The role of Interleukin-7 in hepatocellular immunoregulation

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Zusammenfassung

Die Leber nimmt eine zentrale Rolle im Metabolismus ein. Sie speichert Kohlenhydrate in Form von Glykogen, sie produziert Gallensäuren zur Fettverdauung, sie sorgt für den Transport von Fetten in periphere Körperregionen und entgiftet den Körper z.B. von Alkohol. Aufgrund dieser primär metabolischen Aktivität stellt sich die Frage, warum es Sinn macht, sich bei der Hepatologie auch schwerpunktmäßig immunologischen Fragestellungen zu widmen.

Betrachtet man die Anatomie der Leber, so fällt auf, dass sie nicht nur über einen arteriellen Blutzufluss verfügt, der nährstoffarmes aber sauerstoffreiches Blut führt. Die Leber besitzt auch einen venösen Zufluss, die Portalvene. Diese Vene transportiert sauerstoffarmes, aber nährstoffreiches Blut aus dem Gastrointestinaltrakt zur Leber, damit die Nahrungsbestandteile verwertet werden können. Daneben findet sich in diesem Blutzufluss auch eine vergleichsweise hohe Konzentration an Metaboliten bakteriellen Ursprungs, die von unserem großteils kommensalem Mikrobiom im Darm stammen. Dadurch ergibt sich die Situation, dass das Immunsystem der Leber konstant Molekülen ausgesetzt ist, die normalerweise eine Immunreaktion auslösen würden. Daher sorgen die residenten Immunzellen in der Leber stets für einen Zustand der Toleranz, d.h. es findet keine pro-inflammatorische Antwort auf den jeweiligen Stimulus statt. Zu diesen Immunzellen gehören sowohl Zellen des angeboren Immunsystems, wie die als Kupffer-Zellen bezeichneten Makrophagen, als auch des adaptiven Immunsystems, wie T-Zellen. Da einige Pathogene Leberzellen als Zielzellen haben, wie bei Hepatitisviren, ist das Immunsystem der Leber, in Zusammenarbeit mit den Immunzellen in der Zirkulation, in der Lage, Infektionen zu bekämpfen.

Belastungen der Leber z.B. durch Alkoholkonsum, zu fettreiche Nahrung, Infektionen oder Toxine können zu schweren chronischen Leberschäden führen. Dabei geht funktionelles Lebergewebe verloren und die Leber wird bindegewebig umgebaut. Zu den schwersten Erkrankungen gehört die Leberzirrhose, wobei die Leber bereits stark vernarbt ist. Die Leberzirrhose gilt als kompensiert, solange noch keine klinischen Folgeerscheinungen aufgetreten sind. Dazu gehören u.a.

Aszites, gastrointestinale Blutungen oder eine hepatische Enzephalopathie. Ab Auftreten dieser Symptome gilt die Zirrhose als dekompensiert. Das akut-auf-chronische-Leberversagen ist die schwerste klinische Folgeerscheinung, die eine Mortalität von 50% der Patienten zur Folge hat. Interessanterweise zeigen Forschungsergebnisse sowohl Anzeichen von einer Überreaktion des Immunsystem als auch einer Immunparalyse bei den Patienten. Dies ist ein weiterer Beweis für die Bedeutung der Immunologie in der Hepatologie.

Kommunikation in der Immunologie verläuft großteils über lösliche Mediatoren. Zentral sind dabei die Interleukine, Protein, die Immunreaktionen entweder auslösen oder unterdrücken können. Ein bekannter Vertreter ist das Interleukin-7. Interleukin-7 ist von zentraler Bedeutung bei der Reifung von Zellen des adaptiven Immunsystems im Knochenmark, da die Stimulation mit Interleukin-7 für diese Zellen ein Überlebenssignal darstellt. Auch in Leber und Gastrointestinaltrakt sorgt Interleukin-7 für den Aufbau und Erhalt von verschiedenen Immunzellpopulationen, wobei der Einfluss von Interleukin-7 auf Zellen des angeborenen Immunsystems nicht sehr gut verstanden ist. Eine Studie in Mäusen konnte zeigen, dass sich in den Lebern der Tiere Interleukin-7 induzieren ließ, wenn den Tieren bakterielles Lipopolysaccharid in die Portalvene gespritzt wurde. Anhand dieser Datenlage ergaben sich die drei zentralen Fragestellungen dieser Doktorarbeit. Erstens: Lässt sich die Induktion von Interleukin-7 auch in humane Zellen auslösen und wie wird die Proteinexpression gesteuert? Zweitens: Welche Zellen des Immunsystems sind in der Lage auf Stimulation mit Interleukin-7 zu antworten und welche funktionellen Auswirkungen hat die Stimulation mit Interleukin-7 auf Makrophagen? Drittens: Hat Interleukin-7 eine Relevanz in Patienten mit Leberzirrhose und kann es im Zusammenhang mit dieser Erkrankung als vorteilhaft oder nachteilig charakterisiert werden?

Um diese Fragen zu klären, wurden großteils Versuche in Zellkultur genutzt. Humane Hepatozyten und Leberendothelzellen wurden mit verschiedenen Interferonen (IFNs) stimuliert. Diese lösen generell einen antiviralen Zustand bei Zielzellen aus und werden in verschiedene Typen eingeteilt. Im Anschluss wurde die Expression von Interleukin-7 auf RNA Ebene im Zelllysat oder auf

Proteinebene im Zellkulturüberstand überprüft. Mittels siRNA Transfektion wurden gezielt Mediatoren der Interferon Signalkaskade supprimiert, um Mediatoren der Interleukin-7 Expression zu identifizieren. Für Fragestellungen der Proteinlokalisation wurden mittels sequentieller Lyse Proteine aus Zytosol und Zellkern separate aufgereinigt und analysiert.

Für den zweiten Teil wurden verschiedene Immunzellen aus humanem Blut mittels Ficoll Dichtezentrifugation aufgereinigt und ggfs. mit Wachstumsfaktoren in Zellkultur ausgereift. Auch aus Aszitesflüssigkeit von Patienten mit Leberzirrhose wurden Makrophagen aufgereinigt. In verschiedenen Immunzellen z.B. T-Zellen, Makrophagen und dendritischen Zellen, wurde die Präsenz der Rezeptorproteine für Interleukin-7 überprüft. Auch wurde die Funktionalität der Signalkaskade untersucht. Im Anschluss wurde bestimmt, wie die Interleukin-7 Stimulationen die Funktionalität von Makrophagen beeinflusst. Untersuchte Parameter waren dabei der zelluläre Metabolisms anhand von Glukose, Laktat und ATP, die zelluläre Viabilität sowie die Toleranz gegenüber Lipopolysaccharid (LPS), gemessen an der Expression pro-inflammatorischer Zytokine Interleukin-1β (IL-1β), Interleukin-6 (IL-6) sowie Tumor necrosis factor α (TNFα). Im letzten Teil wurde die Interleukin-7 Konzentration im Serum von Patienten in verschiedenen Stadien der Leberzirrhose bis hin zum akut-auf-chronischen-Leberversagen und von gesunden Kontrollen bestimmt. Zusätzlich wurde der Gehalt an Immunzellen, die den Interleukin-7 Rezeptor tragen, durchflusszytometrisch analysiert. In einigen wenigen Patienten wurde bestimmt, ob sich die Komponenten der Interleukin-7 Signalkaskade auch in der geschädigten Leber, also in deren Biopsaten, nachweisen lassen.

Unsere Ergebnisse zeigen, dass die Expression von Interleukin-7 nur durch bestimmte Typen von Interferon ausgelöst wird. IFNα und IFNγ sorgten für erhöhte Interleukin-7 RNA und Proteinspiegel, während dies bei IFNλ2 nicht der Fall ist. Dies ließ sich sowohl für Hepatozyten als auch für Endothelzellen beobachten. Gleichzeitig ist die IFNλ2 Kaskade aber intakt, da in der Literatur bereits bekannte Zielgene hochreguliert werden. Wir konnten IRF1 als einen zentralen Mediator der Interleukin-7 Expression identifizieren, da eine Suppression von IRF1 die

Expression von Interleukin-7 unterbindet. Stimulation von Hepatozyten mit IFNα und IFNγ, aber nicht mit IFNλ2, führten zu einer gesteigerten Transkription, Translation und nukleären Translokation von IRF1 über mehrere Stunden. Diese Akkumulation von IRF1 führte bei mehrfacher IFNγ Stimulation wiederum nochmals zu massiv gesteigerter Interleukin-7 Expression. Allen Interferon gemein war deren Vermögen den Zellzyklus von Hepatozyten in ähnlichem Umfang zu beeinflussen, sodass dieser Signalweg nicht mit der Interleukin-7 Expression verknüpft zu sein schien.

Wir konnten die Rezeptorkomponenten für die Signaltransduktion von Interleukin-7 auf verschiedenen Zellen des angeborenen und des adaptiven Immunsystems nachweisen. Auch ließen sich alle Zelltypen mit rekombinanten Interleukin-7 stimulieren, wobei in den verschiedenen Zelltypen verschiedene Signalkaskaden angeschaltet wurden. Bei Makrophagen und dendritischen Zellen war für eine erfolgreiche Interleukin-7 Stimulation eine vorherige Behandlung mit LPS notwendig. Passend dazu konnten wir zeigen, dass die Expression einer Rezeptorkomponente, CD127 genannt, durch LPS in Monozyten und Makrophagen induziert werden konnte. Zentrales Molekül bei der Signaltransduktion von Interleukin-7 in Makrophagen war die Glycogen synthase kinase-3 (GSK3), die durch Interleukin-7 phosphoryliert und inhibiert wurde. In Folge dessen zeigten Interleukin-7 stimulierte Makrophagen eine Reduktion ihres intrazellulären ATP-Spiegels. Zusätzlich reduzierte sich auch die Viabilität der behandelten Zellen. Dennoch zeigten die Makrophagen eine stark erhöhte Genexpression der pro-inflammatorischen Zytokine IL-1β, IL-6 und TNFα.

Patienten mit Leberzirrhose haben einen reduzierten Anteil an funktionellem Lebergewebe, dennoch ließen sich mit IRF1 und GSK3 zentrale Mediatoren der Interleukin-7 Kaskade in Leberbiopsaten nachweisen. Der Serumspiegel von Interleukin-7 war in Patienten mit Leberzirrhose sehr stark erniedrigt. Passend dazu, war der Anteil der CD127 positiven myeloiden Zellen erhöht. Die Menge an CD127 positiven T-Zellen hingegen war erniedrigt, wobei diese Patienten generell niedrige Raten an Lymphozyten aufwiesen.

Unsere Daten charakterisieren Interleukin-7 als ein vergleichsweise pro-inflammatorisches Zytokin für Zellen des angeborenen Immunsystems. Die Expression von Interleukin-7 in Hepatozyten und Endothelzellen zeigt die Bedeutung von Nicht-Immunzellen für die hepatische Immunoregulation. Die selektive Expression durch bestimmte Interferone ist von Bedeutung, da IFNλ beispielsweise als Therapeutikum der Hepatitis B getestet wird. In gesunden Makrophagen bricht Interleukin-7 die LPS-Toleranz dieser Zellen, während es gleichzeitig die zelluläre Fitness reduziert. Diese Fakten sind für die Frage, ob Interleukin-7 für Zirrhotiker Freund oder Feind ist, zwiespältig zu betrachten. Studien haben gezeigt, dass hohe Spiegel von IL-6 und TNFα mit der Schwere des akut-auf-chronischen-Leberversagens korrelieren, daher würde eine weitere Produktion dieser Zytokine wahrscheinlich die lokale Schädigung in der Leber weiter vorantreiben. Allerdings treten bei Zirrhose und akut-auf-chronischem-Leberversagen erhöhte Zahlen von Monozyten auf, deren Fitness durch Gabe von Interleukin-7 reduziert werden könnte. Zusätzlich könnte Interleukin-7 auch die Menge an T-Zellen wieder auf das Niveau von Gesunden heben, daher wäre eine therapeutische Intervention mit Interleukin-7 wie bei Sepsis eventuell eine Option. Dies wird dadurch bestätigt, dass sich trotz des niedrigen Zytokinspiegels regulatorische Elemente der Signaltransduktion aus den in *vitro* Daten auf das Patientenmaterial übertragen lassen.

Unsere Daten basieren großteils auf *in vitro* Experimenten, daher sind weitere Untersuchungen nötig, um die therapeutische Nutzbarkeit von Interleukin-7 weiter zu evaluieren. So sollten die Effekte von Interleukin-7 in Ko-Kulturen oder Organoidmodellen von humanen Zellen weiter charakterisiert werden. Außerdem wäre es wichtig, ob sich die in gesunden Makrophagen gefundenen Effekte auch in Zellen von Patienten nachweisen lassen. Generell sollte der Immunstatus von Patienten weiter charakterisiert werden, um die Effektivität einer möglichen Interleukin-7 Therapie weiter zu evaluieren. Schlussendlich sollte in der Zukunft versucht werden, mehr mit Lebergewebe zu arbeiten. Dadurch dass einige Arten von Immunzellen gewebsspezifisch und nicht auch im Blut auftreten, sollte man lokale und systemische Antworten auf das Interleukin-7 charakterisieren, um das therapeutische Potential von Interleukin-7 weiter auszuloten.

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List of abbreviations

ACLF	Acute-on-chronic liver failure
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BV510	Brilliant violet 510
BV605	Brilliant violet 605
CD	Cluster of differentiation
CLD	Chronic liver disease
CO_2	Carbon dioxide
$C_{24}H_{39}NaO_4$	Sodium deoxycholate
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate-Buffered Saline
DTT	1,4-Dithiothreito
ECM	Endothelial cell medium
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GBP5	Guanylate binding protein 5
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK3	Glycogen synthase kinase 3
HBSS	Hank’s balanced salt solution
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene

$KHCO_3$	Potassium bicarbonate
LPS	Lipopolysaccharide
MACS	Magnetic cell separation
M-CSF	Macrophage colony-stimulating factor
MDM	Monocyte-derived macrophage
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NaF	Sodium fluoride
$Na_4P_2O_7$	Sodium pyrophosphate
Na_3VO_4	Sodium orthovanadate
NH_4Cl	Ammonium chloride
NP-40	Nonidet P-40
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE-Cy5	Phycoerythrin cyanine 5
PMSF	Phenylmethylsulfonyl fluoride
rcf	Relative centrifugal force
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
STAT	Signal transducer and activator of transcription protein
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylendiamin
TNFα	Tumor necrosis factor α
7-AAD	7-Aminoactinomycin

Summary

The liver as the biggest endocrine gland of the human body plays a central role in many metabolic pathways such as detoxification, storage of carbohydrates and distribution of lipids. As the liver receives blood supply from the gut by the portal vein, liver cells are often challenged with high concentrations of nutrients and components of our commensal microbiota. Therefore, the immune system of the liver induces a tolerant state, meaning no or low inflammatory reactions to those constant stimuli. Yet, as various pathogens target the liver, the hepatic immune system also needs the capability to induce strong immune responses quickly. Chronical damage to the liver, which can be caused by alcohol, pathogens or toxins, might lead to liver cirrhosis, where the amount of functional liver tissue is decreased dramatically. This pathology can worsen and lead to acute-on-chronic liver failure, whose high mortality is due to high inflammation and multi-organ failure. Interleukin-7 is a cytokine known for its pro-survival functions especially in lymphopoiesis. However, it is also very important for maintenance of mature immune cells in the liver. As mouse experiments have demonstrated an induction of Interleukin-7 in the liver as a response to bacterial lipopolysaccharide, we aimed to characterize the role of Interleukin-7 in hepatic immunoregulation in both health and disease.

The experiments were mostly based on *in vitro* approaches. Induction of Interleukin-7 in liver cells was analyzed using ELISA, quantitative PCR, and Immunoblotting. Knockdown of signal transduction components was performed by siRNA transfection. Primary immune cells isolated from healthy donor buffy coat were studied for their ability to respond to Interleukin-7. Activation of downstream signal transduction was assessed by Immunoblotting. Functional consequences of Interleukin-7 signaling, such as alterations in cellular metabolism, cellular survival and endotoxin tolerance, were studied in monocyte-derived macrophages. Finally, serum concentrations of Interleukin-7 and frequencies of Interleukin-7 receptor positive immune cells were quantified in patients with compensated or decompensated liver cirrhosis or acute-on-chronic liver failure.

Interleukin-7 expression could be observed in human hepatic cell lines and primary hepatic sinusoidal endothelial cells when stimulated with IFNα or IFNγ, but not IFNλ. IRF-1 was identified as a key regulator of Interleukin-7 expression, as its transcription, translation and nuclear translocation were induced and enhanced upon IFNα or IFNγ, but not IFNλ treatment. We identified LPS-primed macrophages as innate immune target cells of Interleukin-7, which responded by an inhibitory phosphorylation of GSK3. This signal transduction led to enhanced production of pro-inflammatory cytokines and abolished endotoxin tolerance. In parallel, cellular fitness was reduced as demonstrated by reduced intracellular ATP concentration and intracellular WST-1 staining. Finally, we could identify components of the *in vitro* signal transduction also in liver cirrhosis patients. However, Interleukin-7 serum concentrations were significantly in liver cirrhosis patients compared to healthy controls. In addition, the frequencies of Interleukin-7 receptor positive immune cell populations differed in patients and controls.

We identify Interleukin-7 as a pro-inflammatory cytokine in hepatic immunoregulation. It is part of a cascade where its induction is regulated by type I and type II Interferons and mainly restricted by the presence of IRF1. We demonstrate the importance of Interleukin-7 also for innate immune cells, where the abolishment of endotoxin tolerance may provide an interesting strategy of liver cirrhosis patients. In addition, reduced viability of macrophages in response to Interleukin-7 is a striking contrast to the well-described survival functions in lymphocytes. The decrease of serum Interleukin-7 levels and alterations of Interleukin-7 receptor positive immune cell populations suggest an important role for Interleukin-7 also in the diseased liver. Due to the identified mechanisms of action, Interleukin-7 may be an interesting candidate for immunotherapeutic approaches of liver cirrhosis and acute-on-chronic liver failure.

