How TAM receptors on monocytes and macrophages regulate the immune system in liver cirrhosis

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Dekan

Dedicated to my parents

Ulrich Krawinkel and Christa Brenig

«Alles Wissen und alle Vermehrung unseres Wissens endet nicht mit einem Schlusspunkt, sondern mit Fragezeichen.»

Hermann Hesse

Summary

Infectious complications substantially contribute to the high morbidity and mortality in patients with cirrhosis. Increased susceptibility to infection has been attributed to immuneparesis and monocyte dysfunction. The underlying immune-pathophysiology and the onset of immuneparesis during cirrhosis progression remain elusive. TAM receptors (Tyro-3, AXL, MERTK), expressed predominantly on monocytes and macrophages, are important regulators of innate immune responses to microbial challenge and promote phagocytosis of apoptotic cells named efferocytosis. Monocytic-myeloid-derived suppressor cells (M-MDSCs) have been defined as $CD14^{+}CD15^{-}CD11b^{+}HLA\text{-}DR^{-}$, inhibit T cell activation and dampen immune responses in inflammatory conditions.

We sought to evaluate differential TAM receptor expression on circulatory monocytes in patients with cirrhosis in relation to monocyte function and disease severity. In addition, we investigated the function and derivation of M-MDSCs in the circulation of patients with compensated and decompensated cirrhosis. In sum, we aimed to understand how distinct monocytic subsets alter innate immune functions in patients at different stages of cirrhosis and to identify potential immunotherapeutic targets.

For this, blood specimens, peripheral blood mononuclear cells, and clinical parameters were collected from patients with cirrhosis and compared to healthy subjects. Phenotypic and functional characterisation of monocytes and M-MDSCs was assessed ex vivo using diverse techniques including flow cytometry. Models to generate and modulate different monocytic subsets were developed in vitro.

We observed an expansion of both AXL-expressing monocytes and M-MDSCs in patients with cirrhosis, increasing with disease severity. Both subsets were almost absent in healthy subjects. Numbers of M-MDSCs were increased in compensated as well as decompensated cirrhosis and substantially elevated in acutely decompensated cirrhosis and acute-on-chronic liver failure. AXL-expressing monocytes similarly expanded from early to advanced cirrhosis but were almost not present upon acute decompensation. AXL-expressing monocytes characterised as $CD14^{+}HLA\text{-}DR^{+}CD16^{+}$ cells were phenotypically different from M-MDSCs

($CD14^+CD15^-CD11b^+HLA\text{-}DR^-AXL_{low/neg}$). Functionally, these two subsets shared certain immune-suppressive properties manifested as impaired secretion of pro-inflammatory cytokines in response to lipopolysaccharide, and impairment of T cell proliferation. However, in contrast to M-MDSCs, which displayed impaired bacterial uptake, AXL-expressing monocytes showed enhanced pathogen uptake and efferocytosis. Whereas both, AXL-expressing monocytes and M-MDSCs could be generated upon exposure to specific toll-like receptor ligands and inflammatory factors in vitro, AXL up-regulation on monocytes followed also pathogen and apoptotic cell uptake. The expansion of both subsets strongly correlated with disease severity scores and indicated infection susceptibility, morbidity and mortality. Immune responses were restored by AXL inhibition and down-regulation reversing TNF-α responses and administration of poly(I:C) reducing proportions of M-MDSCs.

We hereby characterised two distinct immune-regulatory monocytic subsets which evolved with disease severity and substantially contributed to immuneparesis and may in turn explain the high susceptibility to infection and poor clinical outcome in patients with cirrhosis. Clinical associations underline their potential value as prognostic markers. Immunotherapeutic modulation of AXL-expressing monocytes and M-MDSCs may represent options which deserve future evaluation to augment immune responses and reduce infection susceptibility, morbidity and mortality in cirrhosis.

Contents

Abbreviations

ACLF	acute-on-chronic liver failure
AD	acute decompensation
AFP	alpha-fetoprotein
ALT	alanine aminotransaminase
ALF	acute liver failure
AP	alkaline phosphatase
AST	aspartate aminotransaminase
ALD	alcoholic liver disease
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
CCR	chemokine receptor
CD	cluster of differentiation
CLD	chronic liver disease;
DAMP	damage-associated molecular pattern
DC	dendritic cell
GALT	gut-associated lymphoid tissue
GAS6	growth arrest-specific 6
GGT	gamma glutamyl transferase
HBV/HCV	hepatitis B/C virus
HBeAg	hepatitis B e antigen
HCC	hepatocellular carcinoma
HLA-DR	human leukocyte antigen – DR isotype
HSC	hepatic stellate cell
HVPG	hepatic venous pressure gradient
IFNAR	interferon-α/β receptor

IFN	type I interferon
IL	interleukin
INR	international normalised ratio
KC	Kupffer cell
LC	Langerhans cell
LPS	lipopolysaccharide
MELD	model for end-stage liver disease
M-MDSC	monocytic myeloid-derived suppressor cells
MoMF	monocyte-derived macrophage
NAFLD	non-alcoholic fatty liver disease
PAMP	pathogen-associated molecular pattern
PDGF	platelet-derived growth factor
PS	phosphatidylserine
ROS	reactive oxygen species
sAXL/sMERTK	soluble AXL/MERTK
SOCS1/3	suppressor of cytokine signalling proteins 1/3
STAT1	signal transducer and activator of transcription 1
TAM receptors	Tyro-3, AXL, MERTK receptors
TGF-β	transforming growth factor β
TIPS	transjugular intrahepatic portosystemic shunt
TLR	Toll-like receptor
TNF-α	tumour necrosis factor α

1 Background

1.1 Liver cirrhosis

1.1.1 Epidemiology

Cirrhosis of the liver is a major public health problem and causes increasing rates of morbidity and mortality[1]. Cirrhosis results in 1.26 million deaths per year worldwide[2], 170'000 per year in Europe[1], and is the 11th most common cause of death globally[3].

The main aetiologies for cirrhosis in developed countries are alcohol abuse which causes alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD) with an increasing prevalence, and viral infections such as hepatitis B and C (hepatitis B/C virus, HBV/HCV)[3]. Infection with HBV is the most common reason for cirrhosis in Sub-Saharan Africa and parts of Asia[4]. Less common aetiologies are primary biliary or sclerosing cholangitis, autoimmune hepatitis, Wilson's disease, hemochromatosis and drug-induced liver injury[3].

In early stages of cirrhosis before the onset of clinical decompensation symptoms may be absent or mild. Therefore, the disease often stays undiagnosed and prevalence is difficult to assess. Estimated prevalence in France between 2006-2008 was 0.3%[5]. Cirrhosis incidence in the United Kingdom increased between 1998 and 2009 from 23.4 to 35.9 per 100'000 person years[6]. Precise numbers for Switzerland are unknown.

Therapeutic strategies in patients with cirrhosis aim to treat the underlying chronic liver disease (CLD). If the underlying disease is advanced and/or cannot be treated and reversed, the only curative therapy of cirrhosis is liver transplantation. Currently, in Europe more than 5500 liver transplants are performed per year. However, this procedure is technically challenging and ethically complex and thus, only applicable to a limited number of patients. Donor shortage is the most limiting factor[1].

1.1.2 Pathogenesis of cirrhosis

Cirrhosis has been considered to be a common final form of liver damage arising from a wide range of CLDs[7]. Hepatocyte damage through harmful agents (e.g. alcohol, viruses) results in an excessive repair process creating non-functional scar

tissue, termed fibrosis. Cirrhosis represents the end stage of fibrosis and is characterised by the formation of regenerative nodules of hepatocytes surrounded by fibrous septa (**Figure 1.1.**)[4].

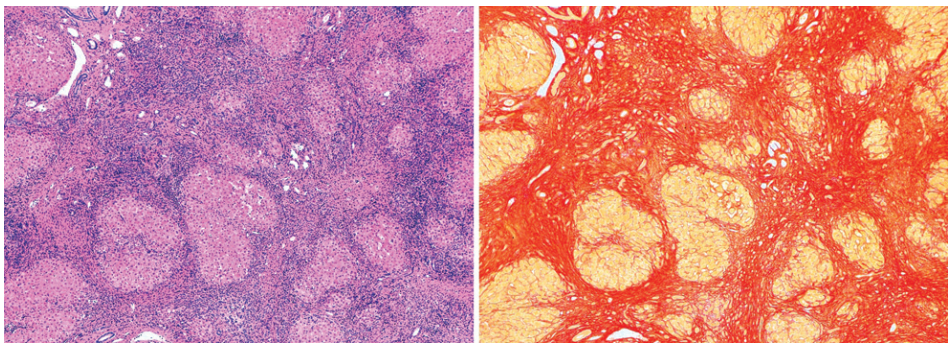

Figure 1.1: Histology of cirrhosis

Liver biopsy of a patient with alcoholic liver disease and advanced cirrhosis showing small regenerative cirrhotic nodules, thick internodular fibrous septa, and a large amount of fibrous tissue. Haematoxylin and eosin stain (left) and picro-sirius red stain (collagen fibers stained red; right). (Adapted from Tsochatzis et al., Lancet, 2014).

Fibrosis and cirrhosis are characterised by pathological systemic and intrahepatic processes such as chronic systemic inflammation, vascular and architectural hepatic adaptions that are triggered by the activation and impairment of parenchymal (i.e. hepatocytes), and non-parenchymal cells[4]: hepatic stellate cells (HSCs) get activated by diverse stimuli including inflammatory cytokines such as transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), tumour necrosis factor-alpha (TNF-α), and interleukin-one (IL-1). In CLD, activated HSCs are pivotal in the initiation and progression of fibrogenesis through the deposition of collagen and extracellular matrix[7]. Intrahepatic macrophages, which mainly consist of Kupffer cells (KCs), mediate inflammation and interact with HSCs. KC-mediated immune responses in cirrhosis are dysfunctional due to defects in different compartments (i.e. change of the intestinal barrier, pathological bacterial translocation, hepatocyte damage etc.), and are favoured by portal hypertension[7] (detailed in **Chapter 1.2.2.1**). Impaired liver sinusoidal endothelial cells and activated HSCs are involved in microvascular remodelling, formation of intrahepatic shunts, and hepatic endothelial dysfunction[4].

These functional and anatomical impairments in cirrhosis result in increased hepatic resistance to portal blood flow. Splanchnic vasodilation, being an adaptive response to intrahepatic hemodynamic alterations, further aggravates the increase of portal pressure. Together, portal hypertension and splanchnic vasodilatation cause the development of complications such as ascites, portal-hypertensive gastropathy, variceal bleeding, portosystemic shunts creating encephalopathy, and hepatorenal or hepatopulmonary syndromes[4].

1.1.3 Diagnosis

Diagnosis of cirrhosis is made using a combination of clinical, laboratory, radiologic and histological criteria. In advanced stages of cirrhosis with portal hypertension, impaired imaging by ultrasonography combined with altered parameters of liver function can be sufficient to make a diagnosis. However, to confirm the diagnosis, liver biopsy is often necessary and represents the gold standard. As mentioned above, clinical symptoms in early compensated cirrhosis are rare. In decompensated cirrhosis symptoms include ascites, infection, sepsis, variceal bleeding, encephalopathy and jaundice.

Aminotransferases (alanine-aminotransferase, ALT; aspartate-aminotransferase, AST), alkaline phosphatase (AP), gamma-glutamyl transferase (GGT), bilirubin, and albumin are typical laboratory measurements and can be assessed in routine laboratory tests. In addition, they may hint at the aetiology of cirrhosis.

Imaging methods such as ultrasonography, CT, and MRI may detect irregular and nodular liver structure, and/or splenomegaly and evidence of portosystemic shunts. Another procedure, which is performed is esophagogastroduodenoscopy as the gold standard for the diagnosis of oesophageal varices. Examination by transient elastography (FibroScan®) may non-invasively provide information about the grade of hepatic fibrosis by assessing liver stiffness[8]. Whereas FibroScan has been validated in chronic viral hepatitis, especially in hepatitis C, its clinical utility in CLDs with different aetiologies remains to be approved[9]. False high values during FibroScan suggesting increased fibrosis may be caused by multiple limitations such as operator experience, operating and disease specific conditions which increase the stiffness of the liver independent of fibrosis[9].

A liver biopsy can be taken using different approaches and is generally associated with a low, but not negligible risk of bleeding (0.6%) for the percutaneous approach[10]. The liver biopsy provides additional information about the aetiology, exclusion of accompanying liver diseases, grading of inflammation and staging of fibrosis[11]. From the practical point of view the percutaneous liver biopsy represents the easier procedure. The transjugular approach provides additional prognostic information about the hepatic venous pressure gradient (HVPG) and is advantageous in the context of acquired coagulopathy[12]. To discriminate between advanced forms of liver fibrosis and early stages of cirrhosis, a liver biopsy should be taken, since ultrasonography may lead to false-negative results[13].

Non-invasive direct and indirect biomarkers may be helpful to distinguish between little or no fibrosis and cirrhosis[14,15]. Biomarkers for staging of cirrhosis are topics of ongoing investigations[16].

Of course, other liver diseases with sometimes similar symptoms and appearance such as congenital hepatic fibrosis, nodular regenerative hyperplasia and non-cirrhotic portal hypertension, must be considered in a differential diagnosis[4].

1.1.4 Natural course and clinical presentation

Cirrhosis has previously been regarded as the final form of CLDs with varying underlying aetiologies. Nowadays it is known that cirrhosis is a dynamic condition and its clinical course is no longer considered to be unidirectional[17]. Reduction of fibrosis and regression of early forms of cirrhosis have been observed when the underlying liver diseases was successfully treated[17,18]. However, treatment of the underlying disease did not always revert or prevent complications such as oesophageal varices, decompensation, and development of hepatocellular carcinoma (HCC)[19].

Depending on the curability of the underlying disease, cirrhosis remains compensated for a variable period of time, with progression, stagnation or regression of fibrosis and portal hypertension[19]. In compensated cirrhosis the risk of decompensation is 5% per year and it is typically characterised by the presence of ascites and/or gastro-oesophageal varices with or without variceal bleeding[19]. An acute decompensation (AD) of cirrhosis is triggered by precipitating events

including infection, variceal bleeding, alcohol excess, portal-vein thrombosis or surgery[4,19].

Variceal bleeding is a major critical emergency with a mortality rate of 10-20%[19]. Bleeding risk can be significantly reduced by non-selective beta-blockers and endoscopic variceal ligation. In selected patients, early transjugular intrahepatic portosystemic shunt (TIPS) reduces the risk of recurrent variceal bleeding[20].

Development of ascites is a hallmark of decompensation and has a five-year mortality rate of 50%[21]. Ascites can potentially become refractory due to progression of the hyperdynamic circulation. Refractory ascites has a 2-year mortality rate of 65% and is another indication for TIPS in selected patients[22].

Also hepatic encephalopathy and/or jaundice occur as clinical signs of decompensated cirrhosis, are often present in advanced stages of cirrhosis and are associated with a five-year survival of 20%[23]. Similarly, renal dysfunction can occur in patients with advanced cirrhosis and manifests as acute kidney injury and hepatorenal syndrome[24].

Infections represent precipitating events for decompensation and are associated with organ failure and poor long-term prognosis[19,25]. Increased susceptibility to infection in patients with cirrhosis is assigned to immune dysfunction[26], which is discussed in detail in **Chapter 1.2.2**.

HCC develops on average in 2-8% of patients per year and is markedly more frequent in patients with oesophageal varices and decompensated cirrhosis. Median survival after HCC detection is nine months in untreated patients and approximately two years in treated patients[19].

Acute-on-chronic liver failure (ACLF) is defined as an acute decompensation of cirrhosis with consecutive development of organ failure(s), and has a high short-term mortality. ACLF is one of the most frequent causes of death in patients suffering from cirrhosis with a 28-day mortality ranging from 10% to 87%, depending on the number of organ failures. ACLF can develop in both compensated and decompensated cirrhosis[27,28]. For the diagnosis and severity of organ failure(s) (liver, renal, coagulation, cerebral, respiratory and circulatory) the

Chronic Liver Failure-Sequential Organ Failure (CLIF-SOFA) score was established[27,28].

To classify compensated as well as decompensated[29] patients with cirrhosis according to their outcome, several prognostic scores have been developed[23,30,31]. Their criteria are based on clinical parameters and complications. The Child Pugh classification is the most widely used and was established in 1964 (**Figure 1.2.A**)[31]. The Model for End-Stage Liver Disease (MELD) score is calculated using concentrations of creatinine and bilirubin, and the international normalised ratio (INR). The MELD score has been established to assess the need for transplantation and accurately predicts 3-month mortality[30]. D'Amico et al. developed a classification which defines four stages within the natural history of cirrhosis, each with distinct clinical features and a different prognosis (**Figure 1.2.B**). The classification takes into account the fact that there is no predictable sequence of such clinical states and that they may not be considered as progressive disease stages[19,23].

1.1.5 Treatment options

Therapeutic strategies in patients with cirrhosis aim to treat the underlying CLD and prevent and ameliorate complications of cirrhosis. Treatment options involve the elimination of the underlying trigger (lifestyle changes, cessation of alcohol intake, antiviral therapy, iron depletion etc.), and the management of complications by variable combinations of non-selective betablockers (reduce portal hypertension, prevent varices and variceal bleeding), antibiotics (for infections triggered by increased susceptibility to infection), diuretics (for ascites), albumin (for ascites, spontaneous bacterial peritonitis, hepatorenal syndrome), and lactulose (for hepatic encephalopathy)[4]. Novel (immunomodulatory) strategies are currently being investigated. For example, molecules targeting monocyte function may improve innate immune responses in patients with AD/ACLF in the future[32,33].

A

Child Pugh Classification

	1 Point	2 Points	3 Points
Bilirubin	< 34	34–51	> 51 µmol/L
Albumin	> 3.5	2.8–3.5	< 2.8 g/dL
INR	< 1.7	1.7–2.3	> 2.3
Ascites	None	Mild	Severe
Hepatic Encephalopathy	None	Grade I-II	Grade III-IV

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

B

Natural Cirrhosis History according to D'Amico et al.

Compensated Decompensated Death

Clinics	No varices No Ascites	Varices No Ascites	Ascites +/- Varices	Bleeding +/- Ascites
Stage	1	2	3	4
1-year mortality	1%	3.4%	20%	57%

Figure 1.2: Cirrhosis classification scores

(A) The Child Pugh Classification is the most widely used score based on clinical parameters. (B) D'Amico et al. defined a classification based on the natural history of cirrhosis. (Adapted from Pugh et al., The British Journal Of Surgery, 1973; D'Amico et al., Journal of Hepatology, 2006).

1.2 Cirrhosis-associated immunity

Immune responses play pivotal roles in the pathogenesis of cirrhosis, a condition which is not restricted to impaired liver architecture and functions but represents a systemic disease. The roles of the immune system in cirrhosis are ambiguous: it mediates hepatocyte damage and is associated with fibrogenesis through the activation of HSCs. However, cirrhosis itself results in immune dysfunctions that are characterised by inadequate immune responses to pathogens resulting in a marked susceptibility to infections[26].

1.2.1 Innate immune responses of circulatory monocytes and hepatic macrophages

In the context of immune responses to inflammation, innate immune cells have major functions in the development of CLD. Depending on disease progression and compartment, monocytes and macrophages are described to contribute in both pro- and anti-inflammatory manners to cirrhosis. Their functions have traditionally been assigned as inflammatory (M1) or anti-inflammatory (M2). This dogma is widely used but oversimplified, and more complex and versatile according to latest knowledge[34]. As an alternative, when referring to a specific condition it has been suggested to describe the origin of macrophages, definition of activators, and a consensus collection of markers[35]. Monocytes and macrophages are very plastic and adapt their phenotype according to signals derived from their microenvironment (e.g. danger signals, fatty acids, phagocytosis of cellular debris), which explains their manifold and even opposing functions in liver disease such as inflammatory, restorative, immunogenic, and tolerogenic[36,37].

Novel unbiased large-scale techniques, such as single-cell RNA sequencing allow for an in depth view of the complexity and plethora of monocyte and macrophage phenotypes[34]. Given the marked heterogeneity of monocytes and macrophages, fascinating possibilities of immunotherapeutic strategies emerge. Shaping and even reprogramming monocyte and macrophage populations towards the desired phenotype and function in order to treat liver diseases including cirrhosis might be promising options in the future.

1.2.1.1 Liver macrophages

The liver as an organ is not an isolated compartment. A major part of its blood supply coming from the gut through the portal vein contains nutrients but also potential pathogens. The liver also acts as a filtering barrier for many toxic substances transported from the circulation and is in contact with peritoneal fluids[34]. In pathophysiological conditions like CLD, these features are disturbed resulting in an ubiquitous state of liver inflammation, where hepatic macrophages have crucial roles.

Liver macrophages mainly consist of two populations including liver-resident KCs and bone marrow-derived recruited monocytes (monocyte-derived macrophages; MoMFs). Both subsets possess the hallmarks of macrophages which are phagocytosis, danger signal recognition, cytokine production, antigen processing, and orchestration of immune responses. However, they express different markers and perform distinct functions depending on their microenvironment. According to their task they can also be classified as inflammatory or pro-resolution macrophages by different surface markers (**Table 1.1.**)[34,38].

KCs are pivotal cells in homeostasis because of their ability to clear pathogens derived from the intestine[39] and to regulate the iron metabolism. Their morphology characterised by long cytoplasmic expansions and fixed positions in the liver helps with their sentinel functions[34,40]. In homeostasis, KCs are constantly renewed independently of bone marrow progenitors but can also differentiate from MoMFs in case of liver insult[34].

MoMFs play a role in both acute and chronic liver injury. As their name suggests, they derive from circulating monocytes of the bloodstream, and studies on murine models showed that they are generated from a chemokine (C-X3-C motif) receptor 1 (CX3CR1)-positive bone marrow progenitor population. In contrast to KCs, they have few cytoplasmic expansions and patrol the liver as an “emergency response team”[34].

Recently, a distinct subset of liver capsular macrophages has been described within the murine liver, a subset which differs from resident KCs and MoMFs and restricts pathogen invasion from the peritoneal cavity[41].

Myeloid population	Murine markers	Human markers	Role
Kupffer cells	$CD11b^+$ $CD68^+$ $F4/80^{++}$ $CLEC4f^+$ $TIM4^+$ $CX3CR1^-$ $TLR4^+$ $TLR9^+$ $CRIg^+$	$CD68^+$ $CD14^+$ $TLR4^+$ $CX3CR1^-$	Promote tolerance under steady-state conditions to restrict immune response against food-borne antigens; Activated during tissue damage; main source of cytokines / chemokines governing local inflammation
Monocyte-derived macrophages	$CD11b^+$ $Ly6C^{+/-}$ $F4/80^{+/-}$ $CCR2^+$ $CX3CR1^+$ $CD64^+$	$CD14^+$ $CCR2^+$ $CD16^{+/-}$	*See subsets below*
Inflammatory macrophages	$Ly6C^{high}$ $CD11b^+$ $CCR2^{++}$ $CX3CR1^+$ $iNOS^+$ TNF^+	$CD14^{++}$ $CD16^-$ $CLEC5A^+$ $S100A9^+$	Pro-inflammatory, massively recruited during liver injury; elicits tissue damage; drive fibrogenesis by maintaining inflammation and activating fibrosis effector cells; can undergo phenotypic switch to restorative macrophages
Pro-resolution macrophages	$Ly6C_{low}$ $CD11b^+$ $CCR2^+$ $CX3CR1^{++}$ $CD206^+$ $MMP9^+$ $MMP12^+$	$CD14^-$ $CD16^+$ $CD163^+$ $CCR2^+$ $CX3CR1^{++}$ $Stabilin\text{-}1^+$ $(MERTK^+)$	Anti-inflammatory; restorative function in liver fibrosis; promote tissue repair after acute damage; in humans $CD16^+$ rather linked to fibrosis progression

Table 1.1: Liver macrophage markers and their corresponding role
(Adapted from Weston et al., Front. Immunol., 2019)

1.2.1.2 Circulating monocytes

Circulating human monocytes are subdivided into three subsets according to CD14 (the co-receptor for bacterial lipopolysaccharide, LPS) and CD16 (a low affinity type III Fc receptor for IgG) expression: classical ($CD14^{++}CD16^-$), intermediate ($CD14^{++}CD16^+$), and non-classical monocytes ($CD14^+CD16^{++}$)[42]. They initiate innate immune responses by phagocytosis and killing of bacteria, inflammatory cytokine production in response to bacterial challenge, antigen presentation, and recruitment and activation of other immune effector cells. Thus, they regulate the anti-microbial defence, perpetuation and cessation of inflammation and tissue injury, liver fibrogenesis and tumorigenesis.

1.2.1.3 Compartmentalisation of monocytes and macrophages in specific disease conditions

It is believed that tissue-specific environmental triggers are needed for circulating monocytes and macrophages in order to change their phenotype and function. CX3CR1 expression has been shown to determine monocyte orientation towards inflamed or non-inflamed compartments[43]. A similar monocyte potential for transition has been shown in experiments using deuterium labelling in humans. In response to bacterial endotoxin challenge, replenishment of circulating monocytes was accomplished through a release of classical monocytes from the bone marrow which showed potential to differentiate towards intermediate and non-classical monocytes[44]. Another surface marker that has recently been shown to regulate compartmentalisation and homing of circulating monocytes back to reservoirs is CXCR4[45].

