Crosstalk between Hepatocytes and CD4$^+$ T cells in Autoimmune Hepatitis

Dissertation

with the aim of achieving a doctoral degree

DOCTOR RERUM NATURALIUM

Faculty of Mathematics, Informatics, and Natural Sciences

Department of Biology

University of Hamburg

submitted by

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Hamburg, March 2024

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Date of oral defense: 4th October 2024

Abstract

Autoimmune hepatitis (AIH) is a chronic liver disease characterized by severe inflammation and resulting in liver cirrhosis. The exact mechanisms leading to an overshooting immune response damaging hepatocytes and loss of tolerance remain unidentified. However, several studies have shown that dysregulated T cell mediated responses against hepatic autoantigens are considered to contribute to AIH. Specifically, these studies have identified enhanced frequencies of pro-inflammatory TNF and IFNγ producing $CD4^+$ T cells in AIH. However, the initial pro-inflammatory signals that activate autoimmune $CD4^+$ T cells and trigger cytokine secretion remain unknown.

Innate immune responses activated by pathogen-associated molecular patterns (PAMPs) are important regulators of T cell responses. Toll like receptors are pathogen recognition pattern receptors, recognizing foreign structures such as double-stranded (ds) RNA typically derived from viruses. Activation of TLRs initiates inflammatory immune responses to control pathogens. However, a dysregulated TLR response can result in an inappropriate production of inflammatory cytokines and contribute to an initiation of autoimmune diseases. Recent studies have suggested that TLR3, TLR4 and TLR5 may be involved in liver diseases.

We hypothesize that in AIH, dysregulated TLR responses result in an elevated expression of pro-inflammatory cytokines that may activate local immune cells, such as T cells, resulting in tissue damage of hepatocytes. Therefore, we first investigated hepatocyte functionality in AIH and secondly determined the consequences of the dysregulated T cells in AIH on hepatocytes.

Recently, organoid systems have emerged as *in vitro* models to study diseases in humans. For this, we established 3D human liver organoids from livers from individuals with and without AIH.

First, we determined the phenotypic characteristics of human liver organoids to appraise their use as a model to study liver diseases, including the expression of hepatocyte markers, such as albumin and Cyp3A4. Next, TLR signaling was examined using organoids from AIH affected individuals and non-AIH individuals, which included the quantification of TLR expression, followed by the stimulation of human liver organoids with TLR3 ligand poly I:C to quantify the resulting cytokine and chemokine expression. These results showed that *TLR3* is expressed by hepatocytes in low levels, however, expression was similar between organoids generated from AIH-derived livers and non-AIH affected individuals. Furthermore, although a trend was observed towards higher expression of pro-inflammatory cytokine *IL-6* and chemokine *CXCL9* by organoids from AIH affected livers, these results did not reach significance. However, the activation of TLR3 and the following induced cytokine and chemokine production then may attract T cells to the liver.

TNF and IFNγ, which are increased produced by T cells in AIH affected livers showed a disrupted liver-organoid growth suggesting that these cytokines prevent hepatocyte regeneration. Co-cultures with AIH affected liver Tissue Resident Memory (TRM1) T cells and human liver organoids showed that the combined effect of TNF and IFNγ, secreted by T cells had a negative impact on organoid regeneration.

Taken together, the findings of this thesis suggest that hepatocytes can produce pro-inflammatory cytokines and chemokines upon TLR stimulation. Furthermore, cytokines produced by T cells, such as TNF and IFNγ have detrimental effects for the health and regenerative capacity of hepatocytes. Thus, dysregulated T cell responses are a critical mediator in the inflammation and tissue damage in the liver of individuals with AIH.

Zusammenfassung

Autoimmunhepatitis ist eine Entzündung der Leber. Durch eine kontinuierliche Fehlsteuerung der Immunantwort attackiert das Immunsystem die Hepatozyten, was zur Zerstörung des Lebergewebes führt. Die Gründe, warum es zum Verlust der Immuntoleranz kommt und das eigene Immunsystem die Leberzellen angreift, sind bisher unklar. Verschiedene Studien wiesen darauf hin, dass eine fehlregulierte Immunantwort gegen Autoantigene, die von T Zellen ausgelöst wird, zu AIH führen kann. Diese Studien deuteten vor allem auf ein erhöhtes Vorkommen von T Zellen hin, die die pro-inflammatorischen Zytokine TNF und IFNγ produzieren.

Immunreaktionen werden unter anderem durch Erkennung von Pathogen-associated molecular patterns (PAMPs) ausgelöst. Die Aktivierung von TLRs durch die Erkennung von PAMPs, wie doppelsträngige (ds) RNA von Viren, lösen die Produktion von Zytokinen aus, um den Wirt zu schützen.

Um die Immuntoleranz im Gewebe, wie innerhalb der Leber aufrechtzuerhalten, wird die Signalübertragung der TLRs streng kontrolliert, damit nur die benötigte Menge an pro-inflammatorischen Signalmolekülen produziert wird. Eine Fehlregulation von TLRs kann jedoch zu einer unangemessenen Produktion von pro-inflammatorischen Zytokinen führen, was die Entstehung von Autoimmunerkrankungen unterstützen kann. Frühere Studien haben gezeigt, dass insbesondere die drei Rezeptoren TLR3, TLR4 und TLR5 an Lebererkrankungen beteiligt sein können.

Basierend darauf, haben wir die Hypothese aufgestellt, dass bei Autoimmunhepatitis eine fehlregulierte TLR Immunantwort zu einer erhöhten Produktion von pro-inflammatorischen Zytokinen führt. Diese können Immunzellen wie T-Zellen aktivieren, die wiederum zum Zelltod der Hepatozyten führen können. Aus diesem Grund, haben wir zunächst die Funktionalität der

Hepatozyten bei AIH untersucht. Des Weiteren, haben wir die Auswirkungen der pro-inflammatorischen Zytokine, die von $CD4^+$ T Zellen produziert werden, auf die Hepatozyten analysiert.

Um dies beurteilen zu können, haben wir dreidimensional (3D) wachsende menschliche Leberorganoide aus Lebern von Patienten mit AIH und nicht-AIH Patienten etabliert. Organoide sind ein *in vitro* Model, das genutzt werden kann, um menschliche Krankheiten zu untersuchen. Aus diesem Grund, haben wir zunächst phänotypische Merkmale von Hepatozyten untersucht, die wie Expression von den beiden Hepatozytenmarkern Albumin und Cyp3a4. Nach Charakterisierung der Organoide, wurde die TLR Expression vor und nach Stimulation mit dem TLR Agonisten Poly I:C bestimmt. Hierbei konnte gezeigt werden, dass die Organoide geringe Mengen von TLR3 in nicht stimulierten Konditionen exprimierten, während sich die Expressionslevel nach TLR3 Stimulation erhöhten. Organoide von Individuen, die an AIH erkrankt sind, wiesen dabei einen Trend zu einer erhöhten TLR3 Expression nach Stimulation auf im Vergleich zu nicht AIH Organoiden. Darüber hinaus wurde ein Trend zu einer höheren Expression des pro-inflammatorschen Zytokins *IL-6* und des Chemokins *CXCL9* in Organoiden von Individuen mit AIH beobachtet. Allerdings waren die Ergebnisse nicht signifikant. Die Aktivierung von TLR3 und die darauffolgende induzierte erhöhte Zytokin- und Chemokinproduktion könnte dadurch T-Zellen in die Leber locken.

Da aus vorherigen Studien bekannt ist, dass TNF und IFNγ mit Autoimmun Hepatitis assoziiert sind, untersuchten wir als nächstes die Auswirkungen der beiden pro-inflammatorischen Zytokine, die von $CD4^+$ T Zellen in AIH produziert werden, auf die Hepatozyten. TNF und IFNγ zeigten Beeinträchtigungen des Wachstums der Leberorganoide, was darauf hindeutet, dass beide Zytokine die Regernation der Hepatozyten beeinträchtigen können. Co-Kulturen mit Organoiden und Tissue Resident Memory (TRM1) T-Zellen aus Lebern von Individuen mit AIH, zeigten ebenfalls, dass das von den T-Zellen ausgeschüttete TNF und IFNγ die Regenerationsfähigkeit der Organoide hemmt.

Zusammenfassend zeigten die Ergebnisse dieser Arbeit, dass Hepatozyten bei einer TLR Stimulation pro-inflammatorische Zytokine und Chemokine produzieren. Des Weiteren, haben TNF und IFNγ, die von T-Zellen exprimiert werden, schädliche Auswirkungen auf die Regenerationsfähigkeit der Hepatozyten. Daraus lässt sich schlussfolgern, dass fehlregulierte T-Zell Immunantworten ein entscheidender Faktor für Entzündungen und Gewebeschäden innerhalb der Leber von Menschen sind, die an AIH erkrankt sind.

Content

List of Abbreviations

°C	Celsius
3D	three-dimensional
ACC	Acceleration
AKB	Asklepios Hospital Barmbek, Hamburg
APC	antigen presenting cell
AIH	Autoimmune hepatitis
ALD	Autoimmune liver disease
ALT	alanine aminotransferase
ANA	antinuclear antibody
Anti-LC1	anti-liver cytosol type 1
Anti-LKM1	anti-liver kidney microsomal type 1
AST	aspartate aminotransferase
BECs	Biliary epithelial cells
BME2	Basement membrane extract, Type 2
BSA	Bovine Serum albumin
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
cDCs	classical dendritic stells
cDNA	complementary DNA
CO_2	carbon dioxide
CXCL	chemokine (C-X-C mofic) ligand
Cyp3A4	Cytochrome P450 family 3 subfamily A member 4
DAMPs	damage-associated molecular pattern
DC	Dendritic cells
DEC	Deceleration
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethyl sulfoxide

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fw	Forward
GWAS	Genome-wide association studies
h	hours
HGF	hepatocyte growth factor
HHCH	Healthy cohort Hansestadt Hamburg
HLA	human leukocyte antigen
HSC	Hepatic stellate cell
IAIHG	International Autoimmune Hepatitis Group
IF	Immunofluorescence
IFNß	Interferon beta
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	interleukin
KC	Kupffer cell
LGR-5	leucine rich repeat containing G protein coupled receptor 5
LPS	Lipopolysaccharide
LRR	leucine-rich repeat
LSECs	Liver sinusoidal endothelial cells
LTX	Liver transplantation
MHC	major histocompatibility complex
µl	microliter
min	minutes

ml	milliliter
mM	millimolar
mRNA	messenger RNA
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor kappa B
ng	nanogram
ON	overnight
OWB	Organoid washing buffer
P/S	Penicillin/Streptomycin
PAMPs	pathogen-associated molecular pattern
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PHHs	Primary human hepatocytes
pDCs	plasmacytoid dendritic cells
Poly I:C	Polyinosinic-polycytidylic acid
PRRs	pattern recognition receptors
PSC	primary sclerosing cholangitis
Rev	reverse
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	room temperature
RT-PCR	Real-time polymerase chain reaction
SMA	anti-smooth muscle actin antibody
SOX9	SRY-box transcription factor 9
TGF-α	Transforming growth factor alpha
TGF-ß	TGF-ß: Transforming growth factor beta
Th	T helper
TIR	Toll/ interleukin (IL)-1 receptor

TLR	Toll like receptor
TNF	Tumor necrosis factor
TRM1	Tissue resident memory cell 1
UKE	University medical center Hamburg-Eppendorf

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1 Introduction

1.1 The human liver

As one of the largest organs in the human body, the liver represents around 2 – 5% of the body weight (Abdel-Misih & Bloomston, 2010; Hansel et al., 2014; Kuntz & Kuntz, 2006; Si-Tayeb et al., 2010). As the liver is directly linked to the gut, the liver is permanently exposed to a large number of antigens through the portal vein blood stream that contains harmless antigens but also can contain pathogens, such as bacteria and viruses. Due to the high exposure to foreign antigens, the liver needs to have an increased ability to maintain immune tolerance (Cheng et al., 2021; Crispe, 2014; Doherty, 2016; Parlar et al., 2023; Racanelli & Rehermann, 2006; Tiegs & Lohse, 2010). Furthermore, the liver performs numerous biological functions, including the storage of nutrients, detoxification, synthesis and secretion of serum proteins, such as albumin and fibrinogen, the production of the bile, the production of several hormones, as well as storage of glycogen and vitamins (Ben-Moshe & Itzkovitz, 2019; Boeri et al., 2019; Gebhardt, 1992; Jungermann, 1986; Telles-Silva et al., 2022). Moreover, as the liver can be targeted by specific pathogens, immune responses against invading pathogens are needed that can result in tissue damage. Upon tissue damage, a healthy liver possess a great regenerative capacity and restores its original size and function. These regenerative mechanisms are highly controlled and ensure the maintenance of the liver-to-body weight ratio (Ben-Moshe & Itzkovitz, 2019; Gao et al., 2008; Liaskou et al., 2012; Michalopoulos, 2007, 2013; Michalopoulos & Bhushan, 2021; Michalopoulos & DeFrances, 1997; Yanger & Stanger, 2011; X. Zhu et al., 2021).

1.1.1 Liver anatomy

The liver is a reddish brown organ that weighs around 1.3 – 1.8 kg (Andersen et al., 2015; Chaudhari et al., 2017; Kuntz & Kuntz, 2006). The surface of the liver is covered with a thin collagenous capsule that is called Glisson's capsule (Abdel-Misih & Bloomston, 2010; Rogers & Dintzis, 2018). Anatomically, the entire organ is divided into the left lobe and the right lobe (Figure 1) (Tsung & Geller, 2011).

The liver consists of eight segments (Andersen et al., 2015; Couinaud, 1954). The numbering of the various segments starts in the posteriorly located left lobe with segment I heading to segment II and III in left lateral segment, while segment IV is encompassing the left medial segment. The right lobe consists of segment V to VIII. Segment V and VIII belong to the right anterior lobe whereas segment VI and VII form the right posterior lobe (Figure 1) (Andersen et al., 2015; Couinaud, 1954; Sibulesky, 2013; Sutherland & Harris, 2002; Tsung & Geller, 2011).

Every single segment consists of a portal vein, hepatic artery and a bile duct (Tsung & Geller, 2011).

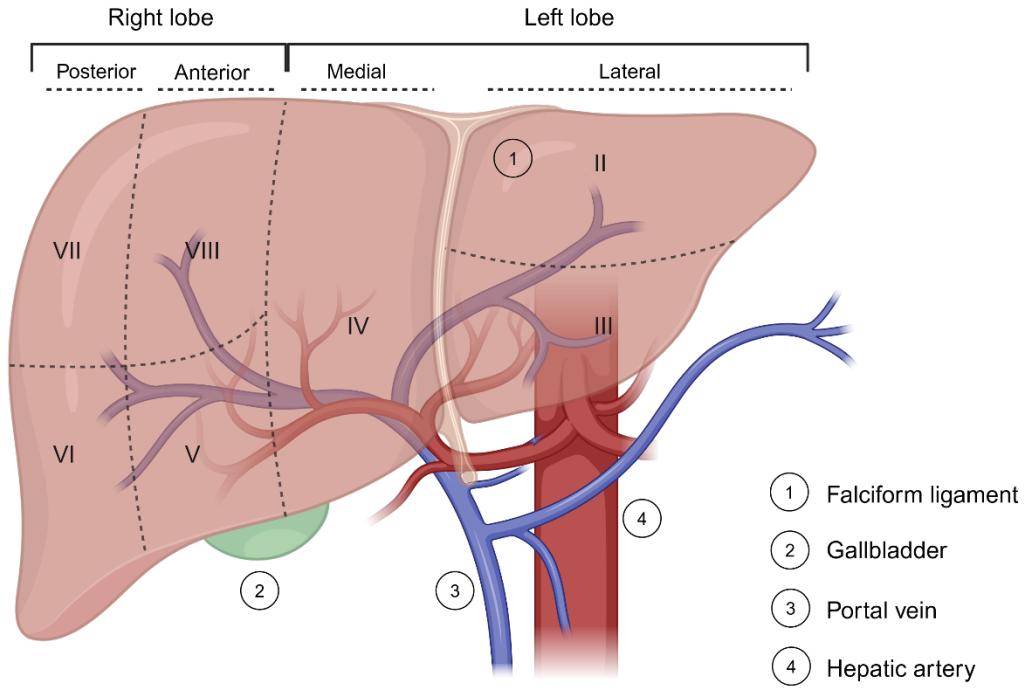

Figure 1: Segmental anatomy of the liver.
The liver is divided into the left and the right lobule, consisting of eight segments. The numeration of the segments starts in the caudate lobe (not shown) going in clockwise direction to segment II. The left lobe include segment I – IV, whereas the right lobe consists of segment V – VIII (Andersen et al., 2015; Couinaud, 1954; Sibulesky, 2013). Figure is adapted and modified from Andersen et al., 2015 and Sibulesky, 2013. Generated via BioRender.

In general, the liver has a dual blood supply via the hepatic artery and hepatic portal vein (Andersen et al., 2015; Tsung & Geller, 2011). The hepatic artery transports around 25% of blood supply by carrying blood with high oxygen to the liver. While the portal vein brings 75% nutrient-rich and antigen-rich blood from intestine, stomach, and spleen allowing direct metabolic exchange between the organs (Abdel-Misih & Bloomston, 2010; Hansel et al., 2014; Invernizzi, 2013; Tsung & Geller, 2011).

1.1.2 Functions of the liver

The liver performs various essential functions. Based on the direct connection to the intestine, the liver is capable to absorb and store several nutrients received through the bloodstream via the portal vein from the gut (Ben-Moshe & Itzkovitz, 2019; Boeri et al., 2019; Rui, 2014; Wahlström, 2019). Because of the immense daily blood flow, the bloodstream transports not only essential nutrients but also toxic substances, such as pathogens to the liver. Therefore, it is essential that the liver carries out its protective functions by removing pathogens in order to

maintain liver homeostasis (Ben-Moshe & Itzkovitz, 2019; Cheng et al., 2021; Parlar et al., 2023).

One of the main activities of the liver is to maintain glucose levels in the blood. Therefore, glucose and amino acids are transported to the liver following digestive processes in the gut. Maintaining the glucose levels within the body is critical for the survival of the organism (Ben-Moshe & Itzkovitz, 2019; Rui, 2014). To this end, the liver stores glucose as glycogen. Under fasting conditions, when the blood sugar levels are too low, glucose is generated from glycogen via gluconeogenesis (Ben-Moshe & Itzkovitz, 2019; Rui, 2014; Trefts et al., 2017). Thus, the liver is critical in strictly regulating glucose homeostasis (Han et al., 2016).

Many important proteins are synthesized in the liver (Goldberg, 1980). Albumin is produced by hepatocytes within the liver (Wada et al., 2017; N. Wu et al., 2024) and performs serval functions (Evans, 2002; Fanali et al., 2012; Kawakami et al., 2006; Setoyama et al., 2017; Shalish et al., 2017; Sun et al., 2019). Amongst others, albumin is necessary for the transport of various compounds, such as bilirubin or fatty acids through the body (Curry et al., 1998; Jacobsen & Brodersen, 1983; Paar et al., 2021).

Furthermore, another fundamental feature of the liver involves accomplishing exocrine and endocrine functions (Si-Tayeb et al., 2010). Exocrine functions include the generation and secretion of the bile via hepatocytes, which is necessary to emulsify lipids in the intestine (Si-Tayeb et al., 2010). Whereas endocrine functions encompasses the secretion of hormones, including insulin-like growth factor 1 (IGF-1) and angiotensinogen (Rhyu & Yu, 2021; Si-Tayeb et al., 2010).

To conclude, many functions are performed by the liver (Duncan et al., 2009; X. Zhu et al., 2021). Hepatocytes, which are the main cell type of the liver, are predominantly involved in performing these functions (Ben-Moshe & Itzkovitz, 2019; Godoy et al., 2013). However, other liver cell types, such as liver endothelial cells, hepatic stellate cells, cholangiocytes and Kupffer cells contribute to perform these functions of the liver and create the environment for optimal liver functioning (Ben-Moshe & Itzkovitz, 2019; Trefts et al., 2017).

1.1.3 Hepatic immune tolerance

The liver is perfused with approximately 1.5 liter of blood per minute (Doherty, 2016; Parlar et al., 2023). Blood is transported to the liver from either the gut via the portal vein of from the lungs via the hepatic artery (Doherty, 2016; Gao et al., 2008; Zheng & Tian, 2019). Thus, the liver is simultaneously supplied with oxygen and nutrients (Böttcher et al., 2011), while on the other hand, a large amount of antigens derived from pathogens as well as harmless antigens, such as food antigens derived from the intestinal tract are transported to the liver (Carambia & Herkel, 2018; Doherty, 2016; Parlar et al., 2023; Zheng & Tian, 2019). Recognition and

elimination of toxins and pathogens from the bloodstream is a major function of the liver. At the same time, due to the constant exposure to antigens, the liver needs to possess the ability of immune tolerance allowing it to avoid tissue damage at steady-state conditions (Invernizzi, 2013; Parlar et al., 2023; Zheng & Tian, 2019).

To maintain immune tolerance, the liver is composed of various non-parenchymal resident liver cells with immunological functions (Tiegs & Lohse, 2010). These liver resident cells include dendritic cells (DCs), hepatic stellate cells (HSCs), Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs) (Tiegs & Lohse, 2010). In particular, both KCs and LSECs are of importance since they function as barriers by recognizing, filtering, and removing pathogens out of the blood to prevent inflammatory immune responses (Bhandari et al., 2021; Gottwick et al., 2022; Jenne & Kubes, 2013; Medzhitov, 2010). To ensure that the antigen-presenting cells (APCs) of the hepatic barrier located in the hepatic sinusoids filter the majority of the incoming pathogens, toxins and damaged cells, a slow blood flow through these vessels is facilitated (Carambia & Herkel, 2018; Gottwick et al., 2022; Racanelli & Rehermann, 2006; Thomson & Knolle, 2010). Nevertheless, liver immune tolerance can be disrupted due to the constant antigen presentation of APCs to lymphocytes, such as B and T cells. Dysregulated immune cell activation subsequently results in liver inflammation and immune mediated liver damage ultimately leading to various liver diseases (Carambia & Herkel, 2018; Doherty, 2016; Parlar et al., 2023; R. Wang et al., 2021).

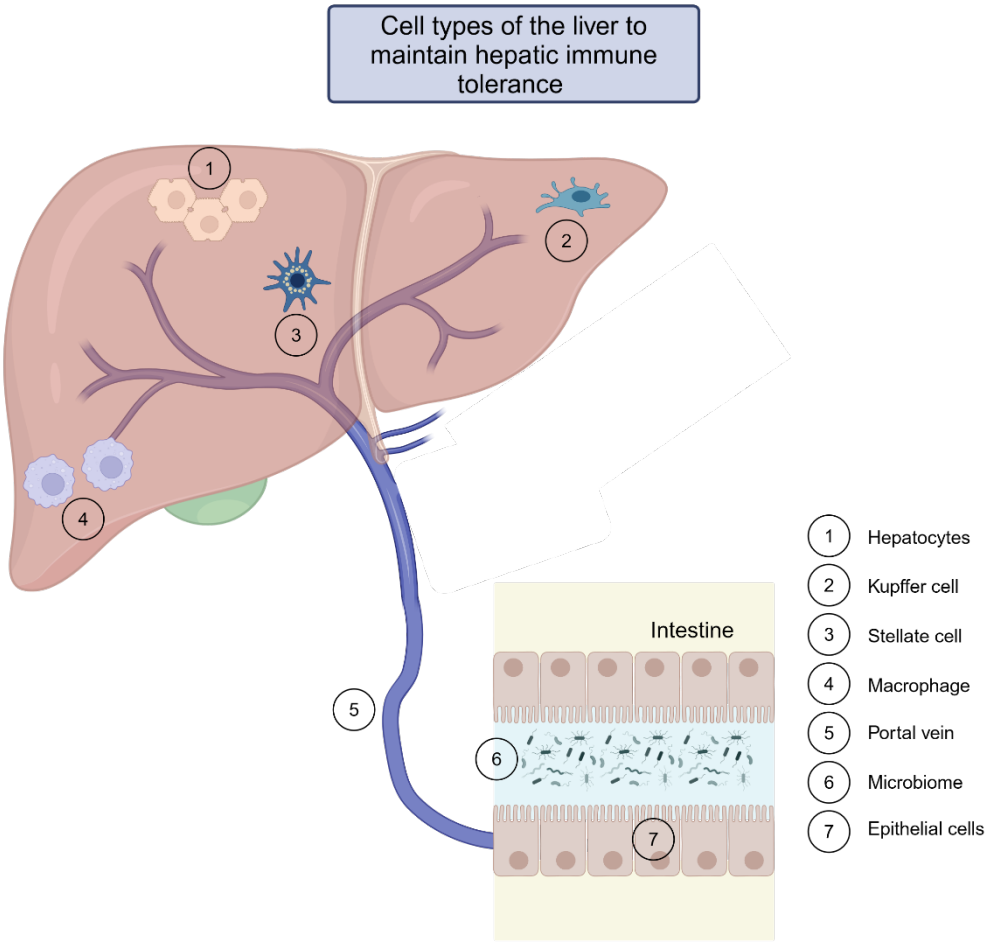

Figure 2: Gut liver axis.

Due to the link to the gut, the liver is exposed to a vast amount of foreign antigens from pathogens as well as harmless antigens such as food antigens derived from the gut through the portal vein (Boeri et al., 2019; Doherty, 2016; Tiegs & Lohse, 2010; Wahlström, 2019). To distinguish between pathogen and self-antigens, the liver needs to balance tolerance and host defense (Carambia & Herkel, 2018; Parlar et al., 2023; Tiegs & Lohse, 2010)*. For this, the liver possesses various cell types that are specialized to maintain liver tolerance (Doherty, 2016). These liver cells include hepatocytes, hepatic stellate cells, Kupffer cells and dendritic cells that function as APCs by presenting antigens to T cells to initiate immune responses for host defense (Doherty, 2016). Figure is adapted and modified from Szabo, 2015. Created with BioRender.*

1.1.4 Histological morphology

The liver consists of hexagonal shaped, repeating units named liver lobules, which are about 1 mm in diameter (Ben-Moshe & Itzkovitz, 2019; Fu et al., 2018; Hoehme et al., 2010; Teutsch, 2005). Each liver lobule consists of six portal triads and a central vein, which is located in the center of the lobule (Figure 3) (Fu et al., 2018; Jungermann & Kietzmann, 1996; Nuciforo & Heim, 2020). The portal triads consist of a hepatic artery, a portal vein and the bile duct, located at the corners of the lobule (Fu et al., 2018; Jungermann & Kietzmann, 1996; Nuciforo & Heim, 2020; Sibulesky, 2013). The blood, incorporated from both, the hepatic artery and the portal

vein is transported through the hepatic sinusoids towards the central vein (Ben-Moshe & Itzkovitz, 2019). Liver sinusoids are providing the hepatocytes with blood. Here, oxygenated blood enters the sinusoids via the hepatic artery, whereas blood rich in nutrients enters the sinusoids from the portal vein transporting the blood to the central vein, where the blood leaves the sinusoids (Gottwick et al., 2022; Hansel et al., 2014; Trefts et al., 2017). Liver sinusoids are lined by liver sinusoidal endothelial cells and are surrounded by hepatocytes, allowing the direct exchange of blood between both cell types for oxygen and nutrient supply (Boeri et al., 2019; P. Lee et al., 2013; P. Li et al., 2017; Maschmeyer et al., 2011a; Moradi et al., 2020). Unlike the blood flow, which flows to the center, the bile, secreted by hepatocytes, is transported in the opposite direction - from the central vein to the intestine (Ben-Moshe & Itzkovitz, 2019).

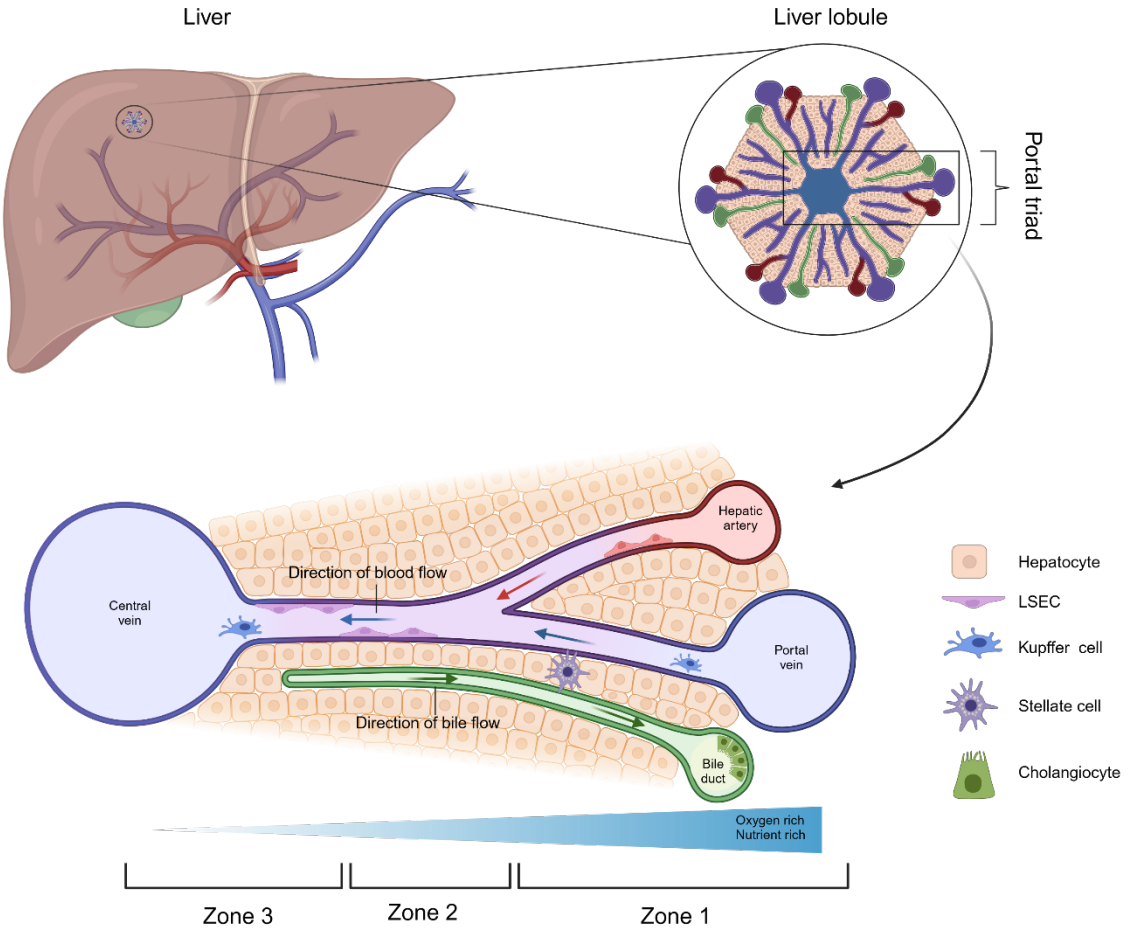

Figure 3: Composition of the liver.

The liver consists of lots of lobules. Each lobule is hexagonal shaped and includes six portal triads that disperse into a central vein in the center of the lobule. Every portal triad contains one hepatic artery, one portal vein and one bile duct. The hepatic sinusoids are separated into three zones and house various hepatic cell types. Figure adapted and modified from Ben-Moshe & Itzkovitz, 2019 and Trefts et al., 2017. Generated via BioRender.

Along the periportal-pericentral axis of the liver lobule, the blood exhibits gradual changes of oxygen, nutrients, and hormone composition (Ben-Moshe & Itzkovitz, 2019; Cunningham & Porat-Shliom, 2021; Jungermann & Kietzmann, 1996). Depending on the location of the hepatocytes along the axis towards the central vein, they are divided into three zones. This separation referred to as “zonation” and is divided into zone 1 (periportal), zone 2 (midzonal) and zone 3 (pericentral). Based on their zones, hepatocytes perform various metabolic functions, including glycogenesis, ß-oxidation of fatty acids or secretion of proteins, underlining their heterogeneity (Figure 4) (Cunningham & Porat-Shliom, 2021; Deane, 1944; Fu et al., 2018; Moradi et al., 2020; Rani et al., 2006; Sasse et al., 1975; Trefts et al., 2017).

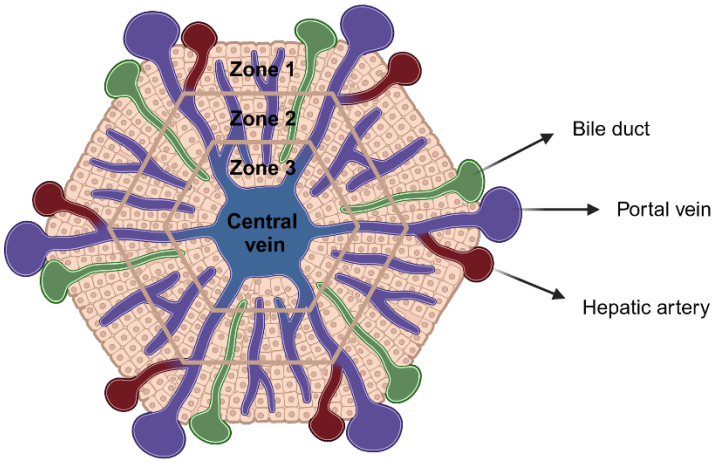

Figure 4: Representation of the zonation of the hepatic lobule.
Representation of the hepatic lobule showing the different zones 1, 2 and 3. The central vein is located in the middle of the hepatic lobule, while the hepatic artery (dark red), portal vein (blue) and bile duct (green) are arranged at each corner of the hexagonal shaped liver lobule. The Figure is adapted and modified from Trefts et al., 2017. Created with Biorender

Due to the gradient change along the periportal-pericentral axis towards the central vein, zone 1 provides the highest concentration of oxygen and nutrients, as it is located closest to the portal vein and the hepatic artery, where the blood enters the hepatic sinusoids. Zone 2 is considered as the mid-zone, whereas zone 3 has the lowest blood and nutrient supply (Cunningham & Porat-Shliom, 2021; Moradi et al., 2020; Usta et al., 2015).