1. Introduction

This part explains why immunologic observations are important in the field of immunology either in the healthy or in the diseased state. Plus, it introduces Interferons and Interleukin-7 as the most important immunological mediators of our experiments.

1.1. The liver from an immunological perspective

The liver is a highly specialized organ with a central role in human metabolism. It provides storage for carbohydrates in the form of glycogen and it organizes uptake of lipids by producing bile acids for fat digestion and by producing lipoproteins for efficient lipid transport e.g. to adipose tissue. In addition, it is necessary for detoxification processes. Even though the need for immunological research does not appear on first sight, hepatic immunology has become important in health and disease even naming the liver a “lymphoid organ” (Crispe et al. 2009).

The reason for the interest in liver immunology can be found when taking a look at the blood supply of the liver. Like any other organ, the liver is connected to systemic circulation and receives blood high in oxygen and low in nutrients. However, the liver is also drained by the portal vein, which transports blood from the intestine to the liver. This blood is low in oxygen but contains high concentrations of nutrients. (Lumsden et al. 1988). Besides, this blood also contains high levels, up to 1 ng/mL, of Lipopolysaccharide (LPS) and other bacterial components, which potentially provoke immune responses (Lumsden et al. 1988). These findings demonstrate that the liver immune cells are constantly challenged with immunogenic metabolites. Therefore, immune reactions in the liver need to be perfectly balanced not to react excessively to portal venous blood components but to be able to fight invading pathogens, such as hepatitis viruses, in a proper way.

The liver contains a high density of resident immune cells. It harbors the single largest population of resident macrophages, also termed Kupffer cells (Jenne et al. 2013). In addition, the liver contains the highest population of NK- and NKT-cells, which displays the need of the liver to kill threats rapidly (Jenne et al. 2013). In addition, large populations of T-cells, both αβ- and γδ-T-cells, and dendritic cells, especially plasmacytoid dendritic cells are found in the liver (Crispe et al. 2009).
There are also non-immune cells in the liver, which contribute to efficient immunoregulation. Liver sinusoidal endothelial cells (termed LSECs) play an important role in antigen-presentation thereby supporting liver dendritic cell populations (Jenne et al. 2013). Hepatocytes also express receptors for pathogen recognition as the liver shows fenestrated epithelium thereby exposing hepatocytes to components in the bloodstream (Jenne et al. 2013). Finally, also hepatic stellate cells, which under healthy conditions provide storage for Vitamin A, express receptors for interaction with T-cells thereby shaping immune responses. Besides the pure liver resident cells, liver resident and peripheral immune cells, therefore, show complex ways of interaction to target evading pathogens (Blériot et al. 2015).

In general, the liver provides a state of tolerance, meaning a reduction in pro-inflammatory cytokines due to constant stimulation (Jenne et al. 2013). Tolerance is needed because concentrations of LPS in the portal vein are higher than in peripheral blood by nature (Lumsden et al. 1988). This tolerance e.g. to LPS affects a wide set of genes (Mages et al. 2007). Kupffer cells contribute strongly to tolerance in the liver, e.g. by the production of Interleukin-10 (Heymann et al. 2015). Mononuclear cells in a tolerant state display an alternative activation state (Pena et al. 2011). Some molecules have also been identified to contribute to establishing and maintaining tolerance. It has been demonstrated that GSK3 is a kinase which is strongly involved in the mechanism of endotoxin tolerance (Park et al. 2011).

Activation of immune responses occurs in different health and disease states. Certain types of immune cells contribute strongly to liver regeneration (Li et al.

2016). This includes especially the Kupffer cells (Tan et al. 2016). Cytokines such as IL-6 play also an important role in liver regeneration (Böhm et al. 2010).
Even mathematical models try to approach the issue of liver regeneration (Furchtgott et al. 2009). The close connection to the gut and thereby high levels of nutrients is always a fact to consider. For example, macrophage activation is closely related to its metabolic state (Langston et al. 2017). Besides the classical M1 and M2 scheme, macrophage polarization has evolved to be more complex and occurs due to a variety of stimuli (Xue et al. 2014). Finally, the importance of the gut-liver-axis can be seen in mouse models of intestinal inflammation, where pro-inflammatory cells then drain into the liver (Mikami et al.2014).

Taken together, the liver shows a high abundance of various immune cells and immunological processes used for various purposes. As also non-immune cells are highly involved, one can easily imagine dysregulation of liver immunology when functional liver tissue is decreasing e.g. in chronic liver diseases.

1.2. Cirrhosis and ACLF as examples of chronic liver diseases

Cirrhosis of the liver has become the fourth most common cause of death in central Europe (Tsochatzis et al. 2014). It is the end stage of chronic liver diseases (Wiegand et al. 2013). Various agents can cause chronic liver damage leading to cirrhosis where alcohol and hepatitis viruses are the most common in Germany (Wiegand et al. 2013). In general, development of cirrhosis from chronic liver disease involves fibrinogenesis as well as changes in the microvasculature and inflammation (Tsochatzis et al. 2014). At first, cirrhosis is asymptomatic, when clinical symptoms occur it is termed decompensated cirrhosis (Tsochatzis et al. 2014). These major complications include ascites, bleedings or hepatic encephalopathy (Moreau et al. 2013). Plus, cirrhosis patients are very susceptible to bacterial and also fungal infections (Fernández et al. 2017). Diagnosis of cirrhosis is mostly based on non-invasive methods such as ultrasound and laboratory parameters (Wiegand et al. 2013).

Acute-on-chronic-liver failure (ACLF) is a syndrome of decompensated liver cirrhosis with single or multiple organ failures (Arroyo et al. 2016). It is feared for its high short-term mortality of 30% to 40% (Jalan et al. 2015 and Arroyo et al. 2015). Therefore, they need to be supervised closely for efficient treatment if possible (Gustot et al. 2015). Recently, new scores were established to get a better estimation of severity of ACLF (Jalan et al. 2014 and Moreau et al. 2013). Of note, especially ACLF is also characterized as a syndrome of systemic inflammation (Bernardi et al. 2015).

Both cirrhosis and ACLF show strong connections to immune dysregulation for several reasons- For example, ethanol is known to strongly impact on the innate immune system (Goral et al. 2008). Certain monocyte subsets impact strongly on the liver in a diseased state (Liaskou et al. 2013). For example, hyponatremia is a common feature of ACLF and as macrophage activation is influenced by salt concentration this might impact on therapy (Zhang et al. 2015). Also, genetic alterations are analyzed for their impact on cirrhosis and ACLF. Mutations in the Interleukin-1 gene family, leading to a decrease of Interleukin-1 family member proteins, have been shown to improve ACLF pathology (Alcaraz-Quiles et al 2017). In this case, a reduction in pro-inflammatory mediators is wanted. However, cirrhosis can also lead to immunosuppression where Prostaglandins are important mediators (O'Brien et al. 2014).

Treatment of liver cirrhosis and ACLF refers to the clinical symptoms observed. Nutrition is mostly needed for many patients while many medications such as beta-blockers are discussed for their benefit (Ge et al. 2016). Due to the severity of liver cirrhosis, many research organizations established guidelines for diagnosis and treatment especially of the acute clinical consequences (EASL 2010, Fukui et al. 2016 and Runyon et al. 2013). Besides that, some treatments refer partially of fully to modulating the immune system. Human serum albumin is a treatment option for decompensated cirrhosis due to its binding affinity to many ligands in the blood even though not all mechanisms are completely understood (Arroyo et al.

2014). Plus, G-CSF is used to mobilize new immune cells from the bone marrow mainly to get rid of infections (Chavez-Tapia et al. 2015).
Taken together, liver cirrhosis and ACLF mark two critical illnesses with high mortality and limited options for effective treatment. Due to the high involvement of the immune system, the local one in the liver and systemic one in peripheral blood, we need to gain a better understanding of immunoregulation in these disease for new effective treatment strategies.

1.3. Interferons as mediators of defense

Interferons comprise a large family of proteins that induce the expression of antiviral factors thereby shaping elimination of pathogens. They are grouped into type I (containing e.g. IFNα and IFNβ) type II (IFNγ) and type III interferons (IFNλ family) which all bind to distinct receptors (Liu et al. 2012 and Kotenko et al. 2003). Interferons are key players in innate and adaptive immune responses. Though their function is partially overlapping, type 1 (mainly IFN-α, -β) and type 3 IFNs (IFN-λ1-3) exert early innate immune responses against viruses such as hepatitis B virus (HBV) or hepatitis C virus (HCV), whereas type 2 IFNs (only IFN-γ) are considered to shape consecutive adaptive immune responses (Heim et al. 2013).

Whereas IFNα had been a therapeutic option for Hepatitis C virus infection, IFNλ also demonstrated strong antiviral properties against this pathogen (Friborg et al. 2015). In mice, IFNα impacts strongly on Kupffer cells in acute hepatitis (Borst et al. 2017). Even though Interferons all “aim” to defend from pathogens the types of answers can be quite distinct (Liu et al. 2012). Importantly, both type I and type III IFNs signal through the JAK-STAT pathway and thereby induce a large number of overlapping, antivirally active interferon-stimulated genes (ISGs), although type I and type III IFNs bind to distinct receptors, the IFN-α receptor (IFNAR) complex, and the heterodimeric IL28-Rα/IL-10R2 receptor complex, respectively (Heim et al. 2013 and Wack et al. 2015). Of note, expression of the IL28-Rα/IL-10R2 receptor complex is restricted to hepatocytes, epithelial cells, and plasmacytoid dendritic cells, in contrast to the ubiquitously expressed IFNAR (Kotenko et al. 2003).

As the restricted expression of the type III IFN receptor results in an improved tolerability of pegylated IFN-λ (PEG-IFN-λ) compared to PEG-IFN-α, PEG-IFN-λ is a potential drug for the treatment of chronic hepatitis B (Chan et al. 2016). Interferon-stimulated genes can also be classified by functionality (de Veer et al. 2001). For better overviews, databases such as Interferome were created (Samarajiwa et al. 2009)

1.4. The cytokine Interleukin-7: Properties. signaling and usage

Interleukin-7 (IL-7) is a non-redundant cytokine required for an appropriate differentiation, maturation and survival of T cells, NK cells and γδ T cells (Mackall et al. 2011). Interleukin-7 also influences the functionality of ILCs (Yang et al. 2018). The best described cellular sources of Interleukin-7 are stromal cells of the primary lymphatic tissues, in which Interleukin-7 is expressed constitutively (Mackall et al. 2011). However, a number of recent reports have revealed that Interleukin-7 expression can be induced by tumor necrosis factor-(TNF)-α, interferon-(IFN)-γ or even cholic acid in several non-lymphatic tissues (Sawa et al. 2009 and Jiang et al. 2018). Yet, the role of locally produced IL-7 in peripheral organs remains poorly understood.

The Interleukin-7 receptor is a heterodimer of an α-chain (termed CD127) and the common γ chain (termed CD132). The Interleukin-7 receptor α-chain can also appear as a soluble protein. Soluble CD127 levels are altered in diseases e.g. in HIV (Crawley et al. 2010). While some publications claim an inhibitory effect of soluble CD127 on Interleukin-7 (Crawley et al. 2010), there is also evidence about soluble CD127 enhancing Interleukin-7 signaling (Lundström et al. 2013). Conformational changes of the Interleukin-7 receptor components are important for efficient signaling (Goh et al. 2017 and McElroy et al. 2012). Interleukin-7 activates several downstream pathways linked to several functional outcomes (Crawley et al. 2014). The most prominent downstream pathways, where

phosphorylation occurs, are the Jak/Stat pathway with Stat5 as the most prominent protein and the PI3K pathway (Crawley et al. 2014).
However, also other downstream targets regarding phosphorylation have been identified and are under further investigation regarding functional outcomes (Aiello et al. 2018). Besides the full-length protein, splice variants of Interleukin-7 have been described (Vudattu et al. 2009). Like the full-length protein, some variants act on adaptive and innate immune cells (Rane et al. 2017). Yet, some splice variants, such as the one lacking exon 5, have adverse effects, such as the promotion of cancer (Yang et al. 2014).

However, like other immunological mediators, Interleukin-7 needs to be restricted as it plays e.g. a major role in murine chronic colitis (Okada et al. 2005). Plus, Interleukin-7 needs to be perfectly balanced as an overshoot or mutations can contribute to autoimmune diseases such as rheumatoid arthritis (Pickens et al. 2011) and Graft-versus-host-disease (Lundström et al. 2012). In addition, mutations in Interleukin-7 pathway genes are found in acute lymphoblastic leukemia of B- and T-cell origin (Lundström et al. 2012). Yet, Interleukin-7 is also of importance and in clinical trials for immunotherapy of cancer (Pellegrini et al. 2009) or sepsis (Francois et al. 2018).

The liver is the place of fetal hematopoiesis and conditional knockout of Interleukin-7 in murine hepatocytes has been demonstrated to affect B cell development in fetal stages (Liang et al. 2012). In the adult liver, abrogation of Interleukin-7 decreases hepatic T and NKT cell populations (Liang et al. 2012). Interleukin-7 can also be used as a biomarker for certain liver diseases such as acute cholangitis (Suwa et al. 2017). In addition, Interleukin-7 is upregulated in primary biliary cholangitis (Jiang et al. 2018).

Of note, Interleukin-7 has been demonstrated to be inducible in the murine liver upon LPS stimulation, which has a severe impact on hepatic T cell responses (Sawa et al. 2009). These findings suggest a role for Interleukin-7 in hepatic immunology and inflammation.

1.5. Aim of the thesis

The thesis aims to understand the contribution of Interleukin-7 to hepatic immunology in health and disease. This is achieved by a sequential approach to target three major aspects. First, we aim to identify the exact cellular source of hepatic Interleukin-7 and mediators of this signaling pathway. Second, we intend to determine immune cells able to respond to Interleukin-7 signals and characterize the functional consequences of this signaling. For that part, we focus on macrophages as an example of innate immune cells as the impact of Interleukin-7 on these cells is not completely understood and because innate immune cells are known to contribute to liver pathology to a large extent. Last, we aim to assess the importance of the Interleukin-7 axis in patients with chronic liver disease. Therefore, we want to address the question whether Interleukin-7 is beneficial or adversely in this type of liver pathology and whether future therapeutic options should enhance or repress the Interleukin-7 signaling pathway.

To answer these questions, we mostly used cell culture approaches. Human hepatocytes and endothelial cells were stimulated with different Interferons and manipulated the expression level of known Interferon responsive factors. We tested various immune cells from peripheral blood for the presence of the Interleukin-7 receptor and for the downstream signaling upon Interleukin-7 binding. As examples of functional consequences, changes in metabolite levels, such as glucose, lactate, and ATP, in cellular viability and endotoxin tolerance were assessed. Finally, levels of Interleukin-7, of Interleukin-7 receptor positive cells and of signal transduction components were measured in CLD patient samples.

2. Material

This section serves as an inventory of all materials used throughout this thesis. It is divided into subsections, where similar types of materials are grouped to provide a better overview.

2.1. General reagents

Table 1 enumerates all general reagents, for example, chemicals used for self-made buffers.

Table 1: List of general reagents

Item	Cat. No	Company
Acrylamide solution	3029.2	Carl Roth
APS	A1142	PanReac AppliChem
Bisbenzimide H 33342 trihydro-chloride	B2261	Sigma Aldrich
Brilliant Stain Buffer	563794	BD
BSA	A7906	Sigma Aldrich
Cell proliferation reagent WST-1	05015944001	Roche
CHIR99021	SML1046	Sigma Aldrich
cOmplete, Mini, EDTA-free protease inhibitor cocktail tablets	11836170001	Roche
Coomassie Plus Protein Assay Reagent	1856210	Thermo Fisher
Cycloheximide	C7698	Sigma Aldrich
$C_{24}H_{39}NaO_4$	D6750	Sigma Aldrich
DMSO	A994.1	Carl Roth
DTT	10 197 777 001	Roche

Item	Cat. No	Company
EDTA	A1103	PanReac AppliChem
EGTA	E4378	Sigma Aldrich
Ethanol	16368	Sigma Aldrich
Etoposide	E1383	Sigma Aldrich
Fluoromount-G	0100-01	Southern Biotech
Glycerol	A1123	PanReac AppliChem
Glycine	G7126	Sigma Aldrich
Goat serum	G9023	Sigma Aldrich
Human Fc-Block	130-059-901	Miltenyi Biotec
IFNα2	Gift from Roche	
IFNγ	300-02	Peprotech
IFNλ2	300-02K	Peprotech
$KHCO_3$	121480	PanReac AppliChem
Laemmli sample buffer 2X	S3401	Sigma
LPS	ALX-581-007-L002	Enzo Life Sciences
Luminata Forte	WBLUF0500	Merck Millipore
Methanol	322415	Sigma Aldrich
$MgCl_2$	A4425	PanReac AppliChem
Monensin	00-4505-51	eBioscience
NaCl	S3014	Sigma Aldrich
Na_2EDTA	443885J	VWR Chemicals
NaF	S1504	Sigma Aldrich
$Na_4P_2O_7$	P8010	Sigma Aldrich
Na_3VO_4	S6508	Sigma Aldrich
NH_4Cl	A3260	PanReac AppliChem
Non-fat milk powder	T145.2	Carl Roth
NP-40	A1694	PanReac AppliChem
Paraformaldehyde	1040051000	Merck Millipore
PhosSTOP phosphatase inhibitor cocktail tablets	04906837001	Roche
PMSF	10837091001	Roche

Item	Cat. No	Company
Ponceau S solution	P7170	Sigma Aldrich
Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards	1610375	Bio-Rad
rhIL-7	200-07	Peprotech
Roti® Histol	6640.4	Carl Roth
SDS	811030	MP Biomedicals
Target Retrieval Solution pH 6	S1700	Dako
TEMED	2367.2	Carl Roth
Tris ultrapure	A1086	PanReac AppliChem
Triton X	3051.3	Carl Roth
Trypan blue solution	T8154	Sigma Aldrich
Tween20	A1389	PanReac AppliChem
Ultra-pure water	10977035	Thermo Fisher
7-AAD	420404	BioLegend

2.2. Cells and cell culture equipment

Table 2 states all cells, culture media and reagents necessary for isolation and culture of mammalian cells.

