermore, MERTK-expressing liver macrophages were found to increasing with cirrhosis progression. Compared to regular monocytes $CD14^{+}DR^{+}AXL^{+}$ and $CD14^{+}DR^{+}MERTK^{+}$ harbor immune-regulatory functions with enhanced efferocytosis capacity, while $CD11b^{+}CD14^{+}CD15^{-}DR^{low/-}$ M-MDSCs fulfill immune-suppressive functions. Inflammatory cytokine (TNF-a, IL-6) production upon TLR4 stimulation with LPS was dampened in AD/ACLF but also – to a lower extend – in stable cirrhosis [68, 83, 114]. AXL-expressing monocytes showed attenuated TNF-a and IL-6 responses to LPS and dampened T-cell activation, but enhanced efferocytosis and preserved phagocytosis of *E.coli* [83]. The underlying mechanisms remain underexplored and detailed characterisation of stage-specific macrophages and monocytic subsets remains unknown. The time and underlying

conditions under which immuneparesis arises and infection susceptibility increases during disease progression remain unknown.

AXL, MERTK and Tyro-3 belong to the TAM receptor tyrosine kinases (RTKs) family and inhibit TLR-signalling, cytokine receptor-mediated monocyte/macrophage activation and apoptotic cell removal [72, 100, 167]. Mice lacking these RTKs develop liver disease characteristic apoptotic cell accumulation [105]. Loss of AXL-expression on antigen-presenting cells (APCs) has been linked to autoimmunity [167].

Here, we for the first time observed that over 90% of liver macrophages expressed AXL in homeostasis. We found a reduction of AXL-expressing resident liver macrophages in parallel with progression of cirrhosis, which highly correlated with cirrhosis severity scores underlining its clinical relevance. Interestingly this process was reversible. Longitudinal liver biopsies taken from patients progressing from Child B to a Child C stage showed a progressive loss of AXL expression on hepatic macrophages, while patients regressing from Child C to a Child B stage showed increased expression of AXL on hepatic macrophages. These examples showed that cirrhosis associated liver macrophage modulation can be reversed in patients transitioning back from a more advanced to a stable cirrhosis stage, increasing AXL expression on liver macrophages again. From a spatial perspective AXL-expressing macrophages were found in hepatic plates while MERTK-expressing macrophages enriched in both areas of fibrosis and hepatic plates. Phenotypically AXL-expressing hepatic macrophages found under physiologic conditions were $CD68^{high}HLA\text{-}DR^{high}CD16^{high}CD206^{high}$, indicating a mature macrophage population. To maintain tolerance at the barrier between the portal and the systemic circulation, phagocytosis capacity of resident liver macrophages is essential. The proportion of macrophages phagocytosing gram-negative *E.coli* was reduced in cirrhosis. Given that AXL-expressing monocytes accumulated in the circulation of cirrhosis patients and AXL was upregulated upon

response to microbial products and efferocytosis *in vitro* [83], we moreover hypothesized whether AXL+ macrophages may also have an increased migratory potential. We could show that AXL-expression was increased on monocytes that had migrated across confluent human liver endothelial cell layers *in vitro*. We furthermore assessed compartment-specific AXL-expression on macrophages in other tissues i.e. the gut, the peritoneum, lymph node and bone marrow. Intestinal macrophages showed reduced AXL-expression with cirrhosis progression, similar to hepatic macrophages.

Peritoneal macrophages showed lower AXL-expression, but higher MERTK-expression compared to monocytes derived macrophages (MDM). Interestingly AXL+ macrophages were not detectable in the bone marrow, but accumulated in the circulation and in lymph nodes of advanced cirrhosis patients, supporting the hypothesis of enhanced migration potential. The AXL-GAS6-axis was previously described and Gas6 was suspected to be produced by HSCs in mice [168, 169]. In patients with cirrhosis plasma levels of GAS6 were increased compared to healthy controls, however. AXL-expression on circulating monocytes did not change upon GAS6 treatment *in vitro* [83]. We were the first describing a decrease of AXL-expression on hepatic macropahges in advanced stages of cirrhosis. Furthermore, we found GAS6 expression to be upregulated by activated HSC in cirrhosis. Mechanistically, activated HSC from cirrhosis patients had the potential to lower AXL-expression on healthy AXLhigh macrophages *in vitro*.