1.2 Liver parenchymal cell types

The liver is composed of five different main types of cells, including hepatocytes, which are parenchymal cells, and non-parenchymal cells, such as cholangiocytes (biliary epithelial cells), liver sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells (Ben-Moshe & Itzkovitz, 2019; L. Guo et al., 2011; Telles-Silva et al., 2022).

Hepatocytes are the most prominent cell type in the liver and perform the majority of liver functions (Ben-Moshe & Itzkovitz, 2019; Godoy et al., 2013). Whereas, non-parenchymal cells represent approximately 40% of liver cells and support hepatocytes performing their function by maintaining liver tissue architecture and regulating immune responses to pathogen (Ben-Moshe & Itzkovitz, 2019; P. Lee et al., 2013; Maschmeyer et al., 2011b; Moradi et al., 2020).

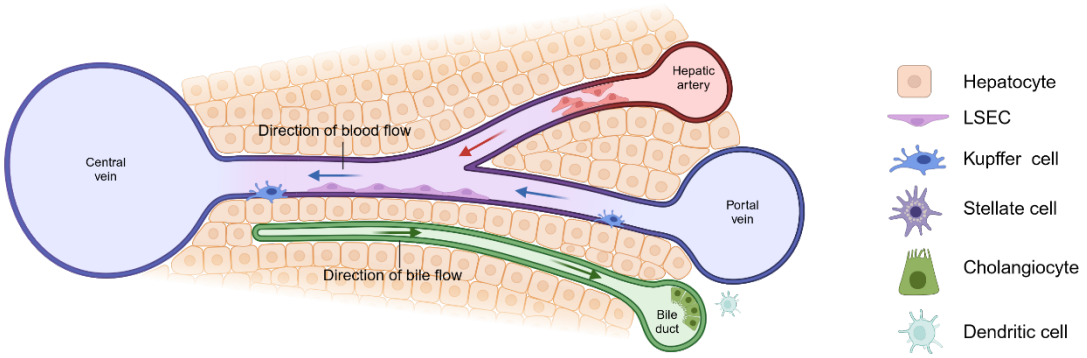

Figure 5: Liver parenchymal cell types within the hepatic sinusoid.
Schematic representation of a portal triad and the associated liver cell types. Hepatic sinusoids are lined by LSECs and are surrounded by hepatocytes. Next to LSECs and hepatocytes, further cell types, such as Kupffer cells, stellate cells, dendritic cells and other immune cells exists within the hepatic sinusoids. The bile consists of cholangiocytes (Gottwick et al., 2022; Tiegs & Lohse, 2010). Figure is adapted and modified from Gottwick et al., 2022 and Ben-Moshe & Itzkovitz, 2019. Figure is created with BioRender.

1.2.1 Hepatocytes

Hepatocytes are diamond-shaped parenchymal cells that encompass most cells in the liver. They are approximately 30 µm in diameter and are functionally as well as morphologically polarized epithelial cells, having the sinusoidal (basolateral) and canalicular (apical) membrane surfaces (Cullen & Stalker, 2016; Materne et al., 2013; Moradi et al., 2020; Tocan et al., 2021; Treyer & Münsch, 2013). The apical surface of hepatocytes forms the bile canaliculus for the secretion of the bile (Tocan et al., 2021; Treyer & Münsch, 2013). The bile is produced by hepatocytes and is subsequently transported into the bile ducts (Duncan et al., 2009). The basolateral surface is either located between adjacent hepatocytes or in the direction to hepatic sinusoids that allow the exchange of nutrients and blood (Duncan et al., 2009; Tocan et al., 2021; Treyer & Münsch, 2013; Wisse, 1970). Hepatocytes perform a variety of biochemical and metabolic functions, such as detoxification, production and secretion of the bile, metabolism of amino acids, protein synthesis of albumin, fibrinogen, and transferrin as well as gluconeogenesis. However, depending on the location of the hepatocytes within the hepatic lobule, the functions that hepatocytes perform differ (Duncan et al., 2009; Gong et al., 2023; Jungermann & Kietzmann, 1996; LeCluyse et al., 2012; Materne et al., 2013; Moradi et al., 2020; Z. Zhou et al., 2016). For example, ammonia metabolism is only performed by the

periportal hepatocytes, which convert ammonia into urea (Duncan et al., 2009; Häussinger et al., 1992; Moorman et al., 1989). In addition, hepatocytes are essential for immune regulation by mediating immune responses against foreign pathogens. Therefore, they act as antigen presenting cells (APCs) (Tiegs & Lohse, 2010). In inflammatory conditions, such as AIH, hepatocytes express major histocompatibility complex (MHC) class I and class II molecules in low levels. Via MHC class I and class II molecules they can interact with lymphocytes and present foreign antigens to $CD4^+$ and $CD8^+$ T cells to initiate immune responses (Bertolino et al., 1998; Gottwick et al., 2022; Herkel et al., 2003; Holz et al., 2008; Tiegs & Lohse, 2010; Warren et al., 2006). Upon recognition of pathogens, hepatocytes can also secrete inflammatory cytokines to the bloodstream, which either directly contribute to pathogen control or indirectly by activating the innate immune system (Gao et al., 2008; Z. Zhou et al., 2016). However, hepatocytes can also be targeted by dysregulated immune responses leading to liver inflammation and hepatocyte death, which is associated with a variety of liver diseases, such as AIH (Gong et al., 2023; Wree et al., 2019).

1.2.2 Biliary epithelial cells (Cholangiocytes)

Biliary epithelial cells (BECs) also called cholangiocytes are epithelial cells and represent 3% of cells in the liver (Masyuk et al., 2018; Si-Tayeb et al., 2010). Cholangiocytes are a heterogeneous cell population that form the three-dimensional intrahepatic and extrahepatic biliary tree. The two biliary trees differ in their function and anatomy (Lemaigre, 2009; Masyuk et al., 2018; Moradi et al., 2020; Raynaud et al., 2011; Si-Tayeb et al., 2010; Strazzabosco & Fabris, 2008; Tabibian et al., 2013). Predominantly, cholangiocytes modify the bile, produced by hepatocytes, during transport through the biliary tree. Bile modification is regulated by various molecules, including hormones, peptides, nucleotides, neurotransmitters and other soluble molecules (Masyuk et al., 2018; Moradi et al., 2020; Tabibian et al., 2013). Cholangiocytes are key to act as APCs (Ronca et al., 2020). Therefore, they can express both classes of adhesion molecules, MHC class I and class II on their surface, allowing them interact with $CD4^+$ and $CD8^+$ T cells (Chuang et al., 2009; Fava et al., 2005; Yoo et al., 2016). Under homeostatic conditions, they express MHC class I molecules (Auth et al., 1993; Ronca et al., 2020). However, in diseased conditions, BECs express MHC class II molecules on their surface. Mostly, this enhanced expression of MHC class II molecules is observed in diseases of the bile duct like PBC (Ayres et al., 1993; Banales et al., 2019; Ronca et al., 2020).

1.2.3 Kupffer cells

Kupffer cells (KCs) are liver resident macrophages, contributing to approximately 20% of the liver resident cell types (Carambia & Herkel, 2018; Dixon et al., 2013; Moradi et al., 2020; Racanelli & Rehermann, 2006). Due to their location within the sinusoidal lumen, KCs are immediately exposed to pathogenic antigens from the gut. This localization enables KCs to

perform on the one hand phagocytosis upon recognition of pathogens, and on the other hand, to function as APCs to maintain liver tolerance (Carambia & Herkel, 2018; Dixon et al., 2013; Gottwick et al., 2022; Moradi et al., 2020; Usta et al., 2015). In healthy conditions, they express low MHC class II molecule levels as well as low levels of co-stimulatory molecules and show a tolerogenic phenotype (Carambia & Herkel, 2018; Dixon et al., 2013; Gottwick et al., 2022; Nguyen-Lefebvre & Horuzsko, 2015; Q. You et al., 2008). However, when performing their tolergenic phenotype, KCs release both anti-inflammatory cytokines IL-10 and TGF-ß to suppress T cells to maintain liver tolerance (Bissell et al., 1995; Carambia & Herkel, 2018; Knolle et al., 1998; Nguyen-Lefebvre & Horuzsko, 2015). However, upon exposure to pathogens, KCs can interact with $CD4^+$ T cells to initiate T cell activation and mediate an inflammatory immune response (Carambia & Herkel, 2018).

1.2.4 Liver sinusoidal endothelial cells

Liver sinusoidal endothelial cells (LSECs) are specialized endothelial cells that represent approximately 3% of the liver volume. Contrary to other endothelial cells, they possess a unique shape and lack a basement membrane (Maslak et al., 2015; Poisson et al., 2017; Regan & Aird, 2012; Sørensen et al., 2015). LSECs line the hepatic sinusoids and represent a permeable barrier allowing the transport of proteins and molecules a through small open pores, also known as “fenestrae” with diameters of around 50 to 150 nm (Braet & Wisse, 2002; Fraser et al., 1995; Maslak et al., 2015; Poisson et al., 2017; Si-Tayeb et al., 2010; Sørensen et al., 2015; Wisse et al., 1985). Furthermore, LSECs perform immunological functions such as endocytosis of pathogens entering the sinusoids and the presentation of antigens (Shetty et al., 2018). Due to their ability to present antigens to lymphocytes to mediate immune responses, they can act as APCs and are essential for maintaining liver tolerance (Gracia-Sancho et al., 2021; Maslak et al., 2015; Shetty et al., 2018; Wohlleber & Knolle, 2016). However, in homeostatic conditions, they express low levels of MHC class II molecules and co-stimulatory molecules, but high levels of co-inhibitory molecules, such as programmed death ligand-1 (Carambia & Herkel, 2018; Diehl et al., 2008; Gottwick et al., 2022; Knolle et al., 1999; Lohse et al., 1996). Once the antigen is presented to the lymphocytes, the expression of MHC class II allows activation of $CD4^+$ T cells to initiate inflammatory immune responses. However, they also have the ability to activate $CD8^+$ T cells through cross-presentation of antigens (Carambia et al., 2013; Carambia & Herkel, 2018; Diehl et al., 2008; Gottwick et al., 2022; Knolle et al., 1999; Limmer et al., 2000; Lohse et al., 1996; Schurich et al., 2009; Shetty et al., 2018)

1.2.5 Hepatic stellate cells

Hepatic stellate cells (HSC) are non-parenchymal cells in the liver (Luo et al., 2021; Schumacher & Guo, 2016). These star-shaped cells are located in the perisinusoidal space

between hepatocytes and liver sinusoidal endothelial cells (S. L. Friedman, 2008; Geerts, 2001; Kamm & McCommis, 2022; Maschmeyer et al., 2011b; Tsuchida & Friedman, 2017; Wake, 1971). Under homeostatic conditions, HSC are inactive and display a quiescent phenotype by showing a low proliferation rate (Huang et al., 2017; Luo et al., 2021; Puche et al., 2013; Tsuchida & Friedman, 2017). However, the main functions of HSCs are to store vitamin A (or retinoid) in lipid droplets, the storage of fat, the production and maintenance of the extracellular matrix (ECM), and to maintain liver tolerance (Kamm & McCommis, 2022; Luo et al., 2021; Moradi et al., 2020; Tsuchida & Friedman, 2017). Furthermore, HSC can additionally function as APCs, by presenting antigens to T cells for their activation (Kamm & McCommis, 2022; Tiegs & Lohse, 2010; Winau et al., 2007). To act as APCs, HSCs express both MHC class I and MHC class II molecules on their surface. Thereby, they present antigens to either $CD4^+$ or $CD8^+$ T cells to mediate inflammatory immune responses for the elimination of pathogens (Tiegs & Lohse, 2010). Whereas under activated conditions, HSCs can contribute to liver fibrosis upon liver injury (Luo et al., 2021). In activated conditions, induced by inflammatory signals or oxidative stress, HSCs exhibit an increased proliferation activity and an enhanced production of ECM. In addition, they lose their lipid-droplet phenotype and differentiate into myofibroblast-like cells (S. L. Friedman, 2008; Gressner & Weiskirchen, 2006; Kamm & McCommis, 2022; Luo et al., 2021; Maschmeyer et al., 2011a).

1.2.6 Dendritic cells

Known as professional antigen-presenting cells (Schwabe et al., 2006), dendritic cells (DCs) are liver resident cells and help maintaining liver homeostasis (Carambia & Herkel, 2018; Thomson & Knolle, 2010). In the liver, DCs are playing a role in innate and the induction of adaptive immunity and are divided into two groups of DCs: classical dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) (Carambia & Herkel, 2018; Ganguly et al., 2013; Thomson & Knolle, 2010). Contrary to the other liver resident cells, DCs are mainly located in the periportal areas (Sumpter et al., 2007; Tiegs & Lohse, 2010). In a healthy environment, DCs exhibit an immature state (Carambia & Herkel, 2018; Thomson & Knolle, 2010). Whereas, under inflammatory conditions, DCs become activated and induce immune responses. On the one hand, pDCs produce type I IFNs upon TLR recognition, while on the other hand, cDCs act as APCs and induce pro-inflammatory immune responses by presenting antigens via MHC class II to immune cells (Carambia & Herkel, 2018; Ganguly et al., 2013; Gottwick et al., 2022; Jenne & Kubes, 2013; Kingham et al., 2007; Reizis et al., 2011).

1.3 Toll like receptors

Toll like receptors (TLRs) belong to the family of pattern recognition receptors (PRRs) and are key to defend the host against foreign pathogens. To initiate a fast innate immune response against various types of pathogens, they sense pathogen-associated molecular pattern

(PAMP) (Aderem & Ulevitch, 2000; Akira & Takeda, 2004; B. A. Beutler, 2009; Chávez-Tapia et al., 2015; Kesar & Odin, 2014; Kiziltas, 2016; Soares et al., 2010). PAMPs are specific structures from various microorganisms, including viruses, bacteria and other pathogens (Aderem & Ulevitch, 2000; B. A. Beutler, 2009; Kesar & Odin, 2014; Soares et al., 2010). Next to PAMPs, TLRs can also recognize endogenous damage-associated molecular pattern molecules (DAMPs), associated with damaged tissue and include for example heat shock proteins (Kesar & Odin, 2014; Mencin et al., 2009; Wheeler et al., 2009). TLRs are type I transmembrane glycoproteins, consisting of an extracellular and intracellular domain. The extracellular domain is made up of leucine-rich repeat (LRR) domains, which differs per receptors by recognizing various PAMPs and DAMPs. While the intracellular Toll/interleukin 1 (IL-1) receptor (TIR) domain plays a role in intracellular signaling (Figure 6) (Booth et al., 2011; Botos et al., 2011; Duan et al., 2022; Kesar & Odin, 2014; Kiziltas, 2016; Lin et al., 2011; Mohammad Hosseini et al., 2015; Soares et al., 2010; H. Y. Wang & Wang, 2012).

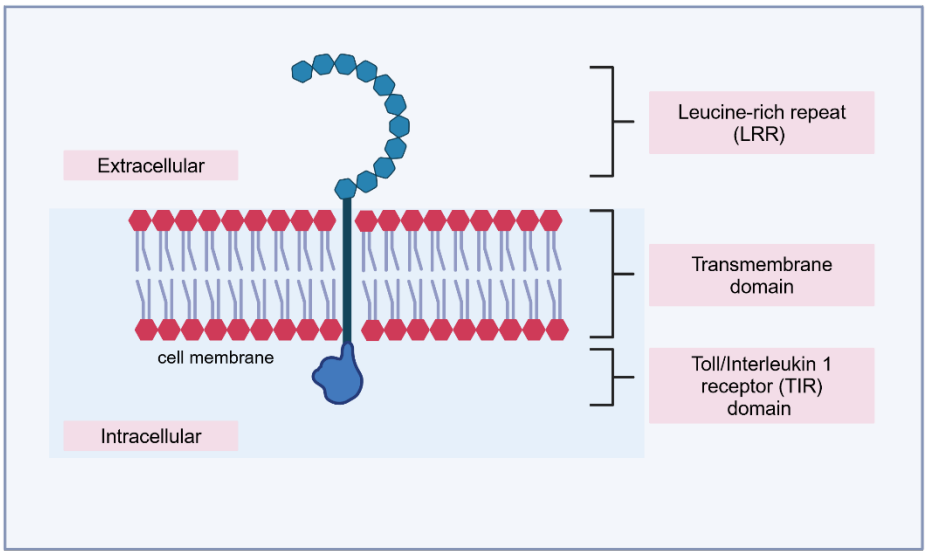

Figure 6: Toll like receptor structure.

TLRs are type I transmembrane glycoproteins. Each TLR consists of an intracellular Toll/Interleukin-1 receptor (TIR) domain and an extracellular domain, containing variable leucine-rich repeat (LRR) domains. Figure adapted from Kesar & Odin, 2014. Generated via BioRender.

TLRs are expressed by immune cells, such as B cells and T cells (Duan et al., 2022; Hua & Hou, 2013; Sutmuller et al., 2006) but also by macrophages, dendritic cells and natural killer cells (Duan et al., 2022; Iwasaki & Medzhitov, 2004; McClure & Massari, 2014). However, next to immune cells, several additional cells of the body including endothelial cells, epithelial cells, and mesenchymal cells can express TLRs (Kesar & Odin, 2014; Kumar et al., 2009).

The recognition of foreign pathogens via TLRs facilitates the immune system to perform a fast immune response (Kesar & Odin, 2014). Therefore, ten TLRs (TLR1 - TLR10) are expressed

in human (Akira & Takeda, 2004; Mencin et al., 2009). They are located either within the cell membrane or intracellular in endosomes, in endoplasmic reticula or in lysosomes (Duan et al., 2022; Kawasaki & Kawai, 2014). Although distributed throughout the whole body, each TLR is highly specific in sensing its corresponding ligand (Kiziltas, 2016; Mohammad Hosseini et al., 2015). TLRs that are expressed on the cell surface include TLR1, TLR2, TLR4, TLR5, and TLR6 (Kesar & Odin, 2014; Pradere et al., 2010). Those TLRs predominantly sense ligands associated with components from bacterial membranes (Akira et al., 2006; Duan et al., 2022). Both TLR4 and TLR5 sense gram-negative bacteria. TLR4 is recognizing LPS, whereas TLR5 can sense flagellin (Akira et al., 2006; Duan et al., 2022; Kesar & Odin, 2014; Soares et al., 2010). TLR3, TLR7, TLR8 and TLR9 are expressed intracellularly. Mainly they sense nucleic acids from bacteria or viruses (Kawai & Akira, 2010; Kawasaki & Kawai, 2014).

Both, TLR3 and TLR7 are recognizing viral-derived patterns (Mencin et al., 2009). TLR3 is sensing viral double-stranded RNA (dsRNA), while TLR7 is activated through viral single-stranded RNA (ssRNA) (Bernard et al., 2012; Kawasaki & Kawai, 2014; Mancuso et al., 2009; Takemura et al., 2014; Zhang et al., 2007). TLR9 is activated through unmethylated cytosine-phosphate guanine (CpG) DNA obtained from bacteria or viruses (Duan et al., 2022; Kawasaki & Kawai, 2014; Lind et al., 2022) (Figure 7). TLR10 is the only receptor that has no identified ligand or function (Kesar & Odin, 2014; Kiziltas, 2016; Soares et al., 2010).

TLRs act either as heterodimers or as homodimers. TLR2 builds a heterodimer in combination with either TLR1 or TLR6, whereas the other TLRs act as homodomers (Figure 7) (Duan et al., 2022; Kawai & Akira, 2010).

Upon dimerization TLRs activate either the MyD88-dependent pathway or the TRIF-dependent pathway, depending on the respective pathogen (Akira et al., 2006; Duan et al., 2022). All TLRs, except TLR3, use the same common downstream MyD88-dependent signaling pathway when binding their specific ligand (Aderem & Ulevitch, 2000; Kesar & Odin, 2014). The MyD88-dependent signaling pathway induces the expression of pro-inflammatory cytokines (such as TNF, IL-1, IL-6, IL-12) (Akira et al., 2006; B. Beutler et al., 2006; Duan et al., 2022; Kiziltas, 2016; Mohammad Hosseini et al., 2015; O'Neill & Bowie, 2007; Seki & Brenner, 2008). Whereas TLR3 activates the TRIF-dependent signaling pathway. The interaction with the adaptor molecule TRIF leads to the production of Type I interferons and the expression of inflammatory cytokines after NF-κB activation (Figure 7) (Akira et al., 2006; Duan et al., 2022; Kawasaki & Kawai, 2014).

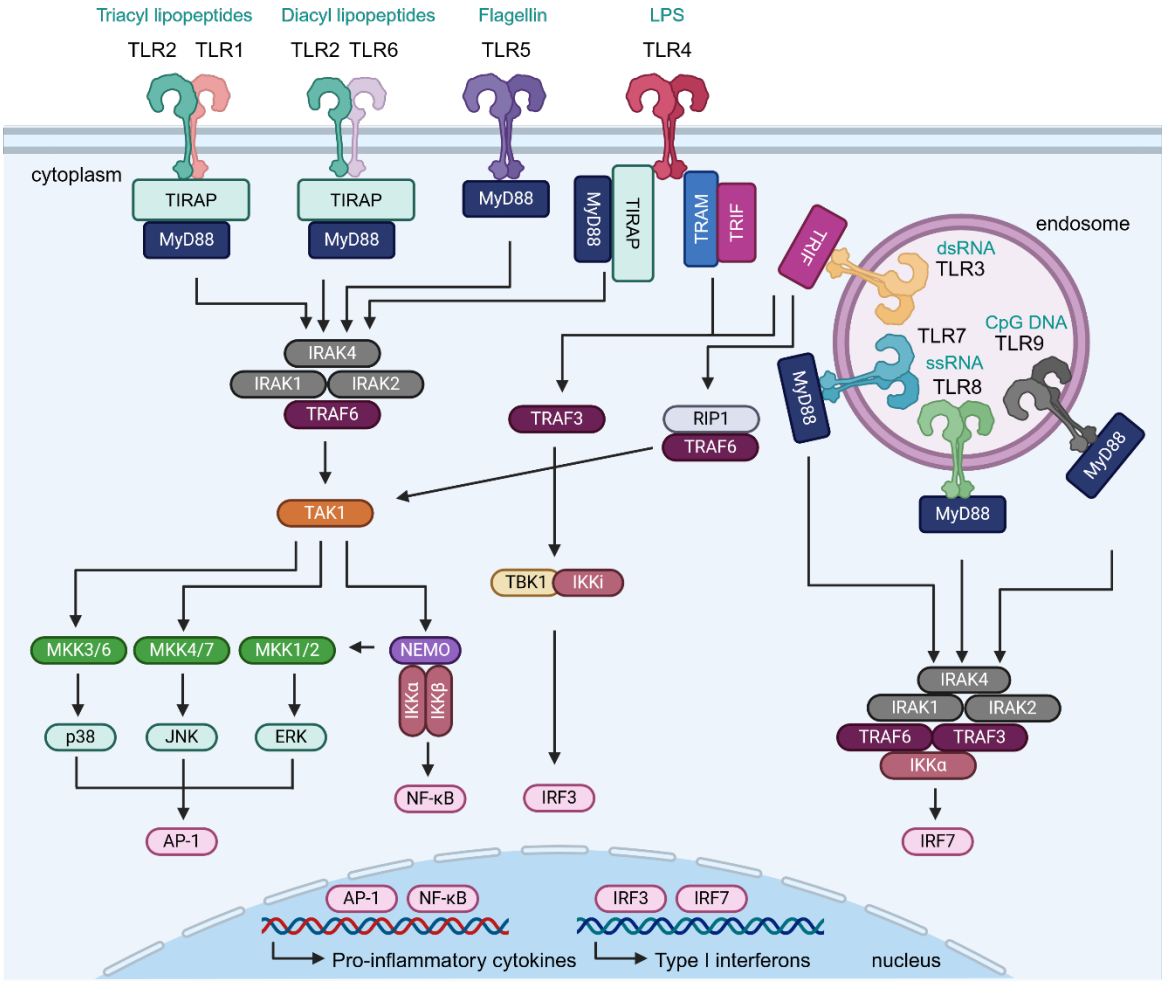

Figure 7: Toll like receptor signaling.

TLR 1/2, TLRTLR2/6 as well as TLR 4 and TLR5 are located on the cell surface. Whereas TLR3, TLR7, TLR8 and TLR9 are localized within the endosome (Duan et al., 2022; Kawasaki & Kawai, 2014). TLR activation upon binding to their specific ligands leads to downstream signaling pathways to produce pro-inflammatory cytokines and type I interferons (Akira et al., 2006; Duan et al., 2022). All TLRs except TLR3 use either just MyD88-dependent signaling or interact with another molecule as TRIF, TRAM or TIRAP for MyD88-dependent signaling, initiating the production of pro-inflammatory cytokines (Akira et al., 2006; Duan et al., 2022; Kawai & Akira, 2010). Whereas TLR3 is interacting with adaptor molecule TRIF for activation of the TRIF-dependent signaling pathway (Aderem & Ulevitch, 2000; Akira & Takeda, 2004; Kesar & Odin, 2014). Through interaction with IRF-3, expression of type I interferons is mediated whereas activation of NF-κB and AP-1 is necessary for pro-inflammatory cytokine expression (Kesar & Odin, 2014; Testro & Visvanathan, 2009). Figure is adapted and modified from Duan et al., 2022. Created with BioRender.

To produce an appropriate amount of cytokines, it is essential that TLR signaling is controlled to maintain liver tolerance, as uncontrolled signaling can initiate autoimmune diseases (Blasius & Beutler, 2010; Duan et al., 2022; Kawai & Akira, 2010; Liew et al., 2005).

1.3.1 TLR Expression in the liver

The liver is continuously exposed to pathogenic and non-pathogenic antigens derived from the gut through the portal vein. Due to this, it is necessary that the TLR signalling is controlled. A dysregulation of the TLR signalling could lead to an increased production of pro-inflammatory cytokines and interferons (Kesar & Odin, 2014; Kiziltas, 2016).

The liver possesses various cell types, classified in parenchymal and non-parenchymal cells (Y. Chen & Sun, 2011; Seki et al., 2011; Seki & Brenner, 2008). Hepatocytes are parenchymal cells, whereas Kupffer cells (KCs), biliary epithelial cells (cholangiocytes), hepatic stellate cells (HSCs), liver sinusoid endothelial cells (LSECs), and dendritic cells (DCs) belong to the non-parenchymal cells (Y. Chen & Sun, 2011; Nakamoto & Kanai, 2014). For a more detailed characterization of the hepatic cells, non-parenchymal can be further classified into immune cells and non-immune cells. Here, B cells, DCs, KCs, natural killer cells (NKs) and T cells are characterized as hepatic immune cells (Seki et al., 2011). However, each hepatic cell type performs its specific function within the liver and exhibits a different TLR expression (Y. Chen & Sun, 2011; Kesar & Odin, 2014; Kiziltas, 2016; Racanelli & Rehermann, 2006).

In homeostatic conditions, the liver shows low TLR mRNA expression levels (Y. Chen & Sun, 2011; De Creus et al., 2005; Nakamoto & Kanai, 2014; Zarember & Godowski, 2002).

Hepatocytes show mRNA expression levels of all TLRs. However, only both receptors TLR2 and TLR4 demonstrate a low responsiveness upon binding their specific ligand (Y. Chen & Sun, 2011; S. Liu et al., 2002; Nakamoto & Kanai, 2014; Seki & Brenner, 2008). In an inflammatory environment, which was induced via either LPS, TNF or IL-1, hepatocytes exhibit an upregulation of TLR2 expression on transcriptional level (Matsumara et al., 2003; Matsumura et al., 2000; Nakamoto & Kanai, 2014).

Biliary epithelial cells express all TLRs at transcriptional level, while they only produce TLR2 to TLR5 at protein level. When in inflammatory conditions with IFNγ, all four receptors TLR2-TLR5 show an upregulation (Y. Chen & Sun, 2011; Harada et al., 2003, 2006; Kiziltas, 2016). Moreover, TLR2 show an upregulated expression in appearance of TNF, however, another pro-inflammatory cytokines, such as IL-6 did not induce an upregulation of TLRs in BECs (Harada et al., 2006).

Hepatic stellate cells (HSCs) are identified to exhibit two different states, quiescent and activated, as described in 1.2.5. In both states, they show mRNA expression levels of all TLRs (Seki et al., 2011; B. Wang et al., 2009). HSCs are associated with liver fibrosis due to an enhanced exposure to bacteria. Upon stimulation with LPS, the activation of TLR4 lead to an upregulation of TGF-ß that following can support liver fibrosis (Kiziltas, 2016; Seki et al., 2007). Furthermore, as immune response upon binding LPS, they produce cytokines, such as IL-6

and IL-8, as well as chemokines like chemokine (C-C motif) ligand 2 (CCL2), CCL3 and CCL4, and intracellular cell adhesion molecule (ICAM-1) and E-selectin (Y. Chen & Sun, 2011; Kesar & Odin, 2014; Kiziltas, 2016; Nakamoto & Kanai, 2014; Paik et al., 2003).

Liver sinusoidal endothelial cells (LSECs) can express TLR1 to TLR9 at transcriptional level. Upon binding their agonist, TLR1, TLR4, TLR6, TLR6, and TLR9 respond by initiating the production of the pro-inflammatory cytokine TNF to eliminate the invading pathogen. Furthermore, activation of TLR1, TLR3 and TLR6 on LSECs lead to proliferation of T cells. However, activation of TLR3 and TLR4 induces an upregulation of the cytokines secretion of IL-6 (Nakamoto & Kanai, 2014; J. Wu et al., 2010).

As Kupffer cells (KCs) are localized within the liver sinusoids. They are key in host defense by inducing inflammatory cytokine responses upon recognition of their agonists (Crispe, 2009; Seki et al., 2011). KCs are able to express all TLRs at transcriptional level and as proteins. However, TLR5 is the only receptor that is not detected, neither on mRNA level nor on protein level (Seki et al., 2011; J. Wu et al., 2010). Furthermore, KCs are able to initiate a pro-inflammatory immune response by secreting both cytokines TNF and IL-6, especially when TLR2, TLR3, and TLR4 gets activated upon binding their ligand (Seki et al., 2011; J. Wu et al., 2010).

In general, hepatic dendritic cells express all TLRs at mRNA level, while no expression of TLR5 is detected (Seki et al., 2011; J. Wu et al., 2010). However, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) exhibit a heterogeneous distribution of the various TLRs within the liver (Y. Chen & Sun, 2011; Edwards et al., 2003; Iwasaki & Medzhitov, 2004). On the one hand, pDCs express TLR1, TLR7 and TLR9 (Edwards et al., 2003), while on the other hand cDCs show another distribution of the TLRs by expressing TLR2, TLR3, TLR4, TLR7, and TLR8 (Y. Chen & Sun, 2011; Edwards et al., 2003; Iwasaki & Medzhitov, 2004; Kiziltas, 2016). As soon as TLR7 and TLR9 are activated, pDCs induce a pro-inflammatory immune response by secreting the cytokines IL-6, IL-12, and TNF (Seki & Brenner, 2008; Shu et al., 2007). Whereas, upon binding the agonists of TLR2, TLR3, and TLR4, cDCs initiate the production of IL-6 and TNF as immune response to eliminate the pathogens (Seki & Brenner, 2008; Shu et al., 2007).

Further immune cells that are necessary within the liver are NK cells, B cells and T cells. NK cells express all ten TLRs on mRNA level (Chalifour et al., 2004; Kesar & Odin, 2014; Schmidt et al., 2004; Sivori et al., 2004). As soon as the TLRs are activated, NKs initiate the secretion of IFNγ, which is necessary to for liver regeneration and hepatic injury (Kesar & Odin, 2014). Other immune cells, such as B cells, express TLR1, TLR7, and TLR9. Whereas T cells show expression levels of TLR1, TLR2, TLR4, TLR5, and TLR9 (Babu et al., 2006; Kesar & Odin, 2014; Lohse et al., 1996)

To date, several studies indicates that a dysregulation of TLR signaling within the liver are associated with the initiation of several liver diseases. These liver diseases include amongst others alcoholic liver diseases (ALD), non-alcoholic fatty liver diseases (NAFLD), hepatic cancer, and autoimmune liver diseases, including autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC) or primary biliary cirrhosis (PBC) (Y. Chen & Sun, 2011; Duan et al., 2022; Kesar & Odin, 2014; Petrasek et al., 2010; Seki & Brenner, 2008; Szabo et al., 2006; Testro & Visvanathan, 2009).

1.4 Autoimmunity

One of the main functions of the immune system is to recognize foreign pathogens as well as the recognition of infected or damaged cells to protect the host. For this, it is important to distinguish between foreign and self to prevent the targeting of self-antigens. Therefore, maintaining immune tolerance is key (Bonilla & Oettgen, 2010; Chaplin, 2010; Marshall et al., 2018; Parlar et al., 2023). In the beginning of the 20th century, Paul Ehrlich was the first one who described autoimmunity as “horror autotoxicus” (Ahsan, 2022; Silverstein, 2005; L. Wang et al., 2015). Autoimmunity is described as a lack of immune tolerance by inducing immune responses to self-antigens that may lead to inflammation and tissue damage followed by initiation of autoimmune diseases (Ahmad & Ahsan, 2022; Ahsan, 2022; Schramm et al., 2022; L. Wang et al., 2015).