Table 2: List of cells and cell culture equipment

Item	Cat. No	Company
Cells (section 3.1.)		
HepG2 cells	HB-8065	ATCC
HLSEC	5000	ScienCell Research Laboratories
Huh7.5 cells	provided by Charles M. Rice, New York	
General cell culture equipment (section 3.1.)		
DMEM	41965039	Thermo Fisher
ECM	1001	ScienCell Research Laboratories

Item	Cat. No	Company
RPMI 1640	21875034	Thermo Fisher
FBS	F9665	Sigma Aldrich
FBS	0025	ScienCell Research Laboratories
Penicillin/Streptomycin	P0781	Sigma Aldrich
Penicillin/Streptomycin	0503	ScienCell Research Laboratories
ECGS	1052	ScienCell Research Laboratories
Fibronectin	8248	ScienCell Research Laboratories
DPBS	14190096	Thermo Fisher
Trypsin-EDTA solution	T3924	Sigma Aldrich
Immune cell isolation and culture equipment (section 3.2.)		
Biocoll	L6115	Biochrom
HBSS	L2055	Biochrom
Human CD14 MicroBeads	130-050-201	Miltenyi Biotec
Human naïve CD4+ T cell isolation kit II	130-094-131	Miltenyi Biotec
LS column	130-042-401	Miltenyi Biotec
MidiMACS separator	130-042-301	Miltenyi Biotec
rhGM-CSF	300-03	Peprotech
rhIL-4	200-04	Peprotech
rhM-CSF	300-25	Peprotech
30µm pre-separation filter	130-041-407	Miltenyi Biotec
siRNA transfection (section 3.12.)		
Lipofectamine® RNAiMAX	13778075	Thermo Fisher
Opti-MEM®	31985070	Thermo Fisher

2.3. Antibodies

Table 3 lists all antibodies used in this thesis with reference to the methods they were used for. Details regarding, for example, the dilution of the antibodies are stated in the methods section.

Table 3: List of all antibodies

Target	Conjugate	Method	Cat.No.	Company
β-Actin	none	WB	A1978	Sigma Aldrich
γ-Adaptin	none	WB	610385	BD Bioscience
CD3	FITC	FC	300452	BioLegend
CD33	BV510	FC	303422	BioLegend
CD68	none	IHC	M0876	Dako
CD127	none	WB, IF	MAB306	R&D Systems
CD127	BV605	FC	351334	BioLegend
CD132	none	WB, IF	AF284	R&D Systems
CK-18	none	IHC	M7010	Dako
GSK3	none	WB	5676	Cell Signaling Technologies
GSK3	none	IHC	Bs7332R	Bioss
IL-7	none	WB	ab103618	Abcam
IRF-1	none	WB	8478	Cell Signaling Technologies
IRF-1	none	IHC	orb253281	Biorbyt
Lamin B	none	WB	sc-365962	Santa Cruz Biotechnology
pGSK3 Ser21/9	none	WB	8566	Cell Signaling Technologies
pStat5 Tyr694	none	WB	4322	Cell Signaling Technologies
Stat5	none	WB	9363	Cell Signaling Technologies
Goat IgG	HRP	WB	sc-2354	Santa Cruz Biotechnology
Mouse IgG	HRP	WB	405306	BioLegend
Mouse IgG	Alexa Fluor 555	IF, IHC	A21422	Thermo Fisher
Rabbit IgG	HRP	WB	NA934	GE Healthcare
Rabbit IgG	Alexa Fluor 633	IF, IHC	A21071	Thermo Fisher

2.4. Primer and siRNAs

All primers listed in table 4 were unmodified DNA Oligos designed with PrimerBlast (Ye et al. 2012) and OligoCalc (Kibbe 2007) and purchased from Eurofins Genomics and were cleaned by HPSF. Upon arrival the lyophilized primers were reconstituted in ultra-pure water at a concentration of 100µM and stored at -20°C until further use. All primers target the human mRNA sequence.

Table 4: List of all primers

Target	Direction	Sequence 5' --> 3'	T (Annealing)
CD127	forward	TGGAGACTTGGAAGATGCAG	58°C
CD127	reverse	AAGCACAGGTCAGTGAGTGC	58°C
CD132	forward	CTTTTCGGCCTGGAGTGGTG	61°C
CD132	reverse	ACGCAGGTGGGTTGAATGAA	61°C
GAPDH	forward	GAAGATGGTGATGGGATTTC	58°C
GAPDH	reverse	GAAGGTGAAGGTCGGAGTC	58°C
GBP5	forward	CGCAAAGGTTGGCGGCGATT	61°C
GBP5	reverse	AGCTGTGCAGCCTGTTCCTGC	61°C
IL-1β	forward	CGAATCTCCGACCACCACTAC	61°C
IL-1β	reverse	GCACATAAGCCTCGTTATCCC	61°C
IL-6	forward	GCCATCCACCTCTTCAGAACG	61°C
IL-6	reverse	CCGTCGAGGATGTACCGAATT	61°C
IL-7	forward	CCTGATCCTTGTTCTGTTGC	58°C
IL-7	reverse	TGATCGATGCTGACCATTAGA	58°C
IRF1	forward	CCACTCTGCCTGATGACCAC	61°C
IRF1	reverse	GGTGCTGTCCGGCACAACTT	61°C
ISG15	forward	TCCTGCTGGTGGTGGACAA	61°C
ISG15	reverse	TTGTTATTCCTCACCAGGATGCT	61°C
TNFα	forward	GGCAGTCAGATCATCTTCTCGAA	58°C
TNFα	reverse	GAAGGCCTAAGGTCCACTTGTGT	58°C

All pre-designed siRNAs listed in table 5 were purchased from Thermo Fisher and were reconstituted at a concentration of 100 µM upon arrival.

Table 5: List of all siRNAs

Name	Assay ID	Cat.No.
Silencer™ Select Negative Control No. 1 siRNA		4390843
Silencer™ Select IRF1 siRNA	S7501	4392420
Silencer™ Select IRF1 siRNA	S7502	4392420

2.5. Buffers

Table 6 lists the composition of all self-made buffers in this thesis as well as the methods they were used for.

Table 6: List of all self-made buffers

Buffer and ingredients	Method
ACK lysis buffer ▪ 150mM NH_4Cl ▪ 10mM $KHCO_3$ ▪ 100µM Na_2EDTA ▪ pH 7.4	Immune cell isolation
Blocking buffer ▪ DPBS ▪ 5% goat serum	Immunofluorescence Immunohistochemistry
Blocking buffer ▪ 1X TBS-T ▪ 5% non-fat milk powder	Immunoblotting
Extraction buffer ▪ 10mM Tris pH 7.4	Cytosol/Nuclear extraction

Buffer and ingredients	Method
▪ 100mM NaCl ▪ 1mM EDTA ▪ 1mM EGTA ▪ 1mM NaF ▪ 20mM $Na_4P_2O_7$ ▪ 2mM Na_3VO_4 ▪ 1mM PMSF ▪ 1% Triton X-100 ▪ 10% Glycerol ▪ 0.1% SDS ▪ 0.5% $C_{24}H_{39}NaO_4$	
Hypotonic buffer ▪ 20mM Tris-HCl pH 7.4 ▪ 10mM NaCl ▪ 3mM $MgCl_2$ ▪ 250µM DTT ▪ 0.5% NP-40	Cytosol/Nuclear extraction
Luciferase lysis buffer ▪ 50mM Tris-HCl pH 7.4 ▪ 150mM NaCl ▪ 2mM EDTA ▪ 1% NP-40	ATP quantification
MACS buffer ▪ DBPS ▪ 0.5% FBS ▪ 2mM EDTA	Immune cell isolation
Resolving gel buffer ▪ 1.5M Tris pH 8.8 ▪ 0.4% SDS	Immunoblotting
RIPA buffer ▪ 50mM Tris pH 8	Immunoblotting

Buffer and ingredients	Method
▪ 150mM NaCl ▪ 1% NP-40 ▪ 0.5% $C_{24}H_{39}NaO_4$ ▪ 0.1% SDS	
Running buffer ▪ 25mM Tris pH 8.3 ▪ 190mM Glycine ▪ 0.1% SDS	Immunoblotting
Stacking gel buffer ▪ 0.5M Tris pH 6.8 ▪ 0.4% SDS	Immunoblotting
Staining buffer ▪ DPBS ▪ 0.5% BSA	Flow cytometry
10X TBS ▪ 200mM Tris pH 7.6 ▪ 1.5M NaCl 1X TBS-T ▪ 10% 10X TBS ▪ 0.1% Tween20	Immunoblotting
Transfer buffer ▪ 50mM Tris-HCl ▪ 40mM Glycine ▪ 130µM SDS ▪ 20% Methanol	Immunoblotting

2.6. Kits

Table 7 enumerates all kits used during the experiments.

Table 7: List of all commercial kits

Item	Cat. No	Company
ATP Bioluminescent Assay Kit	FLAA	Sigma Aldrich
Glucose Colorimetric/Fluorometric Assay Kit	K606	BioVision
Human IL-7 Quantikine HS ELISA Kit	HS750	R&D
Lactate Colorimetric/Fluorometric Assay Kit	K607	BioVision
peqGOLD Total RNA Kit	732-2871	VWR International
PrimeScript RT Reagent Kit	RR037A	Takara Clontech
Proteome Profiler Human XL Cytokine Array Kit	ARY022	R&D Systems
SYBR® Premix Ex Taq™ (Tli RNase H Plus), ROX Plus	RR42LR	Takara Clontech

2.7. Consumables

Table 8 provides an inventory of all consumables/plasticware used in this thesis.

Table 8: List of all consumables

Item	Cat. No	Company
6well cell culture plate	657160	Greiner Bio-One
12well cell culture plate	665180	Greiner Bio-One
96well cell culture plate	353072	Corning
96well Opti Plate	6005290	Perkin Elmer
Cell culture dish 10cm	664160	Greiner Bio-One
Cover slips round 12mm	1-6283	NeoLab
Cryovials	72.694.006	Sarstedt
FACS tubes	352003	Corning
Microscope slides	J1800AMNZ	Thermo Fisher
Monovettes EDTA	01.1605	Sarstedt
Monovettes Serum	01.1601	Sarstedt
Nitrocellulose membrane	10600001	GE Healthcare
PCR plates	72.1978.202	Sarstedt
PCR plates white	MLL-9651	Bio-Rad
Reaction tubes 1.5mL	72.690.001	Sarstedt
Reaction tubes 50mL	227261	Greiner Bio-One
Whatman paper	80-1106-19	GE Healthcare

2.8. Devices

Table 9 list all essential devices used for the experiments. As most companies do not display a catalog number on their websites, only the company name is stated in this section.

Table 9: List of all devices

Device	Company
Analytical/Precision balance	Ohaus
BD LSRFortessa Cell Analyzer	BD Biosciences
BioPhotometer	Eppendorf
Centrifuge 5702R	Eppendorf
EnVision® 2104 Multilabel Plate Reader	Perkin Elmer
FluoView FV1000 microscope	Olympus
Fusion FX imaging system	Vilber Lourmat
GeneAmp PCR system 9700	Thermo Fisher
Heraeus Fresco 17 Centrifuge	Thermo Fisher
NanoDrop 2000 UV-Vis spectrophotometer	Thermo Fisher
PowerPac HC Power Supply	Bio-Rad
Sonoplus Sonicator	Bandelin
StepOnePlus real-time PCR system	Thermo Fisher
Thermoshaker	Universal Labortechnik
Trans-Blot SD semi-dry transfer cell	Starlab
Vortex mixer	NeoLab
4-15C Benchtop Centrifuge	Sigma

2.9. Patients

Between August 2013 and until April 2017, consecutive patients admitted to the University Hospital Frankfurt, Germany, were prospectively included in our liver cirrhosis cohort study if they were diagnosed with compensated liver cirrhosis,

decompensated liver cirrhosis or acute-on-chronic liver failure. The definition of this pathology was stated according to the criteria of the CLIF-EASL consortium (Moreau et al. 2013). Peripheral blood was collected at baseline of admission. Acute decompensation of liver cirrhosis meant the presence of complications of liver cirrhoses, which could manifest in new onset/progression of hepatic encephalopathy graded by West-Haven criteria, gastrointestinal hemorrhage, bacterial infection, or ascites grade II-III. Patients were excluded, if they were younger than 18 years, in case of pregnancy or breastfeeding, if they suffered from of hepatocellular carcinoma (HCC) beyond Milan criteria, if they were infected with human immunodeficiency virus (HIV), or if they went immunosuppressive therapy. All patients provided written informed consent to the study protocol, and the study was approved by the local ethics committee of the University Hospital Frankfurt, Germany.

3. Methods

This section states all methods applied to the question of hepatocellular immunoregulation. The first subsections describe the process of acquiring cells and patient samples as the bases of all analysis. The actual methods thereafter are listed in the order as they appear in the figures of the results section.

3.1 Cell culture

To study the role of Interleukin-7 in the hepatic environment, we used two different human hepatic cancer cell lines. Human Huh7.5 cells (Blight et al. 2002) were provided by Charles M. Rice, The Rockefeller University, New York, NY and human HepG2 cells were purchased from ATCC. Both cell lines were cultured in Dulbecco's modified Eagle medium with 4.5g/L glucose (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin solution (P/S) at 37°C and 5% CO_2. Cells were passaged

into new flasks when reaching a confluency of about 80%, which was about three times a week. The maximum number of passages was limited to 35.

In order to include another cellular entity and to investigate primary cells, human hepatic sinusoidal endothelial cells (HLSEC) were also used for experiments. These cells were cultured in endothelial cell medium (ECM) supplemented with 5% fetal bovine serum (FBS), 1% Penicillin/Streptomycin solution (P/S) and 1X endothelial cell growth supplement (ECGS) at 37°C and 5% CO_2. Endothelial cells were grown in flasks coated with 2µg/cm^2 bovine plasma fibronection according to manufacturer's instructions. Cells were subcultured when they reached 90% confluency, which was about twice a week, and these primary cells were used between passage three and seven.

3.2. Isolation and culture of human immune cells from buffy coat

All primary immune cells were isolated from male healthy donor buffy coats (provided by the German Red Cross Blood Donor Service Baden-Wuerttemberg Hessen) by using Ficoll density gradient centrifugation. The blood was transferred into three 50mL Falcon Tubes and diluted with Hank's balanced salt solution (HBSS) to a final volume of 40mL in each Falcon tube. Four separate Falcon tubes were filled with 20mL of Biocoll Separating Solution and the blood was carefully placed on top of the Biocoll to obtain separate layers. The Falcon tubes were centrifuged at 600rcf for 35 minutes at room temperature with the centrifuge brake switched off. The resulting layers of blood fractions after centrifugation are displayed in figure 1.

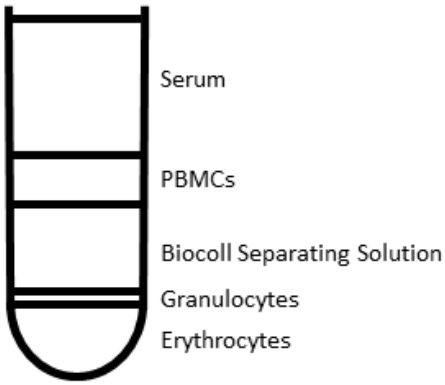

Figure 1: Scheme of blood cell layers resulting of Ficoll density gradient centrifugation.

The cloudy white layer containing the peripheral blood mononuclear cells (PBMC) was carefully transferred into a new Falcon tube, filled with HBSS to 50mL and centrifuged at 300rcf for 10 minutes at 4°C with the centrifuge brake switched on again. The supernatant was aspirated and the pellet was resuspended in 50mL HBSS. For eradication of platelets, the cells were spun down at 200rcf for 10 minutes at 4°C. The supernatant was aspirated and the pellet was resuspended in 5mL of ACK lysis buffer to remove any residual erythrocytes. After 3 minutes of incubation time, 45mL of HBSS was added and the cells were centrifuged at 300rcf for 10 minutes at 4°C. The supernatant was removed, the cells were resuspended in 10mL HBSS and cell count was determined using a Neubauer chamber.

In order to isolate naïve CD4+ T cells and monocytes, the principle of magnetic separation was applied. After cell counting, cells were centrifuged at 300rcf for 10 minutes at 4°C. The supernatant was discarded and the cells were resuspended in MACS buffer. For isolation of naïve CD4+ T cells, the human naïve CD4+ T cell isolation kit II was used. The cells were incubated with 10µL of naïve CD4+ T cell Biotin-Antibody cocktail II per 10^7 total cells for 5 minutes at 4°C. Afterwards, 30µL of MACS buffer per 10^7 total cells were added and the cells were then incubated with naïve CD4+ T cell MicroBead cocktail II for 10 minutes at 4°C. Meanwhile, a 30µm pre-separation filter was placed in an LS Column and both of them were then positioned in a MidiMACS separator. The column was equilibrated with 3mL of MACS buffer before application of the cells onto the column. The flow-through,

which contains the unlabeled naïve CD4+ T cells, was collected. The column was washed with 3mL of MACS buffer and the flow-through was collected again. Cell count was determined using a Neubauer chamber and defined numbers of cells were seeded into cell culture plates depending on the desired experiment.

For the isolation of primary monocytes, the human CD14 MicroBeads were used. The cells were incubated with 10µL of CD14 MicroBeads per 10^7 total cells for 15 minutes at 4°C. Afterwards, MACS buffer was added to a volume of 50mL and the cells were centrifuged at 300rcf for 10 minutes at 4°C. Meanwhile, a 30µm pre-separation filter was placed in an LS Column and both of them were then positioned in a MidiMACS separator. The column was equilibrated with 3mL of MACS buffer before application of the cells, resuspended in 500µL MACS buffer per 10^8 cells, onto the column. The column was washed three times with 3mL of MACS buffer. Afterwards, the column was removed from the separator and the labeled monocytes are eluted by applying 5mL of MACS buffer onto the column and pressing the plunger into the column. Cell count was determined using a Neubauer chamber and defined numbers of cells were seeded into cell culture plates depending on the desired experiment.