AXL-expressing monocytes in the circulation were shown to harbour reduced inflammatory responses to microbial challenges [83]. We propose that this AXL-expressing population, together with MERTK-expressing monocytes appearing in AD/ACLF and MDSC cause the dampened innate immune responses of the monocytic population in AD/ACLF cirrhosis, which may explain the elevated infection susceptibility and poor clinical outcome.

After observing the differentiation and compartmentalization of monocytes and macrophage subsets in relation to cirrhosis severity, our goal was to systemically decode their stage-specific heterogeneity. We aimed to specifically examine the

population changes in circulating monocytes using a high-throughput approach. To achiev this, we selected single-cell RNA sequencing (scRNA-Seq) as our primary unbiased method for identifying transcriptomic profiles of circulating monocytes from patients at different stages of cirrhosis.

We identified seven monocytic clusters, distinguished by similarity of transcriptome and marker gene expression, representing classical, non-classical, intermediate monocytes and M-MDSCs with changing prevalences among different severity stage of cirrhosis. Cluster 1 was defined by expression of Fc-receptors (non-classical monocytes), cluster 2 highly expressed MHC II members (intermediate moncoytes), cluster 3 to 6 (classical monocytes) highly expressed ribosomal proteins, interferon induced proteins, phagocytosis related proteins and proteins involved in cell degradation/recycling. Cluster 7 (M-MDSC) was enriched in S100 proteins, *ITGAM, CD33* but low in MHC II. Interestingly, cluster 1 monocytes (non-classical) decreased in frequency, while cluster 7 (M-MDSC) increased with cirrhosis progession. We identified upregulated genes in specific clusters: *MERTK* (in cluster 1, 3-7), *GAS6* (in cluster 3-7), *S100*-genes (in cluster 1,2) as DE genes in cirrhosis conditions. These findings are consistent with previous studies. MERTK was described to be upregulated in AD/ACLF cirrhosis [114]. Plasma levels of GAS6 were shown to be increased in cirrhosis patients [83] and we found that GAS6 was secreted by activated HSC in cirrhosis [170]. Furthermore S100 proteins were described as markers identifying M-MDSC [171] which is consistent with our findings in the scRNA-Seq data set.

The phosphatidylinositol signalling pathway genes *PLCG2* and *IMPA2* were upregulated, while *PLD4* was downregulated in cirrhosis. WNT signalling related genes *WWC3* and *RHOU* were upregulated, while *SRPRB* was downregulated in cirrhosis. This indicates a central role for GPI-anchored protein CD52 expression on monocytes in the context of cirrhosis. We found CD52 being higher expressed on monocytes from patients with compensated and NAD cirrhosis compared to HC. It is known that CD52 is shed by phospholipase C (PLC), and interestingly we found *PLCG2* being upregulated in our dataset. Furthermore,

CD52 binds to Siglec-10 and we found the family member *SIGLEC12* significantly up-regulated in NAD in our dataset. For these reasons, we further intensified to investigate the potential role of CD52 expression on circulating monocytes in the context of cirrhosis. *CD52^{high}* monocytes showed distinct gene expression patterns depending on cluster affiliation, giving hints for an activated monocyte state through the upregulation of *Fc-receptors, MHC II* members and *interferon induced* genes*,* but also upregulating markers related to M-MDSC-associated *S100* genes. Pathways involved in inflammatory responses, phagocytosis and complement system were upregulated in monocytes of patients in early stage of cirrhosis, but downregulated in later stage, indicating onset of immuneparesis.