Autoimmunity can be classified into two groups: physiological and pathological (Ahsan, 2022; Avrameas & Selmi, 2013; Hang & Nakamura, 1997; L. Wang et al., 2015). Mostly, physiological autoimmunity occurs transiently with no accounted symptoms, whereas pathological autoimmunity is characterized by dysregulated immune tolerance. In pathological autoimmunity, self-derived autoantigens are presented via APCs to self-reactive lymphocytes leading to tissue inflammation and tissue damage (Ahsan, 2022; Rose & Witebsky, 1956; Silverstein, 2005; L. Wang et al., 2015). The lack of self-tolerance results in autoimmune mediated responses, such as the production of autoantibodies producing B cells or the generation of $CD4^+$ T cells to autoantigens (Davidson & Diamond, 2001; Gottwick et al., 2022; Rose & Witebsky, 1956; Silverstein, 2005; L. Wang et al., 2015; Yu et al., 2014).

Around 3 – 5% of the population suffer from an autoimmune disease with a strong female bias (Ahmad & Ahsan, 2022; Ahsan, 2022; Cárdenas-Roldán et al., 2013; Cooper & Stroehla, 2003; Eaton et al., 2007; Jacobson et al., 1997; L. Wang et al., 2015). Furthermore, the incidence of autoimmune diseases is increasing (Bach, 2002; Dinse et al., 2020; Fatoye et al., 2018; Gottwick et al., 2022; Lerner et al., 2015; Mayer-Davis et al., 2017). So far, targeted treatment for autoimmune diseases are still limited. To improve quality of live, current applied treatments are non-specific and target the corresponding symptoms (Gottwick et al., 2022).

1.5 Autoimmune liver diseases

Autoimmune liver diseases (ALD) arise in individuals with a lack of immune self-tolerance, causing tissue damage and tissue inflammation. If left untreated, this may lead to liver cirrhosis (Dinse et al., 2020; Hirschfield et al., 2013; Oo et al., 2010; Richardson et al., 2022; Ronca et al., 2020). The most frequent autoimmune liver diseases are autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) (Gottwick et al., 2022; Hirschfield & Gershwin, 2013; Liaskou et al., 2014; Richardson et al., 2022).

1.6 Autoimmune Hepatitis

AIH was first described in 1950 by Jan Waldenström (Floreani et al., 2018; Manns et al., 2015; Waldenstrom, 1952). AIH occurs in all ages affecting children and adults of both sexes. However, females are affected more often than males. In children, the same female predominance as in adults is observed (Czaja, 2017; Floreani et al., 2018; Gatselis et al., 2015; Manns et al., 2015; Mieli-Vergani et al., 2018; Terziroli Beretta-Piccoli et al., 2022). AIH is a multifactorial disorder with an unidentified cause (Manns et al., 2015; A. Tanaka, 2020). Genetic variations, environmental factors, such as viruses, bacteria or drugs as well as molecular mimicry seem to contribute to AIH (Floreani et al., 2018; Heneghan et al., 2013; Liberal, Selmi, et al., 2016; Manns et al., 2015; Oo et al., 2010). Genetic predispositions in autoimmune hepatitis have been associated with polymorphisms within human leukocyte antigens (HLA) regions that are located on the short arm of chromosome 6. In Europe, Genome-wide association studies (GWAS) reported strong links to HLA-DRB1 alleles such as *HLA-DRB1*0301* and *HLA-DRB1*0401* (de Boer et al., 2014; Donaldson, 2004; Liberal et al., 2011; Manns et al., 2015; Mieli-Vergani et al., 2018). However, depending on the global area, HLA genotype vary (Alvarez et al., 1999; Mieli-Vergani et al., 2018).

The cause of AIH is still unidentified, and it is currently not possible to prevent the development of AIH (Mieli-Vergani et al., 2018). To date, there is no targeted therapy available for individuals affected with AIH. Immunosuppressive drugs are commonly used as standard therapy to prevent progression of liver damage. Therefore, a steroid therapy, such as prednisolone alone or in combination with azathioprine is mainly used (Manns et al., 2010; Pape et al., 2019; Tage-Jensen et al., 1982).

Serologically, AIH can be diagnosed by demonstrating elevated immunoglobulin G (IgG) levels, as well as high liver transaminases levels, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), indicating liver damage, and the appearance of circulating autoantibodies. Histologically, a hallmark of AIH is interface hepatitis (Floreani et al., 2018; Manns et al., 2010, 2015; Mieli-Vergani et al., 2018).

The presence of autoantibodies facilitates the classification of autoimmune hepatitis into two subtypes: AIH type 1 (AIH-1) and AIH type 2 (AIH-2). Autoimmune hepatitis type 1 can affect both, children and adults and is characterized by the presence of antinuclear antibodies (ANA) and/or anti-smooth muscle actin antibodies (SMA). AIH type 2 typically starts in childhood. Here, anti-liver kidney microsomal type 1 (anti-LKM1) and/or anti-liver cytosol type 1 (anti-LC1) autoantibodies are detected (Homberg et al., 1987; G. D. Johnson et al., 1965; Martini et al., 1988; Mieli-Vergani et al., 2018; Terziroli Beretta-Piccoli et al., 2018, 2022).

1.6.1 Pathogenesis of autoimmune hepatitis

The aetiology of AIH remains poorly understood. It is hypothesized that the breach of immune self-tolerance is caused by the interaction of multiple factors. These factors mainly include environmental factors and genetic variations (Floreani et al., 2018; Liberal et al., 2011; Manns et al., 2015; Sucher et al., 2019).

It has been reported that factors as HLA and non-HLA genes, environmental factors, such as viruses like hepatitis A virus, hepatitis C virus, hepatitis E virus or Epstein-Barr virus, as well as toxins, and drugs are potential inducers of AIH (LeCann et al., 1997; Seldin, 2015; Sucher et al., 2019; Vento et al., 1991, 1995, 1997). These factors can lead to activation of the autoimmune reaction that result in T cell mediated immune response upon presentation of liver autoantigens, resulting in an autoimmune attack to hepatocytes (Sucher et al., 2019; Terziroli Beretta-Piccoli et al., 2022).

For an initiation of a $CD4^+$ T cell mediated immune response, self-antigens are presented via HLA class II molecules on APCs (such as DCs, macrophages and B lymphocytes) in presence of co-stimulatory molecules to naïve $CD4^+$ T helper cells (Th0) (Crispe, 2011; Mieli-Vergani et al., 2018; Terziroli Beretta-Piccoli et al., 2022). Upon activation of naïve T cells through antigen recognition and depending on the surrounding cytokines (IL-12, IL-4 or IL-6 and TGF-β), Th0 lymphocytes differentiate either into Th1, Th2 or Th17 $CD4^+$ T helper cells. A differentiation into Th1 T cells takes place, when pro-inflammatory cytokine IL-12 is expressed, whereas Th2 T cells are differentiated upon IL-4 cytokine secretion. In presence of IL-6 and/or TGF-β, a differentiation into Th17 T cells occurs (Liberal et al., 2011; Mieli-Vergani et al., 2018; Sucher et al., 2019; Terziroli Beretta-Piccoli et al., 2022).

Th1 T cells preferentially secrete IFNγ upon activation. IFNγ production induces an enhanced expression of both HLA class I and HLA class II molecules on the surface of hepatocytes. However, the resulting upregulation is implicated in activation of additional T cells and in the progression of liver damage (Lobo-Yeo et al., 1990; Mieli-Vergani et al., 2018; Senaldi et al., 1991). Furthermore, the presentation of antigens via the enhanced expressed HLA class I molecules on hepatocytes through IFNγ secretion result in the activation of cytotoxic $CD8^+$ T

cells. Following, the production of the pro-inflammatory cytokine IFNγ contribute to hepatocyte damage (Bovensiepen et al., 2019; Liberal et al., 2011; Liberal, Krawitt, et al., 2016; Senaldi et al., 1991; Terziroli Beretta-Piccoli et al., 2022).

Th2 cells are marked by the secretion of the cytokines, such as IL-4, IL-13, and IL-21. After secretion, these cytokines the activated B cells differentiate into plasma cells and produce autoantibodies (Liberal, Krawitt, et al., 2016; Mieli-Vergani et al., 2018). Autoantibodies are involved in liver tissue damage via antibody-mediated cellular cytotoxicity and complement activation (Floreani et al., 2018; Liberal et al., 2015; Liberal, Krawitt, et al., 2016; Mieli-Vergani et al., 2018; Moy & Levine, 2014).

Th17 T cells produce the pro-inflammatory cytokines IL-17 and IL-22. Both cytokines are known to be associated with pathogenesis of AIH and induce hepatocytes to produce the pro-inflammatory cytokine IL-6 (Mieli-Vergani et al., 2018; Terziroli Beretta-Piccoli et al., 2022; Thomas-Dupont et al., 2016; L. Zhao et al., 2011).

Accordingly, these sequences of events lead to an impairment of T cell control, resulting in a breakdown of liver self-tolerance and subsequent, in initiation of autoimmune damage (Sucher et al., 2019; Terziroli Beretta-Piccoli et al., 2022; Vuerich et al., 2021).

1.6.2 Diagnosis and treatment of autoimmune hepatitis

The symptoms of AIH can vary between mild and severe (Czaja, 2016). AIH can be asymptomatic or include non-specific symptoms. In some cases, it can occur as acute hepatitis, which can progress to a severe or even to a fulminant hepatitis, resulting in liver fibrosis followed by liver failure (Czaja, 2005b, 2009, 2011a, 2013b, 2016; Czaja & Bayraktar, 2009; Feld et al., 2005; Kessler et al., 2004; Lohse & Mieli-Vergani, 2011; Sucher et al., 2019). Around 25% to up to 75% of AIH affected individuals develop an acute hepatitis, whereas a severe (fulminant) AIH only occurs in 3% to 6% of all AIH cases (Crapper et al., 2009; Czaja, 2013a, 2016; Ferrari et al., 2004; W. S. Lee et al., 2005; Michalska et al., 2003; Nikias et al., 1994; Stravitz et al., 2011).

Typically, AIH presents mild symptoms such as fatigue, malaise or weight loss (Chinese Society of Hepatology, 2017; Gatselis et al., 2015; Terziroli Beretta-Piccoli et al., 2022). In advanced stages of an acute AIH, gastrointestinal problems and bleeding can appear. In AIH, an enlarged liver, a colouring of the skin to yellow and correspondingly enhanced bilirubin levels are detected (Gatselis et al., 2015; Lohse & Mieli-Vergani, 2011; Manns et al., 2015; Sucher et al., 2019). Moreover, it is not uncommon, that next to AIH, additional extrahepatic autoimmune disorders occur, such as Hashimoto thyroiditis, rheumatoid arthritis, Sjögren syndrome, diabetes mellitus type 1, psoriasis, or multiple sclerosis. These disorders may occur at any stage of AIH (Manns et al., 2015; Mieli-Vergani et al., 2018).

1.6.2.1 Diagnosis of AIH

Based on the heterogeneity of AIH, it is still challenging to diagnose AIH (Dalekos et al., 2022). The diagnosis of AIH is based on a combination of several tests, including biochemical, clinical, histological, and serological tests (Dalekos et al., 2022; Sucher et al., 2019). In general, a liver biopsy is necessary to diagnose AIH and to gain insight into the progression of inflammation (Dalekos et al., 2019b, 2022; European Association for the Study of the Liver, 2015; Gatselis et al., 2015; Mack et al., 2020; Manns et al., 2010, 2015; Zachou et al., 2013). Furthermore, it is important to exclude other causes of liver diseases that share mutuality with AIH such as hepatitis A to E, alcoholic liver diseases, drug-induced hepatitis, PBC, PSC or Wilson disease (Dalekos et al., 2019a, 2022; European Association for the Study of the Liver, 2015; Mack et al., 2020; Terziroli Beretta-Piccoli et al., 2022).
A simplified scoring system containing four criteria for diagnosing AIH was published in 2008, based on the scoring system published in 1993 by the International Autoimmune Hepatitis Group (IAIHG) (Hennes et al., 2008; P. J. Johnson & McFarlane, 1993; Sucher et al., 2019). These criteria include the presence of autoantibodies (ANA or SMA), immunoglobulin G (IgG) levels, histology findings, and exclusion of viral hepatitis (Hennes et al., 2008; Manns et al., 2015; Sirbe et al., 2021; A. Tanaka, 2020).

1.6.2.1.1 Detection of autoantibodies

The diagnostic score system published by International Autoimmune Hepatitis Group (IAIHG) incorporates the analysis of autoantibodies to diagnose AIH (Alvarez et al., 1999; Hennes et al., 2008; P. J. Johnson & McFarlane, 1993; Sucher et al., 2019; Terziroli Beretta-Piccoli et al., 2022). Predominantly, indirect immunofluorescence (IIF) or enzyme linked immunosorbent assays (ELISA) are performed to detect autoantibodies (Alvarez et al., 1999; Hennes et al., 2008; P. J. Johnson & McFarlane, 1993; Terziroli Beretta-Piccoli et al., 2022; Vergani et al., 2004). The measurement of autoantibodies is necessary for both, diagnosis and for classification of AIH (Alvarez et al., 1999; Hennes et al., 2008; P. J. Johnson & McFarlane, 1993; Manns et al., 2015; Vergani et al., 2004). In AIH type I, the presence of autoantibodies, such as antinuclear antibody (ANA) and/or anti-smooth muscle antibody (SMA) is observed. Whereas anti-liver kidney microsomal type 1 (anti-LKM-1) and/or anti-liver cytosol type 1 (anti-LC-1) antibodies are more prominent in AIH type II (Liberal, Krawitt, et al., 2016; Terziroli Beretta-Piccoli et al., 2018). In around 65% of AIH type 1 cases, both autoantibodies, ANA and SMA, are measured (Heneghan et al., 2013). ANA autoantibodies are known to be classical antibodies of AIH, and are measured in around 80% of AIH affected individuals. However, this autoantibody is not specific for AIH and is also present in other liver diseases, such as PBC, PSC, in chronic viral hepatitis or non-alcoholic fatty liver disease (Czaja, 2011b, 2015). Importantly, the titre levels of the various autoantibodies differ in every individual affected with

AIH. This means on the one hand that a low level of a specific autoantibody does not automatically exclude the diagnosis of AIH. Equally, on the other hand, an enhanced level of an autoantibody does not directly imply AIH (Czaja, 2005a; Dalekos et al., 2002; Gatselis et al., 2015; Makaritsis et al., 2009; Manns et al., 2010; Vergani et al., 2004; Zachou et al., 2004, 2013).

1.6.2.1.2 Liver histology

Liver biopsies are essential for diagnosis of AIH and to estimate the progression of liver damage (Czaja, 2016; Hennes et al., 2008; Sucher et al., 2019). In liver histology, interface hepatitis is one of the most common characteristics in AIH. Next to interface hepatitis, emperipolesis and the formation of rosettes belong to the histological characteristics of AIH diagnosis, based on the scoring system of IAIHG (Alvarez et al., 1999; Dalekos et al., 2022; Hennes et al., 2008; Mieli-Vergani et al., 2018; Sucher et al., 2019). However, AIH still lacks of specific histological characteristics, as these three characteristics are not disease specific and can also occur in other diseases (Dienes et al., 1989, 2002; Manns et al., 2015; Mieli-Vergani et al., 2018).

1.6.2.1.3 Aminotransferase ALT and AST levels

The last criteria that belong to the diagnostic scoring system of IAIHG is the determination of abnormalities of laboratory features including liver transaminases as well as levels of IgG. As mentioned before, individuals affected with AIH show increased levels of both transaminases ALT and AST as well as IgG levels (Desmet et al., 1994; Hennes et al., 2008; Manns et al., 2015). Especially an upregulation of both, ALT and AST is an indicator for liver inflammation (Desmet et al., 1994; Manns et al., 2015).

1.6.2.2 Treatment of AIH

The goal of AIH treatment is to reach remission. Therefore, it is crucial to decrease serum levels of both ALT and AST, as well as IgG levels, to achieve their normal levels and to prevent progression of liver inflammation (Schramm et al., 2010; A. Tanaka, 2020; Terziroli Beretta-Piccoli et al., 2022).

At present, there is no specific treatment for AIH available. If undiagnosed or untreated, AIH can progress to liver fibrosis, liver cirrhosis or hepatocellular carcinoma. These symptoms may lead to liver failure and require liver transplantation (Mieli-Vergani et al., 2018; Muratori et al., 2023). As soon as AIH is diagnosed, immunosuppressive first-line treatment consisting of steroids such as prednisolone, and azathioprine is recommended to maintain remission (European Association for the Study of the Liver, 2015; Lohse et al., 2020; Muratori et al., 2023; Sirbe et al., 2021). Predominantly, AIH affected individuals respond to standard treatment (European Association for the Study of the Liver, 2015; Mieli-Vergani et al., 2018; Sirbe et al., 2021), including immunosuppressive reagents, such as predniso(lo)ne and

azathioprine (“Autoimmune Hepatitis.,” 2018; Manns et al., 2010; Pape et al., 2019; Terziroli Beretta-Piccoli et al., 2022). However, if they are not responding to first-line therapy, second-line treatment with 6-mercaptopurine (6-MP) or Mycophenolate mofetil (MMF) is applied. Especially 6-MP is known to be successfully used as alternative treatment to azathioprine therapy in inflammatory bowel disease (IBD) treatment (Hindorf et al., 2009; Kennedy et al., 2013; Pape et al., 2019; Terziroli Beretta-Piccoli et al., 2022). In case of tolerance to both, azathioprine and 6-MP, MMF is applied (European Association for the Study of the Liver, 2015; Mack et al., 2020; Terziroli Beretta-Piccoli et al., 2022). When showing an insufficient to second-line treatment, third-line treatment is performed. This is predominantly a combination of various immunosuppressive treatments (Terziroli Beretta-Piccoli et al., 2022). Positive treatment responses are associated with a good survival prognosis in AIH-affected individuals (Muratori et al., 2023).

1.7 Models to study liver diseases

Recapitulating liver architecture, liver function, several processes or pathogenesis of various liver diseases can be investigated by using several *in vivo* as well as *in vitro* model systems (Mieli-Vergani et al., 2018; Moradi et al., 2020; Nuciforo & Heim, 2020; Telles-Silva et al., 2022; X. Yin et al., 2016). Until now, the culture of primary human hepatocytes (PHHs) and mouse models are applied so far (Christen & Hintermann, 2022; Nuciforo & Heim, 2020).

To examine human liver functions that are associated with pathogenesis of various liver diseases, primary human hepatocytes (PHHs) are still regarded as the “gold standard” for cell culture liver models (Faccioli et al., 2021; Green et al., 2017). In 1982, the first PHHs were isolated (Green et al., 2017; Guguen-Guillouzo et al., 1982). Nowadays, PHHs can be obtained from various liver tissues, such as biopsies, whole livers, and explanted livers from individuals undergoing liver transplantation (Faccioli et al., 2021; Stock & Christ, 2017). PHHs are a suitable model to study liver function, as they maintain their characteristics, functionality and genetic background (Huch et al., 2013; Kang et al., 2016; Q. Liu et al., 2022; Nuciforo & Heim, 2020). However, disadvantages of the PHH cell culture model are that they have a limited culture time and exhibit morphological changes after a few days in culture (Huch et al., 2013; Kang et al., 2016; Q. Liu et al., 2022; Nuciforo & Heim, 2020; Xia et al., 2017).

Next to PHHs, mouse models are often used as models for human liver diseases to achieve a better understanding of the pathogenesis leading to AIH (Christen & Hintermann, 2022). However, none of the applied mouse models mimics the complexity of AIH (Christen, 2019; Liberal, Krawitt, et al., 2016; Terziroli Beretta-Piccoli et al., 2022). In addition, mouse models are time consuming and can exhibit variations in their phenotype and their genetic background (Koo & Huch, 2016; Shinozawa et al., 2016; X. Zhu et al., 2021).

Thus, 2D-culturued PHHs do not allow visualization of the three dimensional liver architecture, while AIH mouse models do not sufficiently mimic the complexity of AIH (Christen, 2019; Liberal, Krawitt, et al., 2016; Terziroli Beretta-Piccoli et al., 2022; Xia et al., 2017; Xiang et al., 2019; X. Zhu et al., 2021). Recently, liver organoids have been developed and may present a promising three-dimensional (3D) model (Huch et al., 2013; Lancaster & Knoblich, 2014; X. Zhu et al., 2021).

1.7.1 Organoids

Over the last years, organoids have gained significance as a helpful tool to study human diseases (Yang et al., 2023). In this thesis, human liver organoids were used as model to study AIH. Organoids are defined as three-dimensional (3D) mini organ-like structures (Marsee et al., 2021; Yang et al., 2023) in which organ-specific cell types self-organize themselves through cell-cell interactions, meaning that they are recapitulating the original structure and function of an organ *in vivo* (Y. Chen et al., 2023; Corrò et al., 2023; Huch & Koo, 2015; Lancaster & Knoblich, 2014; Marsee et al., 2021; Prior et al., 2019). Organoids can be generated from a variety of cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or tissue-derived adult stem cells (ASCs) (Corrò et al., 2023; Hofer & Lutolf, 2021; Huch & Koo, 2015; Lancaster & Knoblich, 2014). To be able to self-organize and to grow three-dimensionally, the cells are embedded in an extracellular matrix (ECM) recapitulating the 3D environment of the tissue *in vivo* (Corrò et al., 2023). The ECM is composed of laminin, collagen and heparin sulfate preoteoglans (Corrò et al., 2023). The organoids are cultured in a combination of several growth and niche factors such as epidermal growth factor (EGF), noggin, and R-spondin, promoting self-organization, proliferation, and differentiation (Nuciforo & Heim, 2020). Organoids can be long-term cultured for weeks up to months by maintaining key functions and gene expression without changing phenotype (Corrò et al., 2023; Hu et al., 2018).

In summary, patient-derived organoids may provide a helpful tool for a better understanding of human tissue biology and progression of several human diseases (Lehmann et al., 2019; X. Yin et al., 2016).

1.7.1.1 Human liver organoids

In 2015, Huch and colleagues generated human liver organoids obtained from adult ductal progenitor cells, showing genetically and chromosomal stability over several months *in vitro* (Y. Chen et al., 2023; Huch et al., 2015). Both hepatocytes and cholangiocytes are epithelial cells types in the liver that share the same origin (S. L. Friedman, 2008; Sun & Hui, 2020; Zorn, 2008). During liver organogenesis, hepatoblasts are capable to differentiate into either hepatocytes or cholangiocytes. However, this is dependent on the environmental signals

mediated by factors, such as hepatocyte growth factor (HGF), Wnt signaling and fibroblast growth factor (FGF) (Ober & Lemaigre, 2018; Vargas-Valderrama et al., 2020; F. Wu et al., 2019; X. Zhu et al., 2021).

For this reason, both cell types are present in human liver organoid culture, showing a heterogeneous organoid culture. In this thesis, human liver organoids are generated from patient-derived tissue cells from explanted organs.

1.8 Aims of this study

The mechanisms underlying the pathogenesis of AIH are incompletely understood. Previous studies revealed that TLRs are dysregulated in the liver of individuals affected with AIH (Y. Chen & Sun, 2011; Kesar & Odin, 2014; Mencin et al., 2009; Seki & Brenner, 2008; Soares et al., 2010; Szabo et al., 2006). A dysregulated TLR signaling leads to an enhanced inflammatory cytokine production observed in AIH (Y. Chen & Sun, 2011; Duan et al., 2022; Kesar & Odin, 2014; Kiziltas, 2016; Schwabe et al., 2006; Szabo et al., 2006).

Moreover, it has been shown that TNF and IFNγ producing $CD4^+$ T cells are enhanced in AIH (Bovensiepen et al., 2019; Tiegs & Horst, 2022) and that Th1 cells by means of TNF are key initiators of AIH (H. Chen et al., 2023).

We hypothesize that in AIH, TLR signalling pathways are dysregulated in liver cells due to an overexpression of TLRs. The resulting enhanced inflammatory cytokine production initiates $CD4^+$ T cell activation. In return, the pathogenic effect from the produced cytokines by $CD4^+$ T cells lead to hepatocyte destruction.

The overall objective of this thesis was to understand the mechanisms that lead to loss of immune tolerance resulting in AIH. To achieve a better understanding of the corresponding dysregulated mechanisms, this thesis comprises three specific aims:

1) Establishment of human liver organoids obtained from non-inflamed and AIH diagnosed individuals as new model to study AIH.
2) Assessment of the role of TLR3 in AIH upon stimulation with its ligand poly I:C.
3) Identification of effects of cytokines produced by dysregulated $CD4^+$ T cells derived from AIH affected livers on hepatocytes.

2 Material

2.1 Antibodies

Primary and secondary antibodies used for immunofluorescence staining are listed in Table 1 and Table 2.

2.1.1 Primary Antibodies for immunofluorescence

Table 1: Primary Antibodies.

Name	Fluorochrome	Clone	Manufacturer	Catalogue Nr.	Dilution
Albumin	purified	polyclonal	Bethyl	A80-129A	1:100
Hoechst 33342			Life Technologies	H3570	1:2000
Phalloidin	AF647		Life Technologies	A22287	1:200/ 1:400
TLR3		polyclonal	Invitrogen	PA520184	1:100
Zo-1		polyclonal	Life Technologies	PA5-19090	1:100

2.1.2 Secondary Antibodies for immunofluorescence

Table 2: Secondary Antibodies.

Name	Fluorochrome	Clone	Manufacturer	Catalogue Nr.	Dilution
Donkey anti-Goat IgG (H+L)	Alexa Fluor Plus 488	polyclonal	Life Technologies	A32814	1:500/ 1:1000
Donkey anti-Rabbit IgG (H+L)	Alexa Fluor Plus 555	polyclonal	Life Technologies	A32794	1:500

2.2 RT-PCR Primer

Primers for RT-PCR that are commonly used are listed in the Table below.

Table 3: RT-PCR Primer.

Gene	Sequence
ALBUMIN	Forward 5' – CTGCCTGCCTGTTGCCAAAGC – 3' Reverse 5' – GGCAAGGTCCGCCCTGTCATC – 3'
CXCL9	Fw 5' – TTTTCCTCTTGGGCATCATC – 3' Rev 5' – GAACAGCGACCCTTTCTCAC – 3'
CXCL11	Fw 5' – AGAGGACGCTGTCTTTGCAT – 3' Rev 5' – TGGGATTTAGGCATCGTTGT – 3'
CYP3A4	Fw 5' – TGTGCCTGAGAACACCAGAG – 3' Rev 5' – GTGGTGGAAATAGTCCCGTG – 3'
EPCAM	Fw 5' – GCTGGCCGTAAACTGCTTTG – 3' Rev 5' – ACATTTGGCAGCCAGCTTTG – 3'
GAPDH	Fw 5' – CGGAGTCAACGGATTTGG – 3' Rev 5' – TGATGACAAGCTTCCCGTTC – 3'
IL-6	Fw 5' – TGCAATAACCACCCCTGACC – 3' Rev 5' – TGCGCAGAATGAGATGAGTTG – 3'
IL-12 p35	Fw 5' – CTCCAGAAGGCCAGACAAAC – 3' Rev 5' – AATGGTAAACAGGCCTCCACT – 3'
LGR-5	Fw 5' – GAATCCCCTGCCCAGTCTC – 3' Rev 5' – ATTGAAGGCTTCGCAAATTCT – 3'
SOX9	Fw 5' – GTACCCGCACTTGCACAAC – 3' Rev 5' – TCTCGCTCTCGTTCAGAAGTC – 3'
TLR3	Fw 5' – TAAACTGAACCATGCACTCT – 3' Rev 5' – TATGACGAAAGGCACCTATC – 3'
TLR4	Fw 5' – GAAGCTGGTGGCTGTGGA – 3'

	Rev 5’ –GATGTAGAACCCGCAAG – 3‘
TLR5	Fw 5’ –TTGCTCAAACACCTGGACAC – 3‘ Rev 5’ – CTGCTCACAAGACAAACGAT – 3‘
TNFα	Fw 5’ – CTCTTCTGCCTGCTGCACTTTG – 3‘ Rev 5’ – ATGGGCTACAGGCTTGTCACTC – 3‘

2.3 Kits

All used kits in this work are listed in Table 4.

Table 4: Kits.

Kit name	**Manufacturer**	**Catalogue Nr.**
Ambion™ DNase I (RNase-free)	Invitrogen	AM2222
Human Albumin ELISA	Bethyl	E88-129
CyQUANT™ LDH Cytotoxicity Assay	Invitrogen™	C20301
Luminex Human Discovery Assay (14-Plex) Human Premixed Multi-Analyte Kit	R&D Systems	LXSAHM-14
qScriber™ cDNA Synthesis Kit	highQU GmbH	RTK0104

2.4 Buffer, media and solutions

Buffer, media and solutions are listed in the Table below.

Table 5: Buffer, media and solutions.

Buffer/medium	**Manufacturer**	**Catalogue Nr.**
Advanced DMEM/F-12 (1X)	Gibco	12634-010
DMEM (1X) + GlutaMAX	Gibco	31966021

Dulbecco's Phosphate Buffered Saline	Sigma Aldrich	D8537-500ml
GlutaMAX™ (100X)	Gibco	35050-061
Hank's buffer	Capricorn scientific	HBSS-2A
Hank's Balanced Salt Solution	Sigma Aldrich	H6648-500ML
HEPES Buffer Solution (1M)	Gibco	15630-056
Lymphocyte separation media	Capricorn scientific	LSM-A
RPMI-1640	Gibco	21875-034-500ml

2.5 Cell culture

2.5.1 Cultivation medium and supplements

Media and supplements for cultivation media are shown in Table 6.

Table 6: Cultivation medium and supplements.

Solution	**Media**	**Supplement**
AD+++ (Basal medium)	Advanced DMEM/F-12 (1X)	1 % (v/v) GlutaMAX 10 mM HEPES 1% Penicillin/Streptomycin
D10	DMEM (high glucose, GlutaMAX, pyruvate)	10 % (v/v) FBS 1% Penicillin/Streptomycin
Human liver digestion solution		90 % (v/v) EBSS (sterile) 2,50mg/ml Collagenase D 0,1 mg/ml DNase I
R10	RPMI – 1640 (1X)	10 % (v/v) FBS 1% Penicillin/Streptomycin
Wash Medium	DMEM (high glucose, GlutaMAX, pyruvate)	1 % (v/v) FBS 1% Penicillin/Streptomycin

2.5.2 Organoid cell culture reagents

Used organoid cell culture reagents used in this work are listed in the Table below.

Table 7: Organoid cell culture reagents.

Reagent	**Manufacturer**	**Catalogue Nr.**
A83-01	Tocris	2939
B27 w/o vitamin A	Gibco	12587-010
BME2	R&D	3532-010-02
CHIR99021	Tocris	4423
Collagenase D	Sigma Aldrich	C9407-100mg
DNase I	StemCell	07470
EBSS	Gibco	24010-043
EGF	PeproTech	AF-100-15-500UG
Fetal bovine serum (FBS)	Capricorn scientific	FBS-11A
FGF-10	PeproTech	AF-100-26-500UG
FGF-7	PeproTech	100-19-50UG
Gastrin	Sigma Aldrich	G9145-.5MG
HGF	Peprotech	100-39H-100UG
n-Acetyl-L-Cysteine	Merck	A9165-25g
Nicotinamide	Sigma Aldrich	N0636-100G
Penicillin-Streptomycin	Sigma Aldrich	P4333-100ml
recovery freezing medium	Gibco	12648010
R-spondin conditioned medium (CM)	Produced in-house	Produced in-house
TGF-α	PeproTech	100-16A-100UG
Y-27632	StemCell	72308
Zeocin Selection Reagent	Gibco	R25001

2.5.3 Composition of human liver organoid media

Table 8 lists the composition of the human liver organoid medium.

Table 8: Composition of human liver organoid media.

Reagent	Working [c]	Stock [c]
AD+++	81 % (v/v)	100 % (v/v)
B27 w/o Vit. A	2 % (v/v)	100 (v/v)
N-Acetyl-L-Cysteine	1,25 mM	400 mM
EGF	50 ng/ml	100 µg/ml
R-spondin CM	15 % (v/v)	100 % (v/v)
FGF7	100 ng/ml	100 µg/ml
FGF10	100 ng/ml	100 µg/ml
HGF	50 ng/ml	25 µg/ml
TGFα	20 ng/ml	20 µg/ml
Gastrin	10 nM	10 µM
CHIR99021	3 µM	3 mM
A83-01	2 µM	5000 µM
Nicotinamide	10 nM	1 M
Y-27632	10 µM	10 mM

2.5.4 (Recombinant) proteins for cell culture stimulation

The following Table lists all proteins used for cell culture stimulation.

Table 9: Cell culture stimulants.

Reagent	Concentration	Duration	Manufacturer	Catalogue Nr.
IL-2	50 U/ml	2 h	PeproTech	200-02 500UG
Human IFN-gamma	1 µg/ml	10 d	Bio-Techne	MAB285-500
Human TNF RI/TNFRSF1A	0,5 µg/ml	10 d	Bio-Techne	MAB225-100

Immunocult™ Human CD3/CD28 T cell Activator	25 µl/ 10^6 cells	2 h	StemCell	10991
Poly I :C	10 µg/ml	24 h 7 d	Invivogen	tlrl-pic
Recombinant Human IFNγ	50 Units/ml 5000 Units/ml	14 d	PeproTech	300-02-100
Recombinant Human TNFα Protein	5 ng/ml 500 ng/ml	14 d	Bio-Techne	210-TA-100

2.6 Reagents

Reagents used in this work are listed in the Table below.