1.2.2 Immune dysfunction in cirrhosis

In cirrhosis, impaired peripheral immune responses to pathogenic challenges are summarised by the term “immuneparesis” and favour increased susceptibility to infection[46,47]. Susceptibility to infections is about 5 times more common in patients with cirrhosis than in patients without cirrhosis[48–50]. Similarly, infections account for over 50% of hospital admissions in cirrhotic patients, are the main precipitant for decompensation[27,48,49], and hereby relate to the high mortality rates ranging from 5% up to 77% depending on disease severity[19,27,48–53]. The underlying pathophysiology of immuneparesis is highly complex and heretofore incompletely understood.

1.2.2.1 Pathophysiology of immuneparesis

Processes of immuneparesis are dynamic and involve diverse immune cells and soluble factors in multiple compartments[26,54]. Monocytes, macrophages, neutrophils, lymphocytes (B cells, T cells), and natural killer cells are altered in terms of function and numbers. The same applies for levels of soluble factors including albumin, the complement system (C3, C4, CH50), coagulation factors, hormones, cytokines and administered drugs[54,55]. The complexity of the underlying pathophysiology is further enhanced by the fact that immuneparesis occurs in

different immunological compartments such as the gut, gut-associated lymphoid tissue (GALT), liver, reticuloendothelial system, circulatory blood, peritoneum, and potentially others.

Myeloid-derived cells, the main players of innate immune responses, have crucial roles when it comes to immune dysfunction in cirrhosis. Since impaired monocyte and macrophage function in different compartments (such as circulation and liver) of patients with cirrhosis is the main focus of this thesis, it will be discussed in a separate chapter (**Chapter 1.2.2.2**).

Neutrophils in cirrhosis are impaired in terms of both number and function: neutropenia can be explained by an increased apoptosis rate[56]. Impaired neutrophil function is characterised by decreased phagocytic capacity[57] as well as impaired chemotaxis and ability to migrate to sites of infection[58]. NETosis, a process which defines the formation of extracellular traps by neutrophils to enhance antibacterial defences[59], is described to be impaired in models of alcoholic liver disease and has been linked to defective efferocytotic capacity[60]. In ascites from decompensated cirrhotic patients, neutrophils show reduced phagocytic capacity and oxidative burst activity, which were partially restorable by incubation with autologous plasma[61].

Additionally, adaptive immune responses in cirrhosis are affected. Lymphopenia involves T and B cells[62,63]. B cells are found to be less frequent in cirrhosis and characterised by hyporesponsiveness to toll-like receptor (TLR)-9 activation[63]. Similarly, the number of natural killer cells in cirrhosis is decreased and linked to impaired cytotoxicity and cytokine production[64].

The liver is an important producer of various proteins such as albumin and products of the complement system (C3, C4, CH50). In CLD, serum concentrations of those soluble factors are decreased, resulting in impaired opsonization and phagocytosis of pathogens in different compartments[26,54]. Albumin, the most abundant plasma protein produced by the liver, is necessary to maintain oncotic pressure. In addition, it binds several toxic substances (LPS, reactive oxygen species [ROS]), increases their solubility, transports them to specific sites and thereby contributes to detoxification. Low albumin levels in cirrhosis are related to the development of ascites, renal failure and hepatic encephalopathy. Therefore,

research on human serum albumin is a current hot topic[55,65] and one of the supportive treatments in patients with decompensated cirrhosis[66].

Pathological bacterial translocation from the intestinal lumen to mesenteric lymph nodes and extra intestinal sites negatively modulates the immune system under the influence of pathogen- and damage-associated molecular patterns (PAMPs/DAMPs) in cirrhosis[19,26,54,67]. It is caused by an altered composition of the gut microbiota together with mechanical defects in the intestinal barrier due to epithelial inflammation and defective function of tight junctions. Additionally, porto-systemic shunting, impairments of GALT, mesenteric lymph nodes, as well as the impaired immune responses of circulatory cells described above further contribute to the development of pathological bacterial translocation[67]. The resulting repetitive stimulation by abundant bacterial products initially leads to a pro-inflammatory state in the circulation characterised by pro-inflammatory cytokines, ROS and nitric oxide. Eventually, pro-inflammatory responses switch to a tolerogenic state of chronic immuneparesis, a condition which is typical for advanced cirrhosis[26,54,67]. Pathological bacterial translocation may lead to infection in the peritoneum (called spontaneous bacterial peritonitis), which is also favoured by insufficient pathogen clearance in the context of immuneparesis.

The liver orchestrates immune responses to pathogens derived from the gut by a diverse and very versatile population of macrophages (described above in **Chapter 1.2.1.1**)[34]. Loss or impaired function of these phagocytes in cirrhosis lead to a damaged hepatic reticuloendothelial system resulting in a reduced transhepatic clearance of pathogens[40]. Sinusoidal remodelling, fibrosis driven by HSCs, and portal hypertension with portosystemic shunting further aggravate the process of impaired pathogen clearance[40]. In decompensated cirrhosis, hepatocyte death triggers production of DAMPs which leads to activation and secretion of pro-inflammatory cytokines triggering sterile hepatic inflammation[68]. Thus, the cirrhotic inflamed liver itself is a hallmark of the pathogenesis of immuneparesis.

The grade of immuneparesis is thought to parallel severity of liver disease including progression of fibrosis, portal hypertension, inflammation, and pathological bacterial translocation and increased susceptibility to infections as well as mortality rate **(Figure 1.3.)**[26].

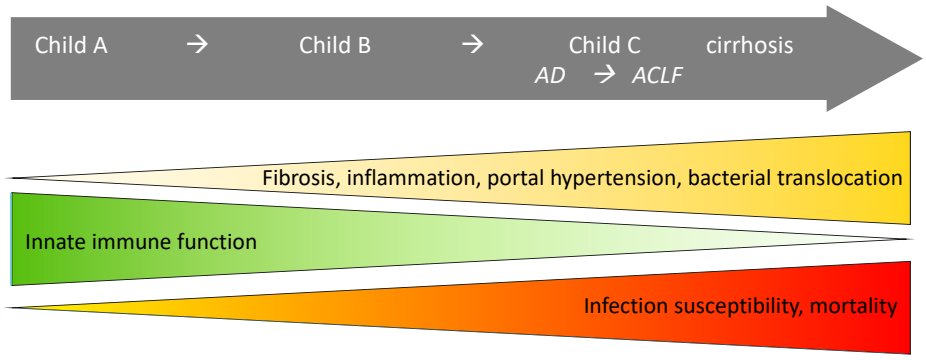

Figure 1.3: Susceptibility to infection in relation to cirrhosis progression
Progression of fibrosis, systemic and hepatic inflammation, portal hypertension and pathological bacterial translocation are paralleled by the impairment of innate immune responses, which results in a high susceptibility to infections and increased mortality rates.
AD, acute decompensation; ACLF, acute-on-chronic liver failure.

A summary of the complex processes contributing to the pathogenesis of immuneparesis is depicted in **Figure 1.4**. Due to its complexity, immuneparesis still remains incompletely understood. Deciphering the underlying mechanisms, identifying the contributing cell subsets in diverse compartments, and the identification of potential immunotherapeutic strategies are all major research topics in this field[38,69,70].

1.2.2.2 Dysfunctional monocytes contributing to immuneparesis

Given the mentioned information above, it seems obvious that systemic and hepatic injury contribute to the differentiation of the phenotype and function of monocytes and macrophages in cirrhosis. Monocyte dysfunction substantially contributes to the underlying pathophysiology of immuneparesis (**Figure 1.4.**).

Dysfunction of circulating monocytes has been described in patients with acute decompensation and liver failure (AD/ACLF) when compared to stable cirrhotics, i.e. reduced expression of HLA-DR and attenuated production of TNF-α and IL-6

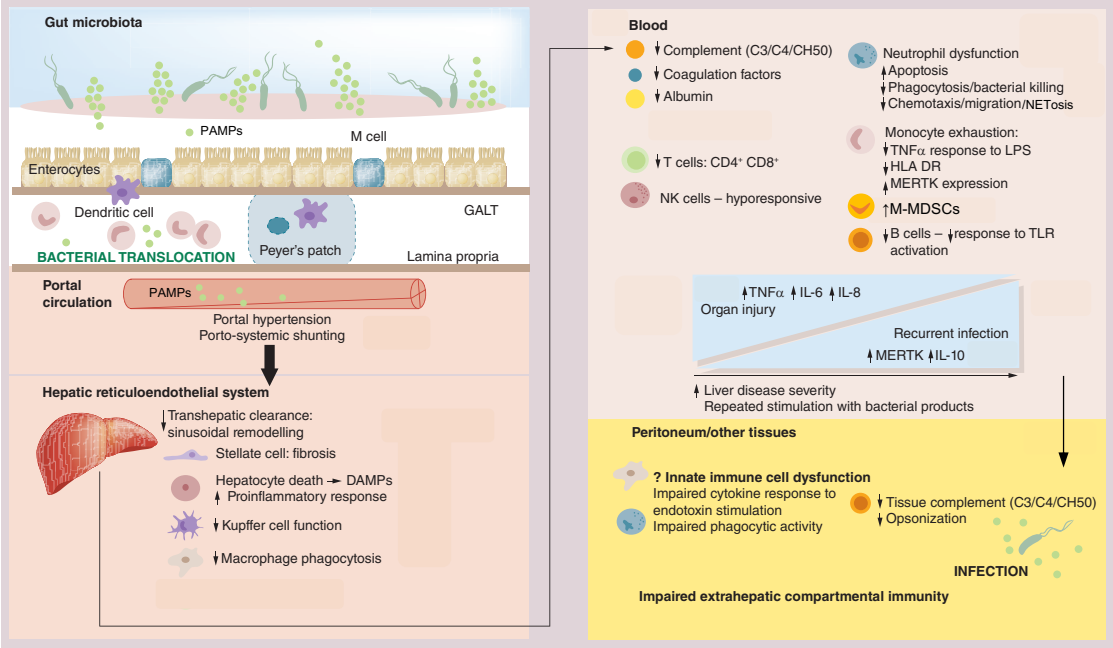

Figure 1.4: Pathogenesis of immuneparesis in cirrhosis

The underlying pathophysiology of immuneparesis involves various defective cells and soluble factors in different compartments: gut, gut-associated lymphatic tissue (GALT), portal circulation, hepatic reticuloendothelial system, circulating blood, peritoneum, and other tissues. Pathological bacterial translocation results in repetitive exposure of different compartments to bacterial products which causes a chronic hyporesponsive state of immuneparesis. (Adapted from Bernsmeier et al., Immunotherapy, 2015).

in response to LPS, and linked to adverse outcome[32,47,71,72]. The attenuation of pro-inflammatory cytokine production might be regarded as a hypo-responsive state to bacterial challenge in response to repetitive microbial exposure, a phenomenon which is termed “endotoxin tolerance”[73]. It is known as a pathophysiological adaptation to compensate excessive inflammatory responses[74].

Since susceptibility to infection is most overt in patients with AD and ACLF, our lab focused on these severe conditions and has recently contributed to the phenotypic and functional characterisation of circulating monocytes in late stages of cirrhosis (AD/ACLF). In our laboratory, we detected the expansion of a distinct MERTK-expressing monocyte subset[32] as well as monocytic-myeloid derived

suppressor cells (M-MDSCs)[33] (**Figure 1.4.**), another immune-suppressive population in patients with AD/ACLF.

MERTK belongs to a family of TAM receptor tyrosine kinases which also includes Tyro-3 and AXL. TAM receptors are important inhibitors of innate immune responses to microbial challenge and promote resolution of inflammation following tissue injury (see **Chapter 1.3**)[75,76]. Bernsmeier et al. discovered an accumulation of immune-regulatory MERTK-expressing cells in various compartments (circulation, liver, lymph nodes, peritoneum) of patients with AD/ACLF, strongly correlating with disease severity and prognosis, and at the same time with the degree of innate immune dysfunction (pro-inflammatory cytokine production in response to LPS) (see **Chapter 1.3.4.1**)[32].

It still remained unclear what mechanisms drive the initiation of susceptibility to infection and disease progression in earlier stages of cirrhosis. During progression of cirrhosis, prior to AD and liver failure, a phenotypic modulation of circulating monocytes towards pro-inflammation and pro-fibrogenesis has been proposed[77]. In parallel an increase in pathological bacterial translocation with disease severity from stage Child A to B/C has been observed[78–80].

1.3 Tyro-3/AXL/MERTK (TAM) receptor tyrosine kinase family

Receptor tyrosine kinases are transmembrane receptors. Their extracellular domain acts as a sensor for ligands, the binding of which triggers receptor dimerization and activation of the tyrosine kinase cytoplasmic domain. The consequence is the recruitment and phosphorylation of various downstream proteins, resulting in specific cellular processes such as differentiation, proliferation, growth, phagocytosis, efferocytosis, or apoptosis.

TAM receptors are expressed on monocytes, macrophages, dendritic cells (DC) and glial cells, as well as epithelial cells of the reproductive system, the retina, various tumour cells[81] and are specific to vertebrates[82]. Accordingly, their function has been described to be related to cell survival, proliferation, migration, adhesion and phagocytosis[81,83]. In myeloid cells, TAM receptor function is described in the context of tissue and immune homeostasis in response to constant cellular turnover and continuous environmental challenge resulting in billions of apoptotic

cells[81]. Specifically, TAM receptors are known as intrinsic negative regulators of innate immune responses to microbial challenge, acting by negatively regulating TLR signalling pathways and promoting phagocytic removal of apoptotic cells (efferocytosis) during resolution phases of inflammation[75,81].

1.3.1 Regulation of TAM receptor activity

Growth-arrest-specific 6 (GAS6) and protein S are reported ligands with different affinities for TAM receptors in a Vitamin K-dependent manner[84,85]. The binding affinity for GAS6 is AXL>Tyro-3>MERTK. Protein S binds and auto-phosphorylates only Tyro-3 and MERTK, but not AXL[81]. Later on, two other ligands have been described: Tubby (only binds MERTK) and tubby-like protein 1 (Tulp1) (binds all TAM receptors) in relation to phagocytosis[86], and galectin-3 (binds MERTK)[87].

These structurally closely related ligands are required as binding molecules in order to enable TAM receptors to recognize apoptotic cells by binding phosphatidylserine on their cell surface. Bridging of TAM receptor-expressing cells and apoptotic cells results in efferocytosis[81,84]. This process, as well as the molecular structure of TAM receptors are depicted in **Figure 1.5.**[81].

The source of GAS6 and Protein S is not clarified in vivo. Both ligands have been found in cell culture supernatants of DCs and macrophages, indicating that TAM receptor signalling might be, to some extent, mediated in a paracrine/autocrine manner[75].

Soluble forms of the receptors are induced upon receptor activation and proteolytic cleavage of the extracellular domain[76]. Soluble AXL and MERTK (sAXL, sMERTK) are measurable in peripheral human blood and linked to diseases and malignancies, where they are suggested to serve as biomarkers[88–90].

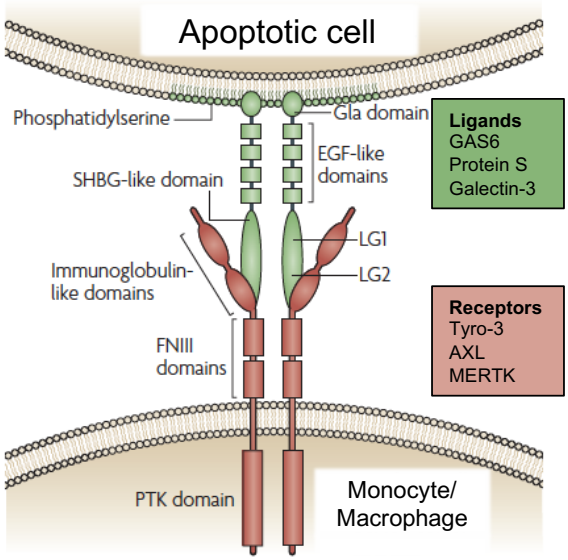

Figure 1.5: Structure of TAM receptors

TYRO3, **A**XL and **M**ERTK are receptor tyrosine kinases which belong to the family of TAM receptors. TAM receptor dimers bind to their ligands, growth-arrest-specific 6 (GAS6), protein S, and Galectin-3 through interaction between the two N-terminal immunoglobulin-like domains of the receptors and the two C-terminal laminin G (LG) regions, which together make up the SHBG (sex hormone binding globulin) domain, of the ligands. The ligands then bind to phosphatidylserine that is displayed on the extracellular surface of the plasma membranes of apoptotic cells via their N-terminal Gla domains, which in turn triggers efferocytosis.
EGF, epidermal growth factor; FNIII, fibronectin type III. (Adapted from Lemke and Rothlin, Nat. Rev. Immunol., 2008).

1.3.2 Signalling pathways of TAM receptors

The response to inflammation is a dynamic process. Innate immune responses to pathogens through myeloid cells must be rapidly triggered, but also terminated again in case of pathogen elimination, a process which must be tightly regulated. Insights in TAM receptor signalling pathways have shown that inhibition of inflammation is regulated via a negative feedback loop mediated by inhibition of TLR signalling pathways through a signal transducer and activator of transcription 1 (STAT1)- and suppressors of cytokine signalling (SOCS1/3)-dependent mechanism[75,81]. The signalling cascade is initiated by the activation of TLRs, which triggers a kinase cascade that results in a first burst of type I interferons (IFNs) and other pro-inflammatory cytokines. Inflammatory cytokines finally trigger the

transcription of TAM receptor genes. The activation of a TAM/type I interferon receptor (IFNAR)/STAT1 signalling cassette consequently induces SOCS1 and 3. SOCS 1 and 3 proteins finally inhibit TLR and cytokine receptor signalling. Monocytes/macrophages and DCs return then to baseline functions (**Figure 1.6.**)[75].

Expression and signalling pathways of respective TAM receptors are suggested to be ligand-, context-, and environment-specific. However, they are yet incompletely studied[76,76,91]. Different studies showed that AXL and MERTK expression is regulated in a reciprocal manner[76,92]. In murine bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs), both receptors acted as phagocytic mediators in vitro, whereas MERTK expression was induced by tolerogenic stimuli and induced tolerance, while AXL was induced by inflammatory stimuli and played a role in the feedback inhibition of inflammation[76].

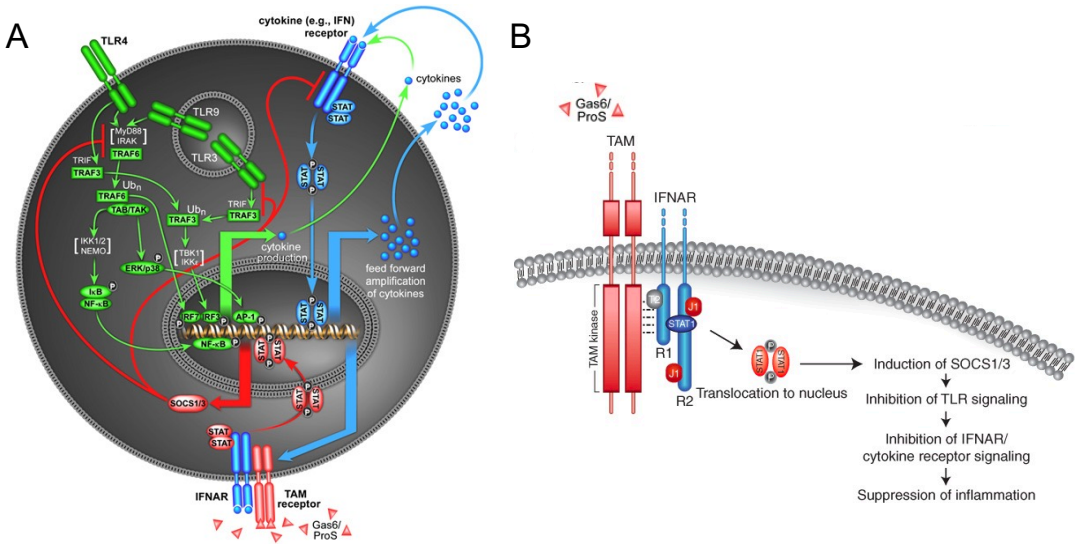

Figure 1.6: TAM receptor mediated inhibition of inflammation

(A) TAM receptors dampen innate immune responses via a negative feed-back loop of Toll-like receptor (TLR) activation. TAM receptor activation triggers the inhibition of TLR signalling pathways and type I interferon receptor (IFNAR)/cytokine signalling pathways through a signal transducer and activator of transcription 1 (STAT1)- and suppressors of cytokine signalling (SOCS1/3)-dependent mechanism. (B) TAM receptor activation follows ligand binding (GAS6, protein S) and requires physical interaction with IFNAR. (Adapted from Rothlin et al., Cell, 2007; Lemke, Cold Spring Harb Perspect Biol., 2013)

1.3.3 Distinct functions of TAM receptors in mice

Depending on the cell type and microenvironment, TAM receptor activation has distinct cellular effects. The most thoroughly described functions of TAM receptors are regulation of innate immune responses and promotion of phagocytic removal of apoptotic cells (efferocytosis), which are essential for a normal cell function. TAM receptor dysfunctions result in the accumulation of cell debris, increasing necrosis, and leads to inflammation. Evidence comes from single, double or triple TAM receptor knockout mice which develop autoimmune diseases and symptoms that are related to lack of apoptotic cell clearance and sustained inflammatory responses by monocytes and macrophages[76,81]. Whilst tyrosine kinase knockout mice often die at embryonic stage[93], TAM receptor knockout mice do not show phenotypic abnormalities, reveal no differences in peripheral lymphoid organs and in the development of both lymphoid and myeloid lineage cells at birth and several weeks after, indicating a minor role of TAM receptors during developmental stages[94,95]. Starting at 4 weeks after birth TAM receptor triple knockout mice develop splenomegaly and enlarged lymph nodes. In parallel, lymphocytes, macrophages, and DCs are observed to be constitutively activated[75,94]. Triple knockout mice eventually develop diverse autoimmune diseases, including autoimmune hepatitis[75,96]. Additionally, the lack of TAM receptors results in hyper-responsiveness to endotoxins, increased production of inflammatory cytokines by macrophages upon LPS stimulation, and impaired efferocytosis[75].

MERTK$^{-/-}$ mice show the most pronounced impairments in terms of phenotypes and functions. MERTK deficiency in mice results in lethal endotoxic shocks in response to LPS, caused by hyper-responsiveness of resident macrophages[97]. Splenomegaly and retinal dystrophy are assigned to defects in efferocytosis by phagocytes under homeostatic conditions[81]. Importantly, phagocytic defects of MERTK$^{-/-}$ mice seem to affect only the clearance of apoptotic cells (efferocytosis) and generally do not impair the uptake of bacteria, yeast, and latex spheres[98].

AXL$^{-/-}$ mice develop splenic DCs showing impaired efferocytotic capacity and have more severe forms of autoimmune encephalopathy[99,100]. Langerhans cells (LCs) are epithelial antigen presenting cells that sense danger signals and in turn trigger specific immune responses. They can arise from CD14^{++}CD16^{-} monocytes and are

specifically characterised by expression of AXL. Lack of AXL receptor on LCs leads to spontaneous skin inflammation in mice, supporting its role in efferocytosis and blocking of pro-inflammatory cytokine production[101]. Fujimori et al. nicely demonstrated the compartmentalisation of AXL expression in the lung of rodents in homeostatic macrophage differentiation; while mouse airway macrophages constitutively express AXL, interstitial macrophages do not. Lack of AXL leads to uncontrolled inflammation in response to infection with influenza virus[102].

Tyro-3$^{-/-}$ mice develop seizures caused by neuro degeneration 7 months after birth[95] and are reported to develop less synovial hyperplasia and bone damage in arthritis[103].

1.3.4 TAM receptor function in humans

Given the pivotal role of TAM receptors in the regulation of innate immune responses, different pathologic immunological conditions have been assigned with dysregulated TAM receptor activity in humans[104]. Increased sAXL and sMERTK levels have been shown in multiple sclerosis[99]. Protein S is reduced in plasma of patients with systemic lupus erythematosus, suggesting a role for reduced TAM signalling in its pathogenesis[105]. In patients with severe sepsis elevated GAS6 plasma levels could be measured[106]. Interestingly, MERTK is overexpressed on monocytes from patients with septic shock compared to trauma patients and healthy controls, and linked to poor clinical outcome. Supporting the hypothesis of differential TAM receptor expression and function, AXL is conversely overexpressed in trauma patients[107].

TAM receptors are also associated with various cancers of different organs including the lung, liver, breast, colon and skin, are linked to poor clinical outcome, but also represent promising therapeutic targets. Their underlying mechanisms in this context have been assigned to proliferation and cell survival of TAM receptor-expressing cancer cells and anti-tumoral immunity by TAM receptor-expressing immune cells[108–110].

1.3.4.1 TAM receptors associated with liver disease

Aberrant TAM receptor functions, i.e. impaired innate immune responses and defective efferocytic capacity, have been recently also described in CLDs ranging from various stages of fibrosis[111] and cirrhosis to AD and ACLF[32], as well as subsequent HCC[16,112,113]

Two recent multicentre studies suggested sAXL as biomarker for advanced fibrosis, cirrhosis and HCC. Outstanding sensitivity and specificity values calculated by receiver operating characteristic curve analysis for the detection of cirrhosis and HCC let them conclude that sAXL may outperform alpha-fetoprotein (AFP) as biomarker for HCC[16,90]. GAS6/AXL pathways have been associated with HSC activation and fibrogenesis in CLD. Collagen deposition in carbon tetrachloride treated mice was reduced by the small molecule AXL inhibitor BGB324[111].