For the generation of monocyte-derived macrophages (MDMs) or monocyte-derived dendritic cells (DCs) cells were seeded at a density of $0.75*10^6$ cells per 6well or $2*10^6$ cells per 10cm dish. For DCs cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin/Streptomycin solution (P/S), 10ng/mL rhGM-CSF and 20ng/mL rhIL-4 for 8 days. These cells resemble immature DCs (iDCs), which can be developed into mature DCs (mDCs) by adding 10ng/mL LPS to the culture media on the last two days of culture. For MDMs culture medium was supplemented with 20ng/ml rhM-CSF for 6 days instead of rhGM-CSF/rhIL-4 for 8 days. After the maturation period, the culture medium was switched to RPMI 1640 containing 10% FBS and 1% Penicillin/Streptomycin and cells were incubated at 37°C and 5% CO_2.

3.3. Isolation of peripheral blood mononuclear cells from patients

For human immune cells, peripheral blood of patients and healthy donors was collected in monovettes containing 1.6mg/mL potassium EDTA. The blood was transferred into one 50mL Falcon tube and residual blood droplets were flushed into the Falcon tube with Dulbecco's Phosphate-Buffered Saline (DPBS). A separate 50mL Falcon tube was filled with Biocoll Separating Solution and the blood was carefully pipetted on top of the Biocoll to obtain two layers. The Falcon tubes were centrifuged at 600rcf for 20 minutes at 4°C with the centrifuge brake switched off. The resulting layers of blood fractions after centrifugation are displayed in figure 1. The PBMCs were carefully transferred into a new Falcon tube, filled with DPBS to 50mL and centrifuged at 300rcf for 10 minutes at 4°C with the centrifuge brake switched on again. The supernatant was aspirated and the washing with DPBS was repeated once. Finally, cells were resuspended in 1mL 90% FBS/10%DMSO and cryopreserved in liquid nitrogen until further usage.

3.4. Isolation of human macrophages from ascites fluid

For the preparation of primary “peritoneal” macrophages about one liter of ascites fluid was divided into 50mL Falcon tubes and centrifuged at 600rcf for 10 minutes at room temperature. 45mL of the supernatant was aspirated and cells were resuspended in the residual 5mL supernatant. All liquids were pooled into two remaining Falcon tubes and centrifuged again at 600rcf for 10 minutes at room temperature. The supernatant was aspirated; cells were washed with DPBS and pelleted at 600rcf for 10 minutes at room temperature. The supernatant was discarded and the cells were seeded into cell culture dishes to adhere for 16 hours. On the next day, suspension cells were discarded by flushing the cell culture dish with DPBS vigorously for several times. Residual adherent cells

resembled the population of human ascites macrophages, which could be cultured in RPMI 1640 supplemented with 10% FBS and 1% Penicillin/Streptomycin.

3.5. Preparation of human serum

For human serum peripheral blood of patients and healthy donors was collected in monovettes containing clot activator. The monovettes were centrifuged at 2000rcf for 10 minutes at 4°C. Cell-free serum was carefully transferred into cryovials and cryopreserved at -80°C until further use.

3.6. Isolation of RNA from human cells

Isolation of total RNA from cell culture was achieved using the peqGOLD Total RNA Kit, which is a column-based method. After stimulation, cells were washed once with DPBS and then lysed in the supplied lysis buffer. Complete lysis was ensured by pipetting up and down and the liquids were transferred onto a DNA-removing-column and then centrifuged at 12000rcf for 1 minute at room temperature. Flow-through was mixed with equal volume of 70% ethanol and applied onto the RNA-binding-column, where the binding was ensured by centrifugation at 10000rcf for 1 minute at room temperature. After several washes, high-quality RNA was eluted in 50µL RNase free water at 5000rcf for 1 minute. RNA concentration was measured by using a Nanodrop spectrophotometer at 260nm and quality was monitored by regarding the 260nm/280nm ratio and the 260nm/230nm ratio.

3.7. Reverse transcription

Before RNA expression could be analyzed via quantitative Real-Time PCR, complementary DNA (cDNA) needed to be synthesized from the template RNA.

To achieve this, the PrimeScript RT Reagent Kit was used, and 500ng was used as a substrate and mixed with the components listed in table 10 in 96wells.

Table 10: Pipetting scheme for reverse transcription

Reagent	**Amount**
Template RNA	XµL (equivalent to 500ng)
5X PrimeScript Buffer	2µL
PrimeScript RT Enzyme Mix I	0.5µL
Oligo dT Primer (50µM)	0.5µL
Random hexamers	0.5µL
RNase free water	Add 10µL

The mixture of components was pulse centrifuged to eliminate air bubbles in these low-volume samples and was incubated in a PCR-Cycler according to the programme listed in table 11.

Table 11: Cycler programme for reverse transcription

Temperature	**Duration**
37°C	15 minutes
85°C	5 seconds
4°C	∞

After the reaction, samples were diluted with 90µL of ultra-pure water before being processed in the PCR reaction.

3.8. qRT-PCR

For semiquantitative analysis of cDNA presence in the samples, SYBR Green PCR was applied. SYBR Green binds double-stranded DNA, therefore PCR products are detected. The mix for the PCR reaction was set up in white PCR-plates as listed in table 12.

Table 12: Pipetting scheme for qRT-PCR

Reagent	**Amount**
Template cDNA	1µL
SYBR® Premix Ex Taq™ (Tli RNase H Plus), ROX Plus	7,5µL
Forward Primer (10µM stock)	0.45µL
Reverse Primer (10µM stock)	0.45µL
Ultra-pure water	5.6L

The mixture of components was pulse centrifuged to eliminate air bubbles in these low-volume samples and was incubated in a StepOnePlus Real-Time PCR system according to the programme listed in table 13.

Table 13: Cycler programme for qRT-PCR

Step	**Temperature**	**Duration**
1: Initial denaturation	95°C	10 minutes
2: Denaturation	95°C	20 seconds
3: Annealing	Dependent on primer	30 seconds
4: Extension	72°C	60 seconds
5: Final extension	72°C	10 minutes

Steps 2 – 4 were repeated for 40 cycles. To confirm product specificity, melt curve analysis was applied. GAPDH was used as a housekeeping control. Calculations of gene expression were based on the Threshold Cycle (ct-value) of the target gene, which was automatically computed by the StepOne Plus. The equation of

$2^{\Delta\Delta ct}$ was used with Δct = ct (gene of interest) – ct (housekeeping) and ΔΔct = Δct (mock) – Δct (stimulus). All experiments were performed according to the MIQE suggestions (Bustin et al. 2009).

3.9. Immunoblotting

Immunoblotting was used to detect distinct proteins in cell culture lysate. To this end, cells were stimulated, washed once with DPBS and then lysed in 50µL RIPA buffer for 30 minutes on ice. Afterwards, lysates were centrifuged at 16000rcf for 20 minutes at 4°C. The supernatant containing the proteins was transferred to a new tube and stored at -80°C until Immunoblotting.

As the next step, proteins were separated using SDS polyacrylamide gel electrophoresis. Usually, a resolving gel containing 10% acrylamide was used for the detection of proteins > 70kDa, otherwise, the concentration of acrylamide was 12%. Table 14 lists all components of the resolving and stacking gel.

Table 14: Components of polyacrylamide gels

Resolving Gel	**Stacking Gel**
Ultra-pure water	Ultra-pure water
Resolving gel buffer	Stacking gel buffer
50% Glycerol	
Acrylamide solution	Acrylamide solution
10% APS	10% APS
TEMED	TEMED

Protein concentration was measured at 595nm on a BioPhotometer after mixing the samples with Coomassie. In most cases, 25µg of protein were mixed 1:1 with 2X Laemmli sample buffer, boiled at 95°C for 5 minutes and applied onto the gel with Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards as a

size control. Gels were immersed in running buffer and were run at 100V for approximately 3 hours (exact time was dependent on the desired protein to detect).

After gel electrophoresis proteins were transferred onto a nitrocellulose membrane in a semi-dry transfer chamber. To this end, Whatman paper and the membrane were wetted with transfer buffer and placed together with the gel in the transfer chamber. Electricity was applied at 55mA per gel for 2 hours. The efficiency of the transfer was controlled by Ponceau staining which was washed away with ultra-pure water afterwards.

After transfer, membranes were blocked with blocking buffer for 60 minutes at room temperature. In the meantime, primary antibodies were diluted 1:1000 in TBS-T / 5% BSA. Membranes were incubated with the primary antibodies overnight at 4°C. In the next day, membranes were washed three times with TBS-T before being incubated with the secondary antibody, diluted 1:5000 in TBS-T / 5% milk, for 60 minutes at room temperature. Afterwards, membranes were washed three times with TBS-T. As the secondary antibodies were conjugated to horseradish peroxidase (HRP), protein bands were visualized using 1mL of Luminata Forte Western HRP Substrate on a Fusion FX imaging system.

3.10. Measurement of extracellular Interleukin-7 via ELISA

The levels of secreted Interleukin-7 in cell culture supernatant and human serum were quantified using the Human IL-7 Quantikine HS ELISA Kit. Briefly, undiluted samples were used for Interleukin-7 detection and were incubated for 16 hours at room temperature in the ELISA plate. All other steps were performed according to manufacturer’s instructions. The optical density at 490nm of samples and standards were measured on an EnVision® 2104 Multilabel Plate Reader.

3.11. Membrane-based antibody array for cytokine detection

In order to detect and roughly quantify a large number of different cytokines in cell culture supernatant, the Proteome Profiler Human XL Cytokine Array Kit was applied. This immunoassay provides a nitrocellulose membrane on which different antibodies are spotted. Comparable to immunoblotting, this membrane was first blocked with a provided blocking buffer for 60 minutes at room temperature. Next, the blocking buffer was aspirated and the membrane was immersed in the desired supernatant at 4°C overnight. On the next day, the membrane was washed and incubated with provided detection antibody for 60 minutes at room temperature. The membrane was washed again then immersed with Streptavidin-HRP for 30 minutes at room temperature. After a final washing step, a provided chemoluminescence substrate was applied to the membrane and signals were analyzed on a Fusion FX imaging system.

3.12. Silencing of gene expression via siRNA-Transfection

For efficient knockdown of the desired protein target, Silencer Select siRNAs are used. Huh7.5 cells were seeded at a density of 250000 cells per 6well and were allowed to adhere overnight. On the next day, the normal culture medium was replaced by 2mL Opti-MEM®. Cells were incubated for one hour at 37°C at 5% CO_2 as priming for transfection. Meanwhile, the transfection complexes were prepared. Per 6well, 4µL of Lipofectamine® RNAiMAX Transfection Reagent was used to ensure transfection. The reagent was mixed well with 24 pmol of Silencer Select siRNA for IRF-1 knockdown or negative control in 400µL Opti-MEM®. This mix was incubated for 20 minutes at room temperature before being added dropwise to the cells. This cell culture was maintained for 24 hours before the Opti-MEM® was replaced by the normal culture medium. After another 24 hour incubation period the cells were lysed and efficient knockdown was verified by Immunoblotting, see section 3.9.

3.13. Analysis of cytosolic and nuclear protein fractions

The generation of separate cytosolic and nuclear protein fractions from cell culture was achieved by lysing cells sequentially in different lysis buffers. Huh7.5 cells were stimulated and washed with DPBS. The first lysis was performed in hypotonic buffer for 15 minutes on ice, which only disrupted the cell membrane but left the nucleus intact. Lysates were vortexed thoroughly and centrifuged at 10000rcf for 10 minutes at 4°C. The supernatant containing cytosolic proteins was transferred to a separate tube and stored at -80°C. The nuclear extract pellet was washed at least five times with DPBS. Afterwards, the pellet was lysed in extraction buffer and was kept for 30 minutes on ice with vortexing after every 10 minutes. For efficient lysis, pellets were sonicated before centrifugation at 20000rcf for 30 minutes at 4°C. The supernatant then contained the nuclear proteins and was stored at -80°C. Proper separation of the two fractions, as well as presence of desired proteins, was determined via Immunoblotting using γ-Adaptin and Lamin B as loading controls for cytosol and nucleus, respectively (see section 3.9.).

3.14. Immunofluorescence of cultured macrophages

The presence of the two subunits of the Interleukin-7 receptor, which are CD127 and CD132, was confirmed via immunofluorescence. To achieve this, about 10^5 monocyte-derived macrophages were matured on round coverslips placed in 12wells according to the protocol in section 3.2. After the maturation period, cell culture medium was removed and macrophages were fixed with 4% Paraformaldehyde for 15 minutes at room temperature. Afterwards, the fixing agent was removed and cells were washed three times with DPBS for five minutes each. To ensure efficient binding of antibodies, cells were permeabilized with 0.25% Triton X in DPBS for 10 minutes at room temperature. Next, cells were washed again and unspecific staining was inhibited by incubation with blocking buffer for 60 minutes at room temperature. Meanwhile, the primary antibodies

were diluted 1:100 in blocking buffer. After blocking the primary antibody solution was applied to the cells and the cells were incubated overnight at 4°C with constant agitation. On the next day, unbound antibodies were washed away with DPBS. The secondary antibodies were diluted 1:200 in blocking buffer. The antibody solution was applied to the cells and incubated for 60 minutes at room temperature in darkness. Afterwards, unbound antibodies were washed away again with DPBS. Nuclear counterstaining was achieved by incubating the cells with bisbenzimide H 33342 trihydrochloride (diluted 1:5000 in ultra-pure water) for 30 minutes at room temperature in darkness. After one last washing procedure, coverslips containing the MDMs were mounted onto microscope slides and fluorescence intensity was evaluated using an Olympus FluoView FV1000 microscope.

3.15. Quantification of glucose/lactate in cell culture supernatant

To get a first impression about macrophage metabolism, the Glucose Colorimetric/Fluorometric Assay Kit and the Lactate Colorimetric/Fluorometric Assay Kit. Both kits are based on the same principle. The desired molecule is metabolized by a provided enzyme mix and the resulted product reacts with a provided probe. The final reaction creates a product detectable either in a colorimetric or fluorometric way. In our experiments, MDMs were stimulated for different time points. Afterwards, the supernatant was transferred to a new tube and centrifuged at 300rcf for 10 minutes to remove cellular debris. The reaction mixes for the two assays were set up in 96wells as described in table 15.

Table 15: Pipetting scheme for the glucose and lactate assay

Component	Glucose Assay	Lactate Assay
Cell culture supernatant	2µL	50µL
Assay buffer	94µL	46µL
Enzyme Mix	2µL	2µL
Probe	2µL	2µL

The mixes were incubated for 30 minutes at room temperature protected from light. Afterwards, levels of the metabolites were measured at 570nm on an EnVision® 2104 Multilabel Plate Reader. For quantification, a standard curve was generated in the same way.

3.16. Bioluminescent quantification of ATP

To assess intracellular concentrations of ATP, a molecule linking metabolism, viability, and inflammation, the ATP Bioluminescent Assay Kit was used. The emission of light is catalyzed by oxidation of luciferin by the enzyme luciferase. As this reaction uses ATP, the amount of detectable light is proportional to the concentration of ATP present in the samples. In our experiments, MDMs were stimulated for different time points. Afterwards, cells were washed once with DPBS and lysed with 200µL of Luciferase lysis buffer for 15 minutes on ice. The lysates were then centrifuged at 17000rcf for 1 minute and 100µL of the supernatant were mixed with 100µL of ATP Assay Mix (diluted 1:500) in white 96well plates. Emission of light was measured on an EnVision® 2104 Multilabel Plate Reader and quantification was enabled by establishing an ATP standard curve.

3.17. Colorimetric WST-1 cellular viability assay

To assess changes in macrophage viability, cell proliferation reagent WST-1 was used. The assay based on an enzymatic reaction with the tetrazolium salt WST-1 as a substrate of mitochondrial dehydrogenases. The reduced reaction product is Formazan, a dark red compound that can be measured with a spectrophotometer. For our purposes, macrophages were maturated in 96wells as described in section 3.2. Afterwards, they were stimulated for different time points. 30 minutes before the end of stimulation, 10µL of WST-1 was added to the 96wells and incubation at

37°C and 5% CO_2 was continued. Afterwards, levels of synthesized Formazan were measured at 450nm on an EnVision® 2104 Multilabel Plate Reader.

3.18. Fluorescent immunohistochemistry analysis of liver sections

The presence of the several proteins involved in Interleukin-7 signal transduction was confirmed via fluorescent immunohistochemistry. Human paraffin-embedded liver tissue was provided by the pathology and was cut in 10µm slices and placed on microscope slides. Tissue was deparaffinized by immersing the slides in Roti® Histol three times for five minutes each. Afterwards, the tissue was rehydrated with descending concentrations of ethanol (95%, 70%, and 50%) and finally with ultra-pure water (all two times for 10 minutes each). Next, epitope retrieval was performed by boiling the slides in Target Retrieval Solution pH 6 for 20 minutes. Slides were washed three times with DPBS for five minutes before being blocked with blocking buffer for 60 minutes at room temperature. Meanwhile, the primary antibodies were diluted 1:100 in blocking buffer. After blocking, the primary antibody solution was applied to the cells and the cells were incubated overnight at 4°C. On the next day, unbound antibodies were washed away with DPBS. The secondary antibodies were diluted 1:200 in blocking buffer. The antibody solution was applied to the cells and incubated for 60 minutes at room temperature in darkness. Afterwards, unbound antibodies were washed away again with DPBS. Nuclear counterstaining was achieved by incubating the slides with bisbenzimide H 33342 trihydrochloride (diluted 1:5000 in ultra-pure water) for 30 minutes at room temperature in darkness. After one last washing procedure, coverslips were mounted onto the liver section slides and fluorescence intensity was evaluated using an Olympus FluoView FV1000 microscope.