Table 10: Reagents.

Reagent	**Manufacturer**	**Catalogue Nr.**
2-propanol/ isopropyl alcohol	Th. Geyer	I9030-500ML
ACK lysing buffer	Gibco	A10492-01
AlbuMAX™ II Lipid-Rich Bovine Serum Albumin (BSA)	Gibco	11021-037
Aqua ad iniectabilia Braun	B. Braun Melsungen	2351744
Chloroform	Th. Geyer	39553-250ML
Dimethyl sulfoxid (DMSO)	Sigma Aldrich	D5879-100ml
Ethanol absolute reinst.	Th. Geyer	100983.2500
Ethanol 99 % vergällt mit MEK, IPA and Bitrex	Th. Geyer	2212-5L
Ethylenediaminetetraacetic acid (EDTA), 0.5 M	Promega	V4231

GlycoBlue ™ Coprecipitant (15 mg/ml)	Invitrogen™	AM9516
Paraformaldehyde, 4 %	Sigma Aldrich	P6148-500G
Recovery™ Cell culture Freezing Medium	Gibco	12648010
RNAlater™	Invitrogen	AM7020
RNase AWAY	Geyer Th. GmbH	6227799
RNaseOUT™ Ribonuclease Inhibitor	Invitrogen	10777-019
SYBR® Green	highQU GmbH	QPD0450
TRIzol Reagent	Life Technologies	15596018
Trypan blue solution, 0.4 %	Sigma Aldrich	T8154-100ml
TrypLE Express	Gibco	12605-028
Trypsin-EDTA solution	Sigma Aldrich	T3924-100ML
Tween® 20	Th. Geyer	P2287-100ML

2.7 Cell lines

Table 11 lists the eukaryotic cell lines used in this thesis.

Table 11: Cell lines.

Cell line	**Origin**	**Cell line**	**Description of modification**
HA-R-Spondin1-Fc 293T	Hubrecht Institute (generated by Calvin Cuo lab)	HEK293	production of R-Spondin1-Fc
HepG2-NTCP	Ulrike Protzer, München	Human hepatic cell line	Transfected for NTCP expression
MRC-5	ATCC® CCL-171™	Human diploid fibroblast cell line	

2.8 Equipment and Software

Applied technical devices and software are listed in Table 12 and Table 13.

2.8.1 Equipment

The following Table lists all technical devices used in this work.

Table 12: Equipment.

Technical device	**Company**
EVOS™ M5000 Imaging System	Thermo Fisher Scientific
LightCycler 480 Instrument II	Roche
Spectrophotometer NanoDrop 1000	PeqLab
TC20™ Automated Cell Counter	Bio-Rad
Tecan Safire^2	Tecan
TI2 spinning disk microscope	Nikon

2.8.2 Software

Data analysis and figure preparation was performed by using the following software shown in the Table below.

Table 13: Software and website.

Software/website	**Company**
Organauts GOAT (Bremer et al., 2022)	In house
BioRender	BioRender
GraphPad Prism, Version 9 and 10	GraphPad Software Inc.
LightCycler® 480 SW 1.5	Roche
Magellan6	Tecan
Mendeley	Mendeley Ltd
Microsoft Office 2016	Microsoft
ND-1000 V3.8.1	PeqLab
NIS-Elements	Nikon

SpectroFlo	Cytek Biosciences

3 Methods

3.1 Cell culture

3.1.1 Cultivation of cell lines

All cell lines in this work (Table 11) were cultured in their respective cultivation medium (Table 6) in an incubator at 37 °C and with 5% CO_2. HepG2 NTCP cells were cultured at $5x10^6$ cells/ml in R10 in T75 cell culture flasks. The cells were split after reaching a confluence of about 80 - 90%. Generally, they were split in a ratio up to 1:5 every 3 - 4 days. For splitting, the old medium was removed and the cells were washed with PBS to remove dead cells. Then, 0.05% (v/v) Trypsin-EDTA was added to detach the cells and they were incubated for around 5 min at 37 °C. Immediately after detaching, cultivation medium was added and the cells were centrifuged to remove Trypsin-EDTA and were cultured with fresh cultivation medium for further cultivation.

3.1.2 Cell thawing

Frozen vials containing cryopreserved cells were thawed at 37 °C in the water bath until the freezing medium was half thawed. After completely thawing of the cell pellet, the cell suspension was transferred dropwise into a 15 ml Falcon tube containing 9 ml of cultivation medium and immediately centrifuged at 500 g and 22 °C for 5 min. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 10 ml of pre-warmed cultivation medium to culture the cells at the desired density in the respective cell culture flask. The cells were cultured at 37 °C and 5% CO_2 in an incubator.

3.1.3 Cryopreservation of cells

Cryopreservation was performed for long-term storage of cells in liquid nitrogen tanks at - 160 °C. Usually, 10 - $50x10^6$ cells/ml were cryopreserved in 1 ml of freezing medium (FBS supplemented with 10% DMSO). For freezing adherent cells, these were trypsinized using Trypsin-EDTA solution, incubated at 37 °C until the cells were detached and immediately centrifuged at 500 g for 5 min at 22 °C. The cell pellet was resuspended after centrifugation in freezing medium, transferred into a cryotube, and placed in a 4 °C pre-cooled freezing container filled with isopropanol. The freezing container was stored overnight in a - 80 °C freezer, which allows to lower the temperature of the cells slowly down at a rate of 1 °C per minute. This process preserves the viability of the cells before transferring the cells to a liquid

nitrogen tank for long-term storage. For freezing freshly isolated liver cells, cell recovery freezing medium was used instead and $10x10^6$ cells were resuspended in 1 ml of cell recovery freezing medium.

3.1.4 Quantification of the cell counts in suspension

To determine viable cell concentrations, cells were counted by using Bio-Rad TC20™ Automated Cell Counter (Bio-Rad) and Trypan blue. Trypan blue is a dye that is absorbed by dead cells through their disrupted cell membrane. The Bio-Rad TC20™ Automated Cell Counter can distinguish trypan blue dyed cells. To count the cells, they were diluted in a ratio of 1:1 with trypan blue (10 µl of cell suspension mixed with 10 µl trypan blue) in a 96-well v-bottom plate (Sarstedt). Then, 10 µl of the cell-trypan-blue mixture was pipetted inside the Bio-Rad Dual-chamber cell counting slide. The gating was set on 6 – 17 µm to count PBMCs. The Bio-Rad TC20™ Automated Cell Counter gives the cell counting in cells per ml.

3.1.5 Isolation of peripheral blood mononuclear cells

Whole blood samples/PBMCs were obtained from individuals through the Healthy Cohort Hansestadt Hamburg (HCHH) with provided written informed consent. These studies were approved by the ethics committee of the Medical Association of Freie Hansestadt Hamburg (Ärztekammer Hamburg).

Peripheral blood mononuclear cells (PBMCs) were isolated using a density gradient centrifugation from fresh human peripheral blood. Briefly, fresh blood from human donors was collected in EDTA whole blood tubes and it was transferred into 50 ml Falcon tubes. These tubes were filled up until with Hank's buffer until a volume of 35 ml prior gently layering the blood on 15 ml of Lymphocyte separation media, which was added to a fresh 50 ml falcon tube for building a Ficoll layer underneath the blood. After layering, the blood was centrifuged at 500 g and 22 °C for 30 min with an acceleration (ACC) set on 1 and the lowest level of deceleration (DEC) on 0. After centrifugation, the samples exhibited individual layers. The interphase between Ficoll and plasma, that contains the PBMCs, was carefully harvested with a 10 ml serological pipette into a new 50 ml falcon tube. The tubes were then filled up to 50 ml with Hank's buffer to wash the cells and they were centrifuged at 500 g and 22 °C for 5 min. After centrifugation, the cell pellet was resuspended with 3 ml of ACK lysis buffer and immediately incubated for 3 min at 22 °C for lysing the remaining erythrocytes which were not washed out in the previous steps. In the following, the cells were washed again by filling up the falcon tube to 50 ml with Hank's buffer and repeated the centrifugation as before. After centrifugation, the cells were resupended in 10 ml of pre-warmed R10 and they were ready for further experiments.

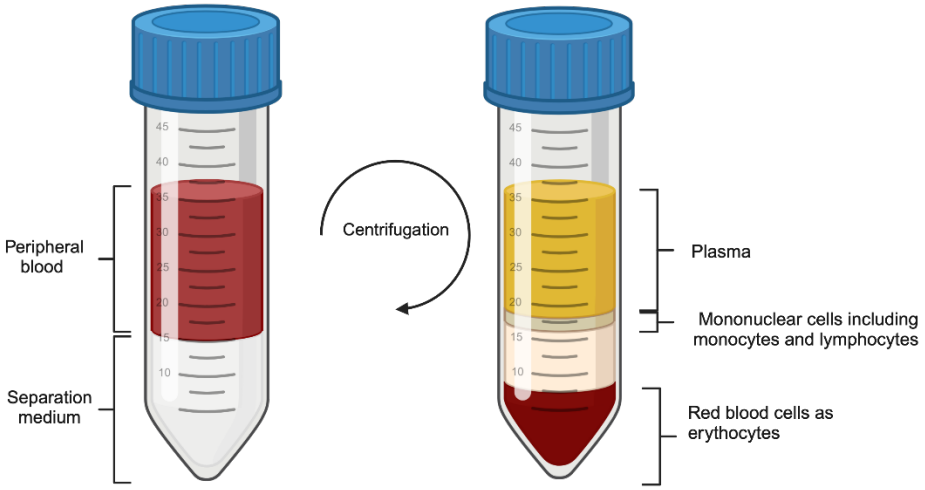

Figure 8: Workflow of isolation of peripheral blood mononuclear cells.

For the isolation of PBMCs, a method called density gradient centrifugation is used. Here, cellular components of the human blood are separated by their size. For separation, the blood is centrifuged to build several layers depending on their size. At the top, a layer with plasma is built, followed by a layer including monocytes and lymphocytes and then and the bottom of the 50 ml Falcon tube, a pellet with red cells as erythrocytes is seen. Figure is created with BioRender.

3.1.6 Generation of Rspo1 conditioned medium

The HEK293T-HA-Rspo1-Fc cell line was originally generated by the lab of Calvin Cuo to produce R-spondin conditioned medium. After generation, it was used for the human liver and intestinal organoid medium. Briefly, $5x10^6$ of these cells were cultured in D10 supplemented with 300 µg/ml of Zeocin Selection reagent in T175 cell culture flasks until 100% of confluence. Cells were then split once and expanded into new T175 flasks with D10 medium with Zeocin. After reaching confluency, the cells were split again into new T175 flasks with D10 medium without Zeocin. After four days of culture, the old cultivation medium was replaced with AD+++ and the cells were cultured for further 8 days. The R-spondin conditioned medium was harvested by transferring the medium in 50 ml falcon tubes, centrifuging at 500 g and 22 °C for 5 min and sterile filtering through a 0.2 µm filter afterwards. The medium was stored up to 12 month at - 20 °C.

3.2 Human liver organoid processing

3.2.1 Liver sample processing

Liver tissue samples were obtained from adult individuals with autoimmune hepatitis undergoing liver transplantation (LTX) due to end-stage liver disease from University Medical Center Hamburg-Eppendorf (UKE). Liver samples that served as non-inflamed controls were collected from individuals from Asklepios Hospital Barmbek (AKB) undergoing a partial liver resection surgery for liver metastases. Here, the tumor-free sections of the liver were used.

Ethic protocol approval for these studies were obtained from the local ethics committee of the Ärztekammer Hamburg. Patients provided written informed consent for use of liver tissue removed for clinical purpose for the proposed studies. The liver samples were processed according to two different protocols as described below.

3.2.2 Mechanical isolation of cells from liver sample

For mechanical isolation of liver cells, the tissue was cut into small pieces of around 5x5x5 mm in a 10 cm petri dish and the tissue was hashed into single cells using a gentleMACS Dissociator (Milteny). This dissociator is a semi-automated machine that dissociate tissues into single cells. This process was repeated 6 times for 36 seconds. Following, the cell suspension was filtered through cell strainers with different sizes, starting from 500 µm down to 40 µm into new 50 ml Falcon tubes after every filter step. After filtering through the decreasing filter sizes, the cell suspension was filled up to 50 ml with PBS and centrifuged at 500 g and 22 °C for 10 min. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 10 ml of R10. The isolated cells were then counted, washed and cryopreserved until further use as described above.

3.2.3 Enzymatic isolation of liver cells

For enzymatically isolation of liver cells, the liver tissue was weighted and afterwards minced into small pieces of around 0.5 mm using tweezers and a scalpel in a 10 cm petri dish. The minced tissue pieces were transferred into a 50 ml Falcon tube. To remove remaining blood and fat, the small pieces were washed twice with 25 ml of washing medium (Table 6) by pipetting up and down with a 25 ml serological pipet. After the tissue settled, the supernatant was completely removed and 5 ml/g of human liver digestion solution was added to the tissue pieces. To digest the tissue and to generate single cells, the liver tissue pieces were incubated for 30 – 90 min at 37 °C in the water bath in the human liver digestion solution. Once single cells were detected by using a light microscope (Evos M5000, 4x objective), 25 ml of pre-chilled wash medium was added to the cell and tissue suspension to stop the digestion. The cell suspension was next filtered through a 70 µm cell strainer and centrifuged at 300 g and 4 °C for 5 min. After centrifugation, the supernatant was discarded and the cells were washed twice with cold wash medium. The cells were either directly seeded to generate liver organoids or were frozen in cell recovery freezing medium (Gibco).

3.3 Human liver organoid culture

Human liver organoids from adult liver samples were generated from single cells after enzymatic based cell isolation as described in 3.2.3. For this, a fraction of the obtained digested liver cells were centrifuged after filtering at 300 g and 4 °C for 5 min. The cell suspension was resuspended in 1 ml of AD+++ in a 1.5 ml Eppendorf tube and immediately centrifuged for 1

min on a desk centrifuge prior resuspending in cold human liver organoid medium mixed with basement membrane extract 2 (BME2) in a 1:4 ratio (22.5 µl BME and 7.5 µl medium). BME2 is an extracellular matrix allowing the isolated epithelial cells to grow in 3D structures to human liver organoids. The BME2/cell-suspension was seeded in 30 µl droplets by seeding one drop per well in a pre-warmed 24 well plate containing 60.000 single cells isolated from liver tissue. To solidify the BME2, the plate was incubated at 37 °C for 15 min. Once the droplets were solidified, 500 µl of human liver organoid medium containing various growth factors, differentiation factors, niche factors and nutrients (Table 7 and Table 8) were added per well. Freshly seeded liver cells were cultured at 37 °C and with 5% CO_2 in an incubator. Medium was refreshed every two to three days for up to 10-14 days until first passaging, depending on the growth of the organoids.

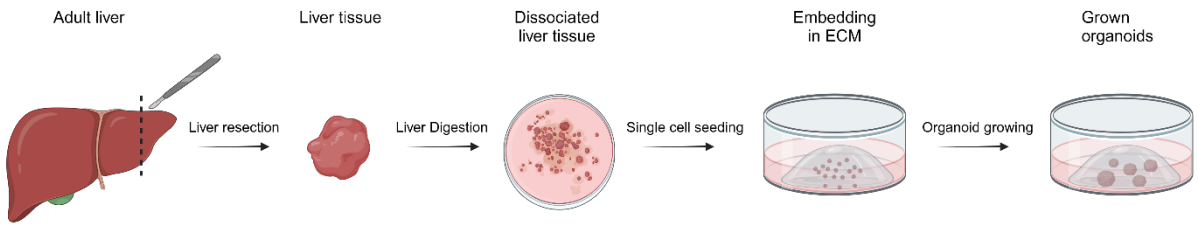

Figure 9: Workflow of the generation of human liver organoids.
Human liver organoids were derived from adult human liver tissue undergoing liver resection. After mincing the liver tissue into small pieces and enzymatic digestion, the generated single cells were subsequently seeded into 30 µl droplets of BME to let them grow to organoids in their corresponding human liver organoid culture medium. Figure is generated via BioRender.

For passaging of the liver organoids, the organoid medium was removed and 1 ml of 4 °C cold AD+++ was added to each well to dissolve BME2 droplets. Then, three wells of organoids were transferred to a 15 ml falcon tube placed on ice and AD+++ was added up to 10 a total volume of 10 ml. The tubes were centrifuged at 300 g and 4 °C for 5 min. After centrifugation, the supernatant was discarded and the cell pellet was resuspended with 300 µl of cold AD+++, transferred to a 1.5 ml tube and immediately placed on ice. The organoid suspension in 300 µl AD+++ was then dissociated by pipetting up and down around 100 times first with a 100µl pipet tip and then another 100 times by using a 10µl pipet tip. The tubes were filled up to 1 ml with AD+++ afterwards. Depending on the confluence of the organoids, the organoids were split and seeded in a ratio from 1:4 to 1:8. If many organoids were generated from a tissue, a subset of them could be frozen in 500 µl of recovery freezing medium. To thaw cryopreserved liver organoids, the frozen organoids were thawed at 37 °C in a water bath. The cell suspension was transferred dropwise to 9 ml pre-warmed AD+++ and centrifuged at 300 g and 4 °C for 5

min. After one washing step with additional 10 ml AD+++, the organoids were seeded as described above.

To generate single cells of the human liver organoids and to start an experiment for examining growth kinetics, the organoids were harvested as described above. After centrifugation and removing the supernatant from the 15 ml falcon tube, 300 µl of TripLE Express were added and the cell suspension was incubated at 37 °C for 15 min. To ensure the dissociation of organoids to single cells, a small amount of cell suspension was transferred into a fresh 24 well plate to check cells under the light microscope. Single cells were washed in AD+++ by centrifuging at 300 g and 4 °C for 5 min. After centrifugation, the cells in suspension were counted using a Bio-Rad TC20™ Automated Cell Counter and 60.000 cells were seeded per well in 22.5 µl of BME2 and 7.5 µl human liver organoid medium per well as described before. For the assessment of the effects of cytokines or TLR ligands on organoids, these stimuli (Table 9) were added to the human organoid culture medium.

3.3.1 Cytokine stimulation of human liver organoids

Single cells were seeded in a 24 well plate. The cell cultures were then stimulated either with 5 ng/ml or 500 ng/ml of human TNF, with 50 Units/ml or 5000 Units/ml of human IFNγ or both cytokines in combination for 14 days. Organoid growth kinetics were determined at the end of the experiment by imaging the BME droplets with Evos M5000 light microscope, measuring, and counting the organoids with the AI organauts GOAT (Bremer et al., 2022).

For investigating the cytokine and chemokine production of human liver organoids, fully grown and confluent organoids were stimulated with 10 µg/ml poly I:C supplemented in human liver organoid culture medium according to Table 9. Following, the organoids were subsequently incubated for 24 hours or for 7 days.

Due to limited amount of the AIH samples, replicated of AIH donors were performed in different experiments on different days. In addition, to generate technical replicates from the same donors, multiple samples were thawed on the same day, cultured and stimulated on different days and time points.

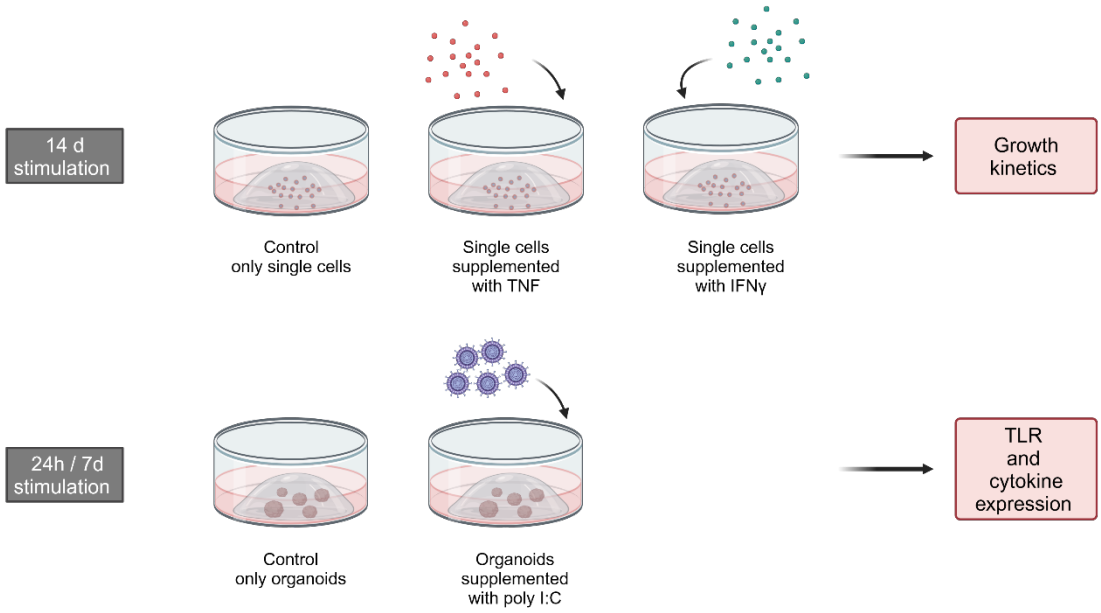

Figure 10: Workflow stimulation.

To determine growth kinetics upon stimulation for 14 days, single cells were seeded and stimulated either with 5 ng/ml or 500 ng/ml TNF or 50 U/ml or 5000 U/ml IFNγ. Single cells without a stimuli were used as control. To examine TLR expression and the cytokine and chemokine production upon stimulation, human liver organoids from non-inflamed livers as well as organoids generated from AIH affected livers were stimulated either for 24 hours or 7 days with poly I:C. Unstimulated organoids were used as control. Figure is generated with BioRender.

3.3.2 Co-Culture of human liver organoids and AIH tissue resident memory 1 T cells

Human liver organoids were co-cultured with fresh or cryopreserved sorted and expanded tissue resident memory 1 (TRM1) T cells from individuals affected with AIH. The TRM1 T cells in this study were generated in the lab of Nicola Gagliani. These T cells were obtained from liver biopsies and were expanded to yield sufficient cells for the co-cultures as follows. These TRM1 T cells were activated at 37 °C for 2 hours with Immunocult™ Human CD3/CD28 T cell Activator (StemCell) by using 25 µl of the activator per $1x10^6$ cells/ml in R10 cultivation medium. For the T cell liver organoid co-cultures, fully confluent human liver organoids were split in a ratio of 1:6 and they were seeded together with $2x10^5$ pre-activated T cells in a 30 µl BME2 droplet. For co-cultures, human liver organoid culture medium was supplemented with, 50 U/ml of IL-2 to support T cell survival. Liver organoids co-incubated with TRM1 T cells were supplemented with either 0.5 µg/ml of TNF RI/TNFRSF1A antibody alone to block TNF signaling or with 1 µg/ml IFNγ antibody combined with 0.5 µg/ml of TNF RI/TNFRSF1A antibody to block TNF and IFNγ signaling. Unstimulated organoids were used as a control

condition. After 10 days of co-culture, the supernatant was harvested for further analyses and the growth kinetics were calculated by using the GOAT.

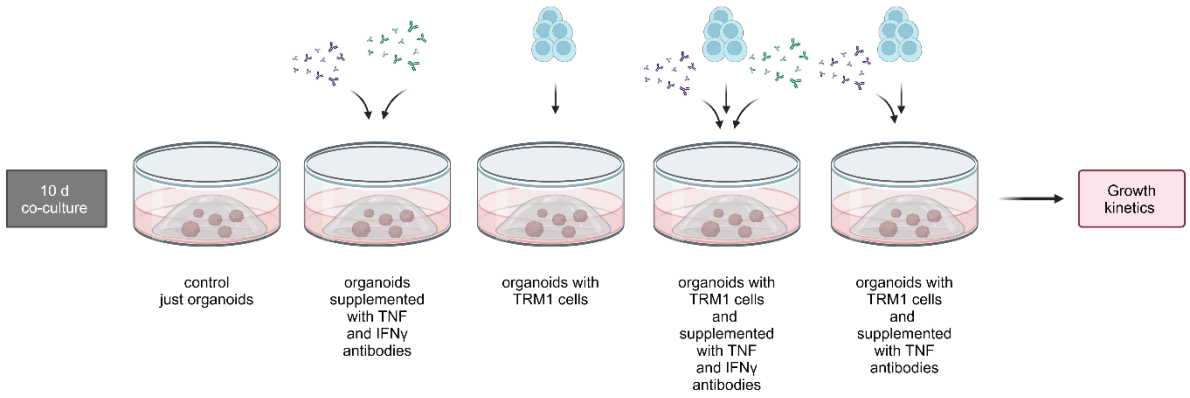

Figure 11: Workflow liver organoid co-culture with TRM1 cells.
To examine the effect of TMR1 cells upon their activation on human liver organoids, co-culture of liver organoids with TRM1 cells was performed. Organoids from non-AIH livers and organoids derived from AIH affected livers were supplemented either with only TMR1 cells, with TRM1 cells supplemented with both, TNF and IFNγ antibodies or with TRM1 cells and TNF antibody alone. TNF and IFNγ antobodies were used to block the corresponding signaling pathways. Figure is created with BioRender.

3.4 Fluorescence staining

All antibodies which were used in this thesis and the corresponding dilutions are listed in Table 1 and Table 2.

3.4.1 Immunofluorescence staining of liver organoids

In this study, immunofluorescence based analyses were used to determine the albumin expression, which is expressed by hepatocytes, or to examine toll like receptor 3 (TLR3) expression in human liver organoids. Immunofluorescence analyses of human liver organoids was performed as described in Dekkers et al., 2019 in Nature Protocols. Briefly, the medium was removed and the liver organoids were carefully detached from each well before adding ice-cold cell recovery solution to the wells. The organoids were then incubated for 1 h at 4 °C by shaking on a horizontal shaker. The organoids were transferred to a pre-coated 15 ml Falcon tube with 1% (wt/vol) PBS-BSA and washed with cold PBS for 3 min at 70 g at 4 °C. Following, the cells were fixed in 1 ml of 4% PFA for 45 min at 4 °C. Then, the cells were washed in 4 °C cold PBS-Tween (PBT (0.1% (vol/vol)) for 10 min at 4 °C. After fixation and a following washing step, unspecific binding of antibodies to the cells in the organoids was blocked by resuspending the pellet in 400 µl of cold organoid washing buffer (OWB) (OWBs ingredients: 1 ml of Triton X-100, 2 g of BSA, 1 L of PBS). The cells were incubated overnight

with the primary antibodies (Table 1) at 4 °C on a horizontal shaker. The liver organoids were washed three times with OWB. Each washing step comprised an incubation time of 2 hours each. Then, the organoids were stained with Hoechst and the corresponding secondary antibodies (2x concentration) as written in Table 2 overnight. On the next day, the organoids were washed again three times in OWB as described before. After washing, they were collected in 50 µl fructose-glycerol clearing solution (60% (vol/vol) glycerol and 2.5 M fructose) at RT. After resuspending in clearing solution, the organoids were incubated for 20 min at RT and they were then transferred to coverslips. They can be stored at - 20 °C for at least 6 month until imaging. Confocal microscopy was performed using a Nikon Ti2 spinning disk microscope equipped with an Andor iXON888 EMCCD camera (Andor Technology). The setup was equipped with 405, 488, 561, and 640 laser lines. Image acquisition was carried out with NIS-Elements.

3.4.2 Immunofluorescence staining of adherent cells

To analyze albumin expression in human liver organoids, MRC-5 cells were used as negative control for albumin production, as these cells are not able to produce albumin. HepG2 NTCP cell line was used as positive control for albumin staining. This cell line is a hepatocellular carcinoma cell line that expresses albumin. For immunofluorescence staining of adherent cells, the cells were harvested and seeded at $1x10^5$ cells per well on a glass coverslip in a 24-well plate for 24 h in their corresponding culture medium. Next, the cells were washed in PBS and were fixed in 1 ml of 4% PFA for 30 min at 37 °C. After fixation, unspecific binding of the cells was blocked using blocking buffer (1% BSA in PBS) for 30 min at 37 °C. For this, the cover slip was transferred into a petri dish cell-side-up. Before blocking, the cells were washed with PBS. Subsequently, the blocking buffer was removed and the cells were incubated in 150 - 200 µl of the primary antibody solution (blocking buffer plus primary antibody) for 1 h at RT. Here, albumin was used as primary AB (Table 1). Afterwards, the cells were washed three times with PBS à 5 min per washing step to remove the primary antibody. After washing, the cells were covered with 150 - 200 µl secondary antibody solution for 1 h at RT. In this immunofluorescence staining, the same secondary antibodies as applied in 3.5.1 for human liver organoids IF staining were used (Table 2). Following, the cells were washed three times as before. To prepare the cells for microscopy, the coverslip containing the cells was covered with mounting media and sealed with nail polish. As soon as the polish was dried, the cells were ready for microscopy. They were stored at - 20 °C for at least 6 month until imaging. Confocal microscopy was performed using a Nikon TI2 spinning disk microscope equipped with an Andor iXON888 EMCCD camera (Andor Technology). Image acquisition was carried out with NIS-Elements.

3.5 Molecular biology

3.5.1 RNA extraction and DNAse Treatment

To extract RNA from organoids after culture, the human liver organoid medium was removed and 1 ml of TRIzol™ Reagent was directly added to each well containing the organoids. The extraction was performed as follows. After adding the TRIzol™ reagent, the suspension was transferred into a 1.5 ml tube and was vortexed for 30 seconds to homogenize the samples. Afterwards, 200 µl of chloroform were added to the samples, incubated for 3 min at RT and centrifuged for 30 min at 12.000 g at 4 °C. The centrifugation separates the mixture in three layers: the lowest red phenol-chlorophorm layer, the interphase and a clear aqueous upper layer containing the RNA. This latter was transferred to a fresh 1.5 ml tube. To precipitate the RNA, the samples were treated with 500 µl of isopropanol and left at - 20 °C for a minimum of 20 min. To increase RNA yield, the samples can be stored over night at - 20 °C. After incubation, the samples were centrifuged at 12.000 g for 30 min at 4 °C, the supernatant was discarded and the pellets were washed by adding 1 ml of 75% ethanol. The samples were vortexed to ensure good wash of the pellet and they were centrifuged again for 15 min with the same conditions as before. The supernatant was removed after centrifugation and the pellet was air dried for 10 min before eluting with 21 µl or RNase-free water. RNA concentration was determined by using NanoDrop 1000 Spectrophotometer using 260/280 absorbance ratio.

To remove the small amounts of contaminating genomic DNA, DNAse treatment was performed after RNA isolation. For this, the DNase master mix was prepared as following:

Table 14: DNase master mix for DNase treatment.

Reagent	**Amount [µl]**
Water	0.67 µl
RNaseOUT™ Ribonuclease Inhibitor	1 µl
AMBION Buffer	3.33 µl
DNaseI (2U/µl)	3.33 µl

After preparing the DNase master mix, the RNA extract samples were topped with 8.3 µl of DNase master mix and shortly spinned down before incubation for 15 min at 37 °C. Then 3.3 µl of EDTA 25 mM was added to each sample and were incubated for 10 min at 65 °C. The samples were then immediately used for cDNA synthesis of were stored at - 80 °C.

3.5.2 cDNA synthesis

RNA was reverse transcribed to complementary DNA (cDNA) by using qScriber™ cDNA Synthesis Kit (HighQu). For each sample, 4 µl of 5X qScriber Reaction Mix 1 µl of qScriber Enzyme Blend (20X) were added to 1000 ng/µl RNA template. RNase-free water was added up to a 20 µl per reaction of RNA mix into a 200 µl PCR tube. The 20 µl reaction mix was then incubated 30 min at 42 °C to synthesize the cDNA, followed by 10 min at 85 °C to inactivate the enzyme using a Biometra Thermal Cycler- Biometra TAdvanced (Analytikjena). The cDNA was stored at 4 °C for short-term storage and at - 20 °C for long-term storage.

3.5.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed to determine the gene expression in liver organoids by using LightCycler 480 II (Roche). For each primer set, 1 µl of cDNA template, 5 µl of SYBR® Green, 3 µl of RNase-free water, 0.5 µl of 10 µM forward primer as well as and 0.5 µl of 10 µM reverse primer were used per reaction. All reactions were performed in a white 96 well micro PCR-plate and were sealed with an optically clear sealing tape. After sealing, the plate was shortly centrifuged to remove bubbles. The threshold cycle (Ct) was calculated by using the mean of two technical duplicates. These values were normalized to the housekeeping gene (HKG) GAPDH. The used program for RT-PCR is listed in the following:

Table 15: RT-PCR program.

Description	Temperature	Time	Number of cycles
Reactivation	95 °C	05:00	1
Cycling	95 °C	00:10	
(fluorescence acquisition step)	60 °C	00:30	40
Melting curve	95 °C	00:15	1
	60 °C	00:15	1
Cooling	40 °C	00:30	1

LightCycler® 480 SW 1.5 software was used for data analysis.

3.5.4 Enzyme-linked immunosorbent assay (ELISA)

Albumin levels in liver organoid culture supernatant were measured using Human Albumin ELISA Kit (Bethyl) according to the manufacturer's instructions. In brief, 50 µl of the liver organoid culture supernatant was added to the pre-coated 96-well plate and was incubated for 1 hour at RT. Following, the plate was washed four times and 100 µl of anti-albumin detection

antibody were added to each well. The plate was covered and incubated for 1 h at RT again. After repeating the washing steps, 100 µl of HRP Solution A was added and the covered plate was incubated for further 30 min. After washing again the plate, 100 µl of TMB substrate Solution were added and the plate was incubated for 30 min in the dark. To stop the reaction, 100 µl of Stop solution were added and the absorbance was immediately measured at 450 nm with a Safire^2 Tecan plate reader. Next to the samples, a standard was running in duplicates. Concentrations were determined based on the standard curve. Here, HepG2-NTCP hepatocellular carcinoma cell line was used as positive control.