Bernsmeier et al. discovered an accumulation of immune-regulatory MERTK-expressing cells in various compartments (circulation, liver, lymph nodes, peritoneum) of patients with AD/ACLF[32]. MERTK expression on monocytes strongly correlated with disease severity and prognosis, and at the same time with the degree of innate immune dysfunction (pro-inflammatory cytokine production in response to LPS). Inhibition of MERTK in vitro reversed the innate immune dysfunction, indicating a role for MERTK expression in AD/ACLF not only as a prognostic biomarker, but also as a potential therapeutic target[32].

MERTK-expressing cells accumulated not only in patients with AD/ACLF, but also in the circulation and the liver of patients with acute liver failure (ALF)[32] and no underlying liver disease in which they were characterised as a pro-resolution type of cell[114]. Importantly, the authors of both studies suggested MERTK as potential immunotherapeutic target to modulate innate immune responses and resolution capacities of monocytes and macrophages[32,114].

In the context of HCC (and other non-hepatic malignancies[109]) AXL and Tyro-3 overexpression have been also identified and suggested as therapeutic targets[112,113].

TAM receptors do not belong to the consensus markers for monocyte/macrophage differentiation. However, we have recently linked their expression to specific immune-regulatory functions of the corresponding immune cells indicating the state of immuneparesis and the severity of cirrhosis: in this work, we provide first evidence that in patients with cirrhosis immune-regulatory AXL-expressing monocytes accumulate in the circulatory compartment where sensing of danger signals and phagocytosis are required.

1.4 Myeloid-derived suppressor cells

MDSC are elicited under various pathological conditions where they control T cell and innate immune responses[115]. Human MDSCs are a heterogeneous population including a $CD14^{-}CD11b^{+}CD33^{+}CD15^{+}$ polymorphonuclear fraction (PMN-MDSC), and a $CD14^{+}CD15^{-}CD11b^{+}HLA\text{-}DR_{low/-}$ mononuclear fraction (M-MDSC)[116]. Circulating M-MDSCs have been identified in a wide number of hepatic and systemic inflammatory diseases and malignancies, including hepatocellular carcinoma[117,118]. Recently, it has been shown that the Hepatitis B e antigen (HBeAg) induces an expansion of M-MDSCs, which favours a chronic infection of HBV[119]. Due to their immune-suppressive function, MDSCs might also be associated with immuneparesis and may implicate susceptibility to infection in cirrhosis.

In murine models, MDSC occurred in the liver during phases of regeneration[120] and liver inflammation and protected from fibrosis[121]. The distribution of M-MDSCs in the cirrhotic liver has not been described to date. The abundance of M-MDSCs in the circulation of patients with cirrhosis and their derivation in relation to cirrhosis progression prior to the onset AD/ACLF is also unknown.

2 Aim of the thesis

While patients with AD/ACLF have a high likelihood to develop infections with subsequent death from liver failure, patients with compensated cirrhosis still retain a better prognosis. However, at a certain stage of disease progression they also become prone to infection and switch to decompensated disease upon their first infectious period. The time and circumstances under which immuneparesis occurs and infection susceptibility increases along the progression of cirrhosis and portal hypertension remain unknown.

Therefore, we aimed to detail the phenotypical and functional differentiation of monocytes over the time course of cirrhosis progression (cirrhosis at stages Child Pugh A, B, C, and AD/ACLF) contributing to the dynamic pathogenesis of immuneparesis.

- In a first part, we focused on the investigation of differential TAM receptor expression on circulating monocytes using a cohort of patients with stable and advanced cirrhosis (Child Pugh A, B, C) in the absence of acute decompensation in relation to monocyte function and disease severity. We thereby observed and characterised a distinct AXL-expressing monocyte population, which was expanded in advanced cirrhosis.
- In a second part, we sought to evaluate the function and derivation of another immune-regulatory monocytic population in compensated and decompensated cirrhosis that are circulating M-MDSCs. We aimed to explore the abundance and function of M-MDSCs in relation to infection susceptibility and disease outcome. My contribution consisted on one hand of the investigation of M-MDSCs in compensated cirrhosis and on the other hand of developing in vitro models to explore the mechanisms of their derivation.

Given that liver transplantation is still the only curative therapy for advanced cirrhosis, understanding the pathophysiology of monocyte dysfunction and immuneparesis might support the development of future immunotherapeutic approaches in compensated and decompensated cirrhosis as treatment options to prevent infectious complications, decompensation, need for transplantation and death.

3 Material and methods

Materials and methods that were used for the experiments within the manuscript and publication are listed in the respective material and method sections in **Chapter 4**.

4 Results

4.1 Expression of AXL receptor tyrosine kinase relates to monocyte dysfunction and severity of cirrhosis

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Expression of AXL receptor tyrosine kinase relates to monocyte dysfunction and severity of cirrhosis

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Infectious complications in patients with cirrhosis frequently initiate episodes of decompensation and substantially contribute to the high mortality. Mechanisms of the underlying immune-paresis remain underexplored. TAM receptors (TYRO3/AXL/MERTK) are important inhibitors of innate immune responses. To understand the pathophysiology of immuneparesis in cirrhosis, we detailed TAM receptor expression in relation to monocyte function and disease severity prior to the onset of acute decompensation. TNF-α/IL-6 responses to lipopolysaccharide were attenuated in monocytes from patients with cirrhosis (n = 96) compared with controls (n = 27) and decreased in parallel with disease severity. Concurrently, an AXL-expressing (AXL^+) monocyte population expanded. AXL^+ cells ($CD14^+CD16^{high}HLA\text{-}DR^{high}$) were characterised by attenuated TNF-α/IL-6 responses and T cell activation but enhanced efferocytosis and preserved phagocytosis of *Escherichia coli*. Their expansion correlated with disease severity, complications, infection, and 1-yr mortality. AXL^+ monocytes were generated in response to microbial products and efferocytosis in vitro. AXL kinase inhibition and down-regulation reversed attenuated monocyte inflammatory responses in cirrhosis ex vivo. AXL may thus serve as prognostic marker and deserves evaluation as immunotherapeutic target in cirrhosis.

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Introduction

Patients with cirrhosis are at increased risk of infection and consequent acute decompensation (AD) with substantially elevated morbidity and mortality (1). Compared with the overall rate of infections in hospitalised patients (5–7%), bacterial infections occur significantly more frequently in patients with cirrhosis (32–34%) (2, 3). Similarly, infections account for more than 50% of hospitalisations in cirrhotic patients, are the main precipitant for AD without and with organ failure (acute-on-chronic liver failure [ACLF]) (4, 5), and implicate a high mortality (2, 6). Infection susceptibility in cirrhosis has been attributed to a state of immuneparesis, defined by inadequate immune responses to microbial challenge (7, 8, 9).

The pathophysiology of immuneparesis in cirrhosis is highly complex and remains incompletely understood, involving diverse defects in immune cell function, including monocytes, and soluble factors in multiple compartments (7). Circulating monocytes from patients with AD and ACLF compared with stable cirrhosis demonstrated reduced expression of HLA-DR and attenuated production of TNF-α/IL-6 in response to lipopolysaccharide (LPS), which has previously been linked to adverse outcome (10, 11, 12). Moreover, the role of bacterial translocation in the pathogenesis of immune dysfunction and infection susceptibility has been highlighted (13, 14).

TAM receptors (TYRO3, AXL, and MERTK) belong to the family of receptor tyrosine kinases. Among immune cells, they are expressed on monocytes, macrophages, dendritic cells, and glial cells, and additionally on epithelial cells of the reproductive system, the retina, and tumour cells (15). TAM receptors are important regulators of innate immune homeostasis, acting by inhibition of TLR signalling pathways through a signal transducer and activator of transcription 1 (STAT1)- and suppressors of cytokine signalling (SOCS1/3)-dependent mechanism (15, 16) and by promotion of phagocytic removal of apoptotic cells (efferocytosis) (16). Their activation succeeds ligand binding (growth arrest–specific gene-6 [GAS6], PROTEIN S) and interaction with phosphatidylserine on apoptotic cells (15, 16, 17). In murine dendritic cells, activation required interaction with the type I interferon receptor (IFNAR) (16).

We recently identified the expansion of MERTK-expressing monocytes and macrophages in diverse compartments in patients with ACLF that dampened innate immune responses to microbial challenge and

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conferred disease severity and adverse outcomes (18). The expansion of MERTK-expressing monocytes and macrophages was moreover detected in acute liver failure (18) and characterised by both suppressed immune responses and enhanced efferocytic capacities (19). Another immune-suppressive population, expanded in the circulation of patients with ACLF, was monocytic myeloid–derived suppressor cells (M-MDSC) that suppressed T cell activation, innate immune responses, and pathogen uptake (20).

It is not clear when and/or under which circumstances immuneparesis and monocyte dysfunction occurs with an associated susceptibility to infection during the clinical course of cirrhosis and portal hypertension, before the onset of AD. The main emphasis of this study is to detail the expression of TAM receptors on monocytes in relation to monocyte function and disease severity of patients with cirrhosis in the absence of AD using patients with AD, chronic liver disease (CLD) without cirrhosis and healthy controls (HCs) as comparators. We hereby seek to better understand the pathophysiology of immuneparesis development in patients with cirrhosis prior to AD and identify candidates for biomarkers and future immunotherapeutic targets that may preserve innate immune responses.

Results

Patient characteristics

Patients with cirrhosis were distinguished between Child-Pugh A, B, and C and compared with AD, CLD without cirrhosis, and HC. The cohort was characterised by disease severity scores, aetiologies, and diverse clinical parameters (Tables S1 and S2). In patients with cirrhosis without AD, 1-yr mortality rate was 4.5% and rising with Child-Pugh stage: A (0%), B (5.7%), and C (11.8%). 1-yr mortality rate for those with AD was 75%, with two of eight patients dying within 28 d of enrolment. N = 9 patients deceased from cirrhosis-related complications during follow-up of 1 yr (secondary infections [n = 4], ACLF with multiorgan failure [n = 3], hepatocellular carcinoma [HCC] [n = 1], hypovolemic shock due to variceal bleeding [n = 1]), and the cause of one death was unknown. Current infections at hospital admission were seen in 62.5% of patients with AD. Within 4 wk following inclusion into the study, 5.2% of patients (Child B: 5.7%, Child C: 5.9%, and AD 25%) developed infectious complications, adapted from the definition by Bajaj et al (9). Episodes of AD developed in 10.2% of cirrhotic patients (Child B: 17.1% and Child C: 17.6%) within 4 mo following inclusion (Table S1).

Innate immune responses are impaired in patients with cirrhosis and parallel the expansion of an AXL-expressing circulating monocyte population

In patients with AD/ACLF, we recently described impaired inflammatory cytokine production of circulating monocytes to microbial challenges (18, 20). Attenuated responses were also seen in stable cirrhotic patients (18, 20). It, however, remained unknown when and to what extent circulating monocytes develop immune dysfunction over the time course of disease progression. We measured ex vivo inflammatory cytokine production upon LPS treatment of circulating monocytes from patients with cirrhosis at different stages of disease. TNF-α and IL-6 production was reduced in cirrhosis compared with HC and incrementally decreased from Child A to C, and AD but remained preserved in patients with CLD without cirrhosis (Fig 1A and B).

In parallel with increased disease severity and the decline of inflammatory cytokine production in response to LPS, we demonstrated the expansion of an AXL-expressing monocyte population ex vivo in the circulation of patients with cirrhosis (Figs 1C and S1A). The occurrence of AXL-expressing monocytes was independent of the underlying aetiology and other potential confounders (inpatient treatment, current infection, antimicrobial treatment, immunosuppressive therapy, and non-metastatic malignancies; Fig S1B and D). Within monocyte subsets, the expression of AXL was highest in but not restricted to the intermediate subset (cluster of differentiation [CD]14^{++}CD16^{+}) (Fig S2A). AXL expression on monocytes of patients with CLD without cirrhosis was low; a similar pattern was also seen in AD (Fig 1C). Other immune cells such as lymphocytes and granulocytes barely expressed AXL (Fig S2B). Longitudinal follow-up data showed an increase in AXL expression after re-compensation of AD episodes and a change in AXL expression paralleling the evolution of disease severity after 1 yr (Fig S1E and F). Recently, we described a MERTK-expressing monocyte population that was expanded in the circulation of patients with AD/ACLF (18), which was again confirmed in this cohort (Fig 1D). In CLD with and without compensated cirrhosis, however, MERTK and TYRO3 expressions were sparse (Figs 1D and E, and S1A). Circulatory plasma levels of the AXL ligand GAS6 were significantly elevated in cirrhosis compared with HC, independent of the aetiology. GAS6 increased from Child A to C and correlated with AXL-expressing monocytes (Figs 1F and S1C).

Circulating AXL-expressing monocytes in patients with advanced cirrhosis indicate diseases severity, complications, and poor outcome

We next assessed the expansion of AXL-expressing monocytes in relation to clinical parameters, disease severity scores, indicators of complications, and outcome. The proportion of AXL-expressing monocytes strongly correlated with Child-Pugh and model for end-stage liver disease (MELD) scores and the classification of cirrhosis established by D'Amico et al (21) (Fig 2A). AXL-expressing monocytes also correlated with soluble AXL (sAXL) plasma levels. sAXL was significantly elevated in cirrhosis compared with controls, independent of the underlying aetiology, correlated with Child-Pugh and MELD, and predicted the onset of AD episodes within 4 mo following study inclusion (Fig S3A–E).

AXL on monocytes predicted 1-yr mortality with a sensitivity of 80% and specificity of 79.2% for the criterion median fluorescence intensity (MFI) > 440. AXL moreover predicted the onset of AD within 4 mo following inclusion, and the development of infection over the next 4 wk. AXL was also associated with C-reactive protein (CRP) (Figs 2B and S4A, and B). Furthermore, AXL-expressing monocytes were associated with manifestations of portal hypertension (ascites, hepatic venous pressure gradient, varices, hepatic encephalopathy,

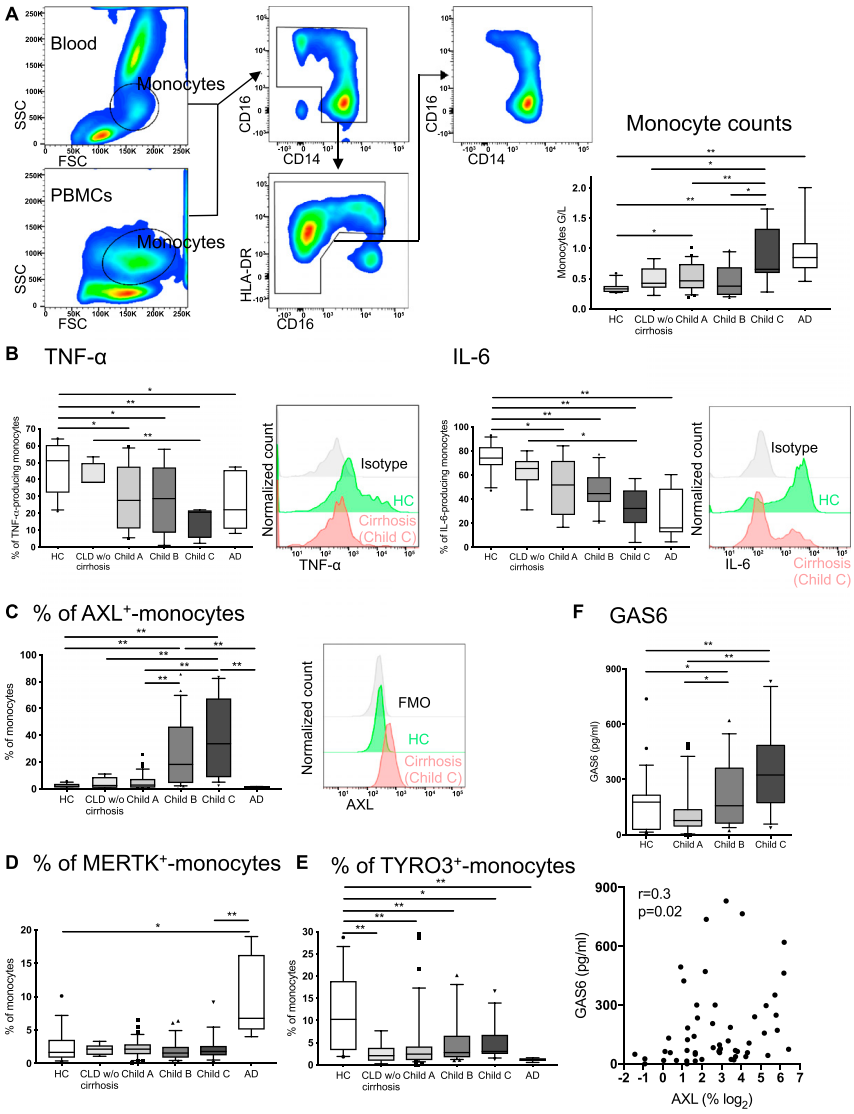

Figure 1. TAM (TYRO3, AXL, and MERTK) receptor expression and functional characterisation of circulating monocytes in cirrhosis.
(A) FACS gating strategy used to identify circulating monocytes in whole blood or PBMCs (left panel). Side scatter (SSC), forward scatter (FSC). Monocyte counts (differential leucocyte count, right panel). **(B)** TNF-α– and IL-6–producing monocytes (%) in response to LPS ex vivo at different stages of cirrhosis and representative FACS histograms (HC, Child C, and isotype). **(C, D, E)** TAM receptor expression on circulating monocytes (%) at different stages of cirrhosis and representative FACS histograms for AXL expression (HC, Child C, and fluorescence minus one). **(F)** GAS6 levels (pg/ml) in HC and cirrhosis (upper panel) and in correlation with AXL expression (% of monocytes, lower panel). HC n = 27, CLD without (w/o) cirrhosis n = 8, Child A n = 36, Child B n = 28, Child C n = 17, and acute decompensation (AD) of cirrhosis n = 8. Data are presented as box plots showing median with 10–90 percentile. *P < 0.05/**P < 0.01 (Mann–Whitney tests, Spearman correlation coefficient).

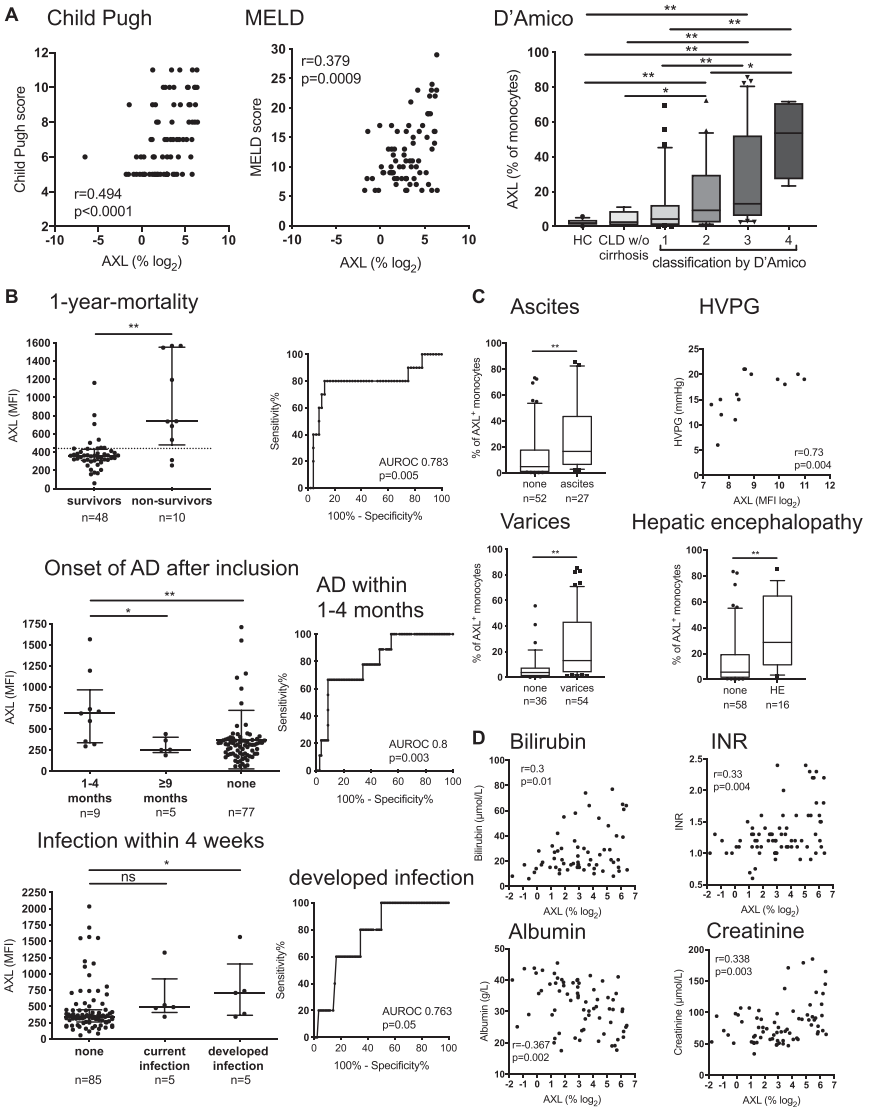

Figure 2. The AXL-expressing monocyte population in patients with cirrhosis in relation to disease severity, complications and prognosis.
(A) Correlations of AXL-expressing monocytes (%) with Child-Pugh (n = 78) and MELD (n = 73) scores and the classification by D'Amico et al (21). HC, CLD without (w/o) cirrhosis. Box plots showing median/10–90 percentile. **(B)** AXL expression predicted 1-yr mortality (criterion MFI > 440, sensitivity 80%, specificity 79.2%), development of further episodes of AD of cirrhosis within 4 mo (criterion MFI > 362, sensitivity 66.7%, specificity 67.1%), and development of infection over 4 wk (criterion MFI > 389, sensitivity 60%, specificity 65.5%). Median/interquartile range (IQR). **(C, D)** AXL-expressing monocytes in relation to portal hypertension (C: ascites, hepatic venous pressure [HVPG, n = 14], varices, hepatic encephalopathy; D: bilirubin, n = 72; INR, n = 74; albumin, n = 72; and creatinine, n = 75). Median/10–90 percentile. *$P < 0.05$/**$P < 0.01$ (Mann–Whitney tests, Spearman correlation coefficient).

and renal dysfunction) and correlated with individual parameters of liver function (bilirubin, international normalised ratio [INR], albumin) (Fig 2C and D). High AXL expression on monocytes may albeit small numbers also predict the need for transplantation, transplantation-free 1-yr survival, and development of HCC within 1 yr (Fig S4C–E).

Phenotype of circulating monocytes in patients with cirrhosis and the AXL-expressing monocyte population

Monocytes from patients with CLD without cirrhosis did not differ phenotypically from HC. Monocytes from patients with cirrhosis, however, showed an HLA-DR_{low} phenotype with decreased expression of Fcγ- and homing receptors ($CD32_{low}CX3CR1_{low}CCR7_{low}$). HLA-DR expression significantly decreased from Child A to C (Fig S5A).

Within this entire population, the expanded subset of AXL-expressing monocytes (AXL^+) (Fig 3A) were $CD14^+CD16^{high}$HLA-DR^{high} indicating a mature monocyte subpopulation with augmented expression of Fcγ-receptor CD32, TLR4, and homing/chemokine receptors (CCR5, CCR7, and CX3CR1) (Fig 3B). There was no difference in viability between AXL^+- and AXL^--monocytes (Fig 3C).

Importantly, the $CD14^+$HLA-DR^+AXL^+ immune cell subset detailed here has to be distinguished from the recently identified immunosuppressive M-MDSCs in patients with cirrhosis and ACLF (20), which we observed expanding from Child A to C in our cohort. M-MDSCs were $CD14^+CD15^-CD11b^+$HLA-$DR_{low/neg}$, as previously defined (22), and expressed lower levels of AXL in comparison with $CD14^+$HLA-DR^+ monocytes (Fig S6A–C).

AXL-expressing circulating monocytes contribute to impaired innate immune responses and suppression of T cell proliferation while retaining phagocytic capabilities for bacteria ex vivo

To investigate the effect of the expanded $CD14^+$HLA-DR^+AXL^+ subset on the impaired inflammatory cytokine responses observed in monocytes from patients with cirrhosis we assessed the functional properties of AXL^+ monocytes ex vivo.

Detailed analyses of the distinct subsets revealed that TNF-α/IL-6 production in response to LPS was decreased in both $CD14^+$HLA-DR^+AXL^+ monocytes and M-MDSCs when compared with $CD14^+$HLA-DR^+AXL^- monocytes from patients with cirrhosis and HC. In detail, TNF-α production decreased from 59.2% (13.8) to 40% (34.6) of monocytes in $CD14^+$HLA-DR^+AXL^- from HC versus $CD14^+$HLA-DR^+AXL^+ from patients (MFI: 2665 [1668] versus 1117 [2812]) and IL-6 from 73.7% (37.3) to 40% (55) (MFI: 1909 [1702] versus 408 [298]; median [interquartile range, IQR]) Figs 4A and S7A, and B). The $CD14^+$HLA-DR^+AXL^- population represented the majority of monocytes in HC, indicating it may be regarded "functionally intact" but was sequentially lost in the circulation of patients with progression of cirrhosis (Fig S6B). In line with previous data detailing TAM receptor signalling pathways (16), we observed higher mRNA levels of SOCS1/SOCS3 in monocytes of Child B/C patients, compared with HC (Fig 4B). Our data thus reveals the determination of functional roles of monocyte subsets in a pathophysiological context such as cirrhosis.