Interestingly, CD52 expression on monocytes was downregulated or absent in patients at AD/ACLF stages of liver cirrhosis. A similar CD52 expression pattern was observed on neutrophils from patients with cirrhosis, but not other circulating immune cells. In the plasma of cirrhosis patients at different stages an inverted picture was seen when observing soluble (sCD52) levels – sC52 levels were high only in AD/ACLF patients whose monocyte expression of CD52 was low. The expression of CD52 on monocytes and the presence of soluble CD52 (sCD52) in the bloodstream, respectively, highlights the significance of this pathway as a clinically relevant biomarker indicating the state of innate immune function of patients with cirrhosis at different stages. Presumably CD52 could also serve as marker for immunefunction in other conditions independent of cirrhosis, which has not been studied to date. By detecting elevated levels of CD52 expression on monocytes and sCD52, healthcare providers might be able to readily monitor innate immune function, and potentially improve patient outcomes through timely immunotherapeutic intervention. Monitoring innate immune function in cirrhosis has long been desired but is not feasible in clinical practice to date. These biomarkers could thus play a crucial role in the management and treatment of liver cirrhosis, offering a less invasive alternative to traditional diagnostic methods.

An increase in CD52-expressing monocytes in compensated and NAD stages of cirrhosis mostly affected classical monocytes ($CD14^{++}CD16^{-}$), while a decrease

in AD/ACLD stages was observed in all monocytic subsets. Phenotypically CD52^{high} monocytes revealed an activated monocyte state (CD16^{high}HLA-DR^{high}CD206^{+}AXL^{+}MERTK^{+}) and a macrophage like phenotype (MNDA^{low-}TIM4^{+}).
CD52^{high} monocytes have increased phagocytosis capacity, elevated cytokine production potential (IL-6, TNF) and enhanced adhesion/migration behaviour, but dampen T cell activation *in vitro*. These findings reflect an alarmed innate immunity in patients with cirrhosis, ready to potentially fight pathogens from BT, while also showing activated innate immunity as the first line of pathogen defense, yet with a somehow dampened activation of adaptive immunity.
Proof of principle experiments by using transduced THP-1 cells stably expressing hCD52 confirmed *ex vivo* findings in regad to activated monocyte state and increased cytokine production.
Treatment with alemtuzumab *in vitro* dampened phagocytosis capacity and cytokine production potential of CD52^{high} monocytes as well as adhesion and migration potential, but restored T cell activation. Downregulation of innate immune function by alemtuzumab treatment is unintended in immunocompromised patients with liver cirrhosis. CD52^{high} monocytes with enhanced innate immune function might be beneficial in cirrhosis given the ehanced phagocytosis capacity and cytokine production. Stabilization or up-regulation of CD52 may represent a favourable treatment option worth exploring in order to enhance immune functions in patients with cirrhosis.
Inhibition of CD52 with alemtuzumab showed no effect on CD52^{low} monocytes and no cytotoxic effects on monocytes. Taken together, CD52 inhibition on monocytes using alemtuzumab resulted in a partly immunosuppressive monocyte state comparable to AD/ACLF.
Deciphering the mechanism initiating CD52 up- or down-regulation in cirrhosis plasma conditioning experiments were performed. Components in compensated/NAD plasma seemed to upregulate CD52 expression on monocytes, while components in healthy and AD/ACLF plasma caused downregulation. To further

investigate into single plasma components that may potentially change CD52-expression levels on monocytes, the effect of bacterial particles and TLR ligands were examined. This experimental setup also reflects the pathophysiologic concept of cirrhosis, a systemic condition where bacterial translocation is increased and bacteria/bacterial products passage into mesenteric lymph nodes and potentially into the systemic circulation. Bacterial particle treatment *in vitro* elevated CD52 expression on monocytes, while LPS or PAM3 treatment did not show clear effects.

Plasma from AD/ACLF patients had the potential to dampen the phagocytosis capacity of monocytes, indicating plasma components not only change expression levels of CD52 on monocytes, but also have the potential to change their function. It is known from literature that PLC cleaved CD52 on T cells and sCD52 is released. Plasma levels of PLC were significantly increased in AD/ACLF patients and mostly under detection limit in healthy, compensated and NAD patients. PLC plasma levels correlated negatively with CD52-surface expression and positively with sCD52 plasma levels. Furthermore, elevated PLC plasma levels were associated with an increased mortality and adverse events such as the presence of infection, ascites and HE. These findings suggest that PLC may be responsible for the cleavage of CD52 on monocytes in patients with AD/ACLF cirrhosis. PLC could be considered an alternative biomarker for immune paresis in cirrhosis and a target for future therapies aimed at stabilizing CD52 on monocytes through PLC inhibition.