3.5.7 Luminex discovery assay

Luminex discovery assay was performed to quantify various cytokine levels in the supernatant of liver organoid cultures after 7 days of poly I:C stimulation by using Human Premixed Multi-Analyte Kit (R&D Systems). This assay was performed following the manufacturer's instructions. Briefly, 50 µl of standard as well as 50 µl of the culture supernatant were added to each well of the pre-coated 96-well plate. Additionally, 50 µl of diluted micro particle cocktail were added to each well and incubated for 2 h at RT on a shaker at 800 rpm. After incubation, the liquid was removed and the plate was washed three times by filling with 100 µl of washing buffer. Following the washing steps, 50 µl of diluted Biotin-antibody cocktail were added per well. The plate was sealed and incubated for 1 h at RT on the shaker at 800 rpm. The washing steps were repeated after 1 h of incubation and 50 µl of Streptavidin-PE was given for 30 min by shaking at 800 rpm. After washing, 100 µl of washing buffer were added and another incubation of 2 min at RT and 800 rpm was performed. Within 90 min, the plate was read using Bio-Plex 200 System (BioRad).

3.5.8 Lactate dehydrogenase (LDH) Cytotoxicity Assay

Lactate dehydrogenase cytotoxicity assay was performed following the manufacturer's recommendations to quantify LDH in the supernatant of the human liver organoid T cell co-cultures. For this, 50 µl of the supernatant was added to a 96-well flat-bottom plate in duplicates followed by adding 50 µl of reaction mixture and incubated for 30 min in the dark after mixing well. As a control, just the human liver organoid medium was used. To stop the reaction, 50 µl of Stop solution was added to each well and immediately measured at an absorbance of 490 nm and 680nm with the Safire^2 Tecan plate reader.

3.6 Data acquisition and statistical analysis

Statistical analyses were performed by using GraphPad Prism 9 and 10 software. Immunofluorescence stainings were acquired on a Nikon Spinning Disk confocal microscope.

4 Results

The results of this thesis are structured in two parts.

In the first part of this thesis, the establishment of a 3D human liver organoid cell culture model from human liver tissue is described. Subsequently, human liver organoids are analysed and assessed for their use to study liver diseases and interactions with immune cells. For this, the characterization of human liver organoid culture system was performed by analysing gene expression and protein levels of human liver markers.

As dysregulated TLR responses have been described to contribute to autoimmune liver diseases (Duan et al., 2022), organoids from AIH affected livers and from livers not affected with AIH were stimulated with poly I:C, which is recognized by TLR3 (Blasius & Beutler, 2010). Upon stimulation, the cytokine and chemokine responses were analyzed to assess liver generated pro-inflammatory responses.

In the second part, the impact of immune cells on human liver organoids is studied in detail by showing the impact of immune mediators and T cells on liver organoids. For this, the organoids were cultured long-term with the pro-inflammatory cytokines TNF and IFNγ, known to be elevated in AIH and produced by T cells in individuals affected with AIH (Bovensiepen et al., 2019). Next, a novel co-culture model of liver organoids with liver-derived tissue resident memory T (TRM1) cells was developed to explore the effects of TRM1 cells from AIH affected livers on growth kinetics of the liver organoids.

4.1 Generation and validation of non-inflamed control and AIH human liver organoid models

4.1.1 Generation human liver organoids

In this work, human liver organoids were generated to investigate pro-inflammatory pathways that are dysregulated in AIH. For this, liver tissue was obtained from individuals undergoing liver resection surgery for liver metastases. The liver organoids were generated from non-metastatic resected border regions. AIH organoids were generated from explanted livers from adult individuals diagnosed with AIH. Liver samples were processed, digested and cultured in an extracellular matrix in order to generate 3D organoids. The size of the organoids ranged between 100 – 500 µm diameters.

Organoids can be cultured in their suitable human liver organoid culture medium for weeks up to month and over several passages. In culture, the liver organoids exhibited a heterogeneous morphology as analysed by bright field microscopy. Some liver organoids attained a solid morphology being dense, whereas others had a more cystic phenotype (Figure 12). This

heterogeneous phenotype was observed in non-inflamed and AIH affected liver organoid cultures. These characteristics have been linked to different liver cell types. Although both originate from the common precursor termed hepatoblast, cells can then differentiate to a hepatocyte-like or a cholangiocyte-like phenotype. The heterogeneity of the organoids differed per donor, with no direct relationship to the disease state of the donor.

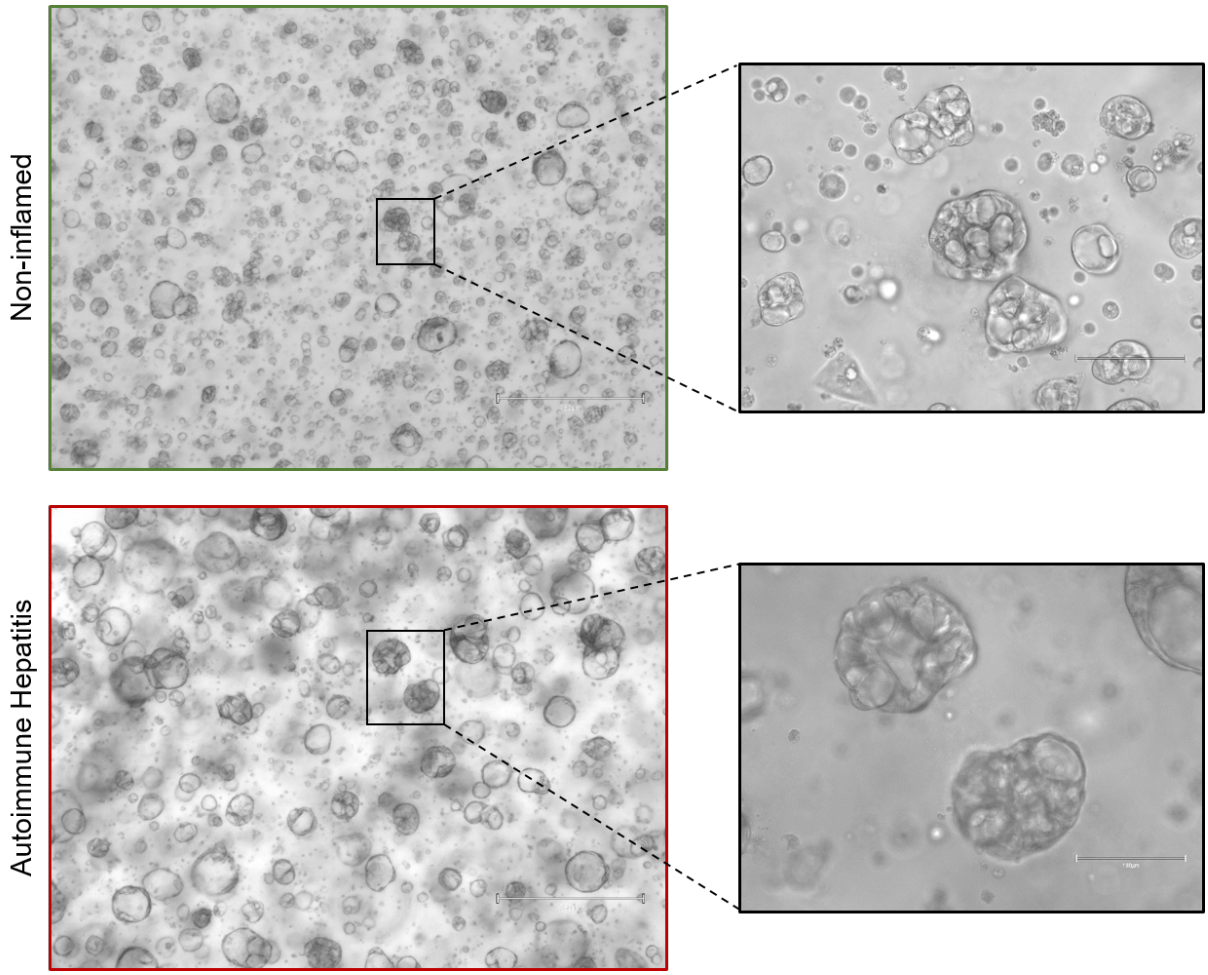

Figure 12: Three-dimensional human liver organoid culture.

Bright field microscopy images showing organoids from non-inflamed livers (top) and AIH-derived liver organoids (bottom) at passage 3 (P3) cultured from cells in digested liver tissue. Lower magnification using a 4X objective (left. black scale bar = 750 µm), higher magnification taken with a 20X objective (right, white scale bar = 150 µm).

4.1.2 Characterization human liver organoids

To investigate the cell types in 3D human liver organoids obtained from livers of non-inflamed individuals and AIH affected individuals, gene expression of characteristics of liver derived cells was quantified. To analyse the cells, the BME2 was dissolved and the human liver

organoids were resuspended in TRIzol™ Reagent for RNA isolation and the following performed RT-PCR analysis.

The most numerous cells in the liver are hepatocytes (Materne et al., 2013; Moradi et al., 2020). Human hepatocytes play a role in various functions, such as protein metabolism, detoxification and activation of immune cells to keep homeostasis in the liver (Gong et al., 2023). To be able to perform all these activities, hepatocytes express proteins like Cytochrome P450 (CYP450) enzymes, known as drug-metabolism enzymes in the liver (M. Zhao et al., 2021) and albumin as transporter of bilirubin and fatty acids (Paar et al., 2021; Sun et al., 2019).

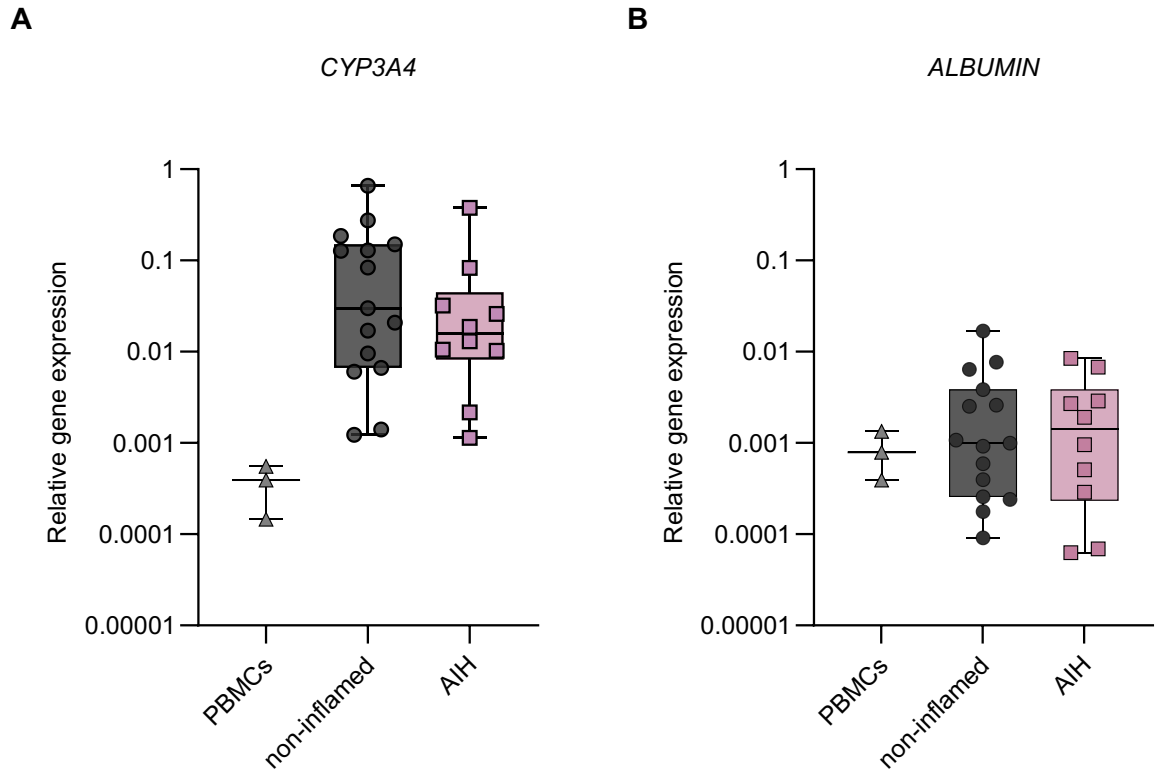

Figure 13: Relative mRNA expression of the hepatocyte markers CYP3A4 and albumin in human liver organoids compared to PBMCs.

A. *CYP3A4 relative gene expression by non-inflamed controls and AIH-derived organoids.* ***B.*** *Albumin relative gene expression by non-inflamed controls and AIH-derived organoids. Values were normalized to the housekeeping gene GAPDH. All genes were measured in duplicates. PBMCs n=3, Non-inflamed controls n=15 and AIH n=10. Mann-Whitney test was used to compare CYP3A4 and albumin expression between liver organoids from non-inflamed control individuals to organoids from AIH affected individuals. No significant differences were observed between non-inflamed organoids and AIH-derived organoids.*

PBMCs were used as control, as these cells would have low expression of these hepatocyte lineage markers. A quantitative real-time PCR analysis was performed of PBMCs and human liver-derived organoids from non-inflamed individuals and AIH affected individuals. Gene

expression was normalized to the housekeeping gene *GAPDH*, which is highly expressed in all eukaryotic cells. Compared to liver-derived organoids, PBMCs showed very low expression of hepatocyte marker *CYP3A4* and *albumin*.

Relative mRNA levels showed that both non-inflamed liver organoids and AIH affected human liver organoids expressed *CYP3A4,* indicating hepatocyte lineage (Figure 13).There were no significant differences in *CYP3A4* expression when comparing non-inflamed control organoids (median of 0.03) to AIH-derived organoids (median of 0.02). The expression of *ALBUMIN* was low in PBMCs, but more variable in liver organoids, with certain liver organoids expressing higher values, whereas *ALBUMIN* was low in others. These findings may reflect the heterogeneous morphology of the liver organoids. The expression of *ALBUMIN* was comparable between liver organoids generated from non-inflamed individuals (median expression of 0.001) and from AIH affected individuals (median expression of 0.001).

Next, quantitative real-time PCR analysis was performed to quantify the expression levels of *LGR-5* (as a marker of regenerating cholangiocytes and hepatocytes) and *EPCAM* (an epithelial cell adhesion molecule) (Huch & Dollé, 2016) within the liver organoids. PBMCs were used as controls and should have low expression levels. As seen in Figure 14, PBMCs showed low expression of *LGR-5* and *EPCAM* compared to liver organoids.

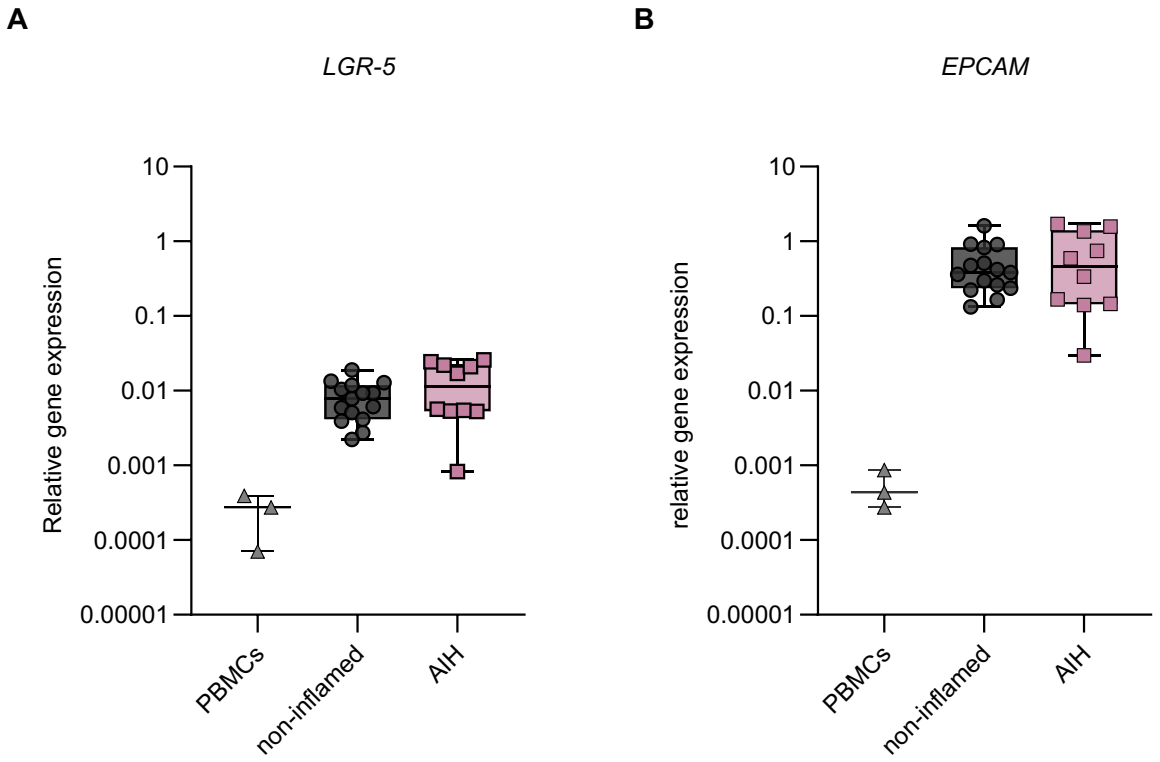

Figure 14: Relative gene expression determined by RT-PCR of LGR-5 and EPCAM in PBMCs and human liver organoids.

A**. Relative gene expression of LGR-5 in non-inflamed controls and AIH-derived organoids.* ***B. *Relative gene expression of EPCAM by non-inflamed and AIH-derived organoids. In both, PBMCs were used as controls for comparisons. The relative level of gene expression data were normalized to the housekeeping gene GAPDH. All samples were measured in duplicates. PBMCs n=3, non-inflamed organoids n=15 and AIH n=10. Mann-Whitney test was used to compare LGR-5 and EPCAM expression in liver organoids between non-inflamed control individuals to AIH affected individuals. No significant differences were observed between non-inflamed organoids and AIH-derived organoids.*

LGR-5 levels were comparable between non-inflamed individuals (median expression of 0.008) and AIH-derived organoids (median expression of 0.011). *EPCAM* tended to be lower expressed in non-inflamed liver organoids compared to AIH-derived liver organoids. However, no significant differences were observed (Figure 14).

These results demonstrate that liver organoids express the hepatocyte marker *CYP3A4*, whereas *ALBUMIN* expression is more variable. Furthermore, mRNA levels of the progenitor marker *LGR-5* and adhesion molecule *EPCAM* were low.

To further assess the albumin secretion of human liver organoids, an Enzyme-linked Immunosorbent Assay (ELISA) was performed. Albumin can be secreted to the exterior, indicating a functional phenotype of the cell (Sleep, 2015; Sun et al., 2019). Therefore, albumin

was measured in the supernatants from non-inflamed control and AIH-derived liver organoids (Figure 15).

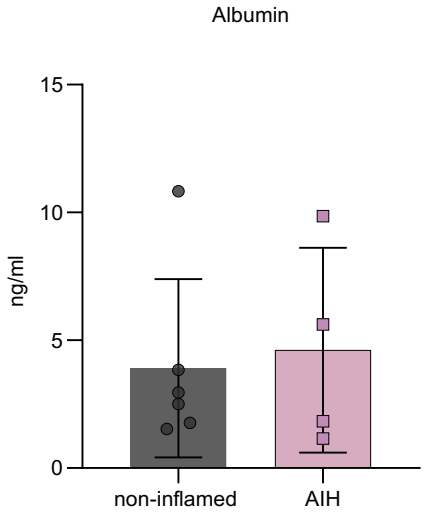

Figure 15: Albumin levels in organoid cultures measured by ELISA.
Cell culture supernatants of the mature organoids were harvested after 48 h of the last medium change. Non-inflamed individuals n=6 and AIH affected individuals n=4. The samples were run in duplicates. Mann-Whitney test was applied to compare secreted albumin levels of liver organoid cultures generated from organoids from non-inflamed livers and organoids from AIH-affected livers. No significant differences were observed between non-inflamed organoids and AIH-derived organoids.

The human liver organoid culture medium alone was measured as control, (protein concentration of 1.19 ng/ml). Little albumin protein secretion was detected in human liver organoid cultures of non-inflamed organoids (median protein concentration of 2.73 ng/ml) and AIH-derived organoids (median protein concentration of 3.72 ng/ml).

To further visualize albumin in cells in liver organoids, albumin was visualized by immunofluorescence staining. The hepatocellular carcinoma cell line HepG2-NTCP was used as positive control. The MRC-5 cell line, which does not produce albumin, was used as a negative control. A “secondary antibody only” control was used to confirm albumin-specific binding (Figure 16).

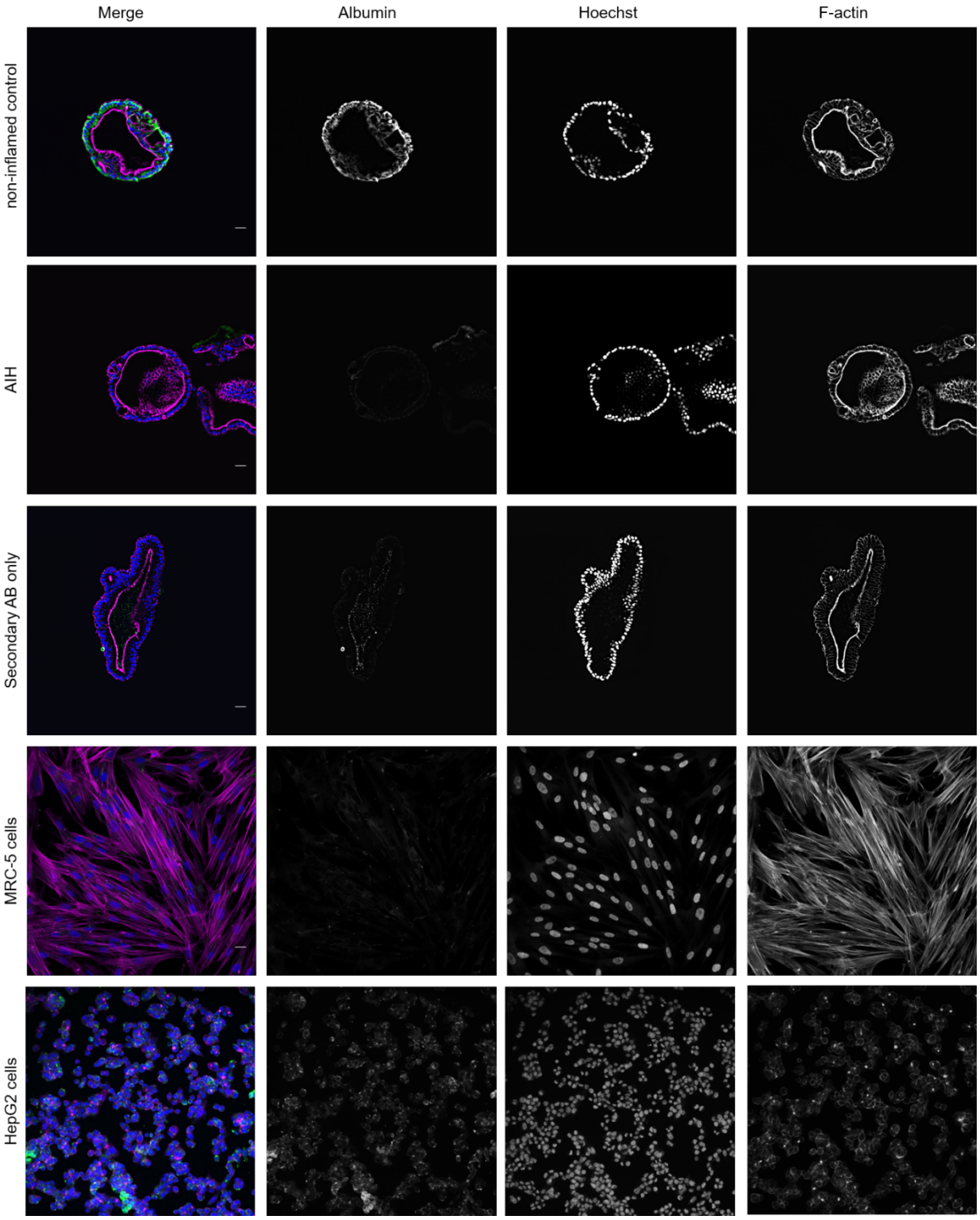

Figure 16: Immunofluorescence staining of albumin.

Immunofluorescence staining of organoids from non-inflamed livers, organoids from AIH-derived livers, MRC-5 cell line (negative control), HEPG2-NTCP cell line (positive control) and "secondary antibody only", from top to bottom. Nuclei were stained with Hoechst (blue). For detection of albumin, AF488 (green) was used, whereas for detection of actin filaments, AF555 labelled Phalloidin (magenta) was applied in all samples. Merged channel shows co-localization. Scale bar for all figures: 50 µm. Confocal microscopy images were performed by using 25x silicone objective at Nikon spinning disc microscope.

Using immunofluorescence imaging, albumin protein was detected in organoids from non-inflamed livers. However, in organoids derived from AIH affected livers, no albumin was detectable by immunofluorescence. This may be due to few organoids images or inherent to AIH affected organoids. The negative control (MRC-5 cell line) and “secondary antibody only” staining further showed absence of unspecific signals.

To conclude, these data showed gene expression and protein levels of liver-specific markers, demonstrating that human liver organoids harbour characteristics of hepatocytes. However, within cultures the phenotype may be heterogeneous. As AIH-derived 3D organoids showed a similar phenotype to the non-inflamed control organoids, these models were next used to perform further studies into hepatocyte dysregulation in AIH.

4.1.3 Toll like receptor expression by human liver organoids in non-activated conditions

In the healthy liver, it has been previously shown that the mRNA expression of the various TLRs is low (Y. Chen & Sun, 2011; De Creus et al., 2005; Kiziltas, 2016; Lichtman et al., 1998; Schwabe et al., 2006; Zarember & Godowski, 2002). As described in 1.3.1, the distribution of the TLR expression varies between die various liver cell types. However, literature describes that hepatocytes are able to express mRNA of all TLRs (Y. Chen & Sun, 2011; Kesar & Odin, 2014; Kiziltas, 2016; Schwabe et al., 2006).

Therefore, the TLR expression on cells in human liver organoids from non-inflamed and AIH-derived liver tissues was assessed (Figure 17). Gene expression was normalized to the housekeeping gene *GAPDH.*

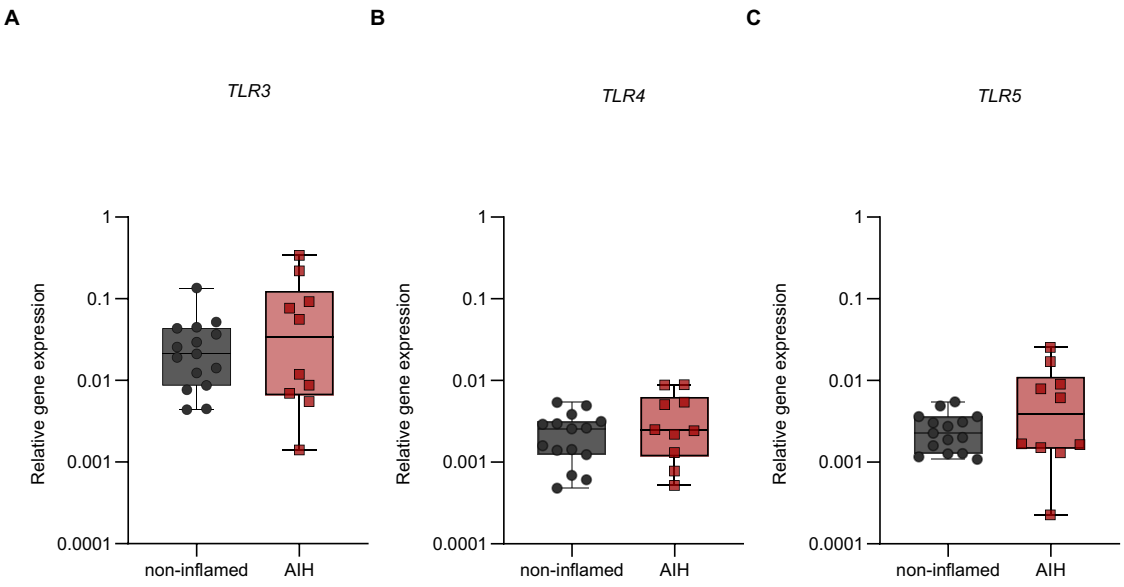

Figure 17: Relative gene expression of TLR3, TLR4 and TLR5 on human liver organoids analyzed by RT-PCR.

*Relative gene expression of **A)** TLR3, **B)** TLR4 and **C)** TLR5 by non-inflamed organoids and AIH-derived organoids. Values were normalized to the housekeeping gene GAPDH. All genes were measured in duplicates. Non-inflamed samples n=15 and AIH samples n=10. Data was analyzed by performing Mann-Whitney test to compare differences in TLR expression of non-inflamed individuals and AIH affected individuals. No significant differences were observed between non-inflamed organoids and AIH-derived organoids.*

All three TLRs were expressed in human liver organoids at low mRNA levels. AIH affected liver organoids displayed similar expression levels as non-inflamed liver organoids for all three receptors analysed. However, *TLR3* tended to be slightly higher expressed by liver organoids from AIH affected livers and organoids from non-inflamed liver in comparison to *TLR4* and *TLR5*. Furthermore, *TLR3* expression levels showed a larger spread in liver organoids derived from AIH affected individuals compared to non-inflamed individuals. For *TLR4*, in non-inflamed individuals, a median expression of 0.0026 was observed, which was similar to liver organoids derived from AIH affected individuals, showing a median of 0.0025, respectively. In line with these findings, *TLR5* was expressed with a median of 0.0023 in non-inflamed livers derived organoids compared to 0.0039 in AIH-derived liver organoids. Overall, neither *TLR3*, *TLR4* nor *TLR5* expression levels differed significantly between organoids from AIH affected livers and non-inflamed control derived liver organoids.

In some cases, mRNA levels do not fully correspond to protein levels. Furthermore, some liver organoids derived from AIH affected livers showed higher expression of TLR3. Therefore, the location of TLR3 was visualized using immunofluorescence (Figure 18).

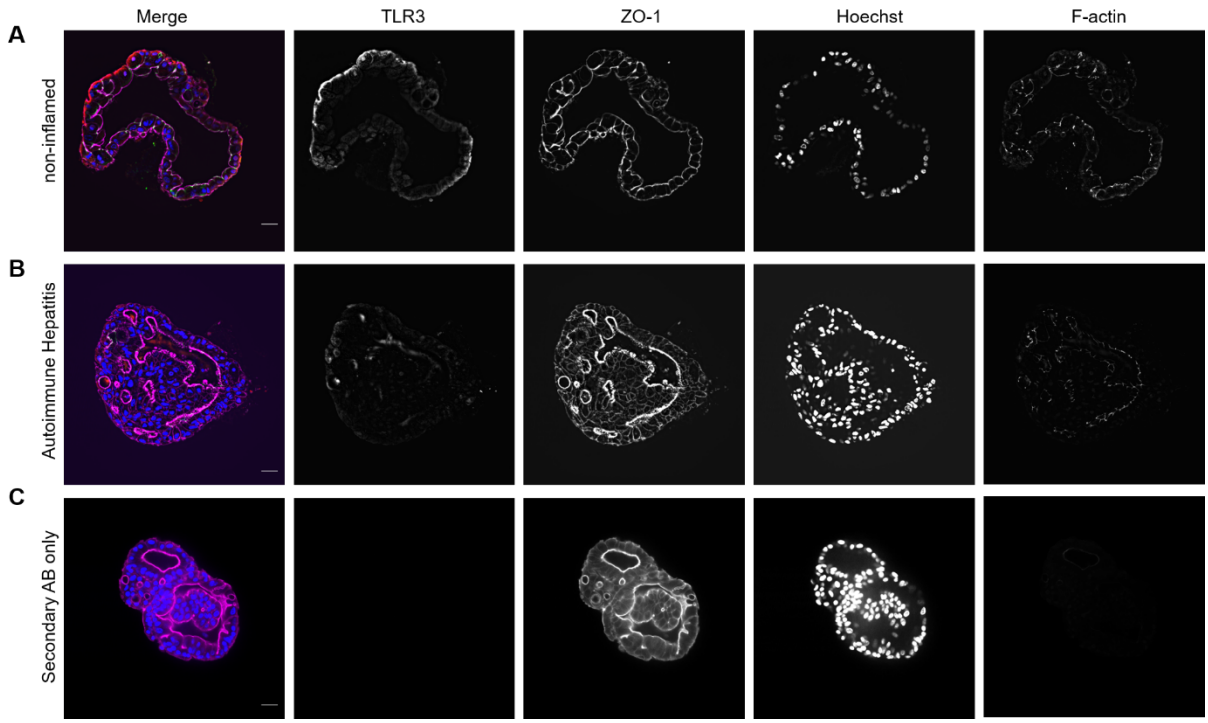

Figure 18: Immunofluorescence staining of TLR3 in liver organoids.
*Immunofluorescence staining of **A)** organoids from non-inflamed livers, **B)** AIH-derived liver organoids, and **C)** secondary antibody only control on human liver organoids. In the merge images, TLR3 proteins (AF555) is shown in red, nuclei were stained with Hoechst and appear in blue. Immunofluorescence staining of tight-junctions protein Zonula occludens-1 (ZO-1) appears in magenta and the cytoskeleton as actin filaments labelled as Phalloidin is shown in green. Merged images showing co-localization. Scale bar represents 50 µm. Images were done by using 40x water objective.*

TLR3 was detected by immunofluorescence analyses in organoids from non-inflamed livers and organoids derived from AIH affected livers (Figure 18).