We further revealed that AXL expression on monocytes in cirrhosis was associated with inhibition of T cell proliferation, when tested in an allogeneic mixed lymphocyte reaction (Fig 4C).

Ex vivo phagocytic capacity of *Escherichia coli* (*E. coli*) bioparticles and live GFP-containing *E. coli* by circulating monocytes did not differ between cirrhotic patients and HC. AXL^+ monocytes showed preserved phagocytosis capacities for *E. coli* bioparticles and live *E. coli* bacteria, whereas M-MDSCs revealed reduced phagocytosis, when compared with $CD14^+$HLA-DR^+AXL^- monocytes from patients with cirrhosis and HC (Figs 4D and S7C).

Considering these observations, the expanded $CD14^+$HLA-DR^+AXL^+ monocyte population in the circulation of patients with cirrhosis (notably, not existing in healthy subjects) remained functionally phagocytic, but prevented T cell proliferation and inflammation (low TNF-α/IL-6 production) in a presumably SOCS1/3-dependent manner, representing an immune-regulatory "homeostatic" monocyte population expanding during cirrhosis progression.

AXL overexpression in THP-1 cells attenuates LPS-induced inflammatory cytokine production in vitro

As proof-of-concept for the observations developed above, in vitro, we overexpressed AXL in the monocytic THP-1 cell line using a retroviral system (Fig 5A). Following transduction, AXL mRNA expression (2.2 ± 0.3-fold; Fig S8A) and protein levels (88% THP-1-AXL^+ cells; Fig 5A and B) were increased. Phenotypic characterisation of the THP-1-AXL^+ model cell line is illustrated in Fig S8B. Consistent with the observations in patients with cirrhosis ex vivo, AXL-expressing THP-1 cells produced less TNF-α and IL-6 in response to LPS when compared with non-transduced THP-1 cells (Fig 5C).

Pathogen-associated molecular patterns (PAMPs), cytokines, bacterial uptake, and efferocytosis induce AXL up-regulation on monocytes

Next, we sought to understand the mechanisms leading to the expansion of the described immune-regulatory AXL-expressing monocyte population. Pathophysiologically, cirrhosis progression involves development of portal hypertension and subsequent pathologic bacterial translocation facilitates microbial products accessing the systemic circulation (8, 13, 14, 23). Hence, we tested PAMPs and damage-associated molecular patterns (DAMPs) for their ability to modify monocyte differentiation and AXL expression in vitro. Stimulation with bacterial products such as TLR ligands (Pam3SK4, LPS, CpG, and poly (I:C)) significantly up-regulated AXL expression in vitro. Similarly, pro-inflammatory factors (IFN-α and TNF-α) induced AXL up-regulation. In contrast, the DAMP high-mobility group protein B1 (HMGB1), TGF-β, and AXL ligand GAS6 did not induce AXL expression. LPS-induced up-regulation of AXL was time dependent (Fig 6A), and those monocytes produced significantly less TNF-α/IL-6 upon LPS when compared with monocytes without prior LPS exposure (Fig S9A). Notably, monocytes incubated with 25% plasma of cirrhosis patients did not show biologically relevant changes in AXL expression (Fig 6B), suggesting that additional factors are required to generate this subset in vivo.

Phagocytosis is required for efficient clearance of pathogenic microorganisms and initiation of various immune responses. AXL^+

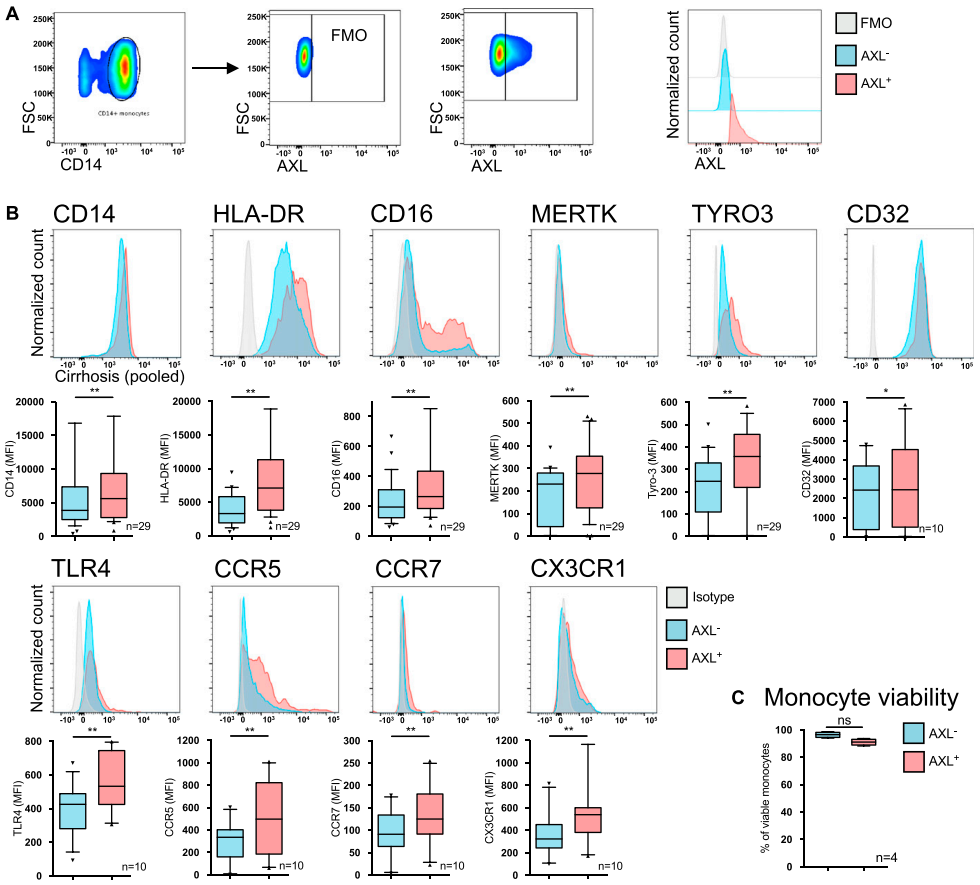

Figure 3. Phenotypic characterisation of the AXL-expressing monocyte subset.
(A) Gating strategy with representative FACS scatter plots and histograms for AXL expression used to distinguish AXL-expressing (AXL^+) from AXL-negative (AXL^-) monocytes. Side scatter (SSC), forward scatter (FSC), fluorescence minus one. **(B)** Immunophenotyping of AXL^+ and AXL^- monocytes in cirrhosis. Glycoprotein CD14, MHC class II receptor HLA-DR, Fcγ-receptors (CD16 and CD32), TAM receptors (MERTK and TYRO3), chemokine receptors (CX3CR1, CCR5, and CCR7), and TLR4. Box plots showing median/10–90 percentile. **(C)** Viability (7-$AAD^-AnnexinV^-$-cells) of AXL^+-/AXL^--monocytes. Median/10–90 percentile. $^*P < 0.05$/$^{**}P < 0.01$ (Wilcoxon tests).

monocytes exhibited preserved phagocytosis capacities (Figs 4D and S7C) and AXL expression significantly increased on monocytes after phagocytosis of *E. coli* and *Staphylococcus aureus* (*S. aureus*) bioparticles as well as of live GFP-*E. coli* bacteria (Fig 6C). Phagocytosis capacity of *E. coli* bioparticles positively correlated with the degree of AXL expression and concurrently negatively with TNF-α production (Fig S7D–E).

Within inflammatory milieus, as prevalent in different compartments of patients with cirrhosis (8), efferocytosis is required to maintain immune homeostasis (24, 25). We, therefore, co-cultured healthy monocytes with neutrophils and HepG2 cells previously labelled with a cytoplasmic cell-tracker and then induced to apoptosis (19, 26). AXL expression on monocytes was up-regulated following co-culture with apoptotic cells. Monocytes that engulfed apoptotic cells (efferocytosing) were characterised by higher AXL expression, compared with monocytes that did not (resting). AXL^+ monocytes showed higher efferocytosis capacity than AXL^- monocytes when co-cultured with apoptotic neutrophils (Fig 6D); following efferocytosis of neutrophils, AXL^+ monocytes produced less TNF-α/IL-6 upon LPS than AXL^- monocytes (Fig S9B), supporting the

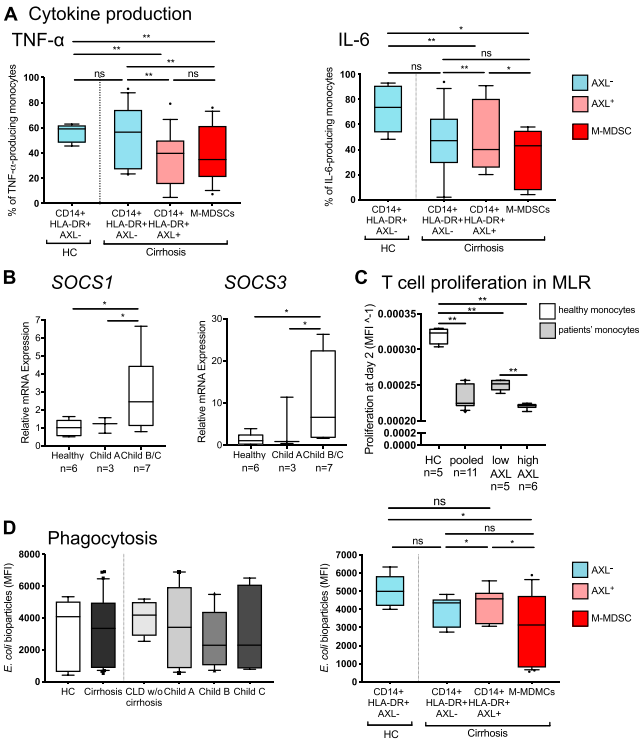

Figure 4. Functional characterisation of AXL-expressing circulating monocytes ex vivo.
(A) TNF-α and IL-6 production upon LPS treatment of $CD14^+HLA\text{-}DR^+AXL^+$, $CD14^+HLA\text{-}DR^+AXL^-$ monocytes and M-MDSCs from HC and patients with cirrhosis (% of monocytes). **(B)** SOCS1/3 mRNA-expression of monocytes from HC and cirrhosis. **(C)** T cell proliferation in co-culture with monocytes at day 2 in a mixed lymphocyte reaction (HC versus cirrhosis; AXL_{low} versus AXL^{high}). Data shown as MFI^{-1} of carboxyfluorescein succinimidyl ester. **(D)** Phagocytosis of *E. coli* bioparticles of the entire monocyte population from different patient groups (HC, CLD without [w/o] cirrhosis, cirrhosis, left panel) and $CD14^+HLA\text{-}DR^+AXL^+$, $CD14^+HLA\text{-}DR^+AXL^-$ subsets, and M-MDSCs from HC and patients with cirrhosis. Box plots showing median/10–90 percentile. $^*P < 0.05$/$^{**}P < 0.01$ (Mann–Whitney, Wilcoxon tests).

accumulation of AXL^+ immune-regulatory monocytes in an inflammatory environment.

AXL inhibitor BGB324 and metformin restore innate immune responses of monocytes from patients with cirrhosis ex vivo

Given the distinct immune-regulatory functions of the AXL^+ monocyte population in patients with cirrhosis and its association with disease severity and infection, we questioned whether inhibition or down-regulation of AXL would reverse the anti-inflammatory properties. BGB324 is a selective small molecule inhibitor of AXL previously tested in clinical studies (27). Metformin, a well-known antidiabetic drug, was previously described to down-regulate AXL expression (28) and to regulate the AXL signalling cascade in the context of cancer (29, 30). Metformin-induced down-regulation of AXL was confirmed in monocytes from patients with cirrhosis ex vivo here. Treatment with BGB324 did not affect AXL expression (Figs 7A and S10A). Both, BGB324 (1 μM) and metformin (10 mM) treatment restored LPS-induced TNF-α production of monocytes from patients with cirrhosis ex vivo. When comparing AXL^+ with AXL^- monocyte populations from patients with cirrhosis following metformin treatment, cytokine production was enhanced in AXL^+ but not AXL^- cells (Fig 7B). Viability of monocytes after metformin treatment was marginally reduced (Fig S10B). Whereas phagocytosis capacity of *E. coli* bioparticles was preserved after BGB324 administration, it decreased upon metformin treatment (Fig 7C).

Discussion

In this work, we detail the characteristics of circulating monocytes in patients suffering from cirrhosis at different stages of disease without signs of AD. We newly describe the expansion of an AXL-expressing immune-regulatory monocyte subset ($CD14^+HLA\text{-}DR^+AXL^+$) along cirrhosis progression and its close association with disease severity, infection susceptibility, development of AD, and prognosis. The AXL-expressing monocyte generation was linked to the abundance of PAMPs and cytokines, phagocytosis, and efferocytosis in the context of recurrent inflammation. Our findings substantially add to the understanding of the pathophysiology of immuneparesis in

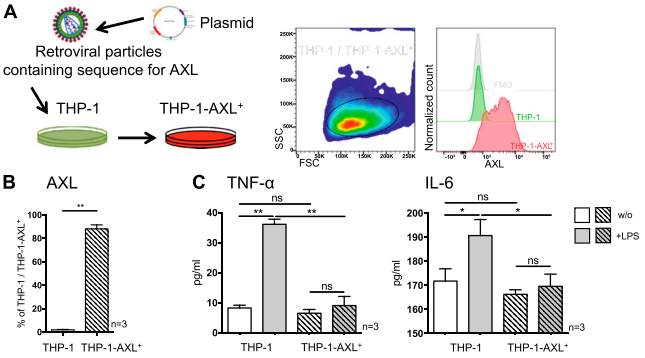

Figure 5. AXL overexpression in THP-1 cells and LPS-induced inflammatory cytokine production in vitro.
(A) Schematic model of retroviral transduction of THP-1 cells and representative FACS histogram of AXL expression in THP-1-AXL$^+$ cells. Side scatter (SSC), forward scatter (FSC). **(B)** AXL expression in THP-1 cells and AXL-expressing THP-1 cells (%). **(C)** TNF-α and IL-6 secretion (pg/ml) in response to LPS in THP-1-AXL$^+$ and THP-1 cells. Bar plots showing mean/SD. $*P < 0.05$/$**P < 0.01$ (*t* tests).

cirrhosis and identify a potential biomarker and immunotherapeutic target.

Although we and others have previously focused on impaired innate immune responses after the onset of AD/ACLF, when infection susceptibility is highest (10, 12, 18, 20), it is barely known when and under which circumstances innate immune dysfunction occurs and infection susceptibility emerges during progression of cirrhosis and portal hypertension. Previous studies addressing phenotype and function of classical (CD14$^+$CD16$^-$) and nonclassical (CD14$^+$CD16$^+$) monocyte subsets in CLD showed inconsistent data regarding cytokine production and phagocytosis (31, 32, 33). We previously showed that inflammatory cytokine production was depressed not only in AD/ACLF but also in stable cirrhosis (18), whereas the underlying mechanism remained unexplained.

Here, we demonstrate the accumulation of circulating CD14$^+$HLA-DR$^+$AXL$^+$ monocytes with attenuated innate immune functions, that is, decreased inflammatory cytokine production (TNF-α/IL-6) and T cell activation, along disease progression of cirrhosis in compensated and chronically decompensated patients. Although dysfunctional monocytes were rarely encountered in Child A, their number substantially expanded in advanced stages (Child B/C) and displaced functionally intact monocytes. The findings were irrespective of the underlying aetiology. Monocyte functions were preserved in patients with CLD without cirrhosis (F ≤ 3). Follow-up data of individual patients showed an evolution of AXL-expressing monocytes in parallel with disease severity scores. However, this requires further evaluation in larger prospective longitudinal studies.

AXL is a member of TAM receptors that mainly function as inhibitors of TLR- and cytokine receptor–mediated monocyte/macrophage activation and promoters of apoptotic cell removal (15, 16). Loss of AXL expression on antigen-presenting cells has been linked to autoimmunity (15). TAM receptors are differentially expressed and exhibit distinct expression, regulation, and activity under specific conditions (34, 35). Although CD14$^+$HLA-DR$^+$AXL$^+$ cells accumulated with worsening stages of cirrhosis, they disappeared upon acute hepatic decompensation. By contrast, CD14$^+$HLA-DR$^+$MERTK$^+$ monocytes remained undetectable in stable cirrhosis and emerged upon AD (18). In addition, CD14$^+$HLA-DR$^+$MERTK$^+$ cells were abundant in the circulation and the liver in acute liver failure where they were characterised as resolution-type monocytes/macrophages (19). This underlines the distinct and counter-regulatory roles of AXL and MERTK in different phases of cirrhosis and inflammation. Our findings in a human disease verify previous murine data that identified differential and reciprocal expression and function between AXL and MERTK on BMDMs/BMDCs (34). The authors described both receptors as phagocytic mediators in vitro, whereas MERTK expression was induced by tolerogenic stimuli and induced tolerance, and AXL was induced by inflammatory stimuli and acted in the feedback inhibition of inflammation (34). The underlying differential signalling mechanisms need to be addressed in future investigations.

Extensive characterisation of the AXL-expressing monocyte population revealed an immune-regulatory subset that emerged in the circulation likely to maintain immune homoeostasis despite rising inflammatory signals during progression of cirrhosis. AXL-expressing monocytes were characterised by increased HLA-DR, CD16 and chemokine-receptor expression, enhanced clearance of apoptotic cells, increased expression of Fcγ-receptor, and preserved phagocytosis of *E. coli*, but attenuated T cell activation and secretion of pro-inflammatory cytokines (TNF-α/IL-6) after microbial challenge presumably in a SOCS1/3-dependent manner, as previously proposed (16). Although phagocytosis of pathogens represents the first line of defence when an organism encounters microbes, clearance of apoptotic cells is required for immune homeostasis during inflammation. Our findings suggest that AXL-expressing monocytes may expand during cirrhosis and progressive portal hypertension in response to the uptake of pathogens and bacterial products in the setting of pathologic bacterial translocation (8, 13, 14, 23), and to clear apoptotic cell debris accumulating in response to chronic inflammation (15, 16). Concurrently, excessive systemic inflammatory responses are inhibited.

Similar to our findings, a recent study described AXL-expressing murine airway macrophages at homeostatic conditions, which increased after influenza infection, thereby preventing excessive tissue inflammation through efferocytosis (35). AXL expression was critical for functional compartmentalisation, as it was not present on interstitial lung macrophages (35). We propose that in cirrhosis,

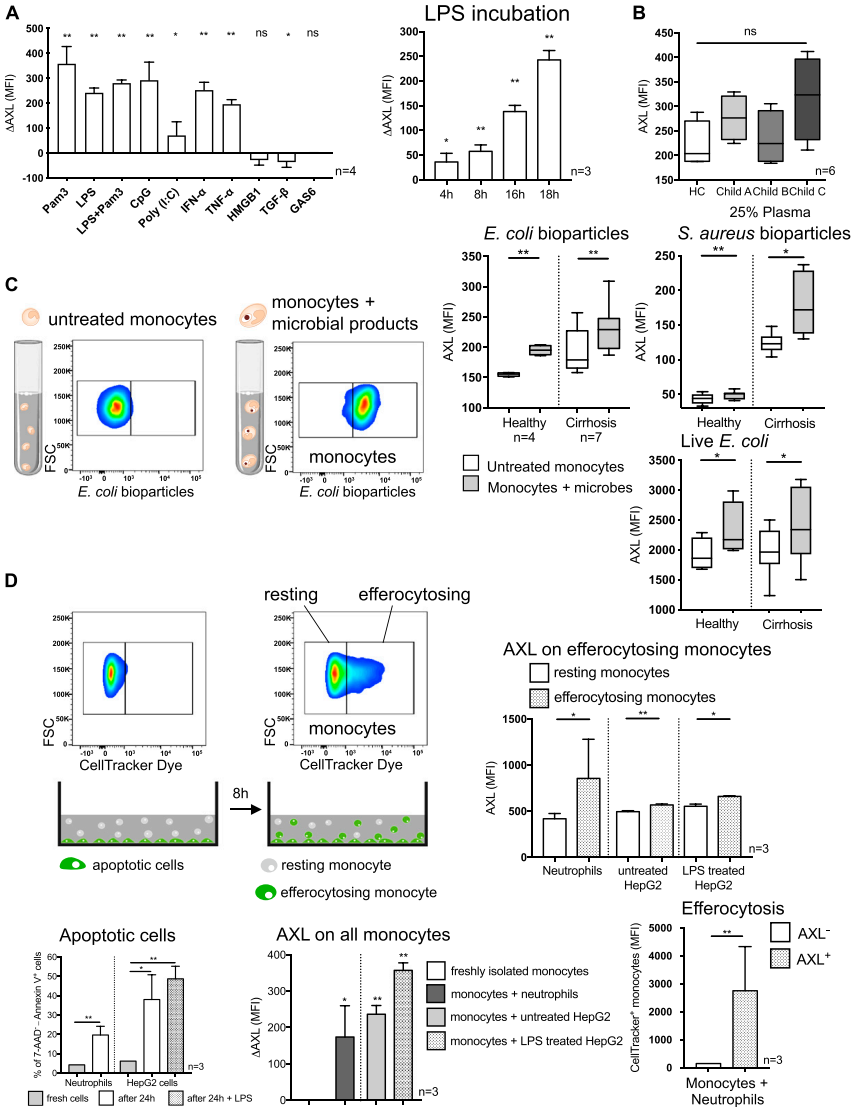

Figure 6. AXL expression on monocytes in response to bacterial and inflammatory stimuli and following phagocytosis and efferocytosis.
(A) AXL expression after incubation with bacterial/inflammatory stimuli as indicated in vitro for 18 h. Time-dependent effect of LPS on AXL expression. Delta AXL (ΔAXL) MFI shows difference to untreated cells. Bar plots showing mean/SD (*t* tests). **(B)** AXL expression after monocyte incubation in 25% plasma of HCs and patients with cirrhosis for 24 h. **(C)** Representative FACS scatter plots for monocyte phagocytosis of microbial products ex vivo. Forward scatter (FSC). AXL expression after *E. coli* and *S. aureus* bioparticle uptake (15 min) and live GFP-*E. coli* ingestion (60 min) on monocytes from HC and patients with cirrhosis. Box plots showing median/10–90 percentile (Mann–Whitney tests). **(D)** Representative FACS scatter plots for resting (CellTracker$^-$) and efferocytosing (CellTracker$^+$) monocytes after

AXL may be operative in settings where the nature of injury is driven by excessive pro-inflammatory responses, as present in diverse underlying aetiologies.

This newly described immune-regulatory, AXL-expressing monocyte population must be clearly distinguished from another recently discovered immunosuppressive cell subset that accumulated in the circulation in stages of AD: M-MDSCs were characterised by suppression of T cell activation, pathogen uptake, and TLR-elicited pro-inflammatory responses to microbial challenge (20). Here, we observed that M-MDSCs started to emerge also in patients with cirrhosis without signs of AD. Considering the reduced inflammatory responses to microbial challenge of both $CD14^+HLA\text{-}DR^+AXL^+$- and M-MDSCs compared with functionally intact $CD14^+HLA\text{-}DR^+AXL^-$ cells in relation to their abundance in the circulation, we propose that these populations together largely explain the depressed innate immune responses of the entire monocytic population at these stages.

Having observed AXL up-regulation on circulatory monocytes in relation to portal hypertension, we hypothesized an underlying mechanism involving pathologic bacterial translocation leading to the abundance of bacterial products, PAMPs (8, 13, 14, 23), and subsequent chronic systemic inflammation (8, 23). At the same time, chronic liver injury leads to release of DAMPs (36). Indeed, we were able to generate AXL-expressing monocytes with dampened innate immune responses by stimulation with selected TLR ligands and pro-inflammatory factors in vitro. These findings coincide with previous data showing AXL up-regulation on murine BMDMs (34, 35) and peritoneal macrophages (35) upon stimulation with inflammatory stimuli (34, 35). In contrast to M-MDSCs, generated in vitro by culturing monocytes in ACLF plasma (20), inflammatory factors in plasma of patients with cirrhosis were necessary, but insufficient to induce AXL up-regulation alone. Our data support the hypothesis that efferocytosis and phagocytosis of bacteria in the circulation are further required to enhance AXL up-regulation on monocytes. The stimulatory effect of pathogen uptake on AXL expression is novel and may explain high AXL expression on circulating monocytes in conditions where pathogens and their products become abundant because of pathologic bacterial translocation such as cirrhosis. TAM receptor activation after efferocytosis had previously been shown on murine BMDMs/BMDCs (34).

Dissecting the complexity of differential monocyte differentiation and activation of effector pathways of particular TAM receptors on monocytes at different stages of cirrhosis will be subject to future investigations, including the use of unbiased large-scale techniques. In a multisystem disorder such as cirrhosis, additional compartments such as the liver, but also the gut, the portal circulation, the peritoneum, and potentially others and their tissue-specific immune systems play crucial roles in the pathophysiology of the underlying immuneparesis. It is the aim of our subsequent investigations to detail the differentiation and immune function of tissue-specific myeloid cells in these compartments, in particular in respect to the immune-regulatory role of TAM receptors.

Moreover, by ex vivo proof of principle experiments treating monocytes from cirrhotic patients with the highly specific AXL inhibitor BGB324 and metformin, which was previously described to target and down-regulate AXL (28), innate immune responses were significantly enhanced, suggesting AXL as potential immunotherapeutic target to augment defence against infections. Whereas BGB324 did not negatively affect phagocytic capabilities, metformin did.