3.19. Multicolor Flow cytometry

PBMCs from patients and healthy controls were analyzed using Multicolor Flow cytometry. Cells were counted using a Neubauer chamber and $1.5*10^6$ cells were transferred into a new FACS tube for the staining procedure. Cells were washed with staining buffer and centrifuged at 500rcf for 5 minutes at 4°C. The supernatant was aspirated and the pellet was resuspended in 50µl of staining buffer. 2µL of Human Fc-Block were added and the cells were incubated on ice for 10 minutes. Meanwhile, all antibodies and 7-AAD displayed in table 16 were mixed in 50µL Brilliant Stain Buffer.

Table 16: Pipetting scheme for multicolor flow cytometry

Target	Fluorochrome	Clone	Cat.No.	Amount [µl]
CD3	FITC	UCHT1	300452	0,5
CD33	BV510	WM53	303422	1
CD127	BV605	A019D5	351334	2
7-AAD	PE-Cy5		420404	2

The staining was incubated on ice for 25 minutes in the dark. Afterwards, cells were washed with 500µL of DPBS/0.5% BSA and centrifuged at 500rcf for 5 minutes at 4°C. The supernatant was aspirated and the cells were resuspended in 300µL staining buffer. Fluorescence was measured on a BD LSRFortessa and the immune cell populations were gated and analyzed as displayed in figure 2.

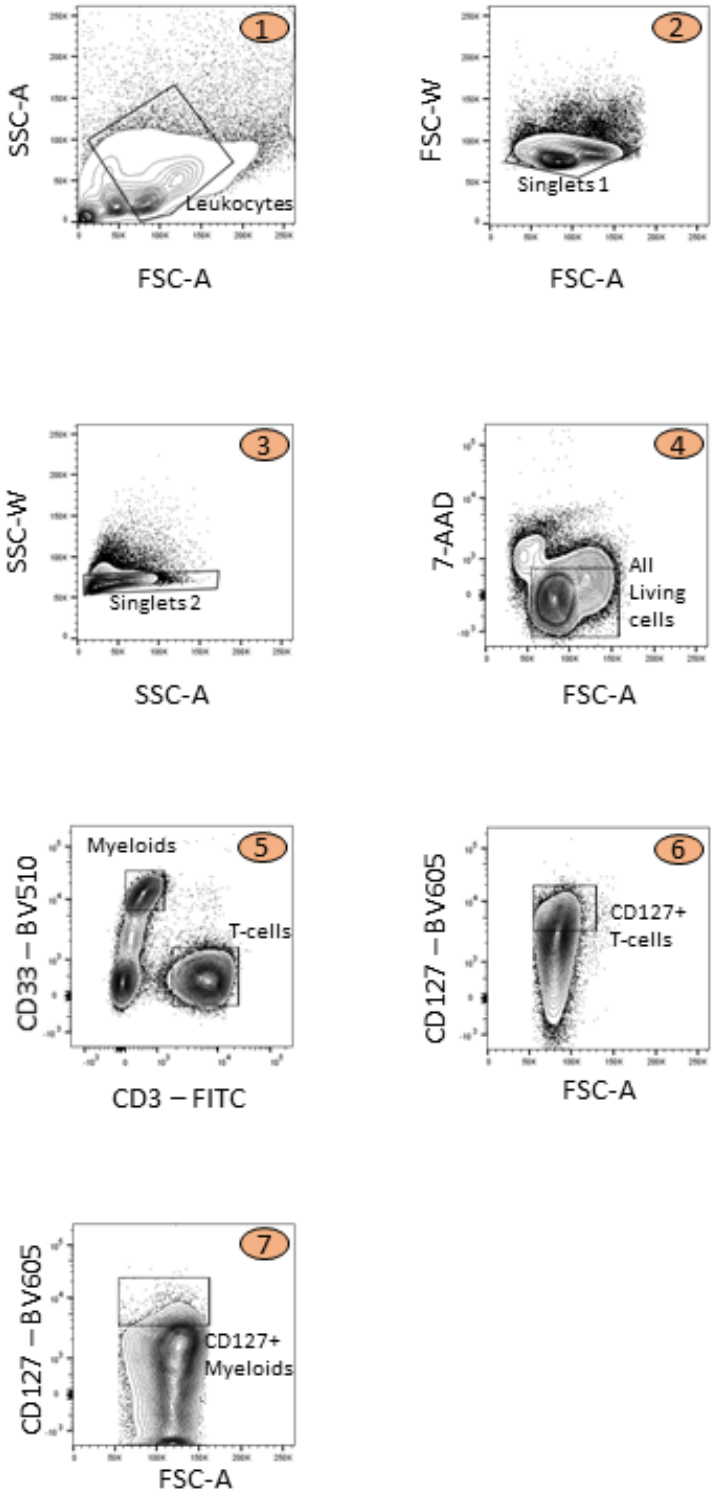

Figure 2: Example of the gating strategy for CD127 positive immune cells. It starts with gating on all myeloid and lymphocyte subsets, thereby termed "Leukocytes" (1) followed by singlet gating (2 and 3). In order to include only "All living cells", live-dead staining 7-AAD was applied (4). Gating on CD3 vs. CD33 reveals two subsets (5): CD3+ cells (T-cells) and CD33+ cells (Myeloids). The expression of CD127 is analyzed in T-cells (6) and in myeloids (7).

4. Results

This part contains all experimental data to provide potential answers about hepatic Interleukin-7 production, possible recipient cells, and clinical consequences.

4.1. Part I: Origin and regulation of hepatocellular IL-7 expression

Regarding the inducibility of Interleukin-7 in murine liver upon LPS stimulation, we investigated whether this phenomenon could also be observed in human cells. In addition, we aimed to identify the exact cellular origin of Interleukin-7 and possible signaling mediators.

4.1.1. IL-7 expression in hepatic cells is limited to distinct types of IFNs

For our first experiment, we aimed to answer the question, which types of interferon were capable to induce Interleukin-7 expression *in vitro*. To this end, we stimulated Huh7.5 cells with IFNα (Type I IFN), IFNγ (Type II IFN) or IFNλ2 (Type III IFN) for six hours. The concentrations used were determined by a former M.D. student in the lab. The results are displayed in figure 3.

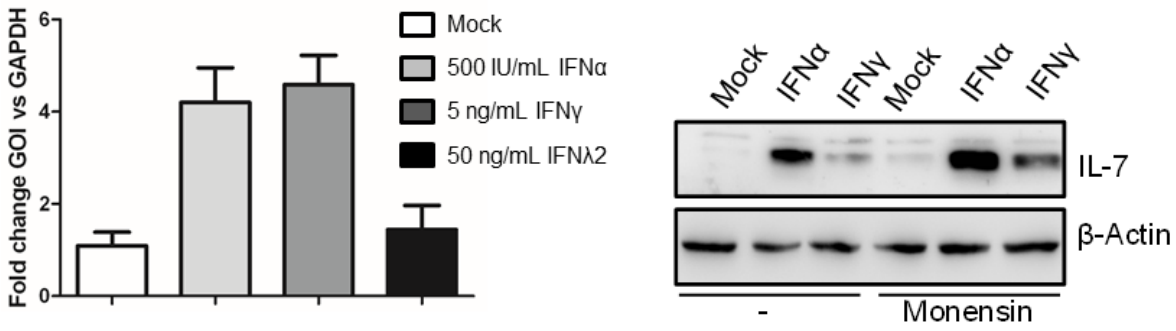

Figure 3: Type I and II IFNs, but not Type III IFN, induce hepatocellular IL-7. Left: Quantification of IL-7 mRNA levels relative to GAPDH mRNA in Huh-7.5 cells, which were treated with 500 IU/mL IFN-α, 5ng/ml IFN-γ or 50ng/ml IFN-λ2 for 6 hours. The IL-7 expression is displayed relative to untreated cells. Standard deviations of 3 independent experiments performed in triplicate are shown. *P < 0.05. f.c., fold change. IU, international units. Right: Detection of IL-7 in HepG2 lysates, which were treated with PBS, IFN-α (500 IU/ml) or IFN-γ (5ng/ml) for 24 hours. For brighter signals. Monensin, a Golgi-inhibitor, was applied.

In our experiments, stimulation of Huh7.5 cells with IFNα and IFNγ induced Interleukin-7 RNA expression. In contrast, IFNλ2 stimulated cells showed IL-7 RNA expression similar to untreated cells, even though IFNα and IFNλ2 rely on the same Jak-Stat components for intracellular signaling. As Interleukins function as proteins, we confirmed Interleukin-7 induction upon IFNα and IFNγ stimulation using immunoblot analysis. HepG2 cells were stimulated with the Interferons for 24 hours and partially incubated with Monensin, a Golgi Inhibitor, for better signal intensity. The immunoblot results are shown in figure 3. For both Interferons, clear Interleukin-7 bands were visible. Both experiments demonstrated the selective Interleukin-7 expression depending on the IFN type used for stimulation.

4.1.2. Inducible IL-7 expression occurs in hepatocytes and endothelial cells

As a next step, we aimed to quantify the amounts of Interleukin-7 secreted by hepatic cells. In addition, we intended to use primary human hepatic sinusoidal endothelial cells (HLSEC) for our study as this cell type also plays a major role in hepatic immunoregulation and is also a target of IFNs (Wohlleber et al. 2016). Therefore, we stimulated Huh7.5 cells and HLSECs with the three IFN types for 6

and 24 hours and assessed the amount of secreted Interleukin-7 in the supernatant via ELISA. The results are displayed in figure 4.

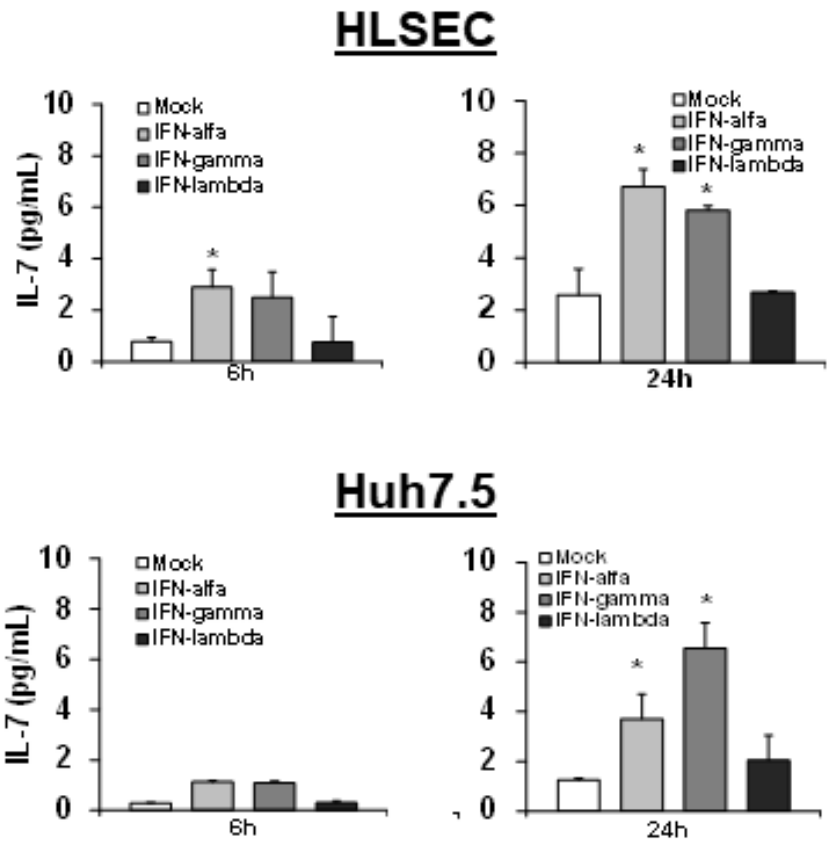

Figure 4: IL-7 is secreted by different cell types. A) ELISA quantification of IL-7 protein level in the supernatant of Huh7.5 and (B) HLSEC, which were treated with 500 IU/ml IFN-α, 5ng/ml IFN-γ, or 50ng/ml IFN-λ2 for 6 and 24 hours. Standard deviations of 3 independent experiments performed in triplicate are shown. *P < 0.05.

For both cell types, an increase of Interleukin-7 levels could be observed after 24h of stimulation with both types of Interferon. As for the RNA data, IFNλ2 stimulation did not lead to an alteration of IL-7 protein expression. The Interleukin-7 protein levels were comparable for both cell types and did also reflect Interleukin-7 levels in human serum (see figure 25 in section 4.3.2.). To sum up, the protein data supported the RNA findings of Interleukin-7 inducibility and suggested two different cell types as sources of hepatic Interleukin-7.

4.1.3. Type III Interferon signaling is intact in human hepatocytes

Murine hepatocytes do hardly express the receptors for IFNλ signal transduction (Hermant et al. 2014). To exclude that the IFNλ2 signaling in our cells is likewise either absent or dysfunctional, we included certain Interferon stimulated genes (ISGs) as a positive control in our RNA data. To do so, we stimulated Huh7.5 cells with the three IFN types for six hours and assessed the fold change in ISG15 and GBP5 RNA expression. The results are displayed in figure 5.

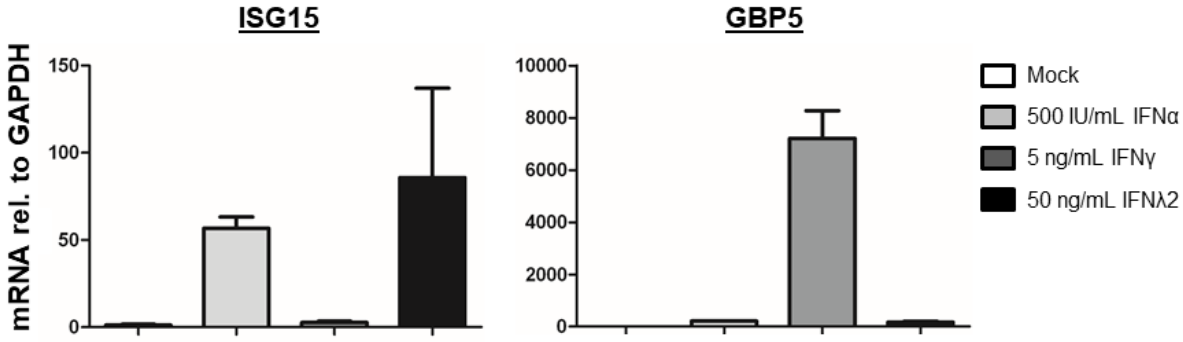

Figure 5: Type III interferon-signaling is intact in Huh-7.5 cells. Quantification of mRNA levels of selected ISGs relative to GAPDH mRNA in Huh-7.5 cells, which were treated for 6h with 500 IU/ml IFN-α, 5ng/ml IFN-γ, or 50ng/ml IFN-λ2. ISG expression is shown relative to untreated cells. Standard errors of 3 independent experiments done in triplicate are shown. *P < 0.05. f.c., fold change. IU, international units.

While high ISG15 expression levels could be observed for IFNα and IFNλ2 stimulated cells, Huh7.5 cells treated with IFNγ showed a strong increase in GPB5 RNA. This provided evidence that IFNλ2 signal transduction was indeed intact in this hepatocyte cell line. For further investigation, we collected the supernatant of stimulated cells and used the Proteome Profiler Human XL Cytokine Array Kit for broad-spectrum protein analysis. The most prominent changes in cytokine expression are shown in figure 6.

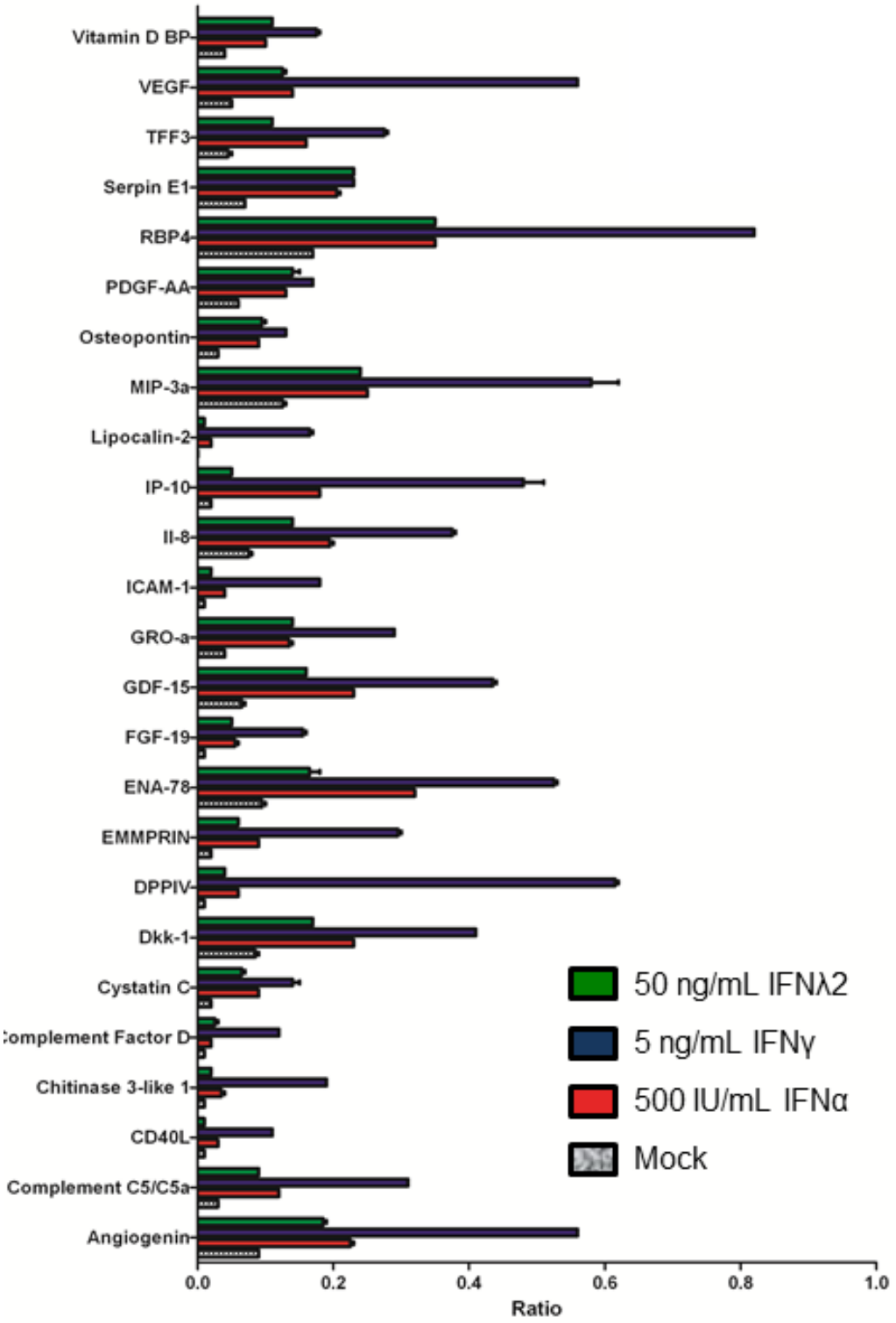

Figure 6: IFNλ2 induces a variety of cytokines in Huh7.5 cells. The liver cell line Huh7.5 was stimulated with 500 IU/mL IFN-α or 5ng/ml IFN-γ or 50ng/ml IFN-λ2 for 2 hours. The medium was changed and the supernatant was collected 22 hours after the medium change. Cytokines were measured using the Proteome Profiler Human Cytokine Array Kit (R&D). Only changes in ratio > 0.1 are displayed.