Overall, under non-activated conditions human liver organoids from non-inflamed livers and from AIH affected livers, TLR3 was highest expressed and detected by immunofluorescence analyses. Furthermore, no significant differences were observed between AIH-derived and non-inflamed liver organoids.

4.1.4 Quantification of mRNA levels of pro-inflammatory cytokines in liver organoids in non-activated conditions

To determine the overall pro-inflammatory set point of hepatocytes in liver organoids generated from AIH affected livers compared to organoids from non-inflamed livers, the expression of pro-inflammatory cytokines including *IL-6*, *IL-12* and *TNF* was determined (Figure 19). These cytokines were chosen as they have been implied in the pathogenesis of AIH (Mieli-Vergani et al., 2018; Sucher et al., 2019).

Gene expression was normalized to the housekeeping gene *GAPDH*.

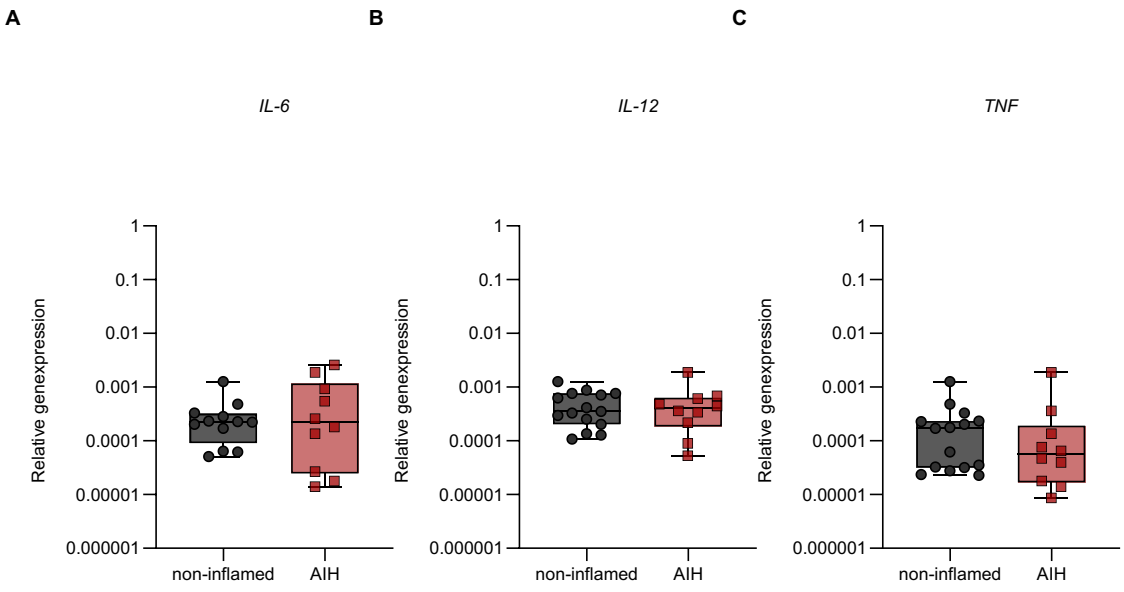

Figure 19: Relative gene expression of pro-inflammatory cytokines IL-6, IL-12 and TNF in human liver organoids analyzed by RT-PCR analysis.

*Relative gene expression of **A)** IL-6, **B)** IL-12 and **C)** TNF of organoids from non-inflamed livers and AIH-derived liver organoids. All samples were assayed in duplicates and normalized to housekeeping gene GAPDH. For A non-inflamed organoids n= 12 and AIH-derived organoids n=10. For B and C non-inflamed organoids n=15 and AIH-derived organoids n=10. Mann-Whitney test was applied for comparison of cytokine expression in non-inflamed individuals to AIH affected individuals. No significant differences were observed between non-inflamed organoids and AIH-derived organoids.*

Gene expression of all cytokines in liver organoids was low. Furthermore, gene expression of *TNF* and *IL-12* was even slightly lower compared to *IL-6* in liver organoids (Figure 19). In both organoids from non-inflamed livers and organoids derived from AIH affected livers, *IL-6* expression was similar by showing a median expression of 0.00023 in non-inflamed livers and 0.00022 in organoids generated from AIH affected livers.

Next, mRNA levels of *IL-12* in liver organoids also did not differ between liver organoids derived from non-inflamed livers (median expression of 0.00035) in comparison to organoids from to AIH affected livers (median expression of 0.00040). However, *TNF* tended to be higher expressed in organoids generated from non-inflamed livers compared to organoids derived from AIH affected livers by showing a median of 0.00017 in non-inflamed organoids compared to 0.00006 in AIH-derived organoids (Figure 19), however, not significant.

To sum up, mRNA expression levels of *IL-6*, *IL-12*, *TNF* at baseline are low and show similar levels between AIH-affected liver organoids and non-inflamed liver derived organoids

In previous studies, it was suggested that along with inflammatory cytokines, chemokines, such as CXCL9 are increased in AIH (Czaja, 2014; Y.-L. Li et al., 2013). To assess whether

liver organoids derived from AIH affected express and produce these chemokines, mRNA expression of both chemokines *CXCL9* and *CXCL11* in non-stimulated conditions was assessed (Figure 20).

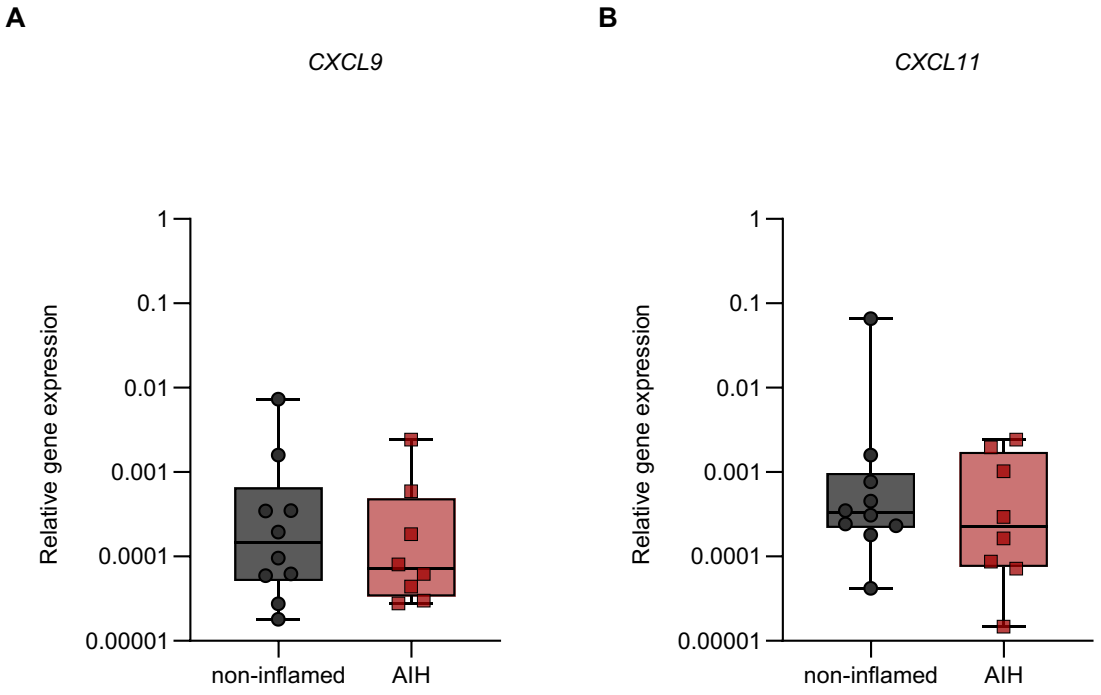

Figure 20: Relative gene expression of chemokines CXCL9 and CXCL11 in human liver organoids analyzed by RT-PCR analysis.

*Quantitative RT-PCR analysis by measuring relative gene expression of chemokines **A)** CXCL9 and **B)** CXCL11 in unstimulated conditions of organoids from non-inflamed livers and AIH-derived human liver organoids. All samples were run in duplicates. Values were normalized to gene expression of housekeeping gene GAPDH. Non-inflamed individuals n=10 and AIH samples n=8. To compare chemokine expression in non-inflamed individuals and AIH affected individuals, Mann-Whitney test was applied. No significant differences were observed between non-inflamed organoids and AIH-derived organoids.*

For both chemokines *CXCL9* as well as for *CXCL11* no statistical differences were detected. A median expression of *CXCL9* of 0.00015 in liver organoids from non-inflamed livers was measured, while in organoids generated from AIH affected livers a median expression of 0.00007 was observed. In line with *CXCL9*, the mRNA expression of *CXCL11* was similar between non-inflamed organoids (median expression of 0.00033) and AIH-derived organoids (median expression of 0.00023).

For validation of the cytokine expression on protein level, IL-6, IL-12, TNF, IFNγ, CXCL9, and CXCL11 were measured in the culture supernatant of unstimulated liver organoids using a Luminex assay. Supernatants were first collected after 24 hours and detection of the molecules

was performed. However, cytokine and chemokine levels were all under the limit of detection (data not shown). Due to this, the experiment was modified by culturing the organoids for 7 days instead of 24 hours. These data are shown in the following in Figure 21.

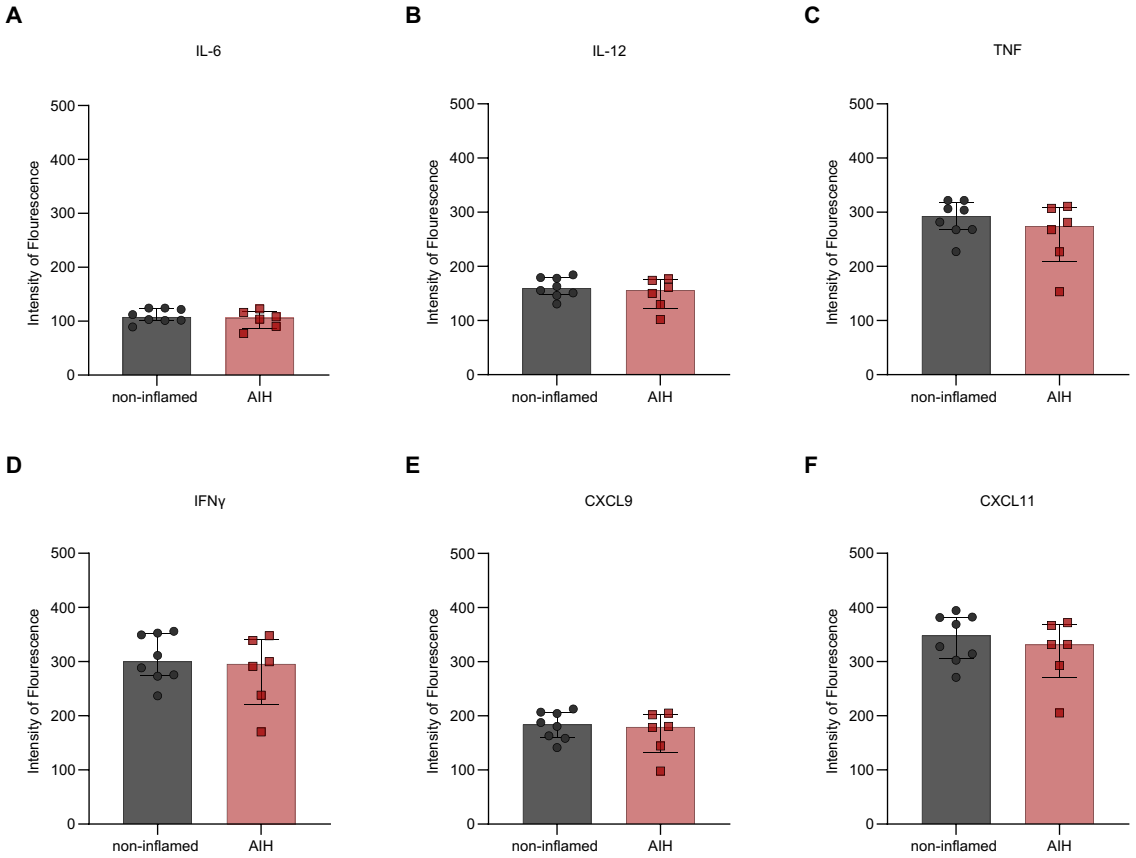

Figure 21: Protein levels of inflammatory cytokines IL-6, IL-12, TNF, IFNγ and chemokines CXCL9 and CXCL11 in unstimulated conditions measured by luminex assay.

*Analysis of intensity of fluorescence of inflammatory cytokines **A)** IL-6, **B**) IL-12, **C)** TNF, **D)** IFNγ as well as chemokines **E)** CXCL9 and **F)** CXCL11 in unstimulated conditions of non-inflamed organoids (n=8. For IL-12 expression n=7) and in AIH-derived liver organoids (n=6. For IL-12 expression n=5). All samples were measured in duplicates. After measuring, the mean of the values was calculated and used for analysis. Mann-Whitney tests were used to compare cytokine and chemokine expression in non-inflamed individuals and AIH affected individuals. No significant differences were observed between non-inflamed organoids and AIH-derived organoids.*

By comparing organoids from non-inflamed livers and AIH-derived organoids, no significant differences between both groups were observed. In non-stimulated conditions, organoids from both non-inflamed livers and AIH affected livers showed nearly the same intensity of fluorescent signal. These data were in line with the similar low mRNA levels of *IL-6*, *IL-12*, *TNF, CXCL9* and *CXCL11.*

Summing up, in non-activated conditions, the non-inflamed liver organoids and AIH-derived liver models were capable to produce low concentrations of pro-inflammatory cytokines, with no significant differences between these two groups.

4.2 Characterization of TLR3 responses by the human liver organoids

4.2.1 Characterization of TLR expression by human liver organoids

It has been reported that a dysregulation in TLR signaling can contribute to autoimmune diseases (Duan et al., 2022). Given that, TLR levels were very low in non-stimulated conditions and pro-inflammatory signals in autoimmune diseases can alter TLR expression. Liver organoids were stimulated with polyinosinic-polycytidylic acid (poly I:C), activating TLR3. In the same line, flagellin and lipopolysaccharids (LPS) were used to activate TLR4 and TLR5, however, flagellin and LPS stimulation did not result in enhancing *TLR4* and *TLR5* expression in liver organoids (data not shown).

Organoids from non-inflamed livers and AIH-derived liver organoids were stimulated with TLR3 agonist poly I:C for 24 hours and harvested to determine mRNA expression of *TLR3*, *TLR4* and *TLR5*. In Figure 22, mRNA TLR levels are shown at baseline level and in stimulated conditions. Gene expression was normalised to the housekeeping gene *GAPDH*.

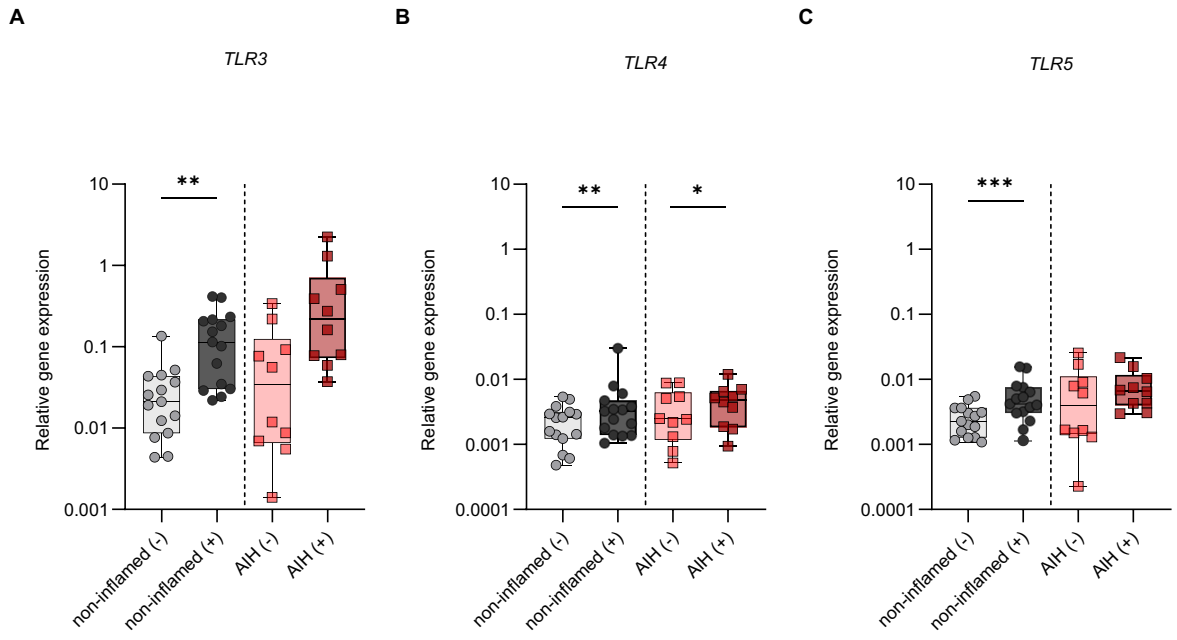

Figure 22: Comparison of mRNA expression of TLRs by human liver organoids in unstimulated and stimulated conditions.

*Quantitative RT-PCR analysis showing the relative gene expression of **A)** TLR3, **B)** TLR4 and **C)** TLR5 in unstimulated conditions (-) and upon 24h poly I:C stimulation (+) in non-inflamed organoids and AIH-derived organoids. All samples were normalized to the relative gene expression of GAPDH. Non-inflamed organoids n=15 and AIH-derived organoids n=10. Mann-Whitney test was performed by*

*comparing TLR expression of non-inflamed individuals with AIH affected individuals upon stimulation. Wilcoxon matched-pairs signed rank tests were used to compare unstimulated with stimulated condition within one group. Only significant p values are shown. *, P <0.05; **, P <0.01; ***, P <0.001.*

A significant increase in *TLR3* expression upon poly I:C stimulation was observed in liver derived organoids from non-inflamed livers (median expression of 0.02 in unstimulated conditions vs. 0.11 in stimulated conditions). TLR3 expression in AIH-derived organoids showed the same trend upon stimulation, however this was not significant.

Although not significant, *TLR3* mRNA expression tended to be higher expressed in liver organoids generated from AIH affected livers (median gene expression 0.22) compared to non-inflamed livers (median gene expression of 0.11) in stimulated conditions. In sum, poly I:C stimulation enhanced *TLR3* expression in liver organoids, with a trend towards higher *TLR3* expression levels in AIH-derived liver organoids compared to non-inflamed livers.

Gene expression of *TLR4* and *TLR5* was also assessed upon poly I:C stimulation of liver organoids. Similar to *TLR3*, poly I:C stimulation of liver organoids significantly increased *TLR4* and *TLR5* levels, especially in organoids derived from non-inflamed livers. Nonetheless, overall *TLR4* and *TLR5* mRNA levels were lower in liver organoids pre- and post stimulation compared to *TLR3*, indicating that TLR3 signaling in hepatocytes might be more relevant compared to TLR4 and TLR5.

In summary, ligands to TLR3 increase *TLR* mRNA levels in liver organoids, however, the expression of *TLR3* resulted in the highest levels upon stimulation with dsRNA virus poly I:C. When binding its agonist, a trend towards increased gene expression of *TLR3* in AIH-derived liver organoids was observed at baseline and after stimulation compared to non-inflamed control liver organoids.

4.2.2 Quantification of pro-inflammatory cytokine and chemokine expression of liver organoids upon TLR3 stimulation with poly I:C

After showing a tendency to higher gene expression of TLR3 in AIH-derived human liver organoids upon poly I:C stimulation in 4.2.1, the human liver organoids generated from non-inflamed livers and from AIH affected livers were used to analyse whether the induction of pro-inflammatory cytokines and chemokines may be increased in hepatocytes in AIH upon TLR3 stimulation.

Therefore, the effect of TLR stimulation on pro-inflammatory cytokine expression by AIH-derived human liver organoids and organoids from non-inflamed livers was investigated. In detail, the mRNA expression levels of *IL-6*, *IL-12*, and *TNF* after TLR3 stimulation were determined. Gene expression levels of the cytokines were normalised to gene expression levels of the housekeeping gene *GAPDH*.

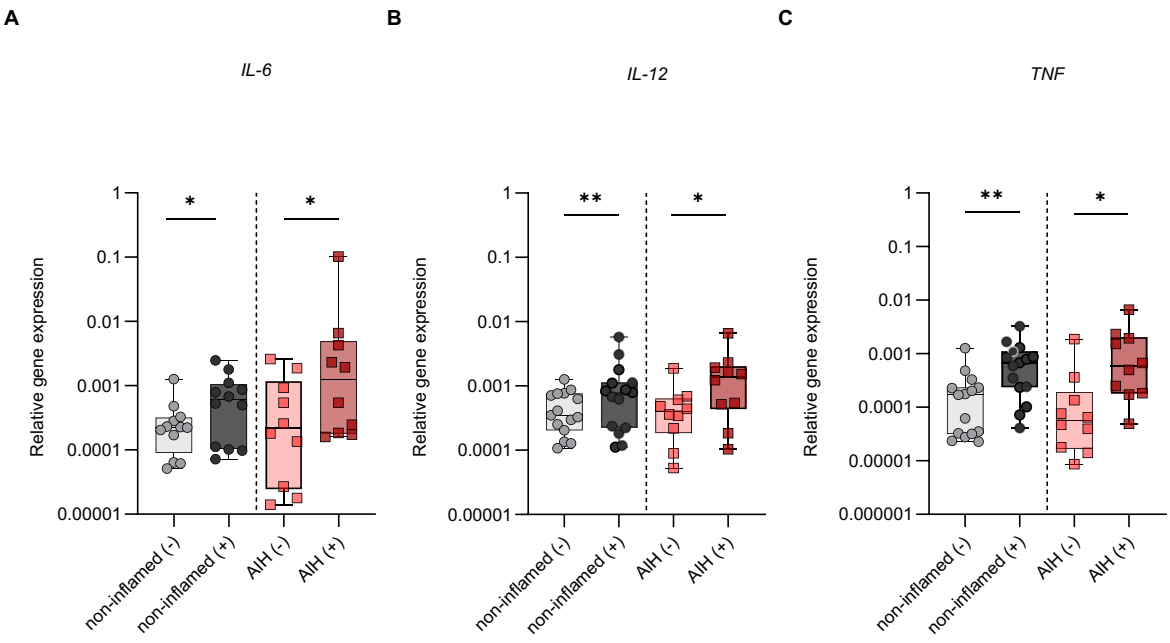

Figure 23: Comparison of mRNA levels of IL-6, IL-12 and TNF after 24h of poly I:C stimulation in human liver organoids.

*Quantitative RT-PCR analysis of mRNA levels of pro-inflammatory cytokines **A)** IL-6, **B)** IL-12 and **C)** TNF post TLR stimulation of non-inflamed liver organoids and AIH-derived liver organoids in non-stimulated (-) and stimulated (+) conditions. (For A in non-inflamed organoids n=12 and AIH-derived organoids n=10. For B and C non-inflamed liver organoids n=15 and AIH-derived organoids n= 10). The samples were normalized to GAPDH. Data was analyzed by performing a Mann-Whitney test to compare cytokines expression of non-inflamed organoids to AIH-derived liver organoids upon 24 hour stimulation with poly I:C. Wilcoxon matched-pairs signed rank tests were used to compare unstimulated with stimulated condition within one group. Only significant p values are shown. *, P <0.05; **, P <0.01.*

Significant changes in relative gene expression were observed in all three pro-inflammatory cytokines between the unstimulated and stimulated conditions in organoids from non-inflamed and AIH affected livers. This indicated that TLR3 activation upregulated mRNA

Specifically, in human liver organoids generated from AIH affected individuals, a significant enhanced *IL-6* expression (median expression of 0.0012) upon stimulation was observed compared to non-stimulated conditions (median expression of 0.0002). In line with these observations, however, not significant, in stimulated conditions, *IL-6* tended to be slightly higher expressed in AIH-derived organoids compared to non-inflamed liver organoids (median gene expression of 0.0012 in AIH-derived organoids and a median gene expression of 0.0006 in non-inflamed human liver organoids) (Figure 23).

Similar observations as for *IL-6* were made in *IL-12* expression. Although no significant differences by comparing non-inflamed organoids and AIH-derived organoids, slightly higher expression levels were observed in AIH-derived organoids (median expression of 0.0014 in

organoids from AIH affected livers compared to non-inflamed liver organoids, showing a median expression of 0.0008).

TNF displayed similar relative gene expression levels in both groups (median gene expression of 0.0006 in liver organoids generated from AIH affected livers vs. a median gene expression of 0.0007 in liver organoids from non-inflamed livers).

Taken together, upon TLR3 activation, an increase in relative gene expression of *IL-6* was observed in liver organoids generated from AIH-affected and non-inflammatory livers. Furthermore, mRNA expression especially of *IL-6* showed a tendency to higher expression levels in the stimulated AIH-derived liver organoids compared to non-inflammatory organoids.

Next to inflammatory cytokines, the chemokines CXCL9 and CXCL11 were investigated (Figure 24).

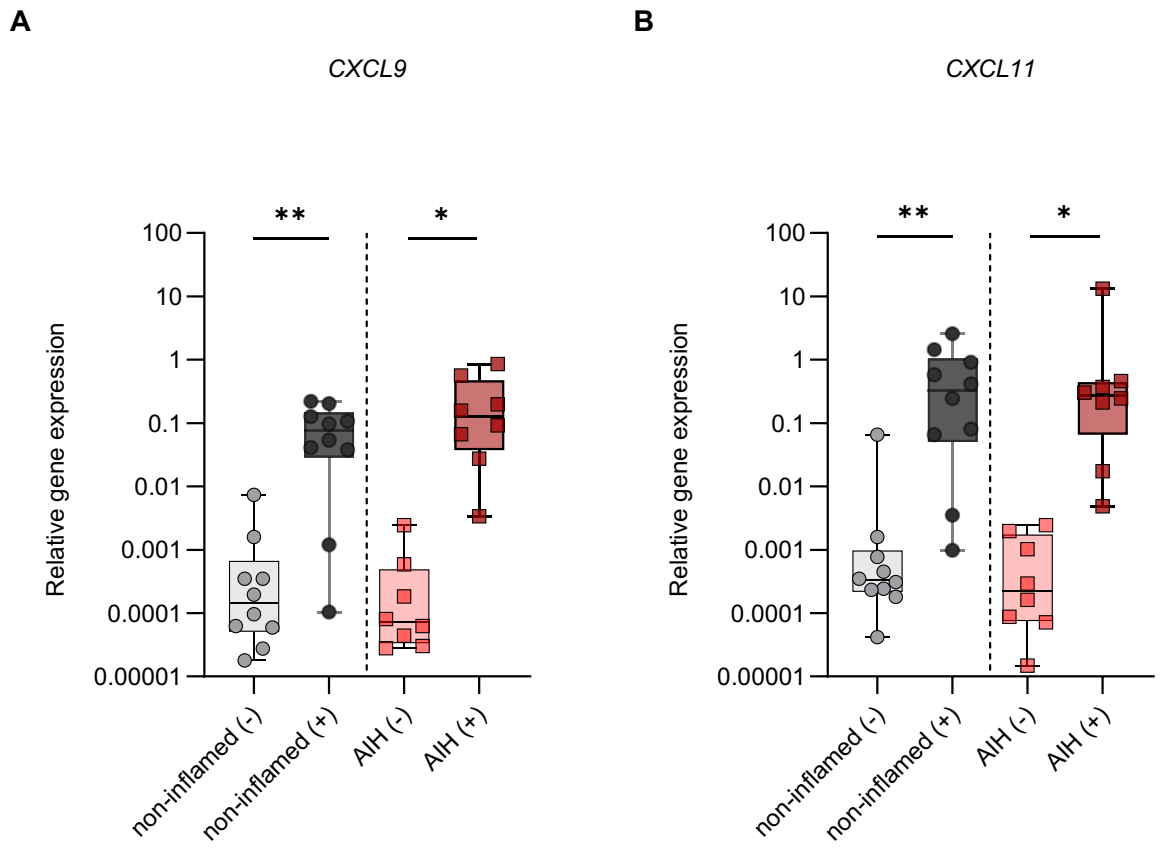

Figure 24: MRNA levels of chemokines CXCL9 and CXCL11 upon 24h poly I:C stimulation in non-inflamed human liver derived organoids and AIH-derived human liver organoids.

*Quantitative RT-PCR analysis showing mRNA expression of **A)** CXCL9 and **B)** CXCL11 in non-inflamed liver organoids and AIH-derived liver organoids in non-stimulated (-) and stimulated (+) conditions. All samples were tested in duplicates and were normalized to GAPDH. Non-inflamed samples n=10 and AIH affected samples n=8. A Mann-Whitney test was used for comparison data of chemokine expression between non-inflamed liver organoids and AIH-derived liver organoids upon stimulation with poly I:C for*

*24h. Wilcoxon matched-pairs signed rank tests were used to compare unstimulated with stimulated condition within one group. Only significant p values are shown. *, P <0.05; **, P <0.01.*

Upon TLR3 stimulation, both *CXCL9* and *CXCL11* showed significant increased relative gene expression levels in comparison to unstimulated conditions within each groups.

In detail, organoids from non-inflamed livers showed a more significant increase of *CXCL9* (median expression of 0.0002 at baseline vs. 0.0755 in stimulated conditions) and *CXCL11* (median expression of 0.0003 in unstimulated conditions and median expression of 0.3302 upon stimulation) contrary to AIH-derived human liver organoids upon stimulation. Both chemokines were also significant increased in AIH-derived human liver organoids upon stimulation with poly I:C. Here, *CXCL9* increased from 0.0001 at baseline level to a median of 0.1247 in stimulated conditions, whereas for *CXLC11*, the expression increased from a median of 0.0002 to 0.2743 (Figure 24).

However, when comparing non-inflamed liver organoids with AIH-derived liver organoids upon TLR3 stimulation, no significant differences were observed in none of the tested chemokines.

In stimulated conditions, in organoids from AIH affected livers, the median gene expression of *CXCL9* was 0.12 compared to a median gene expression of 0.08 in organoids from non-inflamed livers. The relative gene expression in *CXCL11* upon TLR3 stimulation showed a median expression of 0.27 in liver organoids derived from AIH affected individuals compared to 0.33 in non-inflamed control organoids.

Next, protein levels (intensity of fluorescence) of the cytokines and chemokines of interest were measured in the supernatant of liver organoid cultures by Luminex assay upon 7 days of poly I:C stimulation (Figure 25).

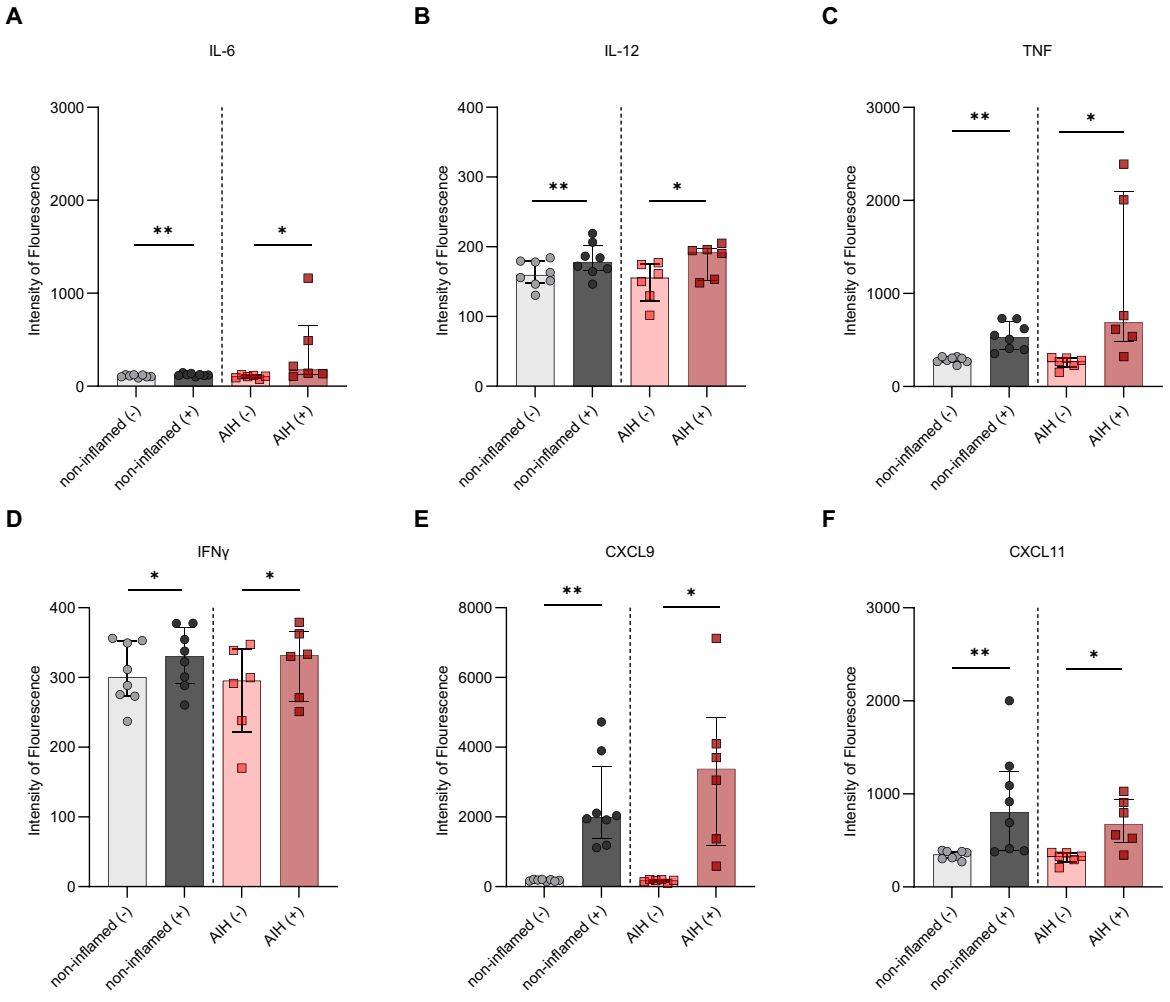

Figure 25: MFI of IL-6, IL-12, TNF, IFNγ, CXCL9, and CXCL11 in the supernatant of non-inflamed liver organoids and AIH-derived organoids 7 days pre- and post stimulation measured by Luminex assay.