BGB324 was originally developed for cancer treatment and is currently tested in clinical Phase Ib/II trials for patients with aggressive and metastatic cancers (37). Interestingly, other studies have examined BGB324 as an anti-fibrotic agent. GAS6/AXL pathways were associated with fibrogenesis in CLD (38) and idiopathic pulmonary fibrosis (39), respectively, and were reversed by BGB324. Multi-tyrosine kinase inhibitors, including AXL, are known for their diverse antitumour effects and are tested in phase III clinical trials for advanced HCC (40). Distinct AXL blockage impacts on tumour progression through immune surveillance by AXL-expressing immune cells and anti-proliferative effect on AXL-expressing tumour cells (37, 40). AXL inhibition by BGB324 may, thus, represent a promising concept with anti-fibrotic, immune-stimulatory, and also anti-tumour effects.

Whereas previous studies described anti-inflammatory, presumably AXL-independent properties of metformin on myeloid cells (41), we observed enhanced immune responses of AXL-expressing monocytes from cirrhotic patients after metformin treatment. Metformin, conventionally used as anti-diabetic drug, exerts various pleiotropic effects acting via diverse downstream signalling pathways (42) and has been reported to be associated with reduced HCC incidence (43) and reduced portal hypertension in cirrhosis models (44). Further studies hint at a potential regulatory effect of metformin on the AXL cascade in the context of cancer (29, 30). As an inexpensive, well-established drug, metformin may represent an interesting immunomodulatory treatment option for patients with cirrhosis and no signs of AD, when AXL-expressing monocytes are frequent and the risk for metformin-associated lactic acidosis is low. Our data are suggestive to further investigate the potential significance of metformin in this context and its underlying signalling mechanism.

As these substances were only tested ex vivo here, subsequent in vivo studies in rodent models are required to systematically investigate target- and off-target effects such as auto-immunity or uncontrolled inflammation. We showed previously that inhibition of MERTK on monocytes of AD/ACLF patients reversed innate immune dysfunction (18). Given the distinct and reciprocal expression profiles of AXL and MERTK in cirrhosis, it further needs to be addressed which receptor to target at which stage of disease and in which compartment.

Finally, strong correlations of AXL expression on monocytes with disease severity and prognosis, that is, i.e. development of infection, episodes of AD, and 1-yr mortality underline its clinical significance. Two recent studies suggested sAXL as a serum biomarker for advanced liver fibrosis, cirrhosis, and HCC (45, 46). Here, we observed strong correlations of AXL-expressing monocytes with

co-culture with apoptotic cells for 8 h. Apoptosis of neutrophils and HepG2 cells after 24 h. AXL expression after efferocytosis, AXL expression of resting and efferocytosing monocytes, and efferocytosis capacity for neutrophils of AXL^+-/AXL^--monocytes. Bar plots showing mean/SD. *P < 0.05/**P < 0.01 (unpaired/paired t tests).

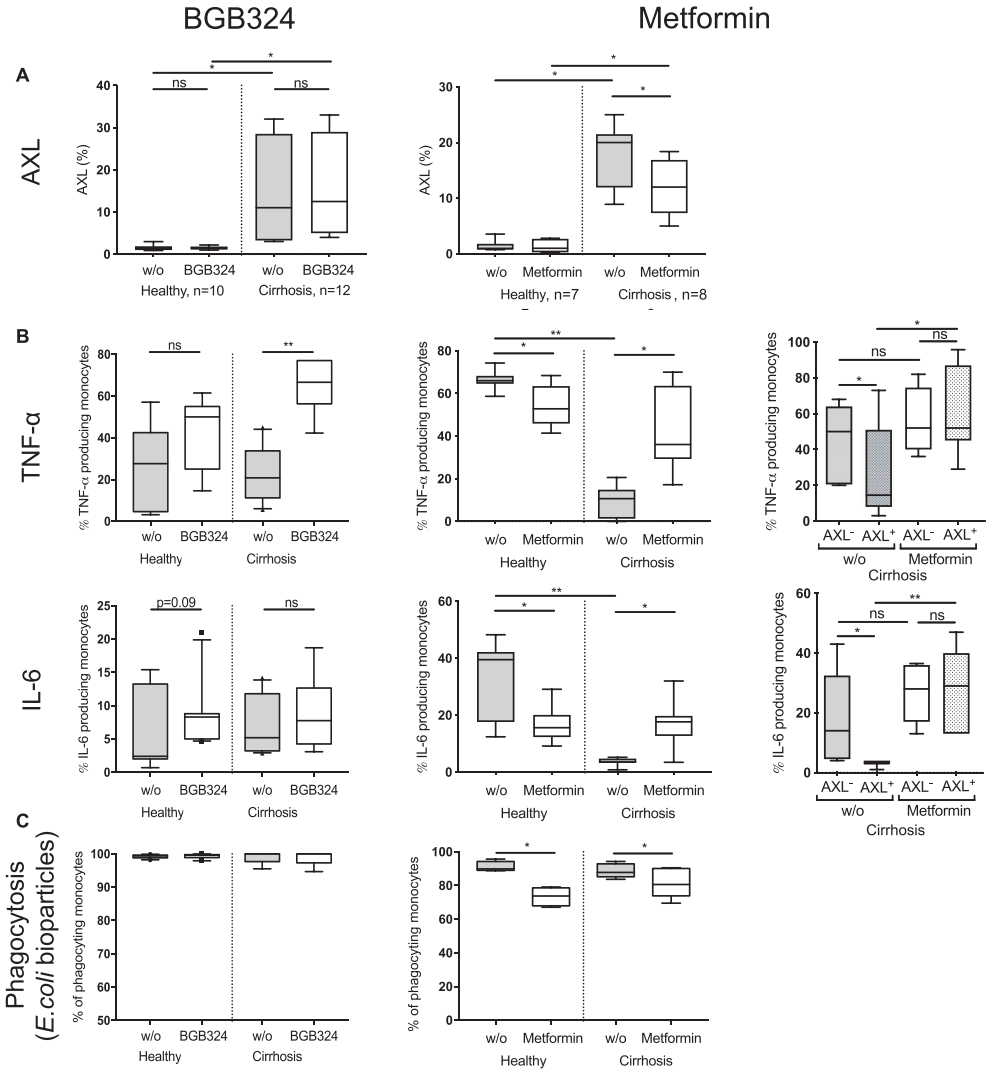

Figure 7. Innate immune responses and phagocytosis capacity of monocytes from patients with cirrhosis after AXL inhibition and down-regulation ex vivo.
(A, B, C) AXL expression (% of monocytes), (B) TNF-α and IL-6 production in response to LPS (total monocyte population and AXL⁺/AXL⁻-cells), and (C) monocyte phagocytosis capacity of *E. coli* bioparticles (%CD14⁺ cells) after small molecule inhibitor BGB324 and metformin treatment compared with untreated cells (w/o) in HCs and patients with cirrhosis. Box plots showing median/10–90 percentile. *P < 0.05/**P < 0.01 (Mann–Whitney, Wilcoxon tests).

the shed receptor sAXL and also liver disease severity scores. Based on our findings, the number of AXL-expressing monocytes in blood count may represent a prognostic biomarker for immuneparesis and cirrhosis and validates further evaluation.

In conclusion, the number of AXL-expressing immune-regulatory monocytes in the circulation of patients with cirrhosis indicated disease severity, immuneparesis, infection susceptibility, AD, and mortality. $CD14^+HLA\text{-}DR^+AXL^+$ monocytes were expanded upon PAMP and cytokine exposure, pathogen,- and apoptotic cell uptake and hallmarked by preserved phagocytosis and enhanced efferocytosis but reduced cytokine production and T cell activation, implying a role in immune homeostasis in a condition defined by pathologic bacterial translocation and recurrent inflammation. Immunotherapeutic modulation of AXL may represent an option deserving evaluation to augment immune responses and reduce infection susceptibility, morbidity, and mortality in cirrhosis.

Materials and Methods

Patients and sampling

A cohort of 96 patients with cirrhosis was identified at the Cantonal Hospital St. Gallen and the University Hospital Basel, Switzerland, between January 2016 and May 2019. Patients were recruited during consultations (Child-Pugh A [n = 36], B [n = 35], C [n = 17]), respectively, categorised according to Child-Pugh and European Association for the Study of the Liver - Chronic Liver Failure (EASL-CLIF) Consortium scores (47). We included HC (n = 27), patients with CLD without cirrhosis (n = 8; Metavir F ≤ 3), and patients with AD within 24 h following hospital admission (n = 8) as comparators. Healthy volunteers from the regions St. Gallen and Basel, Switzerland, were matched by age and sex and served as HCs. Patients' assent was obtained by the patients' nominated next of kin if they were unable to provide informed consent themselves. Cirrhosis was diagnosed by liver biopsy (n = 92, 95.8%) or clinical presentation with typical ultrasound (n = 4, 4.2%). Exclusion criteria for patients were age younger than 18 yr and evidence of metastatic malignancies (including HCC). Five patients with non-metastatic malignancies were included (HCC, Barcelona Clinic Liver Cancer staging system stages A–B [n = 3]; breast cancer, pT1b, pN0, and M0 [n = 1]; prostate carcinoma, Gleason score 7a [n = 1]). Five patients included with AD had infection at inclusion (spontaneous bacterial peritonitis [n = 3]; spontaneous bacterial peritonitis and urinary tract infection [n = 2]) and six patients included were on immunosuppressive therapy (steroids for AD [n = 3]/adrenal insufficiency [n = 1]/allergy [n = 1]; azathioprine for autoimmune hepatitis [n = 2]). Blood specimens were obtained for ex vivo analysis of monocyte differentiation and function, excessive plasma/serum, and PBMCs were stored. Patients were followed-up for 1 yr for adverse events (infection, development of AD after inclusion, mortality, transplantation, and HCC). Evidence of culture-positive/negative infection was documented. The study had been approved by the local ethics committees (EKSG 15/074/EKNZ 2015-308) and recorded in the clinical trial register ClinicalTrials.gov (identifier: NCT04116242) and Swiss National Clinical Trials Portal (SNCTP000003482).

Clinical, haematologic, and biochemical parameters

Routine clinical and laboratory parameters obtained by the clinician such as full blood count, CRP, INR, liver, and renal function tests and other variables were entered prospectively into a database. Differential blood count at the sites was performed using Sysmex XE differential analyser (Sysmex Europe GmbH) (Cantonal Hospital St. Gallen) and ADVIA 2120i hematology systems (Siemens Healthineers) (University Hospital Basel).

Monocyte isolation

Monocytes were isolated from PBMCs using CD14 MicroBeads or Pan Monocyte Isolation Kit (Miltenyi Biotec) as previously described (18). Purity of monocytes was assessed by flow cytometry.

Flow cytometry–based phenotyping of monocytes, assessment of intracellular cytokine responses to LPS stimulation, and viability assay

Phenotyping of monocytes from blood and isolated PBMCs and measurement of inflammatory cytokine production in response to LPS was undertaken using flow cytometry as previously described (18). Antibodies against CD14, CD16, CD163, CD64, CD11b, chemokine receptor (CCR)5, CCR7 (BD Biosciences), CD32, CX3CR1, TLR2, TLR9 (eBioscience), TLR3 (Invitrogen), HLA-DR, CD15, TLR4, TNF-α, IL-6 (BioLegend), TYRO3, AXL, MERTK, and IFNAR (R&D Systems) were purchased from the indicated companies. In addition to ex vivo phenotyping, TNF-α and IL-6 levels were determined after a 5 h incubation of PBMCs with LPS (100 ng/ml) (Invivogen) in X- VIVO medium without complements (Fig S11; Lonza) in a 37°C, 5% CO_2 environment. The Cells were subsequently acquired on BD FACS Canto or BD LSR Fortessa. Flow cytometric gating strategy for circulating monocytes using whole blood or PBMCs was applied as described in reference 48. Flow cytometry data were analysed using FlowJo software (V.10.4.2; Ashland). Results are expressed as the percentage of positive cells and/or MFI.

Cell viability was determined using the Annexin V Apoptosis Detection Kit I (including 7-AAD) according to manufacturer's protocols (BD Biosciences).

Formula for calculating absolute numbers of TYRO3/AXL/MERTK–expressing cells and M-MDSCs

Absolute cell numbers were calculated with the formula: *Frequency of TYRO3/AXL/MERTK-expressing monocytes or M-MDSCs* (*% defined by flow cytometry*) × *monocyte count* (*G/L*).

sAXL, AXL ligands, and cytokines

sAXL (Abcam), GAS6 (Abnova), IL-6, and TNF-α (R&D Systems) were measured using ELISA in plasma or cell culture supernatants as previously described (18).

Mixed lymphocyte reaction

Monocytes from HCs and patients with cirrhosis expressing high (>20%/450 MFI) versus low (<7.5%/300 MFI) AXL levels were isolated

using the Pan Monocyte Isolation Kit (Miltenyi Biotec) and co-cultured with allogeneic $CD3^+$ T cells from a different healthy donor, isolated using the Pan T Cell Isolation Kit (Miltenyi Biotec), in a 1:1 ratio. T cell stimulation was induced with anti-CD2/CD3/CD28 beads (T cell Activation/Expansion Kit; Miltenyi Biotec) as previously described (20). T cells were stained with carboxyfluorescein succinimidyl ester at day 0. Proliferation was assessed at day 2 of co-culture by flow cytometry.

Generation of THP-1 cells stably expressing AXL

pWZL-Neo-Myr-Flag-AXL vector was a kind gift from Hahn's laboratory (#20428; Addgene). Packaging plasmids pUMVC and pMD2.G (a gift from Weinberg's and Trono's laboratories; #8449 and #12259; Addgene) were used for the production of the retrovirus. THP-1 cells were transduced with pWZL-Neo-Myr-Flag-AXL vector, the cells were selected by G418 (Sigma-Aldrich), and THP-1-AXL^+ cells with stably introduced pWZL-Neo-Myr-Flag-AXL were subcloned using MethoCult (StemCell Technologies). The clone with highest AXL expression (for purity see Fig S8) was chosen for phenotypic characterisation by flow cytometry, gene expression analysis by quantitative RT-PCR, and LPS-induced cytokine measurement by ELISA. THP-1 and THP-1-AXL^+ cell lines were cultivated in Roswell Park Memorial Institute 1640 medium (RPMI 1640) (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin (Sigma-Aldrich).

Quantitative RT-PCR

Total RNA of isolated monocytes from patients with cirrhosis, HCs, and THP-1/THP-1-AXL^+ cells in cell culture was isolated using RNeasy Mini Kit (QIAGEN) and reversely transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher Scientific). qRT-PCR was performed with 400 ng of cDNA using LightCycler 480 SYBR Green I Master Mix (Roche). Commercial primers for AXL (Hs_AXL_1_SG QuantiTect Primer Assay) were purchased from QIAGEN. Sequences (5′-3′) of primers are as follows: SOCS1 forward: CCC CTT CTG TAG GAT GGT AGC A; reverse: TGC TGT GGA GAC TGC ATT GTC and SOCS3 forward: ATG GTC ACC CAC AGC AAG TT; reverse: TCA CTG CGC TCC AGT AGA AG. GAPDH was used as endogenous control as previously described (35). qRT-PCR was performed according to the manufacturer's recommendations on QuantStudio Real-Time PCR (Applied Biosystems/Thermo Fisher Scientific).

Whole blood phagocytosis assay

Whole blood was incubated with pHrodo *E. coli* Red BioParticles (Phagocytosis Kit for Flow Cytometry from Invitrogen/Thermo Fisher Scientific) and live *E. coli* NovaBlue carrying the gfp-mut2 encoding plasmid pCD353, which expresses a prokaryotic variant of GFP controlled by a lactac promoter as described in (49) (a kind gift of Prof Dr C Dehio, University of Basel). 5 μl of *E. coli* Red BioParticles were added for 15 min at 37°C to 100 μl of whole blood and processed as previously described (20). Blood with bioparticles was stained with antibodies against CD14, CD16, HLA-DR, AXL, and CD15, and CD3, CD19, and CD56 (BD Biosciences) and acquired on the flow cytometer. *E. coli* bacteria were freshly grown on LB Agar plates supplemented with kanamycin (50 μg/ml; Sigma-Aldrich) and incubated overnight at 37°C. A single colony was picked and grown in LB medium supplemented with kanamycin (50 μg/ml) and IPTG (1 mM; Sigma-Aldrich) for GFP induction at 37°C until early logarithmic growth (OD600 = 0.5–0.6) was reached. After the incubation period, bacteria (1×10^9 bacteria) were centrifuged at 3000*g* for 5 min at 4°C, resuspended in 1 ml PBS, and used immediately. 5×10^7 of GFP-containing *E. coli* were added for 60 min at 37°C to 100 μl of whole blood and processed as previously described (20). Blood with GFP-containing *E. coli* was stained with antibodies against CD14, CD16, and AXL and acquired on the flow cytometer. The rate of phagocytosis was obtained by the proportion of GFP positive monocytes.

In vitro inhibition of AXL

A small-molecule inhibitor of AXL, BGB324 (Selleck Chemicals), and metformin (Stemcell Technologies) were used. Selectivity and mechanism of BGB324 were described previously (27, 38). Metformin was previously described to suppress AXL expression at a concentration of 10 mM (28). We used 0.5×10^6 PBMCs from HCs and patients per well on a 48-well plate and cultured them in X-VIVO medium (Lonza) containing 10% FBS in a 37°C, 5% CO_2 environment. The cells were treated with 1 μM BGB324/10 mM metformin or dimethyl sulfoxide/PBS for 24 h, harvested, and washed two times with PBS before the assessment of inflammatory cytokine production in response to LPS (100 ng/ml, 5 h), phagocytosis capacity, and viability of monocytes by flow cytometry. For the assessment of monocyte phagocytosis from isolated PBMCs in vitro, the harvested cells were incubated with pHrodo *E. coli* Red BioParticles (Invitrogen/Thermo Fisher Scientific) for 60 min, processed, and assessed by flow cytometry as previously described (20). The optimal dose of 1 μM BGB324 was initially defined by a dose finding experiment assessing cytokine production of monocytes in response to LPS (100 ng/ml, 5 h). Cell viability using Annexin V (BD Biosciences) was assessed after BGB324 and metformin treatment (Fig S10B–D).

In vitro models for the generation of AXL-expressing cells

1×10^6 PBMCs per well were cultured on 24-well plates in 1 ml X-VIVO medium (Lonza) in a 37°C, 5% CO_2 environment. Cells were stimulated with or without LPS 100 ng/ml (4, 8, 16, 18, 24 h), Pam3CSK4 5 μg/ml, CpG 10 μg/ml, poly(I:C) 10 μg/ml (Invivogen), IFN-α 250 U/ml (Roche), TNF-α 250 U/ml, GAS6 20 nM (R&D Systems), HMGB1 20 ng/ml (Sigma-Aldrich), and TGF-β 2 ng/ml (PeproTech) for 18 h. The cells were harvested and subjected to immunophenotyping, intracellular staining of cytokine production in response to LPS and viability assays using flow cytometry as described before.

For the experiments incubating healthy monocytes in plasma from HC (n = 4) and patients with cirrhosis (n = 4), 1×10^6 $CD14^+$ cells were cultured in a 24-well plate for 24 h in X-VIVO medium (Lonza) containing 25% of the indicated plasma in a 37°C, 5% CO_2 environment. Subsequently, a fraction of these cells was phenotyped and the remainder was transferred to fresh medium for the assessment of LPS-stimulated TNF-α/IL6 production and phagocytosis capacity of monocytes by flow cytometry as detailed before.

For the experiments measuring AXL-expressing monocytes after treatment of bacteria, whole blood was incubated with pHrodo *E. coli* Red BioParticles, pHrodo *S. aureus* Red BioParticles (Invitrogen/Thermo Fisher Scientific) for 15 min, and with live GFP-containing *E. coli* bacteria for 60 min and processed as described above.

Efferocytosis assay

The experimental design was adapted from Zizzo et al (26) and Triantafyllou et al (19). Human neutrophils were isolated using PolymorphPrep (Axis-Shield) by density-gradient centrifugation according to the manufacturer's protocols, re-suspended at 1×10^6 cells/ml in RPMI-1640 (Sigma-Aldrich) containing 10% FBS (complete medium), labelled with CellTracker Violet BMQC (5 μM in serum-free medium, 45 min, 37°C, dark; Life Technologies, Thermo Fisher Scientific), and incubated for 20 h (37°C in 5% CO_2) in 300 μl complete RPMI-1640 in 24-well plates. HepG2 cells were seeded at 0.4×10^6 cells/ml in 24-well plates, labelled with CellTracker Violet BMQC as described above, and incubated with LPS (1 μg/ml) for 18 h (37°C in 5% CO_2) in 300 μl complete RPMI-1640. After the incubation period, percentage of apoptotic neutrophils and HepG2 cells in culture was determined using Annexin V Apoptosis Detection Kit I according to the manufacturer's protocols (BD Biosciences). Neutrophils and HepG2 cells were re-suspended in the wells and healthy monocytes were added to apoptotic cells (1:4 monocytes to apoptotic cells ratio) for 8 h in 1 ml fresh complete RPMI-1640 (37°C in 5% CO_2). The cells were harvested and washed two times in PBS and subjected to immunophenotyping, intracellular staining of cytokine production in response to LPS (100 ng/ml, 5 h), and viability assays as described above. The rate of efferocytosis was obtained by the proportion of CellTracker-positive monocytes.

Statistical analyses

Statistical evaluation was performed in GraphPad Prism v.7.0a (GraphPad Software). $P < 0.05$ values were considered statistically significant. Data are shown as box and whiskers or scatter dot plots and expressed as median with 10–90 percentile, unless otherwise specified. For data that did not follow a normal distribution, significance of differences was tested using Mann–Whitney or Wilcoxon tests. Spearman correlation coefficients and area under the receiver operating characteristic curve were calculated. Normally distributed data were compared using paired or unpaired *t*-tests.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.201900465.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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4.1.1 Supplementary documents

See online supplementary documents:

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4.2 *CD14+CD15-HLA-DR- myeloid-derived suppressor cells impair antimicrobial responses in patients with acute-on-chronic liver failure*

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ORIGINAL ARTICLE

CD14+CD15-HLA-DR- myeloid-derived suppressor cells impair antimicrobial responses in patients with acute-on-chronic liver failure

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ABSTRACT

Objective Immune paresis in patients with acute-on-chronic liver failure (ACLF) accounts for infection susceptibility and increased mortality. Immunosuppressive mononuclear CD14+HLA-DR− myeloid-derived suppressor cells (M-MDSCs) have recently been identified to quell antimicrobial responses in immune-mediated diseases. We sought to delineate the function and derivation of M-MDSC in patients with ACLF, and explore potential targets to augment antimicrobial responses.

Design Patients with ACLF (n=41) were compared with healthy subjects (n=25) and patients with cirrhosis (n=22) or acute liver failure (n=30). CD14+CD15−CD11b+HLA-DR− cells were identified as per definition of M-MDSC and detailed immunophenotypic analyses were performed. Suppression of T cell activation was assessed by mixed lymphocyte reaction. Assessment of innate immune function included cytokine expression in response to Toll-like receptor (TLR-2, TLR-4 and TLR-9) stimulation and phagocytosis assays using flow cytometry and live cell imaging-based techniques.

Results Circulating CD14+CD15−CD11b+HLA-DR− M-MDSCs were markedly expanded in patients with ACLF (55% of CD14+ cells). M-MDSC displayed immunosuppressive properties, significantly decreasing T cell proliferation (p=0.01), producing less tumour necrosis factor-alpha/interleukin-6 in response to TLR stimulation (all p<0.01), and reduced bacterial uptake of *Escherichia coli* (p<0.001). Persistently low expression of HLA-DR during disease evolution was linked to secondary infection and 28-day mortality. Recurrent TLR-2 and TLR-4 stimulation expanded M-MDSC in vitro. By contrast, TLR-3 agonism reconstituted HLA-DR expression and innate immune function ex vivo.

Conclusion Immunosuppressive CD14+HLA-DR− M-MDSCs are expanded in patients with ACLF. They were depicted by suppressing T cell function, attenuated antimicrobial innate immune responses, linked to secondary infection, disease severity and prognosis. TLR-3 agonism reversed M-MDSC expansion and innate immune function and merits further evaluation as potential immunotherapeutic agent.

Significance of this study

What is already known on this subject?

- Immune paresis has been described in patients with acute-on-chronic liver failure (ACLF) and is postulated to be responsible for infection susceptibility and adverse outcome.
- Low HLA-DR expression on circulating monocytes is associated with poor prognosis.
- Myeloid-derived suppressor cells (M-MDSCs) have recently been defined as CD14+CD15−CD11b+HLA-DR− cells with T cell suppressive function and have been identified to dampen immune responses in sterile and malignant inflammatory diseases.

What are the new findings?

- M-MDSCs are expanded in the circulation in patients with acute liver failure and ACLF.
- Activation of systemic inflammatory response syndrome and circulating pathogen-associated molecular patterns trigger the expansion of M-MDSC in patients with ACLF.
- In addition to suppressing T cell activation, M-MDSCs suppress pathogen uptake and Toll-like receptor (TLR)-elicited proinflammatory responses to microbial challenge.
- Persistence of the M-MDSC phenotype during disease progression confers a poor outcome and is associated with an increase incidence of infections.
- Proof-of-principle data indicate that proportions of suppressive M-MDSC can be reduced and their antimicrobial function augmented in vitro following administration of TLR-3 agonist polyI:C

How might it impact on clinical practice in the foreseeable future?