The analysis of cytokine-expression revealed several proteins induced by IFNλ2 stimulation. Of note, in this experiment we observed several cytokines and growth factors being similarly expressed after IFNα and IFNλ2 stimulation which referred to the shared Jak-Stat pathway. To sum up, Huh7.5 cells show downstream signaling for IFNλ2, which, however, is not connected to the expression of Interleukin-7.

4.1.4. Knockdown of IRF1 abolishes Interleukin-7 expression

Due to the interconnected signal transduction of the Interferon types, we aimed to elucidate, which mediator was responsible for Interleukin-7 expression upon Type I and Type II Interferon stimulation. We focused on Interferon responsive factor 1 (IRF1), as it was strongly involved in Interferon signaling and was shown to induce Interleukin-7 expression (Oshima et al. 2004). Therefore, we silenced gene expression of IRF1 in Huh7.5 cells via siRNA transfection and monitored Interleukin-7 RNA expression upon IFNγ stimulation as displayed in figure 7.

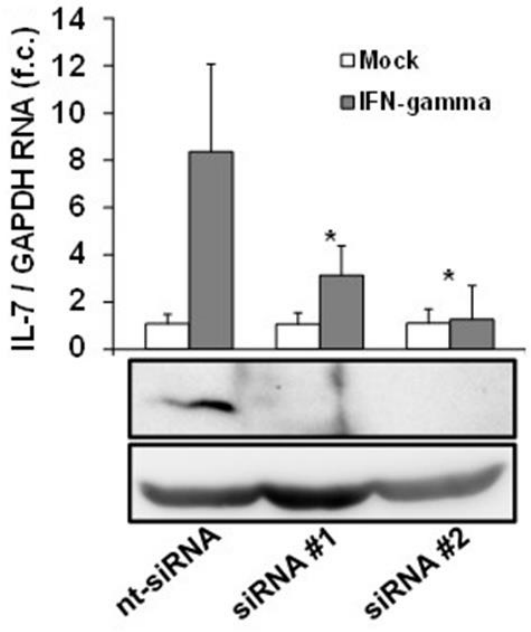

Figure 7: IL-7 induction in the liver is restricted to IRF1. Huh7.5 cells were treated with IRF-1 siRNA or unspecific control siRNA for 24 hours. After siRNA removal, cells were allowed to recover for 24h before being stimulated with 5ng/ml IFNγ for 6 hours. IL-7 mRNA levels relative to GAPDH mRNA are expressed relative to untreated cells. Standard deviations of 2 experiments performed in triplicate are shown. *P < 0.05. f.c., fold change.

We could observe that IFNγ was no longer capable to induce Interleukin-7 expression when IRF1 was silenced. Slight differences were seen for the two different siRNAs, yet, efficient knockdown of IRF1 in both cases was confirmed via immunoblot analysis. This experiment provides a first evidence for the importance of IRF1 for Interleukin-7 expression.

4.1.5. Interferon signaling drives IRF1 translocation to the nucleus

As IRF1 was identified as a transcription factor, nuclear import would be necessary for initiation of gene expression. Therefore, we analyzed, whether translocation of IRF1 could be observed upon IFN stimulation. To this end, we stimulated Huh7.5 cells with all IFN types for different time points and collected the cytosolic and nuclear proteins in separate fractions. The presence of IRF1 was investigated using Immunoblot analysis as shown in figure 8.

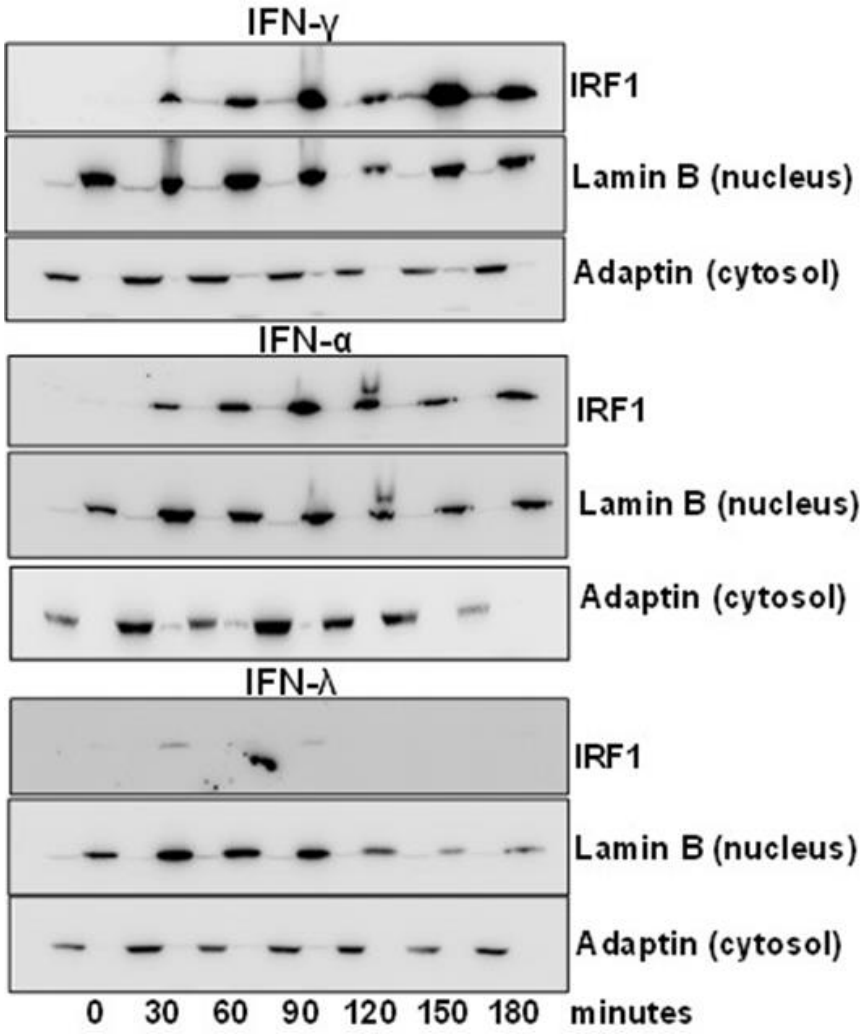

Figure 8: Type I and II, but not type III-IFNs, cause a rapid increase of nuclear IRF1. Huh7.5 cells were stimulated with 5ng/ml IFN-γ (top), 500 IU/mL IFN-α (middle) or 50ng/ml IFN-λ2 (bottom) for the indicated time points. Cells were lysed and cytoplasmatic and nuclear fractions were collected separately as described. The amount of IRF-1 in both fractions was assessed via Immunoblotting, where Lamin B and γ-Adaptin serve as controls for the nuclear and cytosolic fractions, respectively.

Strong bands for IRF1 in the nuclear protein fraction were already detected after 30 minutes of stimulation with IFNα and IFNγ. Treatment with IFNλ2 did not affect the nuclear import of IRF1. Kinetics of nuclear IRF-1 appeared stronger and to last longer after IFNγ treatment. Taken together, IRF1 was transported to the nucleus after Type I and II IFN stimulation, which could enable IRF1 to work as a transcription factor for Interleukin-7.

4.1.6. Inhibition of protein translation reduces nuclear localization of IRF1

The overall strong increase in IRF1 protein band intensity in figure 8 led to the suggestion, that *de-novo* synthesis of IRF1 also played an important role in the signaling pathway. Therefore, we stimulated Huh7.5 cells again with IFNγ, which underwent pre-treatment with cycloheximide, an inhibitor of the ribosome. IRF1 protein levels were again detected via Immunoblotting in cytosol and nucleus as displayed in figure 9.

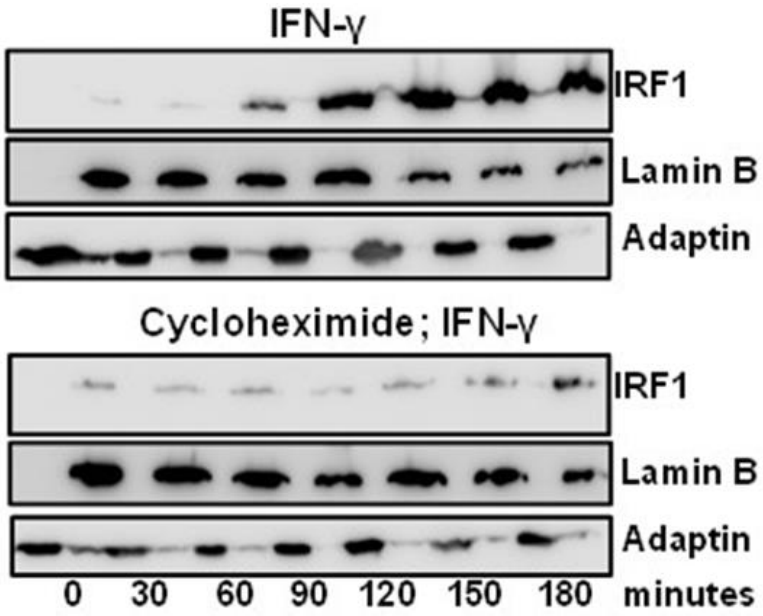

Figure 9: *De novo* translation of IRF1 is needed for high nuclear IRF1 protein levels. Huh7.5 cells were pre-treated with DMSO (top) or 50µg/ml Cycloheximide (bottom) for 6 hours. Afterwards, cells were stimulated with 5ng/ml IFNγ for the indicated time points. The amount of IRF-1 in both fractions was assessed via Western Blot, where Lamin B and γ-Adaptin serve as controls for the nuclear and cytosolic fractions, respectively.

It could be observed, that the effect of a strong IRF1 nuclear influx after IFNγ treatment, was almost completely abolished by cycloheximide treatment. This provided evidence, that nuclear import of IRF1 is dependent on the *de-novo* translation of the IRF1 protein.

4.1.7. Type I and II Interferons drive transcription of IRF1

As protein synthesis also involves the step of transcription, we examined whether IFN stimulation also leads to increased RNA levels of IRF1. This could also be assumed as IRF1 is considered an ISG, meaning that its gene expression would be induced by IFNs (Schoggins 2014). To this end, Huh7.5 cells were stimulated with all types of IFNs for different time points and IRF1 RNA was assessed as shown in figure 10.

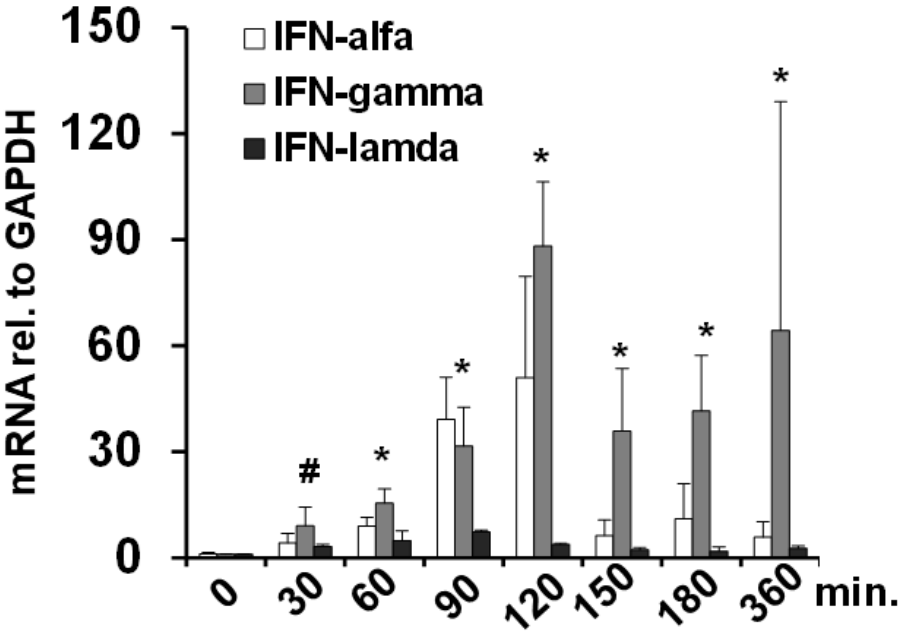

Figure 10: IRF1 transcription is driven by Type I and II IFN signals. Quantification of IRF-1 mRNA levels relative to GAPDH mRNA in Huh-7.5 cells, which were treated for the displayed time points with IFNα (500 IU/mL), IFNγ (5ng/ml), or IFNλ2 (50ng/ml). IRF-1 mRNA relative to GAPDH mRNA is expressed relative to mock cells. Standard deviations of 3 trials done in triplicate are shown. *P < 0.05. f.c., fold change.

We could observe a rapid and strong increase in IRF1 RNA expression for cells treated with IFNα and IFNγ, but not IFNλ2. Comparable to the nuclear protein level, IRF1 RNA expression lasted longer in IFNγ treated cells, whereas the amount of IRF1 RNA in IFNα stimulated cells peaked after 120 minutes of stimulation. To sum up, IRF1 seemed to be essential for signal transduction from IFNs to Interleukin-7, as was de-novo transcribed, translated and translocated to the nucleus upon IFNα and IFNγ stimulation.

4.1.8. Multiple IFN doses amplify hepatic Interleukin-7 expression

The fact, that IFNγ induced a comparably long-lasting effect of IRF1 modulation, raised the question whether multiple doses of IFN amplify the Interleukin-7 expression. To this end, Huh7.5 cells were stimulated with either one single or subsequent doses of IFNγ and Interleukin-7 RNA was monitored as displayed in figures 11.

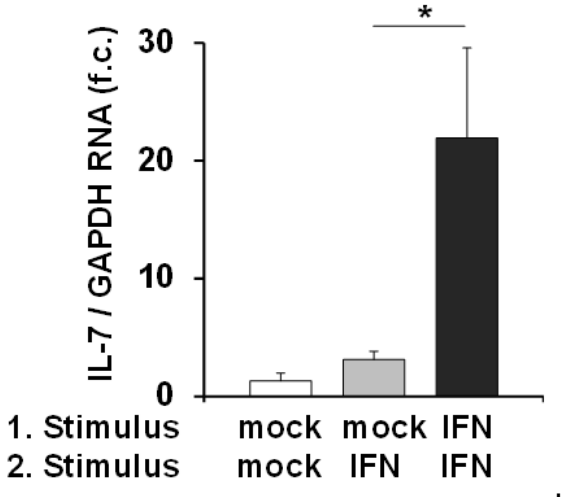

Figure 11: IRF1 transcription amplifies IFN-induced IL-7 induction. Quantification of IL-7 mRNA levels relative to GAPDH mRNA in Huh-7.5 cells, which were stimulated with PBS or one or two subsequent doses (baseline and after 6h) of 5ng/ml IFN-γ for a total of 24 hours. Standard deviations of 2 trials performed in triplicate are shown. *$P < 0.05$. f.c., fold change.

We could indeed observe a much stronger induction of Interleukin-7 expression if we stimulated Huh7.5 with two subsequent doses of IFNγ. It might provide a mechanism to produce a longer-lasting Interleukin-7 expression with higher protein amounts than those measured via ELISA in figure 4.

4.1.9. IL-7 and cell cycle pathways are independent

Besides pure immunology, the different types of Interferons also gained attention regarding eradication of cancer (Takaoka et al. 2003). In addition, also Interleukin-7 itself is used in clinical trials against cancer (Capitini et al. 2009). To study potential side effects of IFN treatment, we observed IFN stimulated HepG2 cells for changes in the cell cycle. We switched to HepG2 cells for this experiment, as they own a wildtype p53 protein (Yoshikawa et al. 1999). Therefore, HepG2 cells were stimulated with all IFN types for 4 hours and the amount of p21 was measured via Immunoblotting as shown in figure 12.