*After the harvest of the supernatant, the MFI of A) IL-6 B) IL-12 C) TNF, D) IFNγ, E) CXCL9, and F) CXCL11 of non-inflamed liver organoids and AIH-derived liver organoids in non-stimulated (-) and stimulated (+) conditions was measured. The samples were running next to the provided standard of the kit in duplicates. Afterwards the mean of the duplicates was calculated and used for the analysis. For A-E except B non-inflamed samples n=8 and AIH samples n=6. For B non-inflamed samples n=7 and AIH samples n=5. For comparison of differences in cytokine and chemokine protein production in non-inflamed individuals and AIH affected individuals upon stimulation, Mann-Whitney test was performed. Only significant p values are shown. *, P <0.05; **, P <0.01.*

Significant enhanced concentration of the MFIs were observed for all tested cytokines and chemokines upon TLR3 stimulation. This was observed in organoids from non-inflamed livers and also in organoids derived from AIH affected livers, when comparing with baseline levels. The strongest effect was observed in CXCL9 in organoids derived from AIH livers, when stimulating with poly I:C (MFI of 180 at baseline compared to median of 3382 in stimulated

conditions). In line with that, similar observations were made in the same chemokine in organoids from non-inflamed livers.

Although not significant, IL-6 showed a trend towards higher protein concentration displayed by higher MFI in organoids derived from AIH affected livers (MFI of 180) compared to non-inflamed organoids (MFI of 120). Similarly, CXCL9 showed a trend towards higher levels (MFI of 3382) in organoids from AIH affected livers vs. in organoids from non-inflamed livers (MFI of 1994). Similar MFI concentrations were measured for IL-12, IFNγ and CXCL11 in both groups of organoids upon stimulation. This mirrors the mRNA expression patterns as described above.

Taking together, upon TLR3 activation by poly I:C, an increase in mRNA expression of IL-6 compared to non-inflamed organoids was observed. Furthermore, on protein level, as measured by concentration displayed by the MFI, IL-6, and CXCL9 exhibited a tendency towards higher expression levels in liver organoids derived from AIH affected livers compared to organoids from non-inflamed livers. These results could indicate that the organoids may imprint the inflammatory environment from which they were extracted by maintaining their environment *in vitro.*

4.3 Characterization of the crosstalk between human liver organoids and immune cells

4.3.1 Enhanced concentrations of TNF and IFNγ decrease hepatocyte regeneration

These studies described above showed that chemokines and cytokines that attract and activate $CD4^+$ T cells were produced by hepatocytes in human liver organoids and that there was a trend towards increased levels in AIH affected human liver organoids. These studies may contribute to observations by other groups, showing that $CD4^+$ T cells that produce TNF and IFNγ are elevated in AIH affected livers (Tiegs & Horst, 2022). Both of these pro-inflammatory cytokines show cytotoxic features that can trigger hepatocyte death (Horras et al., 2011).

To investigate the impact of a T cell mediated immune responses through TNF and IFNγ on hepatocyte organoid regeneration, a long-term dose-dependent stimulation on non-inflamed control organoids and AIH-derived liver organoids was performed. To this end, liver single cells were seeded and stimulated with two different concentrations of TNF (5 ng/ml and 500 ng/ml) or IFNγ (50 U/ml and 5000 U/ml) for a total of 14 days. As control, single cells from both groups were seeded without adding cytokines to the culture. Growth kinetics, including total number

and average size, were evaluated using AI based organoid detection algorithm developed within our lab (Bremer et al., 2022) (Figure 26).

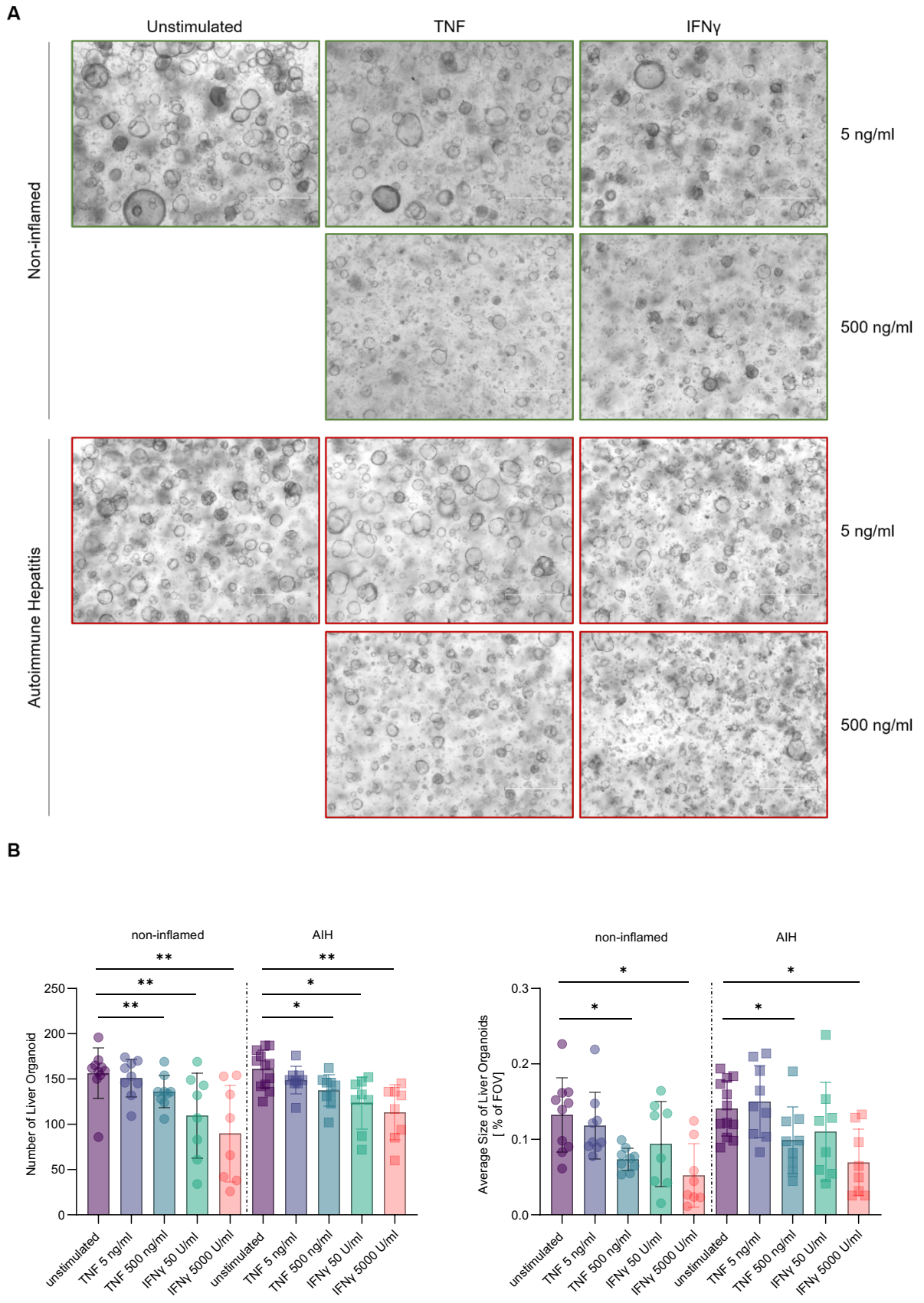

Figure 26: Growth kinetics of organoids from non-inflamed livers and AIH-derived liver organoids upon stimulation with TNF and IFNγ.

A) *Bright field microscopy images showing organoids from non-inflamed livers and AIH-derived liver organoids at passage 3 (P3). The liver organoids were either unstimulated or stimulated with dose-dependent concentrations of TNF or IFNγ. Images were taken using a 4X objective (scale bar = 750 µm). Images were recorded with an Evos M5000 microscope.* ***B)*** *Bar chart plots showing the number of human liver organoids (left) and the average size of liver organoids (right) from AIH-derived livers and non-inflamed livers upon stimulation with 5 ng/ml and 500 ng/ml of TNF or 50 U/ml and 5000 U/ml IFNγ. Analysis was performed by using GOAT. Data was analyzed by comparing stimulated samples to unstimulated samples within the own group in either non-inflamed liver organoids or AIH-derived liver organoids. For this, Wilcoxon matched-pairs signed rank test was performed. Mann-Whitney test was performed to compare differences between non-inflamed control individuals and AIH affected individuals. *P <0.05, ** P <0.01.*

During the 14 days of culturing, the organoids underwent morphological alterations. In unstimulated conditions, the organoids of AIH affected individuals exhibited a more cystic shape than organoids derived from non-inflammatory liver tissue.

Low dose of TNF (5 ng/ml) did not affect the growth the organoids, as no differences neither in organoid number nor in size of liver organoids from non-inflamed livers and AIH-derived livers were observed. However, higher doses of TNF (500 ng/ml) reduced the number of organoids significantly in cultures of liver organoids from non-inflamed livers. This effect was also observed in liver organoids derived from AIH affected individuals. Correspondingly, a trend towards a reduced number of organoids was detected. In line with these observations, organoids were significant smaller when cultured in higher doses of TNF (Figure 26 A and B).

In contrast to TNF, IFNγ demonstrated an effect on organoid growth and numbers in low (50 U/ml) and high (5000 U/ml) doses, leading to a reduced number of liver organoids from non-inflamed livers and AIH affected livers. The strongest effect was observed when stimulating with high IFNγ doses, displaying a significant reduction of organoid size of liver organoids from non-inflamed livers and AIH affected livers (Figure 26 A and B).

4.3.2 Examination of the effect of TNF and IFNγ on the metabolic hepatocyte lineage marker cytochrome P450 3A4

Based on the dose-dependent effects of TNF and IFNγ on regeneration of hepatocytes (Figure 26), the expression of factors relevant to hepatocyte metabolic functionality, such as expression of metabolic marker Cyp3A4, may also be affected. Therefore, Cyp3a4 mRNA levels in liver organoids were measured upon TNF and IFNγ stimulation. Here, the fold change of *CYP3A4* expression was calculated. The gene expression was normalized to the housekeeping gene *GAPDH* and to the average of the corresponding unstimulated controls (Figure 27).

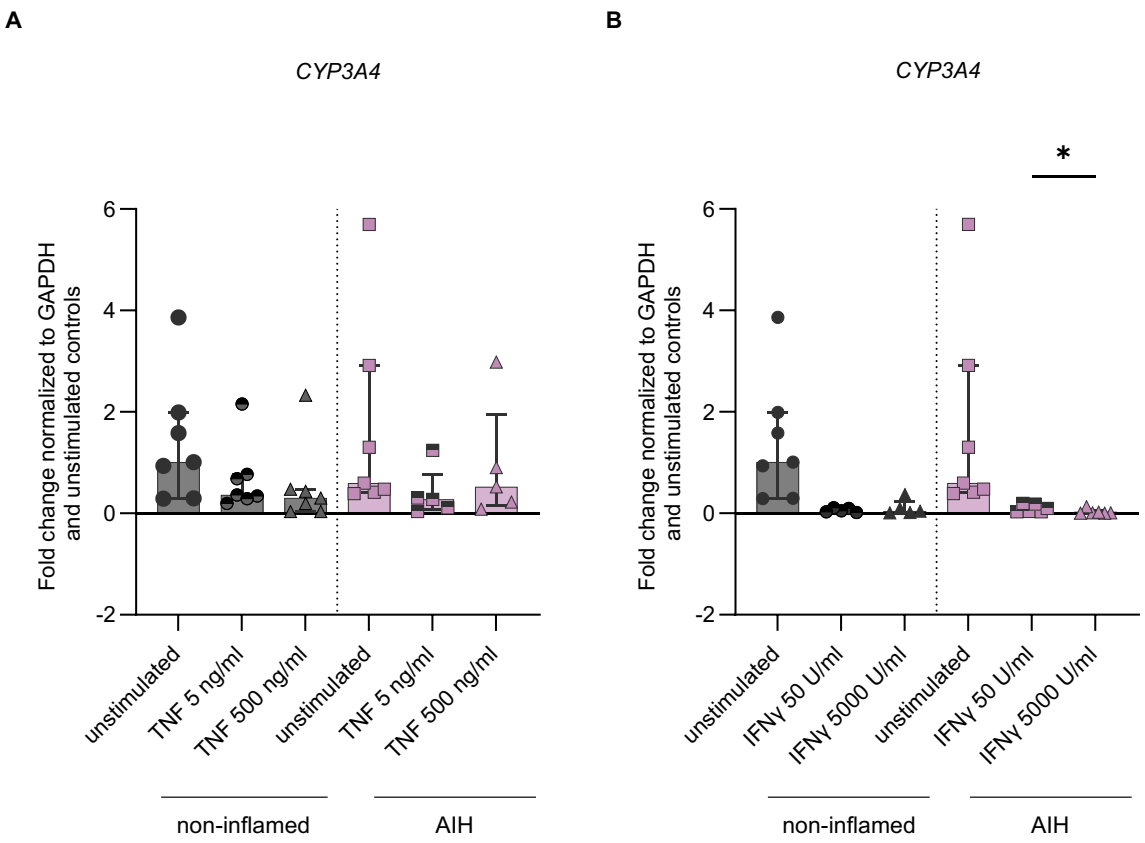

Figure 27: MRNA levels of Cyp3A4 upon long-term stimulation with TNF and IFNγ by human liver organoids.

Quantitative RT-PCR analysis of CYP3A4 post 14 day dose-dependent TNF and IFNγ stimulation of liver organoids from non-inflamed livers and AIH affected livers. ***A)*** *CYP3A4 mRNA expression representing bar chart dot plots in unstimulated conditions and upon dose dependent TNF stimulation.* ***B)*** *CYP3A4 mRNA expression showing bar chart dot plots in unstimulated conditions and upon dose-dependent IFNγ stimulation. All samples were tested in duplicates. The median value of both duplicates was normalized to the housekeeping gene GAPDH and to the average of the corresponding unstimulated controls. Liver organoids from non-inflamed livers n=7 in unstimulated conditions and stimulated with TNF, and n=5 for IFNγ stimulated conditions. For liver organoids from AIH affected livers, unstimulated n=7, n=5 for TNF stimulations; n=6 for IFNγ stimulated conditions. Mann-Whitney test was applied to compare CYP3A4 expression between organoids from non-inflamed livers and organoids from AIH affected livers, while Wilcoxon matched-pairs signed rank test was used to compare TNF or IFNy expression within one group meaning either in non-inflamed organoids or AIH affected organoids. Only significant p values are shown. *P <0.05.*

Neither low nor high doses of TNF stimulation affected significantly *CYP3A4* expression by liver organoids, in both groups. However, low doses of TNF (5 ng/ml) seem to slightly reduce *CYP3A4* expression in liver organoids from AIH affected livers (median gene expression: 0.36 in liver organoids from non-inflamed livers and median gene expression of 0.28 in organoids from AIH affected livers). However, when stimulating with low and high doses of IFNγ, a strong decrease of *CYP3A4* expression in liver organoids in both groups was observed in comparison

to TNF stimulation. The strongest downregulation of the hepatocyte lineage marker *CYP3A4* was detected in liver organoids from AIH affected individuals, when stimulating with high doses of IFNγ. Here, a significant decrease was observed compared to a stimulation with a low dose of IFNγ. High doses of IFNγ seemed to have an even stronger effect in liver organoids from AIH affected livers in comparison to organoids from non-inflamed livers (median expression in liver organoids from non-inflamed livers 0.04 vs. median expression of 0.009 in liver organoids from AIH affected livers). These results matched the previous observations from microscopy pictures where TNF and IFNγ reduced organoid numbers and growth (Figure 26).

To sum up, as observed in microscopy images and validated via RT-PCR analysis, TNF and IFNγ have a cytotoxic effect on hepatocytes. They can suppress hepatocyte regeneration. Additionally, especially IFNγ is capable to downregulate CYP3A4 expression, thereby potentially inhibiting hepatocyte function.

4.3.3 Establishing a human liver organoid co-culture with $CD4^+$ T cells

Given that human liver organoids may provide a new model to represent the human liver hepatocytes, these 3D structures offer the opportunity to examine the crosstalk between immune cells and hepatocytes. Our findings above demonstrated that high doses of TNF and IFNγ can suppress hepatocyte growth and additionally affect hepatocyte proliferation.

To investigate the effect of $CD4^+$ T cells on human liver organoids, co-cultures of acute AIH $CD4^+$ tissue resident memory T cells type 1 (TRM1) with human liver organoids were established. This very novel method was performed in collaboration with the group of Nicola Gagliani. The TRM1 T cells were obtained from liver biopsies from AIH affected individuals. In his group, the AIH-derived TRM1 T cells were isolated, expanded, and cultured.

For the co-culture with the AIH-derived TRM1 cells, the human liver organoid culture medium was supplemented with either human TNF antibody alone or in combination with human IFNγ antibody for 10 days to block the corresponding signaling pathways. Organoids without any supplements and without TMR1 cells were used as control. TRM1 cells were seeded outside the BME to avoid allo-reactions.

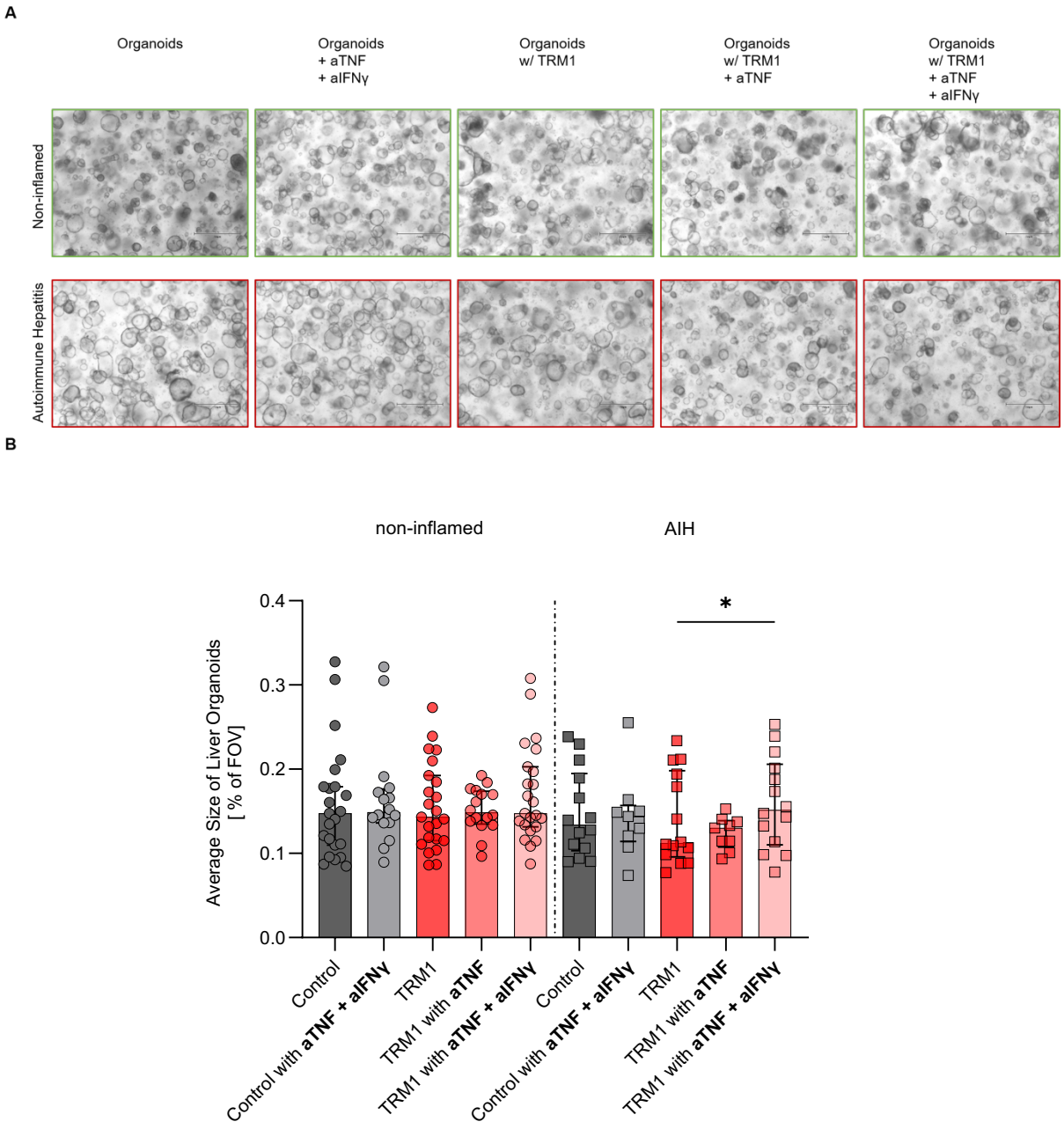

Figure 28: Growth kinetics of liver organoids from non-inflamed livers and from AIH affected livers upon co-culture with AIH derived CD4⁺ TRM1 T cells.

A) *Bright field microscopy images showing liver organoids from non-inflamed livers and organoids from AIH affected livers after 10 days of co-culturing with TRM1 T cells obtained from biopsies from individuals affected with AIH. Organoids were supplemented either with 0.5 µg/ml of TNF RI/TNFRSF1A antibody alone or with 1 µg/ml human IFNγ antibody combined with 0.5 µg/ml of TNF RI/TNFRSF1A antibody. Organoids without any supplements and without TRM1 T cells were used as control. Images were taken at the same time when medium change was performed to document morphology alterations. Bright field images were taken via microscope Evos M5000 by applying a 4X objective (scale bar = 750µm).* ***B)*** *Bar chart plots showing the average size of organoids after 10 days of co-culture. Organoid images were analyzed by using GOAT. Wilcoxon matched-pair signed rank test was used to compare the effect of TRM1 T cells in size of the organoids to the corresponding control. To determine the effect*

*of TRM1 T cells after the usage of the antibodies to block TNF and IFNγ signaling within the non-inflamed individuals and AIH affected individuals, paired t tests were performed. Only significant p values are shown. *, P <0.05.*

Morphological changes were documented via bright field microscopy every 2-3 days in combination with a medium change. Upon 10 days of co-culturing, morphological alterations into more lucid and rounder organoids were observed, when supplementing the organoids with TMR1 T cells (Figure 28 A).

In addition to morphological alterations, the impact of TMR1 T cells and their effect on human liver organoids was analysed by examining the size of the organoids. The resulting effect was verified through bright field microscopy and was also confirmed via GOAT analysis (Figure 28 B).

After 10 days of co-culturing, a reduction in organoid size was observed, especially in organoids from AIH affected livers. When comparing the control organoids with the various applied TRM1 T cell conditions, no significant differences in size in non-inflamed control individuals were noticeable. The same observations were made in liver organoids from AIH affected livers. However, significant differences in the size were seen in organoids generated from livers affected with AIH supplemented with TMR1 T cells (median size of 0.11) and TRM1 T cells, where TNF and IFNγ signalling pathways were blocked (median size of 0.15), indicating that TNF and IFNγ together might had an influence on the hepatocyte size.

Interestingly, these results suggest that the immune response of AIH TRM1 T cells had a negative effect on AIH-derived liver organoids.

As a final step, to further validate the impact of T cell mediated inflammatory immune responses exerted by AIH TRM1 T cells on human hepatocytes, lactate dehydrogenase (LDH) assay was performed. LDH is usually intracellularly localized and released upon plasma membrane damage. Therefore LDH can serve as a surrogate marker for cytotoxicity. The LDH release in human liver organoid culture medium was determined upon 10 days of TRM1 T cell co-culture (Figure 29).

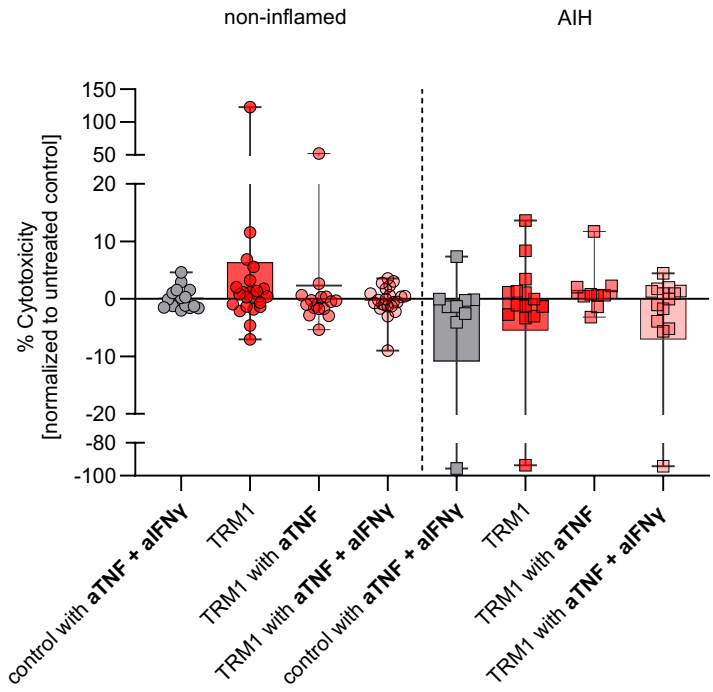

Figure 29: LDH cytotoxicity assay while co-culturing of human liver organoids with TRM1 T cells. *Bar chart plots showing percentage of cytotoxicity (as levels of released LDH) after 10 days of co-culture of human liver organoids combined with AIH TRM1 T cells. Organoids without TRM1 T cells plus any further supplements served as controls. The samples were performed in duplicates and the mean of the out coming values were normalized to untreated organoids. Non-inflamed organoid livers control with TNF and IFNγ and organoids from non-inflamed livers with TMR1 and TNF n=16; non-inflamed human organoids with TMR1, non-inflamed derived organoids with TMR1 plus TNF and IFNγ n=22. AIH-derived organoid control with TNF and IFNγ and AIH-derived organoids with TRM1 and TNF n= 9, AIH-derived organoids with TRM1 and AIH-derived organoids with TRM1, TNF and IFNγ n=14. Mann-Whitney test was applied to compare cytotoxicity between organoids from non-inflamed livers and organoids derived from AIH affected livers, while Wilcoxon matched-pair signed rank test was used to compare the effect of TMR1 T cell immune response to human liver organoids within either non-inflamed individuals or individuals affected with AIH.*

No significant changes were observed after co-culture of non-inflamed or AIH derived liver organoids with TRM1 T cells compared to organoids alone or TNF/IFNγ blocking conditions. The highest cytotoxicity was observed in non-inflamed control organoids exposed to TRM1 T cells by showing a mean value of 6.36%. However, in non-inflamed control organoids co-cultures with TRM1 T cells supplemented with human TNF antibody to block this signaling, a slight decrease of LDH release (mean LDH release: 2.3%) was seen contrary to the organoids supplemented with TRM1 T cells alone (mean LDH release of 6.36%). These observations may suggest that both inflammatory cytokines contribute to cell death in the non-inflamed control organoids.

However, in liver organoids generated from AIH affected individuals, the LDH assay of TRM1 T cells supplemented with TNF antibody showed an increased cytotoxicity mean value of 1.6%, underlining the negative impact of IFNγ to AIH-derived human liver organoids.

Taken together, these data showed that AIH TRM1 T cells have a predominantly negative effect on human liver organoids by attacking them through their initiated pro-inflammatory immune response.

5 Discussion

The overall objective of this thesis was to investigate dysregulated pathways that contribute to the breach of hepatic immune tolerance resulting in AIH. Therefore, I studied mechanisms that contribute to the pathogenesis of AIH and specifically investigated the role of TLRs in hepatocytes and T cells of AIH affected individuals and their associated pro-inflammatory responses upon stimulation. To investigate the dysregulation of hepatocytes in AIH, human liver organoids were generated either from non-inflamed livers or from AIH affected livers after liver explantation. Upon successful organoid generation and characterization, the organoids were stimulated with TLR3 agonist poly I:C, mimicking a virus stimulus, as viral infections are associated and assumed to be a potential trigger of AIH. Next, the potential pro-inflammatory cytokine responses in AIH-derived organoids upon stimulation with poly I:C were analysed.

Furthermore, this thesis focused on investigating the role of both inflammatory cytokines TNF and IFNγ on hepatocyte regeneration, and overall viability, since TNF and IFNγ producing T cells are elevated during AIH pathogenesis. As a first step, long-term stimulations with both individual pro-inflammatory cytokines were carried out in order to analyse their specific impact on the human liver organoids. Both cytokines had a negative impact and impaired hepatocyte proliferation and liver organoid growth.
Finally, a complex $CD4^+$ T cell hepatocyte organoids co-culture system was developed to study the role of TRM1 cells, which were detected within AIH affected livers. These new 3D models allowed to assess specific effects of T cells on hepatocytes and supported a role of TNF and IFNγ by TRM1 cells in hepatocyte dysregulation, potentially contributing to AIH pathogenesis.

5.1 Generation and characterization of human liver organoids

Liver diseases cause around 2 million deaths each year (Asrani et al., 2019). To date, there are several models to study liver diseases, including culturing of primary human hepatocytes (PHHs) and mouse models. Both are helpful to examine pathogeneses of liver diseases but have different limitations (Nuciforo & Heim, 2020; Telles-Silva et al., 2022; X. Yin et al., 2016). PHHs undergo morphological changes, loss of function or cell death within a short time of *in vitro* culture, while mouse models have a different genetic background (Q. Liu et al., 2022; Xia et al., 2017; Xiang et al., 2019; X. Yin et al., 2016; X. Zhu et al., 2021). As both PHHs and animal models may have their drawbacks to study liver diseases, such as AIH, (Christen, 2019; Liberal, Krawitt, et al., 2016; Terziroli Beretta-Piccoli et al., 2022; Xia et al., 2017; Xiang et al., 2019; X. Zhu et al., 2021), a 3D liver model was used in this thesis, which is based on human-derived tissue.

In this work, together with colleagues from the University Hospital Hamburg-Eppendorf (UKE), we established 3D human liver organoids that represent a new model system to investigate the pathogeneses and therapies of various liver diseases and allow co-culture with human immune cells such as $CD4^+$ T cells (Y. Chen et al., 2023; Huch et al., 2015; Lancaster & Knoblich, 2014; Sun & Hui, 2020; X. Zhu et al., 2021). Liver organoids were based on epithelial cells, isolated from resected liver tissue of non-metastatic areas of metastases affected livers and liver tissue from individuals diagnosed with AIH. After isolation of epithelial cells, liver organoids were grown into 3D systems to recapitulate the liver *in vitro*.

AIH is a rare and poorly understood autoimmune liver disease (Dalekos et al., 2022; Manns, 2011). Therefore, the usage of human liver organoids is of importance, as organoids can retain the donor-specific characteristics *in vitro*, such as the genetic and epigenetic background potentially associated with the initiation of AIH (Dotti & Salas, 2018; Shin et al., 2020), whereas other models fail to do so.

Consistent with previous findings, liver organoids generated from liver derived epithelial cells, including hepatocyte-like and cholangiocyte-like phenotypes, could be cultured for long time periods, ranging from weeks up to months (Hu et al., 2018; Huch et al., 2015). These observations agree with findings presented from Huch *et al*. as well as from Hu and colleagues (Hu et al., 2018; Huch et al., 2015). Next to the long-term cultivation, both groups demonstrated structural and chromosomal stability of the organoids throughout the time of culturing (Hu et al., 2018; Huch et al., 2015). These results were reflected in mouse organoids (Huch et al., 2013).

To substantiate the use of liver organoids in studies of liver diseases, we further characterized their hepatocyte phenotype. Therefore, mRNA expression levels of the hepatocyte lineage markers *albumin* and *Cyp3a4* were quantified. Both proteins are identified to execute essential functions in human hepatocytes. Albumin acts as transporter of bilirubin and fatty acids (Paar et al., 2021; Sun et al., 2019), while Cyp3A4 is identified as enzyme involved in drug metabolism (M. Zhao et al., 2021). Lee *et al.* described mRNA expression of both *albumin* and *Cyp3a4* in liver organoids on mRNA level. Consistent with these findings, investigating liver organoids, our generated human liver organoids derived from non-inflamed tissues and organoids from AIH affected livers did show mRNA expression of hepatocyte lineage markers *albumin* and *Cyp3a4* (J.-Y. Lee et al., 2020). Although higher compared to PBMCs, the relative gene expression of the human liver organoids was relatively low in both organoids from non-inflamed tissues and in organoids from AIH-derived livers in comparison to previous studies (Figure 13) (Huch et al., 2015; J.-Y. Lee et al., 2020). With regards to the liver organoids generated from explants, it is well established that individuals who undergo liver transplantation have livers that are not functioning efficiently anymore, which may affect the

liver organoid phenotype. As seen in Figure 13, the *ALBUMIN* expression in AIH-affected livers was nearly equal to organoids from non-inflamed livers. The differences in mRNA expression level from our study compared to *Huch et al*. and Lee *et al.* could be attributed to the possibility that our generated organoids were either no longer capable of expressing these genes in high quantities due to AIH or due to differences in protocols in culturing for both liver organoids from non-inflamed tissues and AIH-derived human livers (Huch et al., 2015; J.-Y. Lee et al., 2020). In addition, in some experiments, Huch *et al.* used $EpCam^+$-sorted cells before culturing. The use of $EpCam^+$ cells results in a different starting material, which could also be a potential indicator for the different hepatocyte marker expression (Huch et al., 2015). However, especially the protocol differences may indicate the weaker mRNA expression in our human liver organoids compared to previous studies (Hu et al., 2018; Huch et al., 2015). Contrary to Huch *et al.* and Lee *et al*., showing higher mRNA levels of both markers in liver organoids, we did not use expansion medium (EM) and differentiation medium (DM) for culturing (Huch et al., 2015; J.-Y. Lee et al., 2020). Huch *et al.* demonstrated, when using EM, in early and late passages, the organoids failed to express both hepatocyte markers *albumin* and *Cyp3a4* expression. However, when switching to DM, both markers were expressed on mRNA and protein level (Huch et al., 2015). This may lead to speculate that the growth factors included in the human liver culture medium might be critical part, showing low mRNA expressions. However, further experiments are required to confirm this.