Having observed AXL expression gradually increasing on circulating monocytes with cirrhosis progression, but in paralell decreaseing on KC, we examined hepatic macrophages in regard to their CD52 expression. Interestingly, lower CD52-expression on isolated macrophages was found in cirrhotic livers compared to non-cirrhotic controls. Furthermore, $CD52^{high}$ hepatic macrophages showed similar phenotypic characteristics like $CD52^{high}$ monocytes ($CD16^{high}HLA\text{-}DR^{high}CD206^{+}AXL^{+}$). Interestingly, phagocytosis capacity of hepatic

macropahges in patients with cirrhosis was dampened, but CD52^{high} hepatic macrophages showed enhanced phagocytosis capacity, similar to CD52^{high} monocytes.
These findings indicate that CD52^{high} cells found in the circulation and in the liver might potentially belong to same population. Furthermore, monocytes which have migrated show significantly higher CD52 expression compared to non-migrated cells. This gives hints that CD52^{high} hepatic macrophages with activated innate immune function might have the potential to migrate into circulation to tackle infections in cirrhosis. The hypothesis warrants verifation in future studies.

Having identified CD52 upregulation in compensated/NAD cirrhosis and a switch to low CD52 expression and elevated sCD52 concurrently with elevated PLC plasma levels, this pathway might serve as potential future immunotherapeutic target. Since inhibition of CD52 on monocytes dampened innate immune responses and most likely reflects the immunosuppressive monocyte state attributed to AD/ACLF. Hypothetically, a stabilization of CD52 on surface of monocytes might be beneficial for cirrhosis patients. Stabilization of surface CD52 might potentially prevent progression of cirrhosis to an AD/ACLF stage. Another point for intervention represents PLC, since PLC plasma levels are elevated in AD/ACLF cirrhosis patients. Inhibition of PLC might be capable to prevent CD52 surface shedding. Both concepts are worth future investigations.
Another remaing question is the mechanism behind release of PLC into plasma and how PLC expression is regulated. Since PLC is an enzyme there may be increased vesicle trafficking initiated upon unknown stimuli resulting in the increased release of PLC. Another possibility is the simple upregulation of PLC expression upon upstream signaling. It also needs to be investigated which cells express PLC in the context of cirrhosis, especially AD/ACLF.

Taken together, dysfunctional circulating AXL-expressing, MERTK-expressing and M-MDSC monocytic subsets were shown to prevail over regular monocytes

in patients with cirrhosis. Here, we demonstrate that i. Using scRNA-Seq we identified 7 clusters of circulating monocytes representing classical, non-classical, intermediate and M-MDSC subsets, corroborating previous findings and identifying novel changes in stage-specific monocyte-differentiation. ii. AXL-expressing hepatic macrophages decreased in a reversible manner along with disease progession, while AXL-expressing immuneregulatory circulating monocytes increased, as previsouly described [83]. Furthermore, we iii. identified CD52-expressing circulating monocytes and hepatic macrophages (Figure 5-1). CD52^{high} circulating monocytes increased in compensated and NAD cirrhosis compared to healthy controls, but were absent in AD/ACLF. CD52^{high} hepatic macrophages, present in physiologic conditions, were reduced in compensated, NAD and AD cirrhosis.
It was previously shown that AXL-expressing monocytes increased in circulation with cirrhosis progression in stages of compensated and NAD cirrhosis, but were absent in stage of AD/ACLF (Figure 5-1) [83]. M-MDSC were found to accumulate at compensated and NAD stages, but peaked in AD/ACLF together with the occurrence of MERTK^{+} monocytes (Figure 5-1) [68, 114]. These dysfunctional subsets prevailed over monocytes with regular immune function [68, 83, 114]. Interestingly, MERTK^{+} hepatic macrophages also increased with cirrhosis progression in parallel to MERTK+ monocytes in the circulation [114]. Here we showed that expression of AXL, expressed on almost all Kupffer cells, by contrast decreased with disease progession, while AXL^{+} monocytes in the circulation on the contrary increased. Of note, AXL-expressing macrophages also decreased in the gut tissue, as observed in the liver, but accumulated in regional lymph nodes, possibly indicating migration of AXL^{+} cells into extrahepatic and extraenteric compartments. AXL^{+} circulating monocytes were characterised by maintained phagocytic function and enhanced efferocytosis capacity [83].
Using scRNA-Seq we comprehensively deciphered the diversity of monocytes in patient at different stages of cirrhosis. We confirmed a reduction of non-classical monocytes and an increase of MERTK-expressing monocytes and M-MDSCs in the circulation of NAD patients using scRNA-Seq. Besides the subsetting into

classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical monocytes ($CD14^{+}CD16^{++}$), we provide evidence for the further partitioning of classical monocytes into 5 clusters with different function, due to distinct marker gene expression. Interestingly, inflammatory responses, phagocytosis and complement system were upregulated in compensated cirrhosis, but inflammatory responses were downregulated at the stage of NAD, indicating unfavourable innate immune response in the NAD stage of cirrhosis. Also, we for the first time described the potential relevance of the CD52-signalling pathway and its regulation on monocytes, with CD52 surface expression as a potential biomarker for intact innate immune function. Furthermore, first insights into the mechanism of CD52 up- and downregulation were presented. At stage of AD/ACLF CD52 was cleaved from circulating monocytes by high levels of PLC releasing sCD52 into the plasma. In respect of these new cognitions, modulating the AXL-GAS6 or CD52-PLC pathways could potentially restore immune responses in cirrhosis patients by preventing infectious complications, progression to AD/ACLF, and should thus be explored as targets to enhance immunity and reduce infection-related morbidity and mortality in the future.

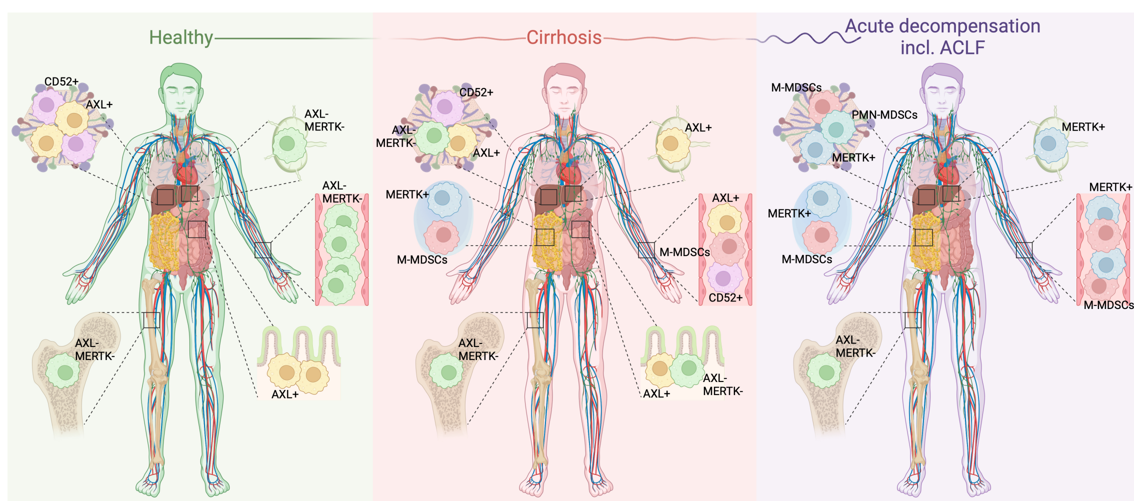


Figure 5-1: Summary
Monocyte and macrophage disease stage specific diversity in cirrhosis

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Publications

Distinct circulating monocytes up-regulate CD52 and sustain innate immune function in cirrhosis unless actue decompensation emerges
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