- M-MDSC may represent mechanistic biomarkers of impaired antimicrobial responses and infection susceptibility in patients with cirrhosis.
- TLR-3 agonism requires further evaluation as an immunotherapeutic strategy to improve antimicrobial responses in patients with ACLF.

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INTRODUCTION

Acute-on-chronic liver failure (ACLF) is characterised as high morbidity and mortality due to profound activation of systemic inflammatory response syndrome (SIRS) and development of multiple-organ dysfunction.[1] Recent reports indicate that activation of SIRS responses in ACLF results in immune dysregulation and is postulated to be responsible for defective immune responses to microbial cues, termed immune paresis.[2 3] Immune paresis is associated with increased frequency of infectious events in cirrhosis[4] and frequently leads to acute decompensation, extrahepatic organ failure and increased mortality.[5] Development of secondary bacterial infections in patients with ACLF is associated with a 30-day mortality of 49% and is highly predictive of adverse outcome.[4]

Over the previous decade, it has emerged that myeloid-derived suppressor cells (MDSCs) are elicited under various pathological conditions. MDSCs were initially defined in murine cells expressing CD11b and Gr-1 antigen, defined by their ability to suppress T cell proliferative and antitumour responses. However, human MDSCs are a heterogeneous population including a $CD14^{-}CD11b^{+}CD33^{+}CD15^{+}$ polymorphonuclear fraction (PMN-MDSC) and a $CD11b+CD14^{+}HLA\text{-}DR^{low/-}$ mononuclear fraction (M-MDSC).[6–8] M-MDSCs have recently been identified in a wide number of hepatic (eg, chronic viral infection, hepatocellular cancer),[9–11] non-hepatic systemic (eg, sepsis),[12 13] organ-specific inflammatory diseases[14 15] and malignancies.[16 17] The increased frequency of this suppressive immune cell population has been associated with impaired T cell responses and serves as an immunological biomarker of disease severity and outcome.

Although the ability of MDSC to supress T cell responses has been extensively documented, their innate immune and antimicrobial responses remain relatively unexplored. Furthermore, while the existence of a $HLA\text{-}DR^{low/-}$ monocyte population has been described in acute hepatic inflammatory disorders,[18–21] little is known about their lineage, innate and adaptive immune function, and their candidacy as a potential immunotherapeutic target to restore antimicrobial responses in patients with ACLF.

We hypothesised that persistent exposure to pathogen-associated molecular patterns (PAMPs), activation of SIRS and the development of ACLF trigger the expansion of immunosuppressive M-MDSCs that serve to impair both innate and adaptive responses to microbial agents thereby contributing to the increased frequency of infections encountered in patients with ACLF. We therefore sought to delineate the presence and function of M-MDSC in patients with ACLF, and explore potential targets to modulate their function in order to augment antimicrobial responses.

MATERIALS AND METHODS

Patients and sampling

From January 2013 to June 2015, we consecutively recruited patients admitted to King's College Hospital for stable cirrhosis (n=22), ACLF (n=41) or acute liver failure (ALF; n=30). Patients with ACLF fulfilled the established diagnostic criteria developed by the European Association for the Study of the Liver-CLIF (Consortium on Chronic Liver Failure).[1] Cirrhosis was diagnosed by a previous liver biopsy or clinical presentation with typical ultrasound or CT imaging. Exclusion criteria were age younger than 18 years, malignancy and immunosuppressive therapy other than corticosteroids. Within 24 hours of admission to hospital, blood was sampled for ex vivo analysis of monocyte differentiation and function. Results were compared with healthy subjects (n=25). For patients with ALF and ACLF, sequential tests were done on days 3, 5, 7 and 14 after admission when feasible. Power calculations indicate that in order to detect significant differences in immune function and phenotype (eg, tumour necrosis factor-alpha (TNF-α) secretion) with 80% statistical power at the 5% significance level, a minimum of 28 patients would need to be recruited into the ACLF and ALF study groups.

Routinely assessed full blood count, international normalised ratio, liver and renal function tests, lactate and clinical variables were entered prospectively into a database. The following disease severity scores were calculated: Child-Pugh, Model for End-Stage Liver Disease (MELD), CLIF-SOFA (Sequential Organ Failure Assessment),[1] North American Consortium for Study of End-stage Liver Disease (NACSELD),[22] Acute Physiology and Chronic Health Evaluation II, Simplified Acute Physiology Score II, SOFA scores; infections and 28-day survival were documented. The study had been approved by the King's College Hospital Ethics Committee (12/LO/0167). Assent was obtained by the patients' nominated next of kin if they were unable to provide informed consent themselves.

CD14+ cell isolation

CD14+ monocytic cells were isolated from peripheral blood mononuclear cells (PBMCs) using the CD14 Microbeads or Pan-Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.[23] Purity of monocytes was assessed by flow cytometry.

Flow cytometry-based phenotyping of monocytes and assessment of cytokine responses to Toll-like receptor stimulation

Phenotyping of monocytes and measurement of inflammatory responses to Toll-like receptor (TLR) stimulation were done based on flow cytometry as previously described.[23] Monoclonal antibodies against CD14, CD16, CD86, CD163, CD64, CD11b, chemokine receptor (CCR) 2, CCR5, CCR7, Annexin V, 7-AAD (BD Biosciences, Oxford, UK); HLA-DR, CD32, CX3CR1 (eBioscience, Hatfield, UK), and hMer (R&D Systems, Abingdon, UK) were purchased from the indicated companies. Results are expressed as the percentage of positive cells or mean fluorescence intensity (MFI). TNF-α and interleukin-6 (IL-6; BD Biosciences) levels were determined after a 4–6 hour incubation of PBMCs with lipopolysaccharide (LPS; 100 ng/mL), Pam3CSK4 (5 μg/mL), CpG-ODN2006 (10 μg/mL) (Invivogen, San Diego, USA). Flow cytometry data were analysed using Flowlogic (Inivai Technologies, Mentone, Australia) or FlowJo software (V.10.2; Ashland, Oregon, USA).

Mixed lymphocyte reaction

CD14+ monocytic cells from a healthy donor were isolated using the Pan-Monocyte Isolation Kit (Miltenyi Biotec) as previously described.[23] Cells were cultured in 25% plasma from patients with ACLF (n=8) or healthy controls (n=2) for 16 hours and cocultured with allogeneic CD3+ T cells from a different healthy donor, isolated using the Pan-T-cell Isolation Kit (Miltenyi Biotec) in a 1:1 ratio. T cell stimulation was induced with anti-CD2/CD3/CD28 beads (T cell Activation/Expansion Kit; Miltenyi Biotec) according to the manufacturer's protocol (2.5×10^{5} cells in 250 μL of Roswell Park Memorial Institute medium, 5% AB serum (Sigma-Aldrich, Gillingham, Dorset, UK), 1% penicillin/streptomycin). Cells were stained

with carboxyfluorescein succinimidyl ester at day 0. Proliferation was assessed at day 3 of coculture. Experiments were performed in triplicate.

Whole blood phagocytosis assay

The pHrodo *Escherichia coli* Red and *Staphylococcus aureus* Green BioParticles Phagocytosis Kit for Flow Cytometry was purchased from Invitrogen, Paisley, UK. The instructions of the manufacturer were precisely followed. CD14+ cells were stained with antibodies against CD14 (APC-H7), CD16 (PercP-Cy5.5), HLA-DR (APC), and CD3, CD15, CD19, CD56 (all fluorescein isothiocyanate [FITC] channel) for 20 min at 4°C. Cells were acquired on BD FACS CANTO II flow cytometer (see online supplementary methods).

Ex vivo phagocytosis assay in PBMC

For the assessment of phagocytosis of PBMCs in vitro, 500 000 healthy PBMCs per well were cultured (24 hours) in complete medium containing 20% healthy controls or ACLF plasma. Cells were stimulated with various agents (polyI:C 10 μg/mL, LPS 1 and 10 μg/mL, CpG-ODN2006 1 and 10 μg/mL) for 4 hours. Harvested cells were supplemented by 10% autologous plasma and pHrodo *E. coli* Red BioParticles (Invitrogen) were added for 60 min (see online supplementary methods).

Phagocytosis of CD14+ cells: Cell-IQ

CD14+ cells were isolated and cultured (24 hours) in complete media with 25% plasma from two healthy subjects and two patients with ACLF. Cells were supplemented with 10% human AB serum and pHrodo *E. coli* Red BioParticles (Invitrogen).

Real-time cell imaging of the uptake of BioParticles was captured by a Cell-IQ system (CMTechnologies), running Imagen software V.2.8.12.0 and analyser V.3.3.0 (see online supplementary methods).

Quantification of bacterial 16S rDNA levels in whole blood using TaqMan qRT-PCR

The procedure was carried out under strict aseptic conditions as previously reported[24] (see online supplementary methods).

In vitro models for the generation of M-MDSC-like cells

The models were adapted from Pena *et al*.[25] 9×10^6 PBMCs per well were cultured on a 12-well plate in 2000 μL X-Vivo medium (Lonza, Basel, Switzerland) in a 37°C, 5% CO_2 environment. Cells were stimulated with or without LPS 10 ng/mL, Pam3CSK4 5 μg/mL (Invivogen) or a combination of LPS and Pam3CSK4, respectively, for 24 hours. After this time, cells were rechallenged with LPS or Pam3CSK4 for a further 4 hours, and then subjected to diverse experiments such as intracellular staining of cytokine production, immunophenotyping, phagocytosis and viability assays as described above.

For the plasma experiments, CD14+ cells were cultured (16 hours) in plasma from healthy subjects (n=3) and patients with cirrhosis (n=3) and ACLF (n=6), respectively. Subsequently, the M-MDSC subset was identified and phenotyped as described above. Parts of the cells were transferred to fresh medium, stimulated with LPS 100 μg/mL (5 hours) and supernatants were used for S100A8/A9 ELISA (Systems).

Phagocytosis PCR array

The RT^2 Profiler phagocytosis PCR array (Cat No 330231 PAHS-173ZA) was purchased from SABiosciences, Quiagen, Manchester, UK. The process from RNA extraction to cDNA synthesis and quantitative RT-PCR was performed exactly as per manufacturer's protocol (see online supplementary methods).

Statistical analyses

Data are expressed as the median/IQR 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 and area under the receiver operating characteristic curve (AUROC) were calculated. Graphs were drawn using Prism 7.0a (GraphPad, La Jolla, California).

RESULTS

Patient characteristics

In comparison to patients with ACLF (n=41), patients with stable cirrhosis (n=22) and ALF (n=30) were included. Clinical characteristics of the different groups including disease stratification scores, the evidence of infection and prognosis are summarised in table 1. A 28-day transplant-free survival was 39% in the ACLF group and 56.6% in the ALF group, while 17.1% and 23.3% were transplanted, 43.9% and 20% died, respectively.

Culture-positive infectious complications occurred in 36.6% (n=15) of patients with ACLF, while n=4 had been admitted with primary infections and n=12 developed secondary infections as previously defined by Bajaj *et al*,[4] and in 23% (n=7) of patients with ALF (primary infections n=2, secondary infections n=5) (table 1). Infectious complications were caused by gram-negative pathogens (*E. coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Serratia marcescens*), gram-positive pathogens (*S. aureus*, *S. epidermidis*, *Enterococcus faecium*, *Streptococcus anginosus*, *Clostridium difficile*), atypical bacteria (*Mycoplasma pneumoniae*) and fungi (*Candida albicans*, *C. tropicalis*).

Increased proportions of circulating immunosuppressive monocytic myeloid-derived suppressor cells in ACLF

Reduced expression of HLA-DR in circulating monocytes from patients with liver failure[18–21] and other systemic inflammatory pathologies has been previously reported and correlated with adverse outcome. Recent advances have identified the existence of an HLA-$DR^{low/neg}$ (CD14+CD15-CD11b+CD64+) expressing myeloid cell population with immunosuppressive capabilities termed monocytic myeloid-derived suppressor cells (M-MDSCs).[6 8 16] In line with recent phenotypic and functional classification of M-MDSC,[8] we show a marked expansion of $CD14^+HLA\text{-}DR^-CD15^-CD11b^+CD64^+$ myeloid cells in ACLF (55% (median; IQR 36%–78%)) and ALF (58.5% (median; IQR 34%–80%)) when compared with patients with stable cirrhosis and healthy subjects (figure 1A) which have a suppressive effect on T cell proliferation when tested in an allogeneic mixed leucocyte reaction (figure 1B).

In comparison to CD14+HLA-DR+ cells, CD14+HLA-DR− cells have reduced expression of differentiation/scavenger (CD163, MERTK), activation/costimulatory (CD86), phagocytosis (CD64, CD32), certain homing markers (CX3CR1, CCR5) but similar expression of myeloid lineage marker CD11b and CCR2 (figure 1C–F). Importantly, this immune cell subset detailed here as M-MDSCs markedly differs from the recently identified suppressive MERTK+ population which is characterised by high expression of differentiation and activation markers such as CD163 and HLA-DR.[23]

Table 1 Clinical characteristics of the cohort including patients with cirrhosis, ACLF and ALF

Parameter	Cirrhosis	ACLF	ALF
	(n=22)	(n=41)	(n=30)
Age (years)	55 (46–62)	48 (38–60)*	35 (27–46)
Child-Pugh	8 (6–9)	12 (11–13)*†	13 (11–13)
MELD	10 (9–15)	31 (22–39)*†	40 (33.5–40)
CLIF-SOFA	3.5 (2–5)	13 (11–16)†	—
NACSELD	0	2 (1–3)†	—
SOFA	NA	13 (12–14)	13 (11–16)
APACHE II	NA	22 (19–26)	20 (13–24)
SAPS II	NA	36 (27–41.5)	40 (28–50)
Transplantation, % (n)	0%	17.1% (7)	23.3% (7)
28-day mortality, % (n)	0%	43.9% (18)	20% (6)
28-day transplant-free survival, % (n)	100%	39% (16)	56.6% (17)
Culture-positive infection at admission, % (n)	0% (0)	9.8% (4)	6.6% (2)
Secondary infectious complications, % (n)	5% (1)	26.8% (11)	16.6% (5)
Bacterial DNA at admission (pg/mL)	3.5 (0.8)	6.7 (5.9)†	NA
Antibiotic treatment at admission, % (n)	5% (1)	87.8% (36)	96.6% (29)
Bilirubin (µmol/L)	27 (19–52)	152 (83–284)*†	83 (49–169)
Albumin (g/L)	34 (31–37)	24 (21–28)*†	25 (24–29)
INR	1.3 (1.2–1.5)	2.0 (1.7–2.7)*†	4.0 (2.74–7.27)
ASAT (U/L)	47 (31–66)	115 (69–288)*†	5250 (1329–7286)
Lactate (mmol/L)	NA	1.8 (1.2–2.8)*	3 (2.2–4.9)
WBC ($\times 10^9$/L)	4.84 (3.45–7.36)	9.82 (6.25–14.37)†	10.97 (8.28–15.81)
Neutrophils	3.03 (2.03–4.92)	7.59 (4.69–12.11)†	8.81 (6.8–14.79)
Monocytes ($\times 10^9$/L)	0.42 (0.3–0.58)	0.51 (0.33–0.88)*	0.25 (0.16–0.51)
CRP (mg/dL)	8.8 (5.7–22.4)	50.5 (23.1–79.2)*†	19.3 (7.4–40.5)
SIRS score	0 (0–1)	2 (1–2)†	2 (1–3)

All data are presented as median (IQR), unless otherwise specified in the corresponding rows.
*Significant difference comparing ACLF to ALF.
†Significant difference comparing ACLF to cirrhosis; p<0.05, Mann-Whitney U tests.
ACLF, acute-on-chronic liver failure; ALF, acute liver failure; APACHE II, Acute Physiology and Chronic Health Evaluation II; ASAT, aspartate aminotransaminase; CLIF, Consortium on Chronic Liver Failure; CRP, C-reactive protein; INR, international normalised ratio; MELD, Model for End-Stage Liver Disease; NA, not applicable; NACSELD, North American Consortium for Study of End-stage Liver Disease; SAPS II, Simplified Acute Physiology Score II; SIRS, systemic inflammatory response syndrome; SOFA, Sequential Organ Failure Assessment score; WBC, white blood cells.

Circulating M-MDSCs in ACLF have impaired innate responses to microbial challenge and refer to poor prognosis and infection susceptibility

Compared with healthy subjects, CD14+ cells from patients with ACLF exhibit attenuated proinflammatory responses (TNF-α/IL-6) to TLR-2 (Pam3CSK4), TLR-4 (LPS) and TLR-9 (CpG) challenge indicating reduced innate responses to several pathogen-induced TLR signalling pathways (see online supplementary figure 1). Compared with classical CD14+HLA-DR+ monocytes, M-MDSCs in ACLF were characterised by defective proinflammatory cytokine production in response to all different TLR ligands (figure 2A). Furthermore, S100A8/A9 protein levels, an inflammatory mediator synthesised and secreted preferentially by M-MDSC,[26] were significantly higher in the plasma of patients with ACLF compared with healthy subjects and patients with cirrhosis (figure 2B).

In our cohort of patients with cirrhosis and ACLF, HLA-DR expression on CD14+ myeloid cells strongly negatively correlated with established scores of liver disease severity Child-Pugh, MELD, CLIF-SOFA (figure 2C), NACSELD score ($r=-0.53$, $p<0.001$, $n=98$) and also SIRS score ($r=-0.46$, $p<0.001$, $n=96$). Moreover, admission HLA-DR expression on CD14+ cells predicted 28-day survival with a sensitivity of 78% and a specificity of 76% for the criterion MFI >1758 (figure 2D).

Median HLA-DR expression over the first 14 days following admission predicted 28-day survival with a sensitivity of 74% and a specificity of 77% for the criterion MFI >1617 and the onset of infection within 28 days of hospitalisation with a sensitivity of 75% and a specificity of 67% for the criterion MFI <1500. Furthermore, it predicted both the presence of primary infection (AUROC 0.82/p=0.04, sensitivity 75%, specificity 71%, criterion MFI <1346; data not shown) and the development of secondary infection (AUROC 0.73/p=0.03, sensitivity 67%, specificity 67%, criterion MFI <1488) (figure 2E). These observations corroborate previous reports linking HLA-DR expression on monocytes with adverse prognosis in patients with ACLF[20 21] and newly suggest an association between the persistence of the M-MDSC population during the course of disease and infection susceptibility.

M-MDSCs display impaired bacterial uptake and clearance in ACLF

Phagocytosis of bacteria is an important function of myeloid cells with regard to effective clearance of invading microorganisms and during sepsis. The proportion of CD14+ cells able to phagocytose *E. coli* particles was significantly reduced in patients with ACLF (median 97.4%) and ALF (median 82.4%; both $p<0.0001$, figure 3A,B) when compared with patients with cirrhosis and healthy controls (median 99%). Also, capacity to phagocytose *S. aureus* particles was reduced in both patients with ACLF and ALF and interestingly, the capacity to phagocytose gram-negative (*E. coli*) particles correlated with capacity to phagocytose gram-positive (*S. aureus*) particles (see online supplementary figure 2A). The production of reactive oxidative metabolites following uptake of *E. coli* was preserved in both patients with ACLF and ALF compared with healthy subjects (see online supplementary figure 2B).

A phagocytosis assay was designed to differentially assess phagocytosis capabilities of CD14+HLA-DR+ monocytes compared with $CD14^+HLA\text{-}DR^-$ M-MDSC (figure 3A). In patients with ACLF and ALF, M-MDSC revealed significantly reduced phagocytosis of *E. coli* particles ex vivo when compared with HLA-DR+ monocytes. Phagocytosis capacity of *E. coli* positively correlated with the degree of HLA-DR expression on all CD14+ expressing cells (figure 3C) and TNF-α production in response to LPS ($r=0.35$, $p=0.04$, $n=36$). Similar to HLA-DR expression, phagocytosis indices correlated with disease severity scores (Child-Pugh score, MELD, SIRS score, CLIF-SOFA score; figure 3D). Taken together, ex vivo experiments reveal that CD14+ cells, in particular the M-MDSC subset, exhibit a marked and persistent deficiency in the uptake and clearance of bacteria in patients with ALF and ACLF (figure 3E,F and online supplementary figure 3).

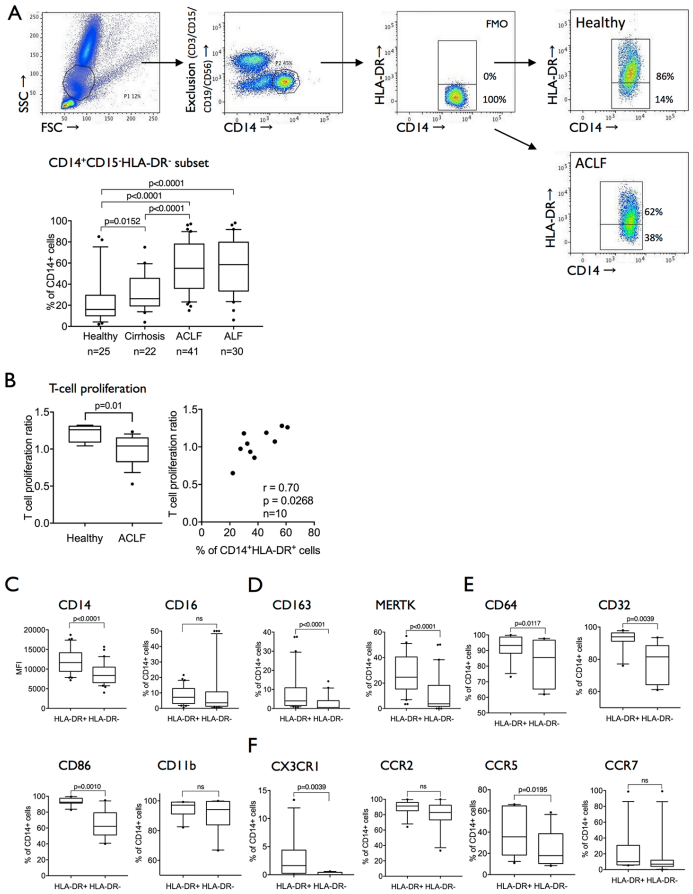

Figure 1 The expanded CD14$^+$CD15$^-$HLA-DR$^-$ population in ACLF is attributed to the previously defined monocytic MDSC subset. (A) Gating strategy to determine CD14$^+$CD15$^-$HLA-DR$^-$ cells: whole blood was sorted, monocytic cells were selected, and non-monocytic cells were excluded by lineage gating (CD3, CD15, CD19, CD56). CD14+, lineage negative cells were divided into HLA-DR+ and HLA-DR- subsets using FMO. Prevalences of the CD14$^+$CD15$^-$HLA-DR$^-$ populations in healthy subjects, patients with cirrhosis, ACLF and ALF. (B) T cell proliferation in a mixed lymphocyte reaction assay is significantly reduced in ACLF and in relation to HLA-DR expression on monocytes in coculture (healthy n=2, ACLF n=8). (C–F). A large panel of myeloid cell differentiation markers was compared between the CD14$^+$HLA-DR$^+$ and CD14$^+$HLA-DR$^-$ populations. The CD14$^+$HLA-DR$^-$ monocytic subset appears to be CD14$^+$CD15$^-$CD11b$^+$HLA-DR$^-$CD64$^+$ in line with the previously defined monocytic MDSC (M-MDSC). (C) Classical markers of monocyte differentiation were reduced in M-MDSC (CD14, CD163, MERTK). (D) FCγ receptors (CD64, CD32) were abundantly expressed in M-MDSC but lower in comparison to CD14$^+$HLA-DR$^+$ cells (n=30) and (E) markers of monocyte activation (CD86, CD11b) were reduced or equally expressed in M-MDSC. (F) Selected chemokine receptor expression (CX3CR1, CCR5) was lower in the M-MDSC population (n=11). Data are expressed as MFI and % of CD14+ cells and shown as box plots; Wilcoxon tests. ACLF, acute-on-chronic liver failure; ALF, acute liver failure; FMO, fluorescence minus one control; FSC, forward scatter; MDSC, myeloid-derived suppressor cells; MERTK, Mer Tyrosine Kinase; MFI, mean fluorescence intensity; SSC, side scatter.