Moreover, our findings in protein levels of the hepatocyte marker albumin assessed via immunofluorescence staining are in line with previous published observations (Hu et al., 2018; Huch et al., 2015), showing expression of albumin in organoids derived from non-inflamed livers. Contrary to non-AIH liver organoids, however, organoids generated from AIH affected livers showed nearly no albumin production via IF staining (Figure 16). Several reports demonstrated that individuals who developed liver cirrhosis or are affected with liver inflammation, showed impaired hepatocellular functions and additionally a decrease in albumin secretion (Carvalho & Machado, 2019; A. N. Friedman & Fadem, 2010; Moshage et al., 1987). As AIH is an inflammatory liver disease, liver inflammation is a typical characteristic of AIH (Terziroli Beretta-Piccoli et al., 2022). Due to end-stadium liver transplant of AIH-affected livers, the lack of albumin visualisation in organoids derived from AIH affected livers could indicate liver function loss. Although not visible in organoids from AIH affected livers in immunofluorescence, the results obtained via ELISA indicated although low amounts of albumin production in the human liver organoid culture medium of organoids derived from AIH affected livers. Here, similar protein secretion of albumin in both organoids from non-inflamed livers and organoids derived from AIH affected livers were observed (Figure 15). These observations raise the question why this protein is almost not visualized in AIH-derived organoids, but detectable as nearly in the same concentration as in organoids from non-AIH

livers. However as only few organoids could be visualized this may be due to the numbers of organoids of AIH affected livers assessed. Distinct antibody clones for albumin visualization or protocol optimization will be necessary to improve albumin detection in immunofluorescence.

Furthermore, next to hepatocyte markers, we additionally measured gene expression levels of *LGR-5* and *EpCam,* which are known to be markers for stem cells and cholangiocytes, respectively, to identify stem cell potential and/or dedifferentiated states of the hepatocytes (Hu et al., 2018; Huch & Dollé, 2016). The human liver organoids expressed both markers on mRNA level, however at relatively low levels. The expression of the markers is in line with the propagation of liver cells in organoids systems as to maintain a stem cell state plays a crucial role in regeneration (Huch & Dollé, 2016). Furthermore as specifically for AIH, these human liver organoids were generated from explant liver tissue, it could be that the stem cell state was activated through liver tissue damage (Huch & Dollé, 2016). Moreover, our results indicated that the human liver organoids can also dedifferentiate into a more cholangiocyte-like phenotype, as they expressed *Epcam* on mRNA level, which is also known as cholangiocyte marker (Hu et al., 2018).

The examination of morphology of the human liver organoids using bright field microscopy showed heterogeneity in the organoid morphology in both groups (Figure 12). Interestingly, we observed that organoids from AIH affected individuals showed an altered phenotype compared to organoids from non-inflamed livers, showing a more cystic phenotype. These findings suggested a dedifferentiated state of liver epithelial cells to generate liver tissue in AIH individuals. This can result in liver fibrosis that is able to progress to liver cirrhosis due to liver inflammation. Those are typical events in autoimmune hepatitis (Jindal et al., 2022; M. Tanaka & Miyajima, 2016). However, for formation of the organoids, the environment of the cultured epithelial cells is a crucial factor (Rossi et al., 2018; X. Zhu et al., 2021). It is important to mention, that two of the components of the human organoid liver culture medium comprise factors that are relevant for hepatocyte proliferation: hepatocyte growth factor (HGF) and the protein R-spondin. R-spondin acts as agonist of the signaling pathway Wnt (Carmon et al., 2011; de Lau et al., 2011; X. Zhu et al., 2021), which is an important pathway in hepatocyte proliferation. Also HFG is known to be associated with hepatocyte proliferation (Hu et al., 2018; Ober & Lemaigre, 2018; Planas-Paz et al., 2016; Prior et al., 2019; B. Wang et al., 2015; X. Zhu et al., 2021). The presence of two crucial components in the human liver organoid culture medium, necessary for hepatocyte proliferation, suggest a stable long-term cultivation of the organoids. The long term cultivation of hepatocytes was also demonstrated by *Guo et al.*(R. Guo et al., 2022). Furthermore, they demonstrated that hepatocyte are capable to dedifferentiate, when adding IL-6 to their hepatocyte culture medium, as IL-6 is identified to be involved in liver regeneration (R. Guo et al., 2022; Michalopoulos & Bhushan, 2021;

Michalopoulos & DeFrances, 1997; Taub, 2004). These finding support our observations by showing differentiated phenotype of organoids from AIH affected livers.

In summary, our findings on mRNA and protein expression indicate that human liver organoids retain a hepatocyte phenotype to a certain extent, but also dedifferentiate into more cholangiocyte-like organoids *in vitro*. Additionally, non-inflamed and AIH derived liver organoids differ morphologically, potentially recapitulating the specific phenotype of their corresponding tissue that they were obtained from.

5.2 Toll like receptor expression in human liver organoids

Toll like receptors (TLRs) are expressed by various cell types within the body. These pattern recognition receptors are necessary for the immune system to maintain homeostasis and to eliminate pathogens by mediating an immune response (Kawai & Akira, 2010; Latorre et al., 2014; Teixeira et al., 2020). However, in case of loss of immune tolerance, there is evidence that dysregulations in TLR signaling lead to initiation of autoimmunity (Farooq et al., 2021; Mohammad Hosseini et al., 2015) and is also associated with the pathogenesis of various liver diseases (Broering et al., 2011; Kesar & Odin, 2014; Kiziltas, 2016).

Literature from Kesar and Odin, and several other reviews, demonstrated that hepatocytes express all ten TLRs (Aderem & Ulevitch, 2000; Akira & Takeda, 2004; Babu et al., 2006; B. A. Beutler, 2009; Botos et al., 2011; Kesar & Odin, 2014; Kumar et al., 2009; O'Neill, 2008; Pradere et al., 2010). Based on these observations, we assessed if human liver organoids express various TLRs. Especially TLR3, TLR4 and TLR5 were of interest in this thesis, as these three receptors have been associated with autoimmune liver diseases, including PSC, PBC and AIH (Honda et al., 2007; Kikuchi et al., 2005; Mao et al., 2005; Seki & Brenner, 2008; Takii et al., 2005; A.-P. Wang et al., 2005). Therefore, mRNA levels of TLR3, TLR4 and TLR5 in human liver organoids were quantified.

Here, we showed that liver organoids, derived from non-inflamed livers and from AIH affected livers, express low mRNA levels of TLR3, TLR4 and TLR5 (Figure 17). Human liver organoids generated from non-AIH livers showed similar mRNA expression pattern of TLR3, TLR4 and TLR5 as organoids derived from AIH-affected livers, suggesting that baseline levels of TLRs do not differ in the specific disease state.

Zhu *et al*, described that autoimmune hepatitis is a multifactorial disorder with unknown aetiology (J. Y. Zhu & Han, 2015). However, environmental triggers, including viral infection seem to play an important role in AIH (Floreani et al., 2018; Sirbe et al., 2021). Lang and colleagues presented evidences that viruses are associated with AIH (Lang et al., 2006). In 2006, Lang *et al.* demonstrated that a viral infection with choriomeningitis virus caused the activation of TLR3 via APCs in mice. This activation subsequently initiated the pathogenesis

of AIH (Lang et al., 2006). In general, little is known about TLR3 in the liver (Y.-S. Lee et al., 2018). Especially studies about TLR signaling in organoids are still missing. However, analyses of TLR3 have been shown that this receptor is associated with liver inflammation (Xiao et al., 2009). Based on growing evidence that TLR3 might be associated with AIH, we hypothesised that this receptor could be a potential trigger to initiate pathogenesis of AIH.

To further assess the role of TLR3 in inflammation in AIH, and to build on previous studies (Lang et al., 2006), TLR3 expression was examined under activated conditions. This was performed to investigate whether activation of this receptor might further amplify dysregulation of signaling pathways in AIH. Upon activation of TLR3, this receptor tended to be higher expressed on mRNA level in AIH affected individuals, however significant differences were not observed, in line with findings from Lang and colleagues (Lang et al., 2006). One could speculate that the lack of significance is due to the low number of AIH donors.

However, the presented trend of the TLR3 upregulation upon stimulation contrary to non-inflamed control may indicate a potential epigenetic memory of the organoids derived from AIH affected livers. Baumdick *et al.* had previously shown that organoids of ulcerative colitis (UC) affected intestinal organoids upon re-stimulation with pro-inflammatory cytokine IFNγ displayed a stronger upregulation of HLA class II molecules compared to healthy intestinal organoids, indicating a type of memory from the inflammatory environment they derived from (Baumdick et al., 2023).

In addition, the expression levels of TLR4 and TLR5 upon stimulation with their respective ligands, LPS and flagellin were determined (Schwabe et al., 2006), as other pathogenic stimuli may contribute to the development of AIH. In contrast to TLR3, an altered TLR4 and TLR5 mRNA expression in liver organoids generated from AIH affected livers compared to the non-inflamed group upon their corresponding stimulus was not observed (data not shown).

From the literature, it is known that some TLRs mediate each other's expression, when recognizing their respective ligand. Although TLR4 is associated with the recognition of LPS, it was shown that TLR2 and TLR9 are also upregulated upon LPS binding (An et al., 2002; Y. Liu et al., 2001). However, the expression of TLR4 and TLR5 compared to non-activated conditions or after poly I:C stimulation was not altered. These result indicate that TLR3 signaling in hepatocytes does not affect the expression of other TLRs. TLR3 is known to use the downstream MyD88-independent signaling pathway, whereas other TLRs use the MyD88-dependent pathway (Kesar & Odin, 2014), potentially explaining the lack of upregulation of other TLRs upon TLR3 stimulation (Figure 22). However, it should be noted that even though there was no increase in TLR4 and TLR5 expression after stimulation, this does not exclude an involvement of both receptors in the pathogenesis of AIH, especially in other cells such as macrophages.

In summary, using RT-PCR analysis of liver organoids, we were able to validate that in non-activated conditions, mRNA expression levels of *TLR3*, *TLR4* and *TLR5* were relatively low (Figure 17). Whereas in poly I:C stimulated conditions, TLR3 tended to show a higher expression in liver organoids generated from individuals affected with AIH (Figure 22). These findings underline that a dysregulation of TLR3 signaling in individuals affected with AIH may contribute to the liver inflammation and liver fibrosis observed in the pathogenesis of AIH. Based on these findings, we decided to exclude TLR4 and TLR5 for further analysis and focus on TLR3.

5.3 Inflammatory cytokine immune responses in human liver organoids upon TLR3 stimulation via poly I:C

Activation of TLR3 via poly I:C initiates expression of type I interferon and pro-inflammatory cytokines such as IL-6, IL-12 and TNF (Alexopoulou et al., 2001; Xagorari & Chlichlia, 2008). However, under homeostatic conditions, hepatocytes typically do not express pro-inflammatory cytokines and show low mRNA expression levels as well as baseline protein levels (Figure 19 and Figure 21). These results indicated that the TLR signaling pathways are not activated at baseline.

To study the cytokine expression of human liver organoids under inflammatory conditions, and to compare alterations in expression upon TLR3 ligand recognition and binding, cytokine expression analyses in non-stimulated and stimulated conditions were performed.

In 2008, Xagoriani and Chlichlia showed that upon binding of poly I:C via TLR3, cytokine expression of IL-6, IL-12, TNF, and IFNγ is induced (Xagorari & Chlichlia, 2008). Contrary to literature and our expectations, no significant changes in cytokine expression upon stimulation with poly I:C in AIH affected individuals in none of the cytokines was detected, neither on mRNA level nor in protein production, although small trends towards increased expression in AIH-derived liver organoids were observed (Figure 23 and Figure 25). Due to the rare occurrence of AIH, we were only able to include four AIH samples available from different individuals, therefore higher AIH sample numbers might need to be included in order to confirm a robust effect.

IL-6 appeared to be the main inflammatory cytokine that tended to be slightly upregulated in AIH-derived liver organoids at mRNA and protein levels (Figure 23 and Figure 25), although not significantly. IL-6 is identified to be key in inflammations (B. Li et al., 2018). IL-6 can be produced by several cell types, including B cells, T cells, macrophages, DCs, as well as by hepatocytes (Choy & Rose-John, 2017; B. Li et al., 2018; Mihara et al., 2012). Especially in inflammatory conditions, IL-6 promotes T cell proliferation (B. Li et al., 2018). Furthermore, IL-6 is one of the main pro-inflammatory cytokines produced by hepatocytes upon inflammation,

therefore it may not be surprising that IL-6 was most pronounced expressed in liver organoids (Figure 23 and Figure 25). Furthermore, when produced by hepatocytes, IL-6 is responsible for the development of Th17 T cells (L. Zhao et al., 2011; L. Zhou et al., 2007). On the one hand, Th17 T cells can produce IL-17, which is associated with hepatocyte destruction in AIH and on the other hand, Th17 T cells are also able to induce further IL-6 secretion by endothelial cells, epithelial cells, fibroblasts, or macrophages (Korn et al., 2009; L. Zhao et al., 2011; L. Zhou et al., 2007). Although we observed a trend towards higher IL-6 expression in organoids derived from AIH affected livers compared to organoids from non-inflamed livers, the difference was not statistically significant. This may indicate that hepatocytes may not be the main drivers for tissue inflammation and initiation of pathogenesis of AIH, but that suggest that also other cell types, such as B cells, T cells, macrophages, or DCs may contribute to IL-6 production and contribute to AIH (Choy & Rose-John, 2017; B. Li et al., 2018; Mihara et al., 2012). However, additional studies are required to further investigate these assumptions.

Furthermore, hepatocytes have also been described to produce chemokines, such as CXCL9 (Czaja, 2014; Poulsen et al., 2022). The production of CXCL9 has been shown is dependent on an inflammatory environment (Lang et al., 2006) and can attract pro-inflammatory T cells.

Lang *et al.,* have been shown that poly I:C stimulation led to an enhanced CXCL9 expression in AIH, triggered by the cytokine production of type I interferons and TNF (Lang et al., 2006). This was also supported by Czaja in 2014 (Czaja, 2014). Wasmuth *et al.* also showed an upregulation of CXCL9 upon IFNγ stimulation and identified this chemokine as a regulator for liver fibrosis (Wasmuth et al., 2009). Furthermore, they demonstrated that the upregulation of CXCL9 secretion depends on the level of fibrosis (Wasmuth et al., 2009). This suggests that CXCL9 production by hepatocytes requires an environment of IFNγ producing T lymphocytes, triggering CXCL9 expression. Our results, also during viral infection, were in line with these studies, reporting that CXCL9 tended to be higher expressed in AIH-derived liver organoids contrary to non-inflamed organoids in both mRNA and protein level, following stimulation (Figure 24 and Figure 25). These results suggest that this chemokine might be a potential recruiter of TNF and IFNγ producing T lymphocytes, supporting hepatocyte damage and death (Lang et al., 2006; S. Yin & Gao, 2010).

Nevertheless, to be able to make conclusions, additional autoimmune hepatitis samples from AIH affected individuals are necessary. Another assumption based on the lack of significance is that our obtained samples from AIH affected individuals are end-stage liver samples, which means that liver cirrhosis is severe in these samples. It is important to note that the liver tissue from AIH affected individuals was derived from AIH end-stage conditions, in which individuals received a liver transplant due to deficient liver function. Correspondingly, it poses the question if human liver organoids derived from end-stage liver samples are able to express the same

quantity of inflammatory cytokines as organoids derived from acute AIH affected individuals. Therefore, we would expect that individuals with an acute AIH may display increased production of pro-inflammatory cytokines, such as TNF and IL-6. However, this has to be investigated in future experiments.

In this study, it was not possible to make any conclusive findings regarding enhanced pro-inflammatory cytokine nor chemokine expression associated with AIH, but a trend to higher IL-6 and CXCL9 expression was shown.

5.4 Pro-inflammatory cytokines TNF and IFNγ expressed by immune cells can influence hepatocyte growth

Autoimmune hepatitis is a liver disease characterized by hepatocyte destruction, liver inflammation, and liver fibrosis that can result in liver cirrhosis and liver failure (Christen & Hintermann, 2016; Koyama & Brenner, 2017; Lapierre & Lamarre, 2015; Mo et al., 2020).

In this study, we have shown that human liver organoids can express an enhanced amounts of *CXCL9* on mRNA and protein level (Figure 24 and Figure 25). In inflammatory conditions, the chemokine CXCL9 recruits Th1 and Th17 T cells to the liver (Czaja, 2014; Lang et al., 2006; S. Yin & Gao, 2010). Both Th1 and Th17 T cells express inflammatory cytokines, such as TNF and IFNγ, as well as IL-17 and that have been described to induce hepatocyte death and liver inflammation (Bettelli et al., 2006; Terziroli Beretta-Piccoli et al., 2022; L. Zhao et al., 2011).

Previously, Bovensiepen *et al.* demonstrated significantly enhanced frequencies of TNF-producing $CD4^+$ T cells in peripheral blood and in the liver in AIH (Bovensiepen et al., 2019). Prior, Behfarjam and colleagues showed that both TNF and IFNγ were significantly increased in blood in individuals affected with AIH (Behfarjam et al., 2017).

Based on these previous studies, we assessed the effect of TNF and IFNγ on hepatocytes in a long-term stimulation with different doses of TNF and IFNγ.

Predominantly, TNF is associated with pathogenic functions, including hepatocyte death, liver inflammation or initiation of autoimmune diseases, such as AIH (Bradham et al., 1998; Grivennikov & Karin, 2011; Tiegs & Horst, 2022; Weiler-Normann et al., 2013; Wullaert et al., 2007). In line, when stimulating with low concentrations of TNF, few morphology changes in organoids derived from non-inflamed livers and organoids from AIH affected livers were observed. In detail, the morphology of the organoids changed from a dense to a rounder phenotype compared to the untreated organoids in both groups (Figure 26 A). The observations of the morphology change suggest a de-differentiation of hepatocytes, which is a common characteristic of liver regeneration (Greenbaum, 2011; Kim et al., 2019; Kung et al.,

2010; Michalopoulos et al., 2005; Yimlamai et al., 2014). This is in accordance to what has been previously described in the literature, demonstrating that TNF is associated in liver regeneration (R. Guo et al., 2022; Michalopoulos & Bhushan, 2021; Michalopoulos & DeFrances, 1997; Taub, 2004).

Here, it was demonstrated that low doses of TNF can prevent hepatocyte death and promote hepatocyte de-differentiation, as it is known from liver regeneration processes by promoting tissue repair. This was supported by a comparison of the number of liver organoids, which showed only a slight reduction compared to unstimulated control (Figure 26 B).

In AIH, TNF is identified to exert either cytotoxic or protective functions by inducing apoptosis or differentiation of hepatocytes (AboElnazar et al., 2020; H. Chen et al., 2023). That TNF has two functions in liver injury, either protective or damaging, was also demonstrated in 2005 by Grivennikov and colleagues (Grivennikov et al., 2005). However, Zhao et al showed that the concentration of TNF is dependent on its performing function (S. Zhao et al., 2020).

Recently, AboElnazar *et al.* demonstrated that TNF can also have a protective function by preventing hepatocyte death. This function of TNF is triggered by the transcription factor NF-κB, which is a component of the TNF downstream signaling pathway (AboElnazar et al., 2020; H. Chen et al., 2023; Papa et al., 2009). However, the interaction between TNF signaling and NF-κB, resulting in the protection of the hepatocytes is less understood and needs to be further investigated (AboElnazar et al., 2020).

Here, we show that a stimulation with high doses of TNF suppressed human liver organoid proliferation or induced hepatocyte death in both, non-inflamed control organoids as well as in AIH-derived organoids. These results are in line with previous studies, showing that an enhanced expression of TNF by T cells in Con A mice led to hepatocellular death (AboElnazar et al., 2020; H. Chen et al., 2023; Yamashita et al., 2011), confirming the pathogenic features of this cytokine. This is also in accordance with Zhao et al. demonstrating that high concentrations of TNF triggered hepatocyte death (S. Zhao et al., 2020). However, in AIH-derived organoids, TNF is not only mediating pathogenic functions by reducing the organoid proliferation. In organoids from AIH affected livers, high doses of TNF inhibited organoid proliferation and suppressed organoids growth. However, contrary to control group, organoids derived from AIH individuals stimulated with high doses of TNF, some human liver organoids proceeded to grow. These observations indicate a protective function of TNF in liver injury that in turn then promotes hepatocyte growth, however, contrary to findings from Zhao *et al.* in high concentrations of TNF (S. Zhao et al., 2020).

Nonetheless, it should be noted, that stimulations with both, low as well as high doses of TNF, resulted in an overall reduction of human liver organoids that predominantly confirmed

cytotoxic effects of TNF on hepatocytes (AboElnazar et al., 2020; H. Chen et al., 2023; S. Zhao et al., 2020).

The function of TNF supporting some hepatocytes to differentiate in liver injury instead of initiating hepatocyte death was further confirmed on mRNA level. Cytochromes P450 are identified to be downregulated in the liver in inflammatory conditions (Aitken et al., 2006; Aitken & Morgan, 2007; Morgan, 1997). Mimura et al. demonstrated a concentration-dependent effect of TNF on *Cyp3a4* mRNA expression in hepatoma cell line. Here, our results are in accordance to what has been described in their study (Mimura et al., 2014). In detail, compared to the unstimulated control, mRNA expression of *Cyp3a4* decreased, when reducing TNF levels. However, when applying higher doses, Cyp3a4 began to increase again (Figure 27 A). This pattern in mRNA expression was also observed in the study conducted by Mimura *et al.* (Mimura et al., 2014).

Regarding the effect of IFN, IFNγ had a stronger effect on human liver organoids in both groups compared to TNF (Figure 27 A and B).

When stimulating with IFNγ, our data demonstrates that low as well as high concentrations of IFNγ exert a pathogenic effect to hepatocytes by inhibiting organoid growth in both, organoids from non-inflamed livers and organoids derived from AIH affected livers (Figure 26 A and B). These observation are in line with published reports, describing the cytotoxic effect of IFNγ to hepatocytes inducing apoptosis or cell cycle arrest (Horras et al., 2011; Kano et al., 1997; Morita et al., 1995). The inhibition of the organoid growth by using low and high doses underlined the anti-proliferative function of this pro-inflammatory cytokine as described in the review from Horras *et al.* (Horras et al., 2011). Especially when using high doses, a strong reduction in the human liver organoid growth was observed, underlining the pathogenic effect of IFNγ in AIH.

In addition, same as TNF, also IFNγ negatively impacted *Cyp3a4,* by showing a decreased trend in mRNA expression within the liver organoids of both group (Figure 27 B), which is in line with the study from Aitken and colleagues, demonstrating a decrease of Cyp3a4 in human hepatocytes (Aitken & Morgan, 2007).

These results additionally underline, that both cytokines have an impact on the regulation of drug metabolism in the liver, as both are decreasing mRNA expression of *Cyp3a4* (Jong et al., 2020), indicating that both pro-inflammatory cytokines are key in inflammation processes within hepatocytes.

Taken together, our results indicate that both pro-inflammatory cytokines secreted by $CD4^+$ Th1 T cell immune response mainly exert a cytotoxic effect on hepatocytes by either destructing hepatocytes or by suppressing hepatocyte growth and proliferation. These findings

suggest that TNF and IFNγ might play a driving role in AIH and provide evidence that both cytokines may be the pathogenic factor in AIH (Bovensiepen et al., 2019).

5.5 Determination the impact of Tissue resident memory (TRM) T cells to human liver hepatocytes

Tissue resident memory (TRM) T cells represent a unique subset of memory T cells that persist in specific tissues such as kidney, liver, lung, gut or brain without recirculating out of their tissue. They are essential in host defense providing a rapid protection by inducing an adaptive immune response to eliminate known pathogens or antigens (Y. Li et al., 2022; H. Wu et al., 2018). However, they can display a pathogenic function and trigger the induction of autoimmune diseases. Accordingly, the pathogenesis of AIH can arise due to an over activation of TRM cells or dysfunction of these cells (Ibidapo-Obe & Bruns, 2023; Y. Li et al., 2022; H. Wu et al., 2018). In 2021, You and colleagues confirmed that TRM T cells are significantly enhanced in the liver of individuals affected with AIH (Z. You et al., 2021). It is known that TRM1 cells are expressing inflammatory cytokines, such as IFNγ, which could be an initiator of hepatocyte death (Guidotti et al., 1996; H. Wu et al., 2018). This assumption led us to perform co-cultures with TRM1 cells derived from acute AIH individuals undergoing liver biopsy and human liver organoids to examine the cross talk of the initiated immune response of TMR1 cells and human liver organoids (Figure 28). Accordingly, we expected that the enhanced pro-inflammatory immune response, resulting in activation of AIH TRM1 cells, would affect the human liver organoids by causing hepatocyte death.

TRM1 are expected to secrete IFNy and TNF during co-cultures that could result in hepatocyte destruction and liver damage (Terziroli Beretta-Piccoli et al., 2022). To study the effect of IFNy and TNF secreted by TRM1 cells in co-culture with organoids in more detail, blocking antibodies for TNF and IFNy respectively were used to block the downstream signaling pathways. Blocking those signaling pathways allowed us to examine if TNF and IFNγ are involved in hepatocyte damage in the context of AIH.

Analysis of the size of organoids from non-inflamed livers revealed that TRM1 cells did not affect the size of the organoids, suggesting that the pro-inflammatory immune response of TRM1 cells did not influence the organoid growth in non-inflamed individuals (Figure 28 B). In AIH-derived organoids, however, the opposite effect was observed. Correspondingly, AIH TRM1 cells reduced the size of the human liver AIH-derived organoids, suggesting that IFNγ impairs hepatocyte growth. Underlining the anti-proliferative function of IFNγ, as described by Horras *et al*. (Horras et al., 2011). This effect was primarily attributable to IFNγ in these co-cultures. These results are in line with previous reports, suggesting the cytotoxic effect of IFNγ to hepatocytes, and that this pro-inflammatory cytokine is able to suppress hepatocyte growth

(Horras et al., 2011; Kano et al., 1997; Tura et al., 2001). To further support these findings, co-cultures with AIH TRM1 cells supplemented with IFNγ antibody alone should be performed to further substantiate the importance of IFNγ in AIH.

The strongest reduction of the organoids contrary to control was observed when adding only TRM1 cells to AIH-derived organoids. Whereas TRM1 cells had a weaker effect on organoid growth in co-cultures supplemented with TNF antibodies to block TNF signaling, indicating less liver damage. In this condition, organoids displayed a better growth and survival compared to AIH organoids culture with TMR1 alone or TRM1 cells in combination with TNF and IFNy. The importance of TNF in AIH is highlighted by the increased number of TNF-producing $CD4^+$ Th1 T cells, as described from Bovensiepen et al. (Bovensiepen et al., 2019). Therefore, it was not surprising that TNF had an influence on hepatocyte proliferation and that this cytokine is able to induce hepatocyte damage. However, individuals diagnosed with AIH showed good responses to immunosuppressive treatment, using TNF blockers, such as infliximab, etanercept, and adalimumab (Efe, 2013).

Anti-TNFα treatment in AIH known to decrease hepatocyte damage by reducing the TNF production (Cassim et al., 2017; Rajanayagam & Lewindon, 2013; Vergani et al., 2021; Weiler-Normann et al., 2009, 2013). Due to this, when supplementing TNF block to the co-culture, it was not surprising to observe less hepatocyte damage and improved organoid growth compared to co-cultures with TRM1 cells alone, underlining the importance of TNF cytokine in AIH.

In summary, these results indicate that both pro-inflammatory cytokines secreted by TRM1 cells have a cytotoxic effect on hepatocytes. It seems that TNF has stronger impact on the number of organoids by inhibiting hepatocyte proliferation, while IFNγ affects the size, preventing cell growth of the organoids.

6 Conclusion

The aim of this thesis was to investigate dysregulated pathways in AIH by examining the functioning of liver cells in a liver organoid system generated from AIH affected livers and the consequences of TNF and IFNγ producing T cells to hepatocytes that are known to be enhanced in AIH.

In this thesis, we were able to show that culturing of 3D human liver organoids and stimulation of those, offer a model to study liver diseases, such as AIH. Additionally, it was investigated that TLR3 signaling may be altered in AIH, as this receptor tended to be higher expressed in liver organoids generated from livers affected with this complex autoimmune disease and the trend towards increased IL-6 production.

Furthermore, in this thesis, we demonstrated that both pro-inflammatory cytokines TNF and IFN expressed by T cells have a cytotoxic effect on hepatocytes, which influence hepatocyte growth and proliferation. Furthermore, both are potential cytokines that trigger hepatocyte death. This was shown in long-term stimulation of human liver organoids with both inflammatory cytokines and was following verified in co-culture of organoids with TMR1 T cells where the corresponding TNF and IFN pathways were blocked.

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Acknowledgement

First, I would like to express my gratitude to Prof. Dr. Madeleine Bunders for her supervision on this exciting project and for giving me the opportunity to conduct my PhD in her group. I am very grateful for your support and your scientific advices during my PhD.

Next, I want to thank Prof. Dr. Julia Kehr for being the first reviewer of my thesis.

Especially, I would like to thank Dr. Annika Niehrs for her kindness, her patience, for introducing me into new techniques, and your scientific support in the liver team. Thank you so much for your mental support during the final stages of writing my thesis.

A special thanks to Heike Hildebrandt for organizing everything in and outside the lab. For all the rapid orders and circumstances, I made sometimes, as well as for your support in the office.

I sincerely thank all members of Abteilung 8 for the wonderful years and the amazing atmosphere in the lab. I am glad that I had the opportunity to meet all of you and many of you became friends over the last years. Thanks for the friendships, answering scientific questions, the hours of invaluable conversations, the mental support, all the coffee we had in the kitchen.

I would like to thank the Asklepios Hospital Barmbek and University Hospital Hamburg-Eppendorf for providing the liver samples. Without these samples, my PhD thesis would not have been possible.

Finally, I would like to express my immense thanks to my parents for supporting me all the time and for making everything possible. Thank you for your encouragement and emotional support. Without you, I would not be where I am today. Thank you for everything.

List of publications

Zecher, B. F., Ellinghaus, D., Schloer, S., Niehrs, A., Padoan, B., Baumdick, M. E., Yuki, Y., Martin, M. P., Glow, D., Schröder-Schwarz, **J., Niersch**, J., Brias, S., Müller, L. M., Habermann, R., Kretschmer, P., Früh, T., Dänekas, J., Wehmeyer, M. H., Poch, T., Sebode, M., … Altfeld, M. (2024). *HLA-DPA1*02:01~B1*01:01* is a risk haplotype for primary sclerosing cholangitis mediating activation of NKp44+ NK cells. *Gut*, *73*(2), 325–337. https://doi.org/10.1136/gutjnl-2023-329524.

Jan P. Bremer, Martin E. Baumdick, Marius S. Knorr, Lucy H.M. Wegner, Jasmin Wesche, Ana Jordan-Paiz, Johannes M. Jung, Andrew J. Highton, Julia Jäger, Ole Hinrichs, Sebastien Brias, **Jennifer Niersch**, Luisa Müller, Renée R.C.E. Schreurs, Tobias Koyro, Sebastian Löbl, Leonore Mensching, Leonie Konczalla, Annika Niehrs, Florian W. R. Vondran, Christoph Schramm, Angelique Hölzemer, Karl Oldhafer, Ingo Königs, Stefan Kluge, Daniel Perez, Konrad Reinshagen, Steven T. Pals, Nicola Gagliani, Sander P. Joosten, Maya Topf, Marcus Altfeld, Madeleine J. Bunders (2022). GOAT: Deep learning-enhanced Generalized Organoid Annotation Tool. bioRxiv, 1-34. https://doi.org/10.1101/2022.09.06.506648.

Parts of this thesis may be published in an ongoing research article.

Declaration of oath

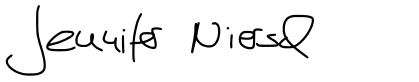
This dissertation was performed in the group of Immune Ontogeny and Viral Infections at Leibniz Institute of Virology (LIV) in Hamburg under the supervision of Prof. Dr. Madeleine Bunders.

First Reviewer: Prof. Dr. Julia Kehr

Second Reviewer: Prof. Dr. Madeleine Bunders

I hereby declare, under oath, that I have written this dissertation independently by my own and that I have not used any additional resources or aids than those acknowledged.

Hamburg, March 20th, 2024



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