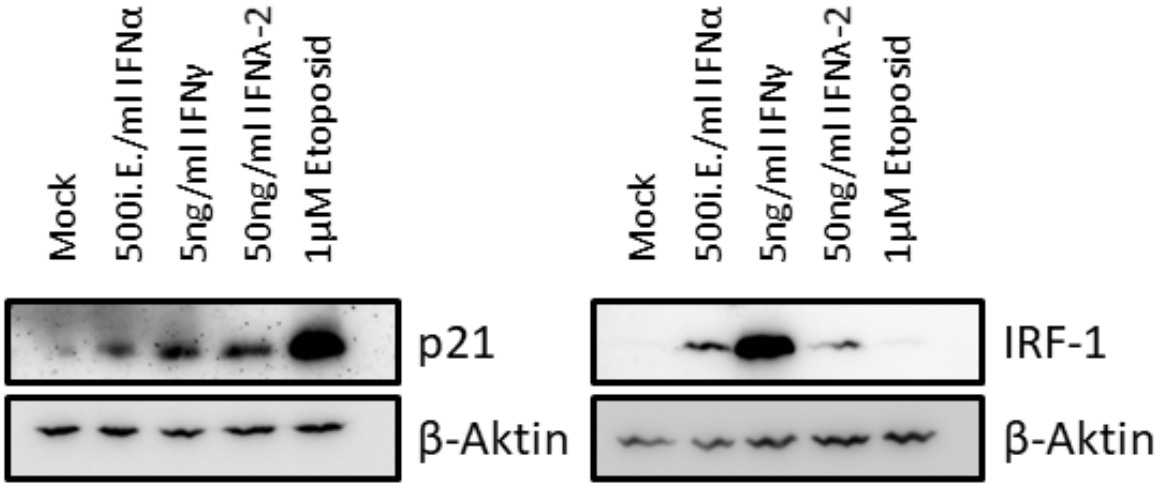

Figure 12: All types of Interferons regulate cell cycle to a comparable extent. HepG2 cells were seeded into cell culture plates and left for 5 hours in the incubator to abolish changes in cell cycle due to changing medium. Afterwards, cells were stimulated with 500 IU/mL IFNα or 5ng/mL IFNγ or 50ng/ml IFNλ2 or 1µM Etoposid for 4 hours. Cells were lysed and p21 (top left) and IRF-1 (top right) expression were assessed by Immunoblotting

We could assess that all IFN types induce p21 expression to a similar extent, yet less strong than Etoposide, a known reagent to induce DNA damage. Therefore, we concluded that the signaling leading to Interleukin-7 is not connected to cell cycle signaling.

Taken together, all results of part I confirm the inducibility of Interleukin-7 that has been identified in murine liver. In addition, they provide IRF1 as the key modulator which restricts responsiveness of the signal pathway to Type I and II Interferons.

4.2. Part II: Identification of IL-7 target cells and consequences

After proper identification of the cellular source of hepatic Interleukin-7, we searched for immune cells carrying the Interleukin-7 receptor complex. Afterwards, we also compared the activity of distinct downstream signaling pathways within these immune cells. Finally, we aimed to identify functional consequences of Interleukin-7 signaling in innate immune cells to describe this cytokine's inflammatory capacity.

4.2.1. The IL-7 receptor components are expressed on various immune cells

To elucidate potential target cells of Interleukin-7, i.e. cells expressing CD127 and CD132, various immune cells were isolated from buffy coats and matured in cell culture as described in section 3.2. Presence of receptors was analyzed via Immunoblotting as displayed in figure 13.

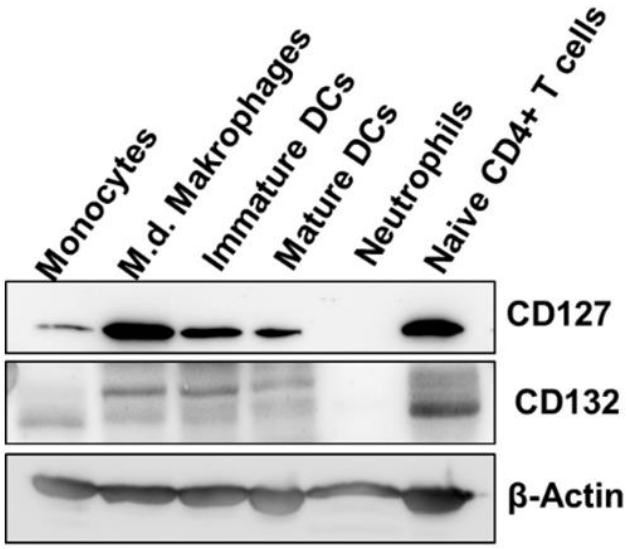

Figure 13: The IL-7 receptor complex is widely expressed. Various innate and adaptive immune cells were isolated from healthy donor Buffy Coats. $CD14^+$ monocytes, neutrophils, and naive $CD4^+$ T cells were lysed just after isolation. Monocyte-derived macrophages, immature and mature DCs were matured from $CD14^+$ monocytes as described. The presence of CD127 and CD132 in these immune cells was assessed via Immunoblotting with β-Actin as a control.

Except for granulocytes, all immune cells showed expression of CD127 and CD132. The band intensity varied between the different cell types and minimal size differences could be observed for CD132. Yet, all cell types could have the ability to answer to Interleukin-7 signaling. To confirm Immunoblotting and to add information about spatial resolution, we performed Immunofluorescence on MDMs as shown in figure 14.

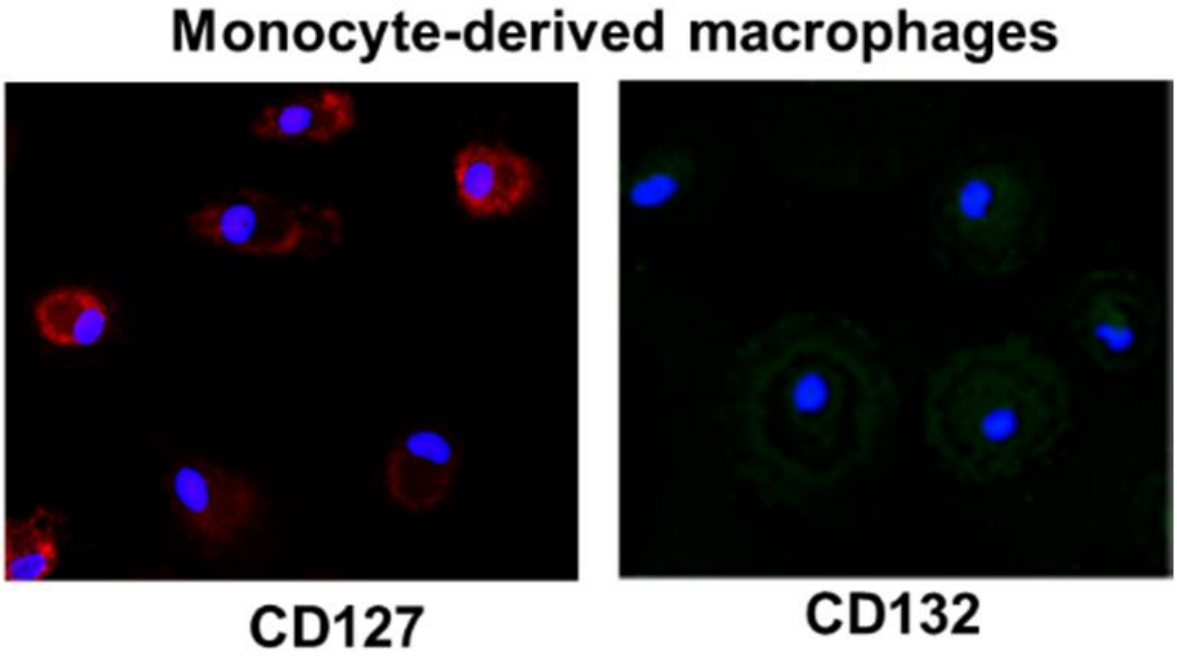

Figure 14: Both components of the IL-7 receptor are expressed on MDMs. Expression of CD127 and CD132 on monocyte-derived macrophages was assessed using immunofluorescence with Alexa Fluor® 633 (red color in left picture) and Alexa Fluor® 555 (green color in right picture) and bisbenzimide H 33342 trihydrochloride as counterstaining (blue color). One representative experiment is shown.

We could observe fluorescence signals for CD127 as well as CD132 in MDMs. Signal intensity was stronger for CD127 but varied between single cells. Taken together, there was a variety of possible Interleukin-7 target cells beyond adaptive immune cells, which were mostly studied in the context of Interleukin-7.

4.2.2. CD127 is an inducible receptor in monocytes and macrophages

Blood from the portal vein draining into the liver is a source of LPS with high concentrations measured in liver cirrhosis patients (Benten et al. 2011). Therefore, we tested whether LPS stimulation altered Interleukin-7 receptor expression in innate immune cells. Monocytes and MDMs were isolated from buffy coat and stimulated with LPS for different time points. RNA expression of CD127 and CD132 was measured as indicated in figure 15.

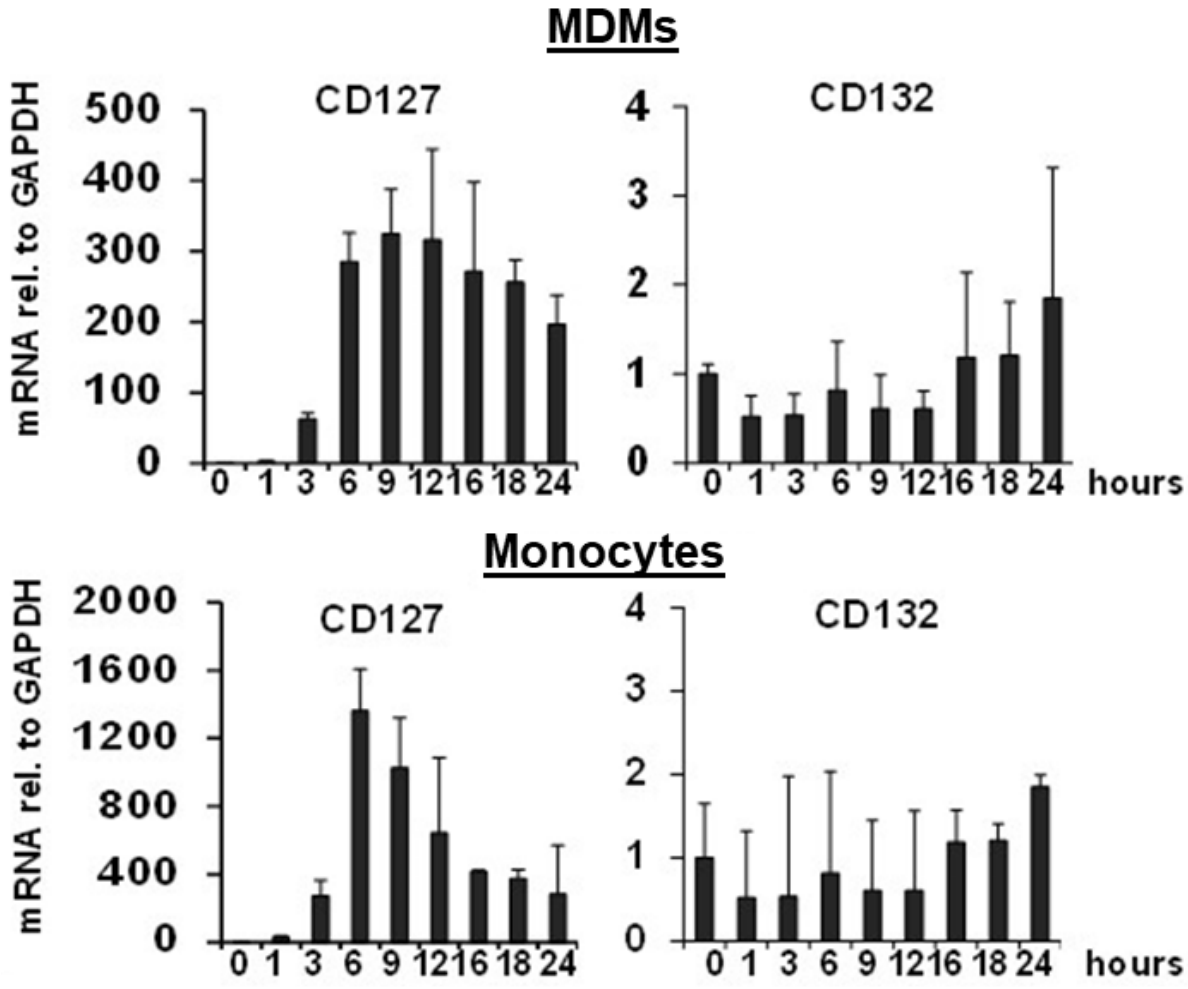

Figure 15: Inducible expression of CD127 in innate immune cells. Quantification of CD127 and CD132 mRNA levels relative to GAPDH mRNA in monocyte-derived macrophages (top) or freshly isolated monocytes (bottom), which were treated with 10ng/ml LPS for the indicated time points. Standard deviations of 3 experiments performed in triplicate are shown.

We could observe high levels CD127 RNA expression especially after 6 hours of stimulation. In contrast, CD132 RNA levels remained unaltered during LPS treatment. As changes in protein level were of interest, we repeated the experiment for the MDMs but collected protein lysates and analyzed CD127 and CD132 via Immunoblotting as displayed in figure 16.

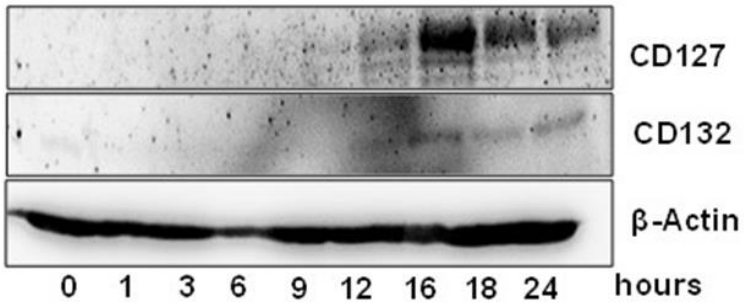

Figure 16: LPS induces CD127 protein content in macrophages. Monocyte-derived macrophages were treated with 10ng/ml LPS for the indicated time points. CD127 and CD132 were assessed via Immunoblotting with β-Actin as a control.

Again, inducibility of CD127 could be shown with a maximum after 16 hours of LPS treatment. Visibility of CD132 protein bands also appeared slightly stronger at the later time points. Taken together, we could demonstrate that CD127 expression was a consequence of LPS stimulation suggesting a regulatory function in the hepatic, high LPS containing environment.

4.2.3. Downstream signaling of IL-7 differs between immune cell types

Interleukin-7 mainly signals through a Jak-Stat pathway, where Stat5 is the classical transcription factor. Besides that, some Kinases such as GSK3 are also involved in downstream signaling. Therefore, we aimed to identify the downstream signals of Interleukin-7 in different immune cell types. To this end, we isolated T cells and monocytes from buffy coat and matured the monocytes into MDMs or DCs according to the protocols stated in section 3.2. The immune cells were stimulated with ascending concentrations of recombinant Interleukin-7 with or without LPS pretreatment. Phosphorylation status of Stat5 (activatory phosphorylation) and GSK3 (inhibitory phosphorylation) were assessed via Immunoblotting as displayed for T cells in figure 17.

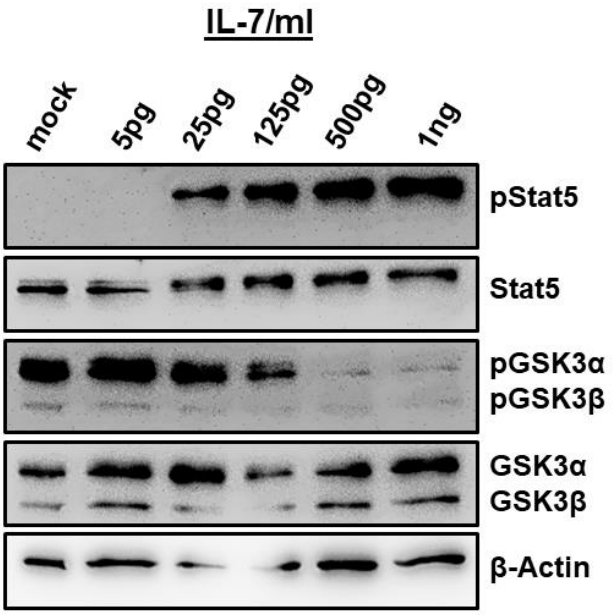

Figure 17: IL-7 signaling is activated in a dose-dependent manner in naive CD4 T cells. Naive CD4 T cells were isolated from buffy coat using magnetic beads. Cells were stimulated with indicated concentrations of recombinant human IL-7 for 15 minutes. Expression of phosphorylated and total Stat5, GSK3 as well as housekeeping control was assessed by Immunoblotting with β-Actin as a control.

Naïve CD4 T cells showed clear bands for phosphorylated Stat5 already at comparably low doses of Interleukin-7. Signal strength intensified in a dose-dependent manner. This showed the relevance of this signal transduction pathway in T cells. On the contrary, GSK3 phosphorylation was slightly decreased meaning a lower inhibitory influence on this kinase. All in all, our T cells mainly relied on Stat5 for signal transduction. The results for mature dendritic cells are shown in figure 18.

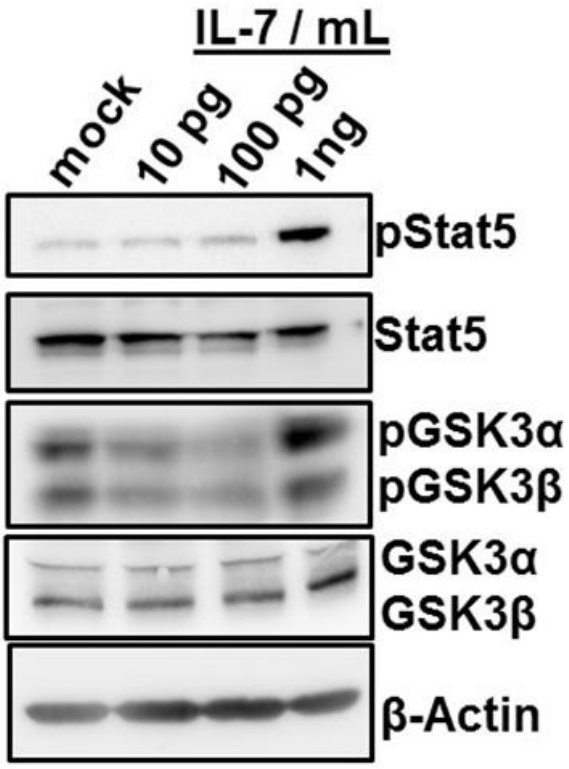

Figure 18: IL-7 signals are present in human dendritic cells. Human monocytes were isolated from buffy coat and were cultured with 10ng/ml rhGM-CSF and 20ng/ml rhIL-4 for 6 days prior to stimulation to obtain monocyte-derived dendritic cells (DCs). Cells were primed with 10ng/ml LPS for 48 hours before stimulation with the indicated concentrations of IL-7 for 15 minutes. Presence and phosphorylation status of proteins was assessed using Immunoblotting with β-Actin as a control.