Transcriptional analysis of circulating monocytes links impaired phagocytosis to downregulation of TLR pathways

In order dissect which intracellular pathways may be involved in defective bacterial clearance and innate responses in ACLF, a phagocytosis-specific RT-PCR array was performed comparing mRNA extracts from CD14+ cells of patients with ACLF bearing an expanded M-MDSC population compared with healthy subjects (figure 4A). Differential expression more than twofold revealed higher expression of three genes (FcγR1/CD64, MERTK and MFGE8) and reduced expression of 16 genes interestingly including TLR-3 and TLR-9, CD14 and interferon-γ. The findings indicate multiple defective pathways of innate immune responses in CD14+ cells from patients with ACLF with marked reductions in

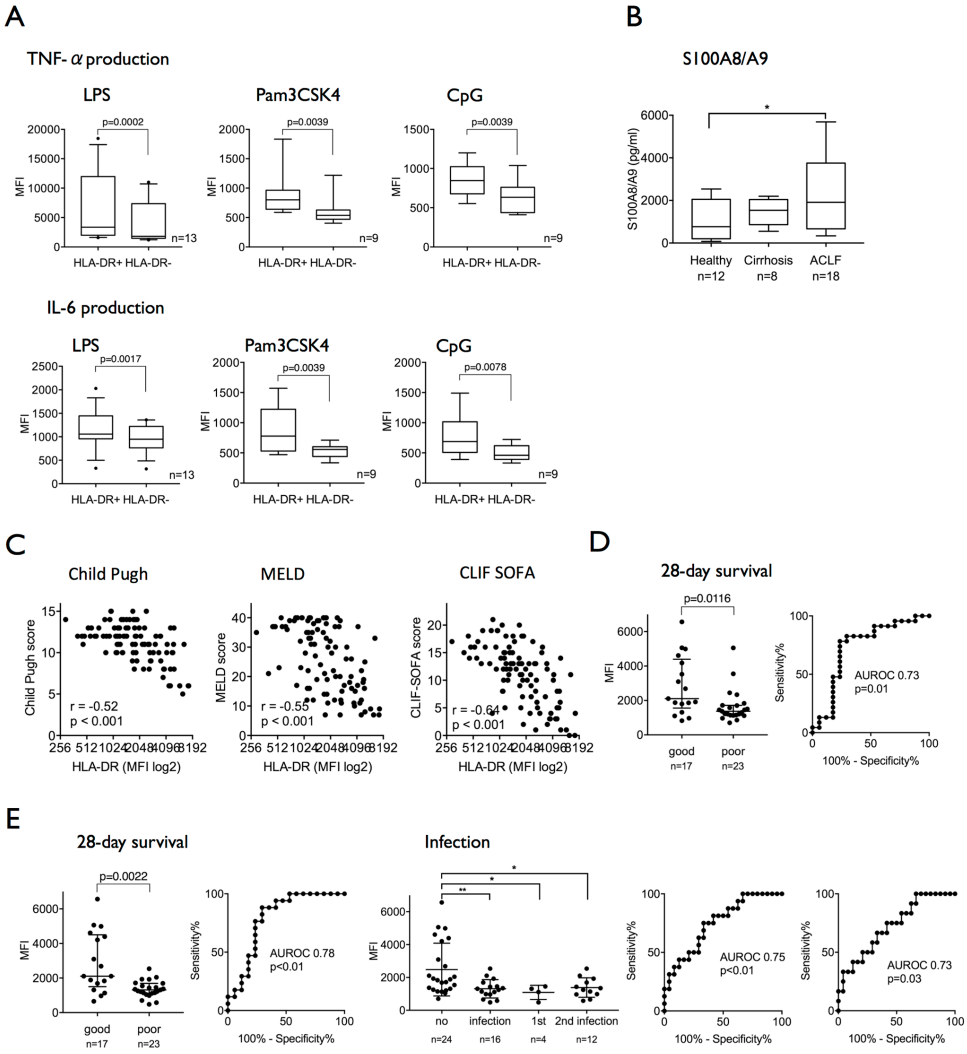

Figure 2 The CD14^{+}HLA-DR^{-} M-MDSC population in ACLF is immunosuppressive and refers to poor prognosis and infection susceptibility. (A) TNF-α and IL-6 production in response to endotoxin stimuli (LPS/TLR-4, n=13; Pam3CSK4/TLR-2, CpG/TLR-9, n=9) ex vivo is significantly reduced in M-MDSC compared with HLA-DR+ monocytes. (B) S100A8/A9 protein levels are significantly elevated in plasma from patients with ACLF. (C) HLA-DR expression on monocytes in patients with ALCF and cirrhosis strongly correlated with validated disease severity scores (Child-Pugh, MELD, CLIF-SOFA; n=98). (D) Admission HLA-DR expression predicted 28-day survival (sensitivity 88%, specificity 77%, criterion MFI >1758). (E) Median HLA-DR expression over 14 days following admission predicted 28-day survival (sensitivity 74%, specificity 77%, criterion MFI >1617) and the presence/development of infection (left AUROC: 75% and a specificity of 67% for the criterion MFI <1500) as well as the onset of secondary infection alone (right AUROC: sensitivity 67%, specificity 67%, criterion MFI <1488). ACLF, acute-on-chronic liver failure; AUROC, area under the receiver operating characteristic curve; CLIF, Consortium on Chronic Liver Failure; IL-6, interleukin-6; LPS, lipopolysaccharide; MELD, Model for End-Stage Liver Disease; MFI, mean fluorescence intensity; M-MDSC, monocytic myeloid-derived suppressor cells; SOFA, Sequential Organ Failure Assessment score; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-alpha.

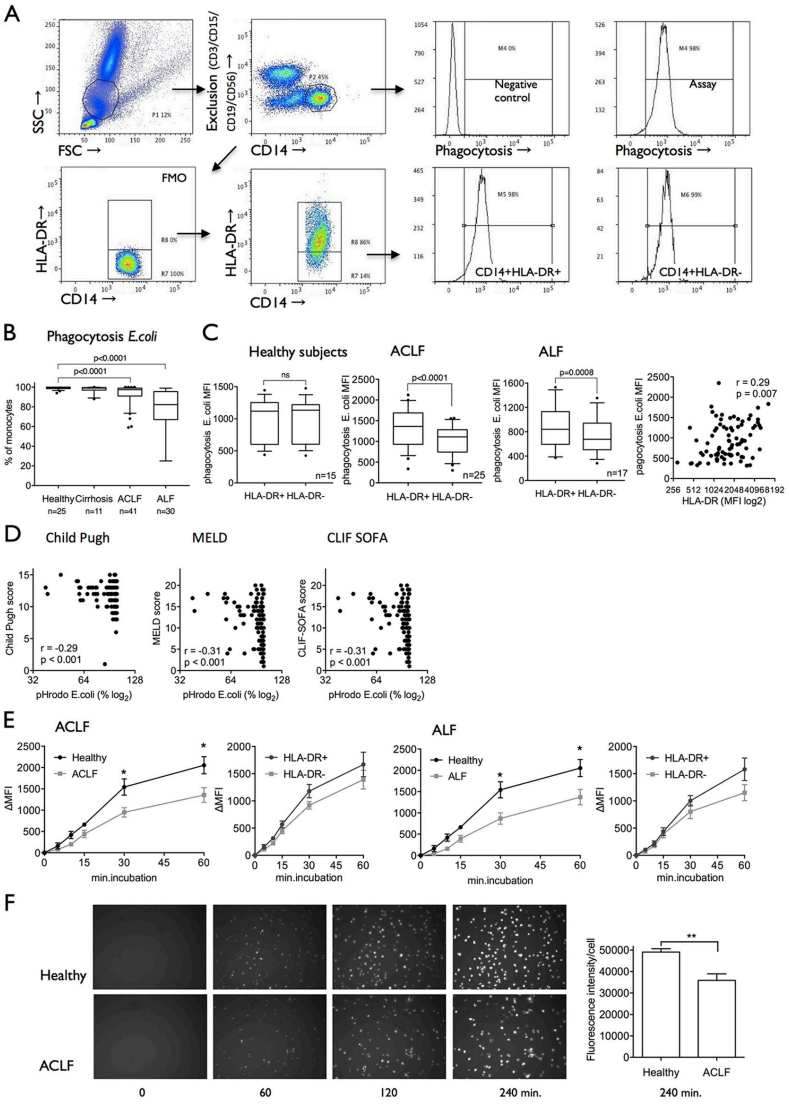

Figure 3 Impaired bacterial uptake and clearance in ACLF is linked to M-MDSC expansion and disease severity. (A) FACS plots showing the methodology of the newly developed whole blood ex vivo phagocytosis assay for different monocyte subsets ($CD14^+HLA\text{-}DR^+$ and $CD14^+HLA\text{-}DR^-$). Using a lineage exclusion panel (CD3, CD15, CD19, CD56), non-monocytic PBMCs were excluded. (B) In liver failure syndromes (ACLF and ALF), capacity to phagocytose *Escherichia coli* particles was significantly reduced. (C) Ex vivo *E. coli* phagocytosis capacity was reduced in M-MDSC versus CD14+HLA-DR+ monocytes in ACLF and ALF; ex vivo HLA-DR expression correlated with phagocytosis capacity (n=84). (D) Ex vivo phagocytosis capacity correlated with disease severity scores (Child-Pugh, MELD, CLIF-SOFA; n=132). (E) Phagocytosis capacity was reduced and decelerated over time (0–60 min) in CD14+ cells from patients with ACLF and ALF. Pathogen uptake is lower and slower in M-MDSC compared with HLA-DR+ monocytes. Results are shown as ΔMFI, healthy n=9, ACLF n=9, ALF n=9. (F) Cell-IQ-based phagocytosis assay revealed marked and persistent deficiency in the uptake of bacteria in ACLF (0–240 min)(supplementary video). ACLF, acute-on-chronic liver failure; ALF, acute liver failure; CLIF, Consortium on Chronic Liver Failure; FACS, fluorescence-activated cell sorting; FMO, fluorescence minus one control; FSC, forward scatter; MELD, Model for End-Stage Liver Disease; MFI, mean fluorescence intensity; M-MDSC, monocytic myeloid-derived suppressor cells; PBMC, peripheral blood mononuclear cells; SOFA, Sequential Organ Failure Assessment score; SSC, side scatter.

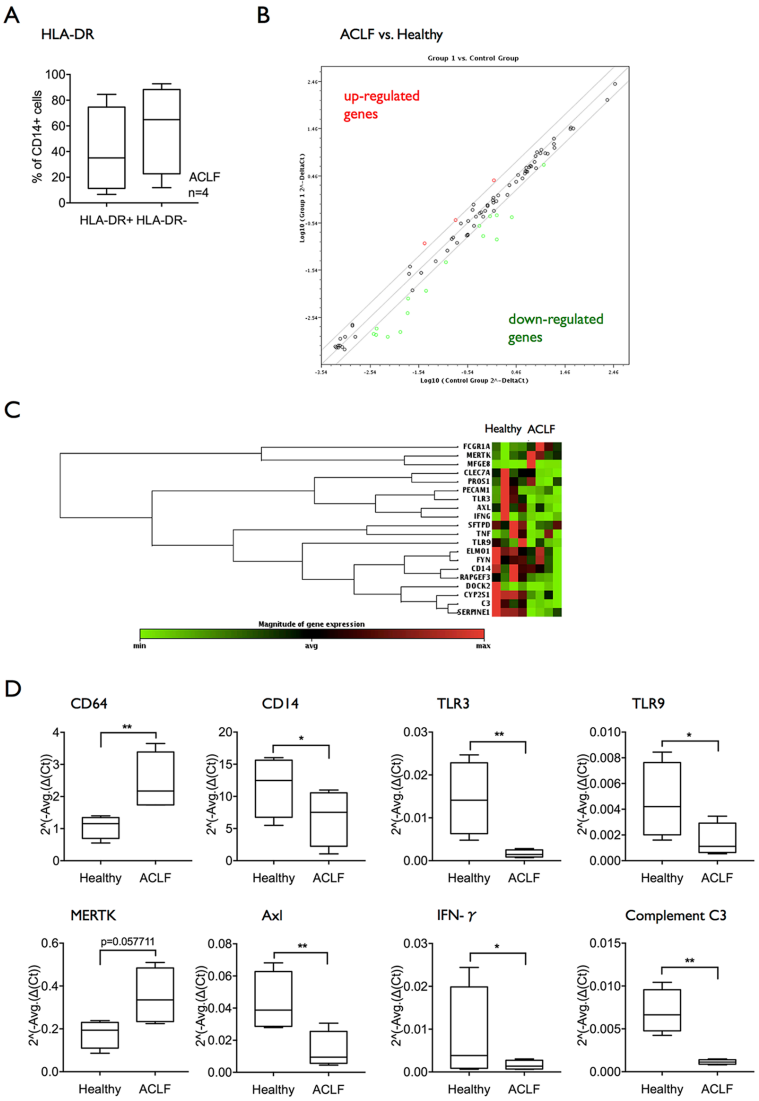

Figure 4 mRNA expression of Toll-like receptors is reduced in CD14+ cells from patients with ACLF. (A) A phagocytosis-specific PCR array was performed using mRNA extracted from isolated CD14+ cells (n=4 healthy subjects; n=4 ACLF). Selected patients with ACLF showed an expansion of the M-MDSC subset. (B) Scatter plot showing genes upregulated or downregulated more than twofold in ACLF compared with healthy subjects. (C) Clustergram showing the differential mRNA expression of genes regulated at least twofold. (D) Differential mRNA expression in selected genes of interest: CD64, CD14, TLR3, TLR9, MERTK, Axl, IFN-γ, complement C3. Data are shown as 2^(-Average(ΔCt)). ACLF, acute-on-chronic liver failure; IFN-γ, interferon-γ; MERTK, Mer Tyrosine Kinase; M-MDSC, monocytic myeloid-derived suppressor cells.

expression of diverse TLRs and in Th1 responses, and simultaneous increases in pathways associated with the clearance of necrotic and apoptotic cells (figure 4B–D, table 2). The striking reduction in expression of transcripts of a number of different TLR pathways led to the hypothesis that reduction in TLR signalling may impair TLR-driven innate immune

Table 2 Genes overexpressed (A) and underexpressed (B) in patients with ACLF versus healthy controls. Quantitative RT-PCR data from n=4 patients of each group in triplicate. Fold difference >1.99

A			B		
Gene symbol	Fold regulation	p Value	Gene symbol	Fold regulation	p Value
FCGR1A	2.3014	0.000418	AXL	−3.8622	0.0001
MERTK	1.9998	0.057711	C3	−5.9677	0.007653
MFGE8	2.7807	0.060632	CD14	−2.1498	0.040977
			CLEC7A	−6.1628	0.536001
			CSF1	−2.4128	0.174415
			CYP2S1	−4.0056	0.000001
			DOCK2	−9.0148	0.322467
			ELMO1	−2.0128	0.066372
			FYN	−2.0566	0.074544
			IFNG	−2.6871	0.048288
			PECAM1	−2.7643	0.033099
			RAPGEF3	−2.3415	0.012308
			SERPINE1	−4.7454	0.006937
			SFTPD	−2.4618	0.00293
			TLR3	−8.5662	0.000047
			TLR9	−3.1654	0.041817

responses and therefore represent a potential therapeutic target.

Generation of the immunosuppressive M-MDSC population in patients with ACLF may result from circulating bacterial products stimulating TLR pathways and circulating cytokines

In addition to impaired pathogen responses and clearance mechanisms, we also detect elevated levels of bacterial DNA in whole blood from patients with ACLF compared with cirrhosis and in cirrhosis compared with healthy subjects. Furthermore, bacterial DNA titres negatively correlated with the expansion of $CD14^{+}HLA\text{-}DR^{-}$ M-MDSCs (figure 5A) and with a number of indices of disease severity (figure 5B).

We therefore hypothesised that continual exposure to PAMPs gives rise to the immunosuppressive M-MDSC with impaired antimicrobial responses as described in vivo. In an in vitro model administering recurrent LPS stimulation[25] as well as recurrent Pam3CSK4 stimulation, we observed (1) expansion of M-MDSC characterised by reduced expression of HLA-DR, CD16, CD163, MERTK, CD64; (2) attenuated proinflammatory responses to LPS; and (3) reduced phagocytosis capacity (figure 5C–E and online supplementary figures 4A–D and 5A–D). Overall the results were more pronounced in the CD14+HLA-DR− MDSC subset (see online supplementary figures 4 and 5).

Simultaneous repeated stimulation with LPS and Pam3CSK4 also results in an expansion of M-MDSC, reduced TLR-evoked proinflammatory responses along with an $HLA\text{-}DR_{low}CD16_{low}CD163_{low}MERTK_{low}CD64_{low}$ phenotype (see online supplementary figure 6). Recurrent PAMP stimulation reduced the numbers of viable HLA-DR+ monocytes (Annexin V-/7-AAD-), but did not significantly alter the survival of M-MDSC (see online supplementary figures 4E, 5E, 6E).

In addition to PAMPs, soluble factors such as cytokines have been reported to induce M-MDSCs. We and others had previously shown that numerous cytokines (TNF-α, IL-6, IL-10, IL-8) are upregulated in ACLF in comparison to cirrhosis and healthy subjects, respectively.[23 27] We therefore studied the effect of ACLF plasma on healthy CD14+ monocytes in vitro and indeed observed the expansion of the M-MDSC population revealing a $CD16_{low}MERTK_{low}$ phenotype. Also, the secretion of S100A8/A9 protein levels significantly increased in the cell culture supernatants of CD14+ cells after incubation with ACLF plasma. Similar to data reported above, the viability of generated M-MDSC was not reduced compared with CD14+HLA-DR+ monocytes. The data implicate the likely involvement of other soluble mediators such as proinflammatory cytokines causing the generation of M-MDSC in patients with ACLF (figure 5E).

In vitro TLR-3 stimulation reduces the frequency of M-MDSCs and augments antimicrobial responses in ACLF

The downregulated expression of TLRs raised the question whether regulation of innate immune function by TLRs was attenuated, and whether targeted TLR agonists may have the potential to reduce the frequency of M-MDSC. Previous reports and studies in man have identified the therapeutic use of TLR-3 agonism therapy as an adjuvant for vaccinations as well as for induction of antitumour immune responses by decreasing the frequency of MDSC.[28–31] Moreover, studies demonstrated that TLR-3 agonist polyI:C promoted phagocytosis of bacteria in vitro.[32]

To explore the effect of different TLR agonists on innate immune responses, we incubated healthy CD14+ cells in plasma from patients with ACLF in the presence or absence of TLR agonists (TLR-3, TLR-4, TLR-9). Interestingly, TLR-3 agonist polyI:C significantly reduced the proportions of M-MDSC population while simultaneously enhancing their phagocytosis capacity in ACLF (figure 6A,B). Conversely, treatment with TLR-4 and TLR-9 agonists or NFκ-B inhibitor N-acetylcysteine did not reduce or in fact further increased proportions of M-MDSC and further impaired bacterial phagocytosis (figure 6C).

DISCUSSION

In this study, we have shown a marked expansion of M-MDSCs[6 8] that impair antimicrobial responses in patients with ACLF. Their biological relevance is highlighted through their strong association with indices of disease severity and incidence of infectious complications in these patients. In addition to impairing T cell responses, M-MDSCs are typified by impaired (1) secretion of proinflammatory cytokines in response to stimulation with a wide range of TLR ligands and (2) phagocytosis of bacteria. Analyses of these cells reveal a distinct immunophenotype characterised by reduced expression of tissue scavenger, costimulatory and phagocytosis receptors (eg, MERTK, CD64, CD86, CD163).

M-MDSCs are not only elevated in patients where primary infection has been the trigger for the development of ACLF but their persistence is also linked with the development of secondary infections. These data echo data from other inflammatory pathologies where the expansion of these cells[6 8] correlates with poor prognosis.[9 10 13–19 33–35] These observations urge to prospectively evaluate the role of M-MDSCs as biomarkers of infection and survival in large cohorts of patients with cirrhosis and ACLF.

Our data also reveal a less pronounced but significant elevation in M-MDSC in patients with cirrhosis. Here, we suggest that they are likely to evolve as a consequence of continual exposure to elevated concentrations of gut-derived PAMPs and proinflammatory mediators that are probably implicated in disease progression. Further studies are required to detail how these cells evolve and their role and migratory patterns in the liver and other tissue sites.

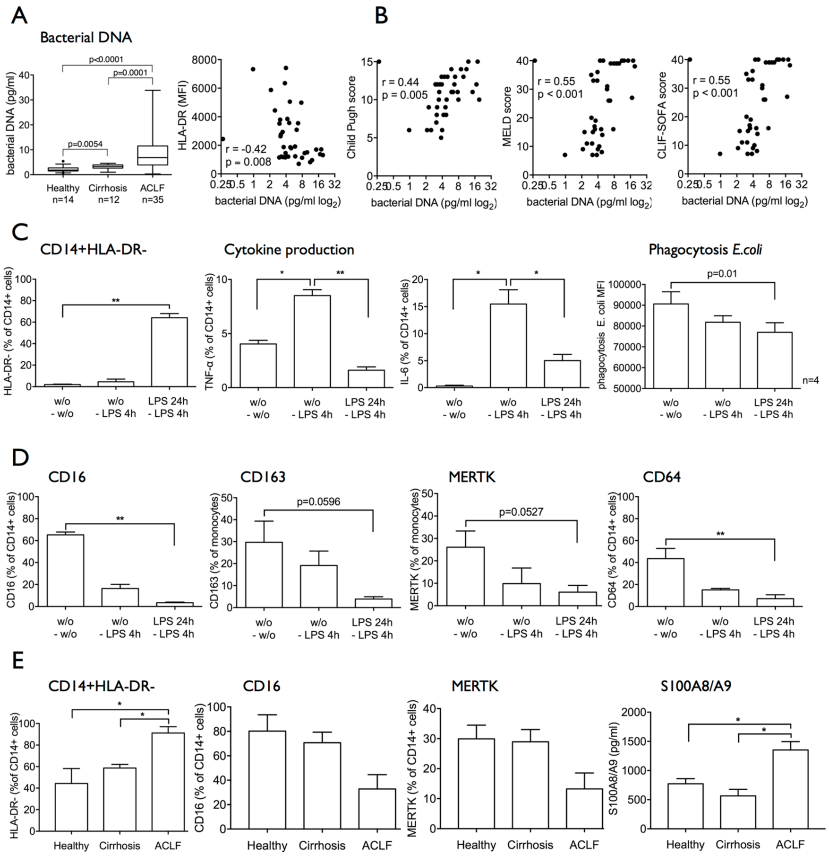

Figure 5 Circulating bacterial products and cytokines in patients with ACLF may lead to the generation of an immunosuppressive M-MDSC-like population. (A) Bacterial DNA levels in whole blood were significantly elevated in patients with ACLF and negatively correlated with HLA-DR expression. (B) Accordingly, bacterial DNA levels in whole blood positively correlated with markers of disease severity (Child-Pugh, MELD, CLIF-SOFA). (C and D) Healthy CD14+ cells were incubated with TLR-4 ligand LPS for 24 hours and subsequent 4 hours (n=4 independent experiments). HLA-DR expression, cytokine responses to LPS, phagocytosis capacity (C) and phenotype (CD16, CD163, MERTK, CD64, TLR-4, TLR-3, TLR-9) (D) were assessed. Recurrent LPS stimulation leads to the generation of an immunosuppressive HLA-DR^{low}CD16^{low}CD163^{low}MERTK^{low} M-MDSC-like population. Data are presented as % of CD14+ cells or MFI, respectively. Paired t-tests. (E) ACLF plasma containing bacterial products and modulated cytokine levels led to generation of an HLA-DR^{low}CD16^{low}MERTK^{low} M-MDSC-like population. S100A8/A9 protein secretion into the supernatants response to LPS was significantly increased. Plasma from n=3 healthy subjects, n=3 cirrhotics, n=6 ACLF, Mann-Whitney U tests. ACLF, acute-on-chronic liver failure; CLIF, Consortium on Chronic Liver Failure; IL-6, interleukin-6; LPS, lipopolysaccharide; MELD, Model for End-Stage Liver Disease; MERTK, Mer Tyrosine Kinase; MFI, mean fluorescence intensity; M-MDSC, monocytic myeloid-derived suppressor cells; SOFA, Sequential Organ Failure Assessment score; TLR-4, Toll-like receptor 4; TNF-α, tumour necrosis factor-alpha.

The generation of M-MDSC involves a complex interplay of numerous soluble circulating factors.[36 37] Using in vitro models, we were able to recapitulate the M-MDSC phenotype with comparable defects of innate immune function, suggesting that the generation and persistence of M-MDSC in ACLF could be related to repetitive stimulation by elevated circulating titres of PAMPs. This hypothesis is further strengthened by our observation of increased bacterial DNA levels in patients with ACLF, which correlated with the frequency of M-MDSCs. Among TLRs, TLR-2/6[38] and TLR-4[39–41] ligands have been reported to promote MDSC population. Consistent with these findings, our data reveal that recurrent stimulation of TLR-2 and/or TLR-4 induces a similar suppressive M-MDSC-like population as described ex vivo.

Given that both ALF and ACLF are conditions characterised by a profound activation of SIRS responses, in addition to continual PAMP exposure, we also propose that increases in circulating inflammatory mediators also contribute to the expansion and differentiation of the M-MDSCs. Our in vitro data support this concept where we demonstrate that incubation of CD14+

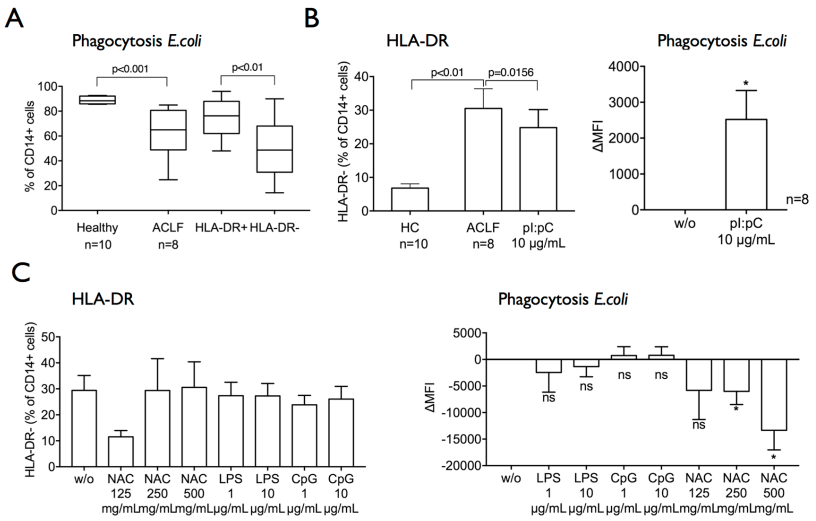

Figure 6 HLA-DR expression and phagocytosis capacity are enhanced by Toll-like receptor 3 agonism. (A) Monocytes cultured in plasma from patients with ACLF compared with healthy plasma (24 hours) develop a defect in phagocytosis capacity, which is more pronounced in the M-MDSC subset. (B) Treatment of CD14+ cells with Toll-like receptor 3 agonist polyI:C (for 6 hours, n=8) significantly reduced the M-MDSC subset and improved phagocytosis capacity. (C) CD14+ cells were treated with LPS, CpG (for 6 hours; n=6) or NAC (for 48 hours, n=7) ex vivo. The CD14+HLA-DR− M-MDSC subset and phagocytosis capacity were assessed. Data are presented as % of CD14+ cells and ΔMFI, in comparison to the untreated sample, respectively; Wilcoxon tests. ACLF, acute-on-chronic liver failure; CpG, CpG oligodeoxynucleotide; HC, healthy controls; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; M-MDSC, monocytic myeloid-derived suppressor cells; NAC, N-acetylcysteine; ns, not significant.

cells in the presence of ACLF plasma induces striking increases in their numbers. Taken together, our observations indicate the expansion and differentiation of myeloid cells into M-MDSC in patients with ACLF occur as a consequence of perpetual immune stimulation from microbial and non-microbial inflammatory cues.

The transcriptional profile of CD14+ myeloid cells in ACLF suggests these cells are functionally reprogrammed to promote uptake of apoptotic cells while concomitantly suppressing T cell activation, TLR-triggered proinflammatory responses and phagocytosis of bacteria. A similar transcriptional profile has been recently reported in patients with septic shock where the leucocyte transcriptome is reprogrammed towards resolution of tissue injury and with concomitant downregulation of genes that prime innate and adaptive antimicrobial responses.[42] Further evidence to support this theory is provided in experimental lung injury models where, following clearance of necrotic/apoptotic cells, macrophages have an attenuated uptake of bacteria and augmented secretion of anti-inflammatory mediators.[43] A similar mechanism may explain how these immunological defects evolve in ACLF, where the host response to severe tissue injury and organ dysfunction is skewed towards tissue repair responses to the detriment of antimicrobial programmes. We therefore hypothesised that selective activation of TLR pathways may represent an immunotherapeutic strategy in order to reverse M-MDSC expansion and improve antimicrobial responses in ACLF.

Deng *et al* had observed that TLR-3 agonist polyI:C promoted bacterial uptake in murine peritoneal macrophages via a TRIF-IRF-3-mediated mechanism.[32] TLR-3 is classically activated by double-stranded RNA from viruses, but may also be released from degraded bacteria and necrotic cells.[44 45] In experimental models, TLR-3 activation was associated with an increased innate immune cell infiltrate and enhanced infection clearance.[46 47] In tumour models, TLR-3 activation reverses the expansion and immunosuppressive function of MDSCs[29 30] and has been used as an immunotherapeutic agent to promote tumouricidal responses.[29 48 49] Consistent with these observations, TLR-3 reduced the frequency of M-MDSC and augments innate immune responses and pathogen uptake in patients with ACLF. These findings may relate to the fact that TLR-3 differentially regulates phagocytosis and may 'rebalance' the phagocytosis processes which are skewed predominantly towards apoptotic cell clearance in ACLF to the detriment of antimicrobial responses. Given that TLR-3 activation has been shown to be protective in experimental models of chronic liver injury, a TLR-3 agonist may improve antibacterial responses without having a detrimental effect on tissue repair processes.[32 50] Further work using in vitro and in vivo models of disease is required to further understand how activation of the TLR-3 pathway modulates inflammatory and antimicrobial responses before considering its use in human studies.

Although M-MDSCs correlate with indices of disease severity, incidence of infectious complications, the data presented in this study strongly suggest but cannot conclusively prove that the increased frequencies of M-MDSC are responsible for reduced pathogen clearance in vivo. Moreover, the immune read-outs developed here do not represent point-of care testing that could be introduced for clinical use in multicentre cohort studies. Future prospective work is therefore required to further evaluate the utility of M-MDSC

as a predictive biomarker of infection susceptibility and an immunotherapeutic target.

In conclusion, we describe a marked expansion of M-MDSC in ACLF. These cells are characterised by attenuated immune responses to pathogens, defective pathogen clearance mechanisms and a higher incidence of overt infectious complications. Perpetual exposure to elevated concentrations of PAMPs and inflammatory cytokines involved in SIRS response play a pathogenic role in the expansion of this immunosuppressive population. In vitro data indicate that TLR-3 agonism may reduce the frequency of M-MDSC and therefore merit further evaluation as potential novel immunotherapeutic strategy to restore antimicrobial responses and reduce infectious complications in patients with ACLF.

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Contributors CB planned the project, obtained funding, acquired and analysed the data, and wrote the manuscript. ET acquired and analysed the data and critically revised the manuscript. RB, FJL, AS, VCP, OTP, WK, RN and RT acquired and analysed the data. CJW, DHA and MRT critically revised the manuscript. JAW conceived the project and critically revised the manuscript. CGA conceived the project, obtained funding, acquired and analysed the data, and wrote the manuscript.

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4.2.1 Supplementary documents

See online supplementary documents:

https://gut.bmj.com/content/67/6/1155 (10th of June, 2019)

5 Synthesis

In this work, we detail as to how distinct circulatory monocytic subsets including AXL-expressing monocytes and M-MDSCs, accumulate and regulate innate immune responses at different stages of liver cirrhosis, thus contributing to the complex pathogenesis of immuneparesis in cirrhosis. Strong associations with liver disease severity scores, development of infections, and increased mortality underline their clinical significance and suggest their prospective evaluation as potential biomarkers and immunotherapeutic targets.

Previous studies addressed the investigation of monocyte dysfunction in severe stages of cirrhosis i.e. AD without or with organ failure (ACLF), when the likelihood of developing infections with subsequent morbidity and mortality is highest[51,53]. Our group previously showed that inflammatory cytokine production upon LPS stimulation was depressed not only in AD/ACLF but to a lesser extend also in stable cirrhosis[32]. The underlying mechanism remained unexplained and a detailed functional characterisation along different cirrhosis stages using scores such as the Child Pugh Score was not performed. Hence, the time and circumstances under which immuneparesis occurs and infection susceptibility increases during progression of cirrhosis and portal hypertension remained unknown.

We hereby describe for the first time evidence of two distinct immune cell subsets with attenuated innate immune functions expanding in the circulation of patients with cirrhosis in parallel with cirrhosis progression prior to the onset of AD/ACLF: AXL-expressing monocytes ($CD14^+HLA\text{-}DR^+CD16^+AXL^+$), which were phenotypically different from M-MDSCs ($CD14^+CD15^-CD11b^+HLA\text{-}DR^-AXL_{low/neg}$). Functionally, these two subsets shared immune-suppressive properties manifested as impaired secretion of pro-inflammatory cytokines in response to LPS, and impairment of T cell proliferation. AXL-expressing monocytes were typified by enhanced capacity for pathogen uptake and efferocytosis, indicating immune homeostatic characteristics. M-MDSCs however were characterised by suppressed pathogen uptake.

Interestingly, M-MDSCs were progressively expanding from stable cirrhosis to advanced decompensated and up to acutely decompensated cirrhosis (AD/ACLF). Immune-regulatory AXL-expressing monocytes expanded similarly from early to

advanced cirrhosis. However, AXL-expressing monocytes were almost absent upon the onset of AD/ACLF, when MERTK-expressing monocytes ($CD14^+HLA\text{-}DR^+MERTK^+$) were numerous. The MERTK-expressing monocyte subset was recently described by our group to be abundant in various compartments (circulation, liver, lymph nodes, peritoneum) in patients with AD/ACLF and ALF showing impaired innate immune responses to microbial pathogens[32]. MERTK-expressing monocytes remained sparse in stable cirrhosis[32], which was confirmed in our cohort.

Our observations highlight the fascinating dynamics of monocyte differentiation and immune regulation during progression of cirrhosis: in healthy subjects, monocytes with low AXL and MERTK expression ($CD14^+HLA\text{-}DR^+AXL^-MERTK^-$) prevailed, and we could show that they have regular innate immune functions. However, this "regular" monocyte subset became progressively replaced by dysfunctional subsets such as AXL-expressing monocytes and M-MDSCs in advanced stages of cirrhosis (Child B/C), and M-MDSCs and MERTK-expressing monocytes upon acute hepatic decompensation (AD/ACLF), together representing up to 90% of the total monocytic cell population in these severe conditions. Considering the reduced inflammatory responses to microbial challenges of these subsets in relation to their abundance in the circulation at different stages of cirrhosis, we propose that these populations together cause the majority of the depressed innate immune responses of the entire monocytic population in decompensated cirrhosis, which may in turn explain the high susceptibility to infection and poor clinical outcome. The complex dynamics of monocytic subset differentiation in the circulation of cirrhotic patients in different stages of disease are summarised in **Figure 5.1**. Considering these cross-sectional observations and preliminary follow-up data showing regression of AXL-expressing monocytes along with decreased severity of cirrhosis, we suggest an evolution in terms of distinct monocyte subset differentiation along the course of cirrhosis. However, these findings have to be verified by long-term longitudinal assessment of monocyte functional and phenotypic differentiation ex vivo in relation to clinical development.

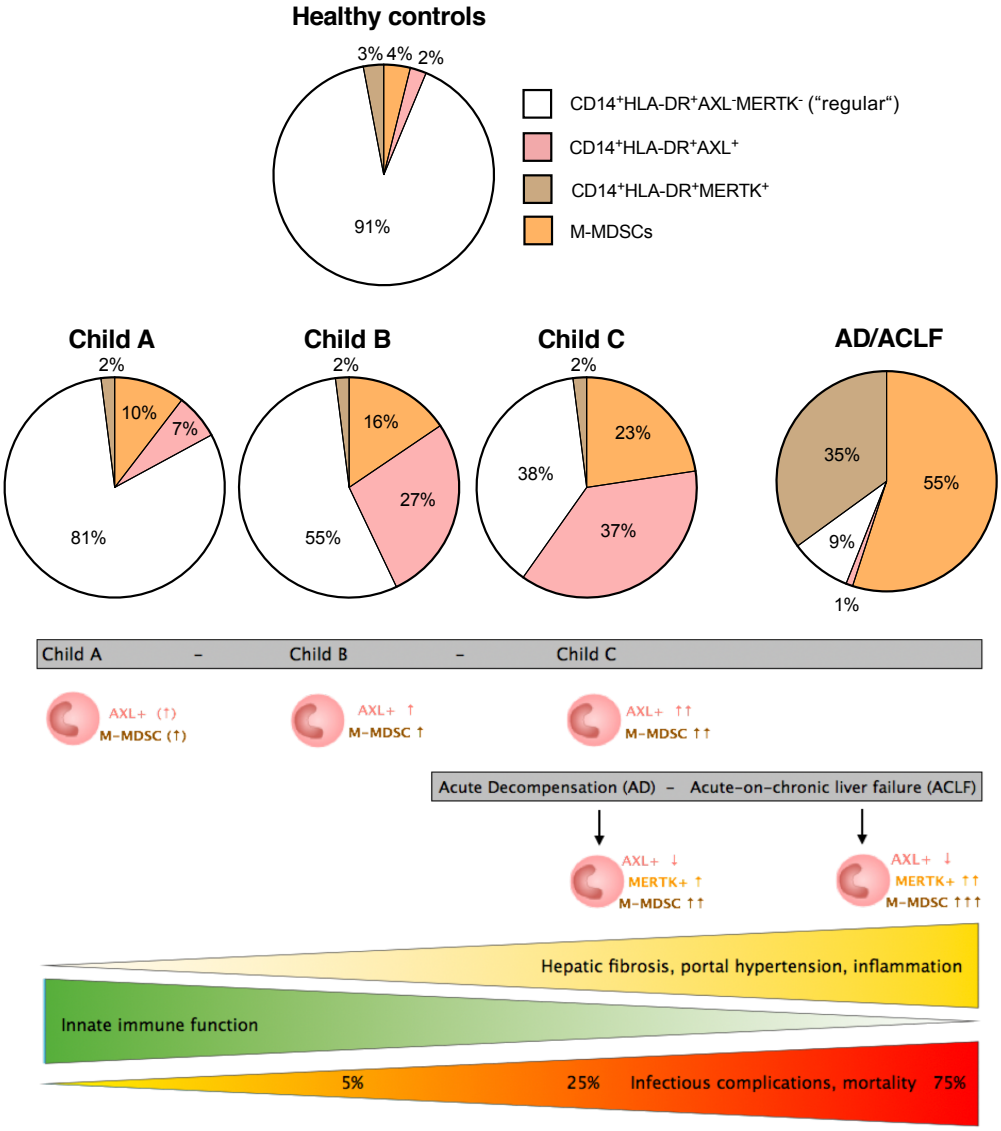

Figure 5.1: Abundance of distinct monocytic subsets in different stages of cirrhosis in relation to innate immune function, susceptibility to infection and mortality rate

The heterogeneous population of MDSCs is elicited under various pathological conditions and can be divided into a mononuclear fraction (M-MDSC) and a polymorphonuclear fraction (PMN-MDSC)[116]. M-MDSCs have recently been identified in a large number of hepatic (e.g. chronic viral infection, HCC)[117,119,122], non-hepatic systemic (e.g. sepsis)[123], and other organ-specific inflammatory diseases and malignancies[124]. An HLA-$DR_{low/-}$ monocyte population has been described in acute hepatic inflammatory disorders[47,125]. However, little is known

about its generation, innate and adaptive immune function, and anti-microbial responses in patients with cirrhosis and AD/ACLF. In our work we newly observed a progressive expansion of immune-suppressive M-MDSCs with an onset as early as in Child A cirrhosis patients, and constantly increasing until they represent the majority of monocytic cells in AD/ACLF patients. According to previous studies in other conditions, M-MDSCs are induced by various soluble circulating factors including different TLR ligands[126–128]. In accordance with these data, we generated M-MDSCs with comparable defects in innate immune responses by recurrently stimulating healthy monocytes with TLR-2 and TLR-4 ligands and by incubation with plasma of ACLF patients in vitro.

AXL is one of TAM receptor tyrosine kinases (Tyro-3, AXL, MERTK) that function as inhibitors of TLR- and cytokine receptor-mediated monocyte/macrophage activation, and promotors of apoptotic cell removal[75,81]. Loss of AXL expression on antigen-presenting cells has been linked to autoimmunity[81]. We showed an accumulation of AXL-expressing monocytes with advanced stages of cirrhosis, irrespective of the underlying aetiology and heterogeneity of the cohort. AXL-expressing monocytes vanished upon acute hepatic decompensation, when a MERTK-expressing monocyte population expanded. We were able to generate AXL-expressing monocytes with dampened innate immune responses by stimulation with selected TLR ligands and pro-inflammatory factors in vitro. These findings coincidence with previous data showing AXL up-regulation on murine BMDMs[76,102] and peritoneal macrophages[102] upon stimulation with inflammatory factors[76,102].

Given the differential functionality and abundance of AXL-expressing monocytes and M-MDSCs depending on cirrhosis stage, we hypothesised individual differentiation mechanisms for AXL-expressing monocytes. According to our observations, M-MDSCs could be generated in vitro by culturing monocytes in ACLF plasma. However, the inflammatory factors present in plasma from patients with cirrhosis were necessary, but insufficient to induce AXL up-regulation alone. Instead, phagocytosis of bacteria and efferocytosis in the circulation were further required to enhance AXL up-regulation on monocytes. The stimulatory effect of pathogen uptake on AXL expression is novel. TAM receptor activation following efferocytosis was also shown on murine BMDMs/BMDCs[76].

Taken together, these observations suggest that the generation and expansion of M-MDSCs and AXL-expressing monocytes might be a consequence of recurrent exposure to circulating microbial and non-microbial inflammatory factors, derived from intestinal sites and inflamed cirrhotic livers, favoured by pathological bacterial translocation during progressive cirrhosis and portal hypertension[26,79,80,129]. To further investigate the composition of circulating microbial and non-microbial products and their effect on the functional differentiation of monocytes, proteomic analysis of patient sera would be a promising next step.

These data also suggest particular roles for AXL-expressing monocytes, that is facilitating the uptake of pathogens and bacterial products in the setting of pathologic bacterial translocation[26,79,80,129], as well as the clearance of apoptotic cell debris accumulating in response to chronic inflammation[75,81] while suppressing excessive systemic inflammatory responses (together with M-MDSCs). The phenomenon of compensating excessive inflammatory responses in response to repetitive microbial exposure as a pathophysiological adaptation has been thoroughly described already in inflammatory conditions such as sepsis[73,74].

Different studies showed that among TAM receptors, AXL and MERTK exhibit differential regulation, activity, and reciprocal expression under specific conditions[76,92,102]. In murine BMDMs and BMDCs both receptors acted as phagocytic mediators in vitro whereas MERTK expression was induced by tolerogenic stimuli and in turn induced tolerance, while AXL was induced by inflammatory stimuli and acted as a feedback inhibition of inflammation[76]. We newly described similarly distinct expression patterns of AXL and MERTK in patients with cirrhosis. AXL-expressing monocytes accumulated with worsening disease but disappeared upon AD/ACLF, when a MERTK-expressing population (which was almost undetectable in stable cirrhosis) emerged. This underlines the distinct and counter-regulatory roles of AXL and MERTK at different phases of cirrhosis and inflammation.

However, the complexity of molecular signalling pathways which underlie the differentiation process of monocytes expressing particular TAM receptors ($CD14^+HLA\text{-}DR^+AXL^+$ vs. $CD14^+HLA\text{-}DR^+MERTK^+$) and M-MDSCs in patients with

cirrhosis in vivo remains underexplored. Also, effector pathways leading to distinct functional profiles of these monocytic subsets are unknown and subject to future investigations. Using novel techniques such as single-cell RNA sequencing to detail the transcriptome of single circulating monocytic cells will help to address their heterogeneity and differential gene expression patterns in patients with cirrhosis at different stages of disease. Understanding the transcriptomes of the distinct monocytic subsets will support the identification of future potential immunotherapeutic targets.

Our data suggesting differentiation of distinct monocytic subsets in different stages of cirrhosis relate to the discussion whether restoration of innate immune function may prevent infection susceptibility and disease progression in advanced cirrhosis. By performing proof of principle experiments in which we treated monocytes from cirrhotic patients with the highly specific AXL inhibitor BGB324 ex vivo, we could enhance innate immune responses without negatively affecting phagocytic capabilities, rendering AXL a potential immunotherapeutic target to augment defence against infections. BGB324 was originally developed for cancer treatment and is currently tested in clinical Phase Ib/II trials on patients with aggressive and metastatic cancers[130]. Other studies reported BGB324 as an anti-fibrotic agent in the context of GAS6/AXL pathway-associated fibrogenesis in CLD[111] and idiopathic pulmonary fibrosis[131], respectively. Previous studies of our lab also showed that inhibition of MERTK on monocytes of AD/ACLF patients reversed innate immune dysfunction[32]. Given the distinct and reciprocal expression profiles of AXL and MERTK in cirrhosis, the question which receptor to target in which stage of disease needs to be addressed.

Interestingly, metformin, which is conventionally used as anti-diabetic drug, has been reported to be associated with reduced HCC incidence[132] and to reduce portal hypertension in cirrhosis models[133]. Metformin also targets and down-regulates the AXL receptor[134], which was confirmed in monocytes from patients with cirrhosis ex vivo here. Treating monocytes from patients with cirrhosis *ex vivo* with metformin enhanced innate immune responses. As an inexpensive, well established drug, metformin may represent an interesting immune-modulatory treatment option for patients with cirrhosis and no signs of acute decompensation,

when AXL-expressing monocytes are frequent and the risk for metformin associated lactic acidosis is low.

Furthermore, using the TLR-3 agonist polyI:C we could reduce the frequency of M-MDSCs and improve innate immune responses and phagocytosis of microbial pathogens in patients with ACLF ex vivo. Originally, TLR-3 agonism has been described in the context of promoting bacterial uptake in murine peritoneal macrophages in a TRIF- IRF-3-dependent manner[135]. TRL-3 is activated by ligands consisting of viral RNA[136] that may also be released from degraded microbial pathogens and cell debris, as present in patients with cirrhosis. Similar to our observations, TLR-3 activation reversed the expansion and immunosuppressive function of MDSCs in tumour models and has been used as an immunotherapeutic agent to promote tumoricidal responses[137,138].

These findings relate to the fact that in patients with cirrhosis TLR-3 agonists, possibly combined with specific AXL or MERTK inhibitors, may improve antibacterial responses without having a detrimental effect on tissue homeostasis and repair processes, which might decrease susceptibility to infection and prevent disease progression. Nevertheless, as these drugs have been only tested ex vivo, additional in vitro and in vivo studies are required to test their influence on efferocytosis and T cell responses, to understand underlying signalling pathways, and to investigate on- and off-target effects such as auto-immunity or uncontrolled inflammation. From knockout mice we know that TAM receptor deficiency can cause autoimmune diseases[75] and hyper-responsiveness to endotoxins, increased production of inflammatory cytokines by macrophages, which resulted in LPS-induced shock[97]. Similar effects may occur and have to be considered when systemically inhibiting TAM receptor-expressing monocytes/macrophages.

Strong correlations with disease severity, infectious complications, and poor prognosis testify to the clinical significance of M-MDSCs and AXL-expressing monocytes and support their evaluation as future predictive biomarkers. Increased numbers of M-MDSCs were reported to correlate with poor prognosis in other inflammatory pathologies[47,117,123] and recent studies suggested sAXL as a serum biomarker for advanced liver fibrosis, cirrhosis, and HCC[16,90]. Here, we observed strong correlations of AXL-expressing monocytes with the shed receptor sAXL and

also liver disease severity scores. Based on excellent sensitivity and specificity values calculated by receiver operating characteristic curve analysis for the prediction of mortality, infectious complications, and onset of future AD episodes, the number of AXL-expressing monocytes in blood count might represent a prognostic point-of-care biomarker for both immuneparesis and cirrhosis severity.

In our work, we mainly focused on monocytic cells in the systemic circulation where they are pivotal for the defence against bacteraemia and sepsis, and in the case of cirrhosis-associated dysfunctionality, contribute to the development of immuneparesis and infectious complications. Since cirrhosis is a multisystemic disorder, detailing the differentiation and expression patterns of the different monocytic subsets in other compartments such as lymphatic tissues and particularly in the liver is obviously necessary and has to be addressed in future studies. Intrahepatic macrophages are crucial with regards to liver disease progression and regulation of systemic inflammation. Balmer et al. showed that liver macrophages may act as a firewall of the systemic circulation facilitating phagocytosis of commensal organisms derived from the intestine[39]. On top of that, it is believed that compartment-specific environmental triggers are needed in order to change monocyte and macrophage phenotype and function[43–45]. In the context of cirrhosis evolution, pathological bacterial translocation and sterile inflammatory responses of tissues are described as contributing to the adaptation of liver macrophages[37,40,68]. Therefore, given their phagocytic properties[76], specific differentiation of AXL- and MERTK-expressing macrophages in the liver might be triggered by microbial pathogens, soluble inflammatory factors, and interaction with parenchymal and non-parenchymal cells in particular. Further evidence to support this hypothesis comes from recently published studies describing AXL expression on murine airway macrophages[102] and disease-associated microglia in the brain[139]. AXL expression was increased following influenza infection or neurodegeneration respectively, and associated with prevention of excessive tissue inflammation through efferocytosis[102,139]. AXL expression on murine airway macrophages was also critical for functional compartmentalisation, as it was not present on interstitial lung macrophages[102].

M-MDSCs on the other hand have been described in murine livers during regeneration[120], and liver inflammation protecting against fibrosis[121]. Considering

their immense expansion in the circulation of patients with cirrhosis, it is mandatory and highly interesting to assess their distribution and function in cirrhotic livers as well.

Assuming a compartment-specific differentiation of the monocytic subsets, it is also mandatory and may not be disregarded in the context of potential future systemic immunotherapies. Similarly, effects of systemic immune modulation on immune homeostasis of tissues need to be evaluated in murine models of chronic liver injury.

In conclusion, we demonstrate that in patients with cirrhosis along disease progression, the monocytic subsets M-MDSC and $CD14^+HLA\text{-}DR^+AXL^+$ (and $CD14^+HLA\text{-}DR^+MERTK^+$, previously described by our group[32]) with distinct immune-regulatory properties incrementally replaced monocytes with regular innate immune function. This process starts as early as in compensated disease and becomes apparent in advanced forms of cirrhosis which may explain the susceptibility to infection and poor outcome in advanced disease stages. Subset differentiation in the circulation is differentially regulated and involves recurrent exposure to microbial products and inflammatory factors (M-MDSC, $CD14^+HLA\text{-}DR^+AXL^+$), as well as pathogen and apoptotic cell uptake ($CD14^+HLA\text{-}DR^+AXL^+$), implying crucial roles in immune homeostasis in a condition depicted by pathologic bacterial translocation and recurrent inflammation. Moreover, we show that the function of these subsets can be modified by targeting AXL and TLR-3 receptors on M-MDSCs, and thus, they deserve evaluation as future immune-modulatory targets to augment immune responses and reduce susceptibility to infection, morbidity, and mortality in cirrhosis.

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