Phosphorylation of Stat5 could also be detected in mature DCs even though higher doses of Interleukin-7 were needed to achieve this effect. In addition, LPS pretreatment was a pre-requisite for this signaling pathway. Interleukin-7 stimulated DCs also displayed higher band intensity for phosphorylated GSK3. Therefore, the kinase was less active. Taken together, high doses of Interleukin-7 influence the Jak-Stat pathway as well as the enzymatic downstream signaling in DCs. The results for LPS pre-treated MDMs are displayed in figure 19.

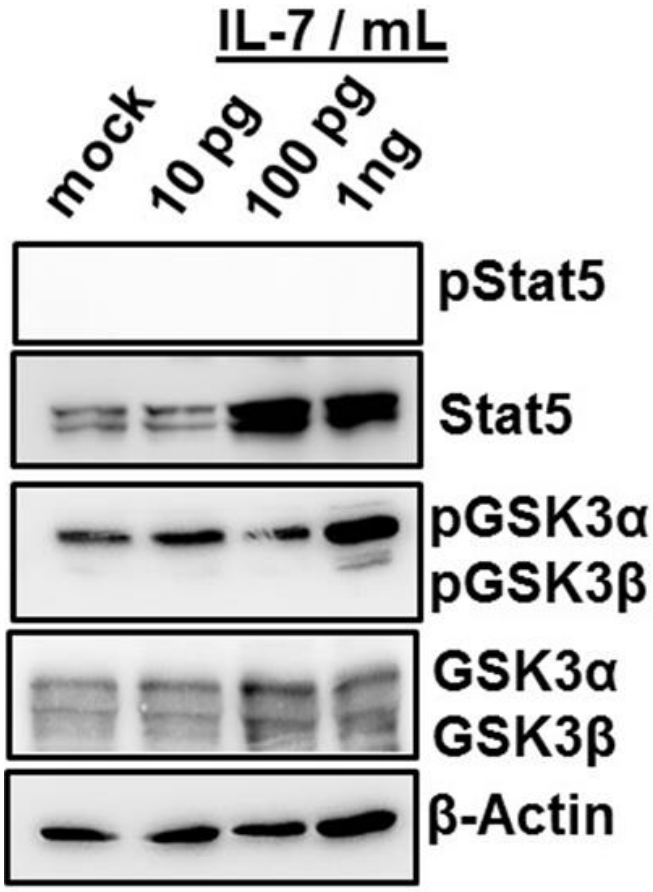

Figure 19: Distinct IL-7 downstream signaling is observed in macrophages. Human monocytes were isolated from buffy coat and were cultured with 10ng/ml M-CSF for 6 days prior to stimulation to obtain monocyte-derived macrophages (MDMs). Cells were treated with 10ng/ml LPS for 48 hours before stimulation with the ascending concentrations of IL-7 (10pg/ml, 100pg/ml and 1ng/ml) for 15 minutes. Presence and phosphorylation status of proteins was assessed using Immunoblotting with β-Actin as a control.

In contrast to T cells and DCs, no Stat5 phosphorylation was observed in MDMs. On the contrary, inhibitory phosphorylation of GSK3 was most prominent in this cell type. Like in DCs, high doses of Interleukin-7 were needed for visible effects and LPS was needed as pre-treatment referring again to a possible induction of CD127 shown in figure 15. To sum up, Interleukin-7 activates distinct downstream signals in different immune cells leading possibly to different functional outcomes.

4.2.4. IL-7 signaling does not affect glucose metabolism of macrophages

GSK3 is a key modulator in glycogen storage and availability of glucose (Beurel et al. 2015). Therefore we assessed, whether Interleukin-7 stimulation of MDMs altered glucose and lactate metabolism. To this end, we isolated MDMs from buffy coat and stimulated them with two doses of Interleukin-7 for different time points. Afterwards, cell culture supernatant was collected and glucose and lactate concentrations were quantified using a commercial colorimetric assay. The results are shown in figure 20.

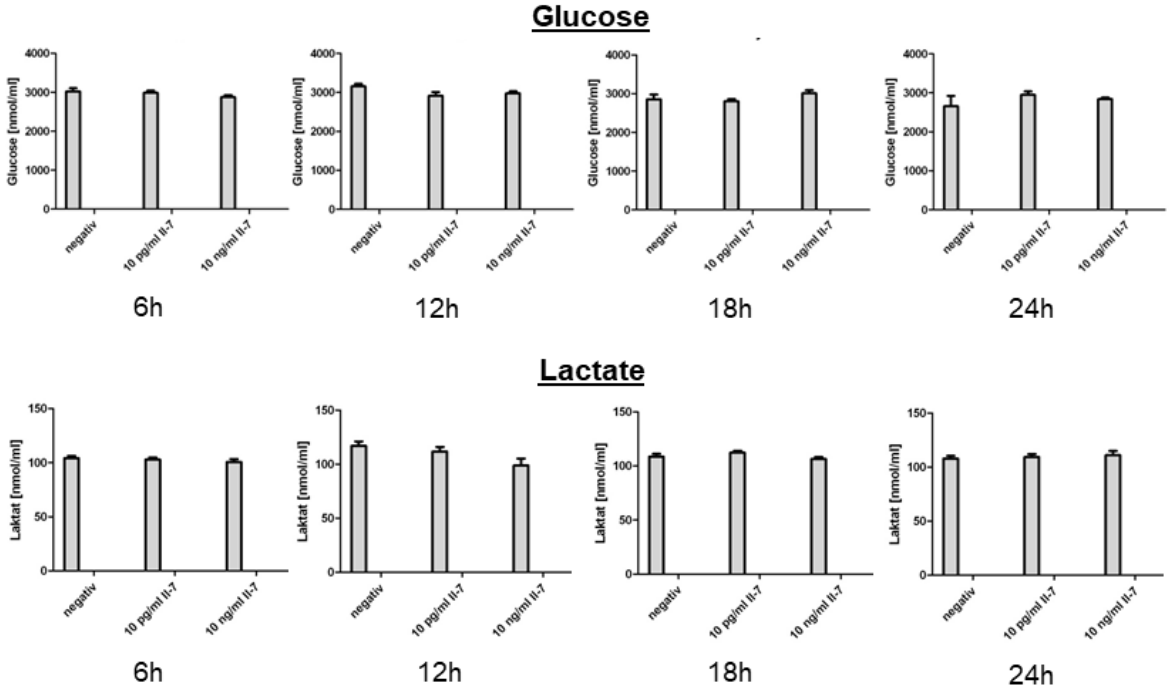

Figure 20: Glycolysis remains unaltered after Interleukin-7 treatment Human MDMs were generated as described and pre-treated with 10ng/ml LPS for 16 hours. Cells were stimulated with 10pg/ml IL-7 or 10ng/ml IL-7 for the indicated time points. The supernatant was collected and analyzed for the amount of glucose or lactate using commercial colorimetric assays.

We did not observe any differences in glucose or lactate concentrations for unstimulated and stimulated MDMs. Also, the overall glucose consumption changed only slightly over time for all durations of stimulation. Therefore, changes in GSK3 phosphorylation status did not affect overall MDM metabolism.

4.2.5. IL-7 decreases intracellular ATP-concentration of macrophages

For a general overview on metabolic state, which is highly interlinked to the inflammatory status in immune cells (Murray et al. 2015 and Williams et al. 2018), we aimed to quantify intracellular ATP concentrations. For this experiment, MDMs were isolated from buffy coat and partially primed with LPS for 16 hours before stimulation with Interleukin-7 or CHIR99021, a GSK3 inhibitor, for different time points. ATP content in cellular lysates was quantified using the ATP Bioluminescent Assay Kit as shown in figure 21

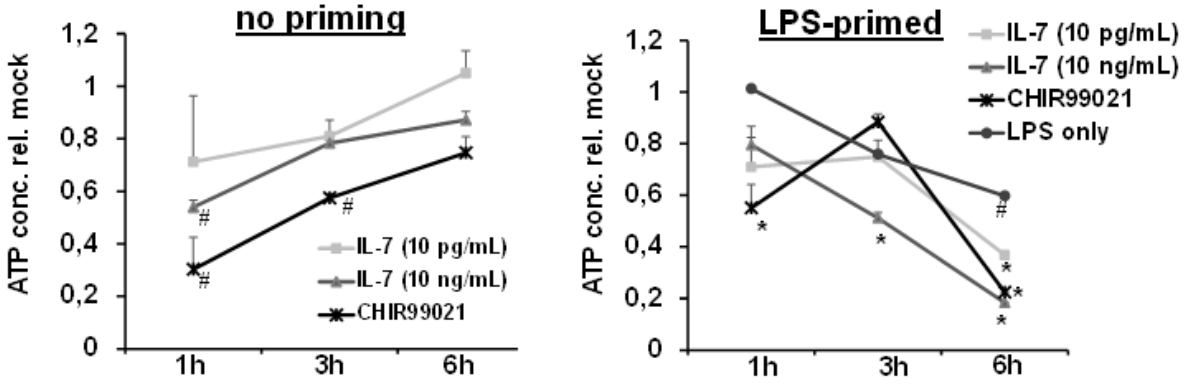

Figure 21: IL-7 reduces intracellular ATP concentration of macrophages MDMs were primed with or without 10ng/ml LPS for 16 hours prior to stimulation with IL-7 or the GSK3 Inhibitor CHIR99021 for the indicated time points. Cells were lysed and intracellular ATP content relative to unstimulated controls was measured.

In LPS primed macrophages low and high dose Interleukin-7 led to a significant reduction of intracellular ATP content. The effect was comparable to CHIR99021. For non-primed macrophages, no reduction in intracellular ATP content was observed. Taken together, alterations in MDM metabolic state after Interleukin-7 treatment can be observed if cells are primed with LPS, even though this finding is limited to ATP and does not necessarily affect glucose and lactate.

4.2.6. Interleukin-7 affects the viability of macrophages

In many biochemical assays, ATP is closely linked to cellular viability. Therefore, we performed a WST-1 viability staining. MDMs were isolated from buffy coat and partially primed with LPS for 16 hours before stimulation with Interleukin-7 or CHIR99021 for 12 or 24 hours. Cells were incubated with WST-1 for 30 minutes and viability according to the absorbance at 450nm was studied as shown in figure 22.

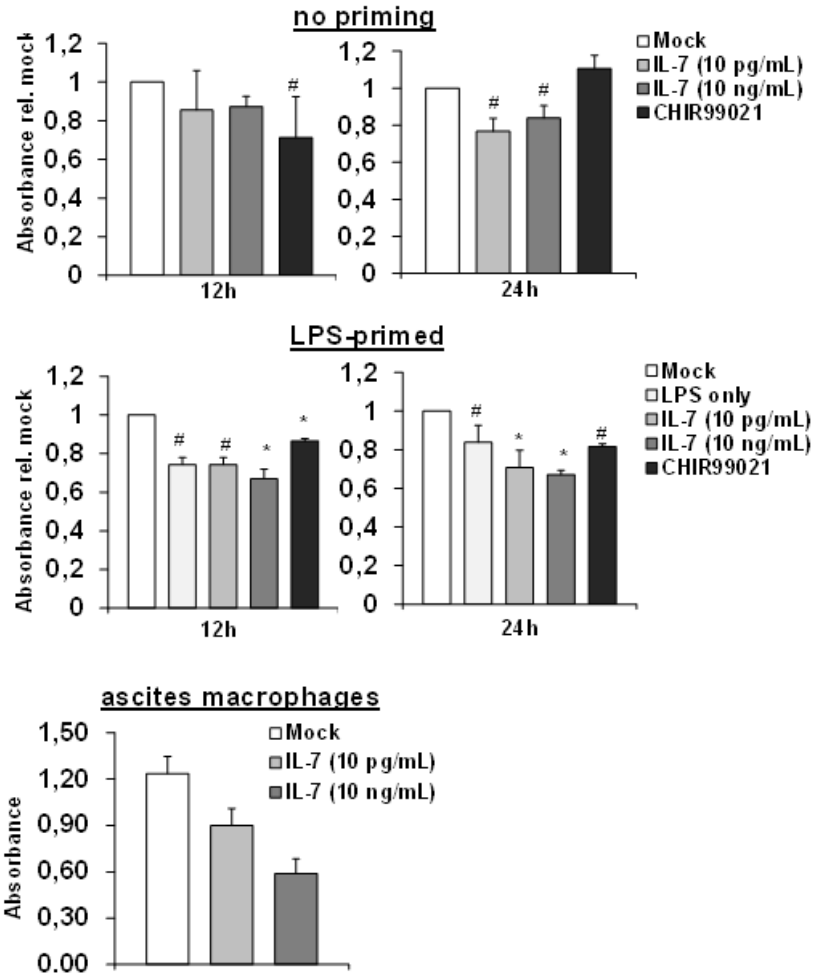

Figure 22: IL-7 reduces the viability of monocyte-derived macrophages. Human MDMs were primed with (middle) or without (top) 10ng/ml LPS for 16 hours followed by stimulation with the indicated concentrations of IL-7 or the GSK3 inhibitor CHIR99021. Cell viability was estimated using WST-1 reagent at 450nm relative to unstimulated cells Standard deviations of 3 experiments performed in triplicate are shown. *P < 0.05. #P < 0.1. Bottom: Macrophages were freshly isolated from ascites of two patients with spontaneous-

bacterial peritonitis and directly cultured and exposed to IL-7 at the indicated doses. Cell viability using WST-1 reagent was assessed after 24h.

Comparable to intracellular ATP content, both doses of Interleukin-7 decreased absorbance, and therefore cellular viability, significantly in LPS primed MDMs only. Again, the effect was similar to viability reduction due to CHIR99021. For non-primed MDMs only a tendency for reduced viability could be observed. The same effect could also be shown for macrophages derived from ascites of spontaneous bacterial peritonitis patients. These results display a role for an axis linking Interleukin-7, GSK3 and macrophage viability, which is the exact opposite of the pro-survival effect of Interleukin-7 in T cells (Unsinger et al. 2010).

4.2.7. Interleukin-7 abolishes endotoxin tolerance in macrophages

GSK3 has also been demonstrated to mediate endotoxin tolerance, a very important feature for liver immunology (Park et al. 2011). Therefore, we aimed to assess, if the inhibition of GSK3 leads to reduced endotoxin tolerance. To this end, we primed MDMs with LPS for 16 hours and stimulated them with Interleukin-7 for 6 hours afterwards. RNA expression of pro-inflammatory cytokines was determined as shown in figure 23.

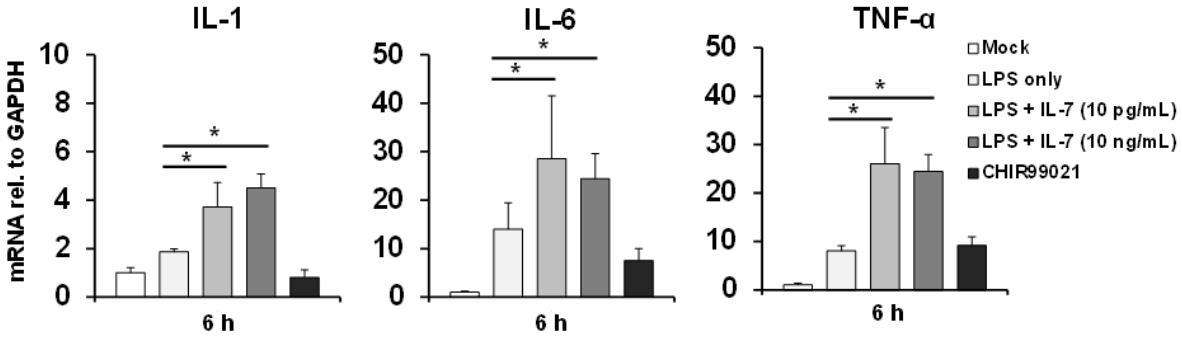

Figure 23: Interleukin-7 augments pro-inflammatory cytokine production Quantification of IL-1β, IL-6 and TNFα mRNA levels relative to GAPDH mRNA in MDMs primed with or without 10ng/ml LPS as indicated, which were then stimulated with IL-7 or the GSK3 Inhibitor CHIR99021 for 6 hours. Standard deviations of 3 experiments performed in triplicate are shown. Standard deviations of 3 experiments performed in triplicate are shown. *P < 0.05. #P < 0.1.

For both doses of Interleukin-7, higher expression of IL-1β, IL-6, and TNFα was observed. The effect was much stronger compared to CHIR99021. Therefore, Interleukin-7 abolished endotoxin tolerance which was possibly mediated not only by GSK3. Taken together, these experiments demonstrate a pro-inflammatory role of Interleukin-7 in innate immunity on the cost of cellular lifespan, which is highly relevant for liver immunology due to its dependency on LPS pre-treatment.

4.3. Part III: Importance of IL-7 in cirrhosis patients

As it was demonstrated in this thesis that Interleukin-7 shapes the inflammatory properties of macrophages, we analyzed, whether the immunoregulatory axis of Interleukin-7 plays a role in patients with severe chronic liver disease. For that reason, we investigated serum levels of Interleukin-7 as well as the populations of CD127-positive innate and adaptive immune cells in patients and healthy controls. Baseline characteristics of our patient cohort are listed in table 17.

Table 17: Summary of baseline patients' characteristics
BMI, body mass index; CRP, C-reactive protein; INR, international normalized ratio;

	ACLF (n=41)	decompensated (n=49)	compensated (n=27)
Age (years), mean (SD)	55 (10)	55 (11)	55 (12)
Male gender, n (%)	30 (73.2)	37 (75.5)	17 (63.0)
BMI (kg/m^2), mean (SD)	27.6 (6.8)	25.1 (5.9)	26.2 (5.7)
Leucocytes (/nl), mean (SD)	11.0 (6.1)	8.6 (4.55)	6.2 (5.0)
Hemoglobin (g/dl), mean (SD)	9.3 (1.8)	10.6 (2.9)	10.9 (2.5)
Platelets (/nl), mean (SD)	110 (73)	132 (95)	113 (66)
CRP (mg/dl), mean (SD)	4.9 (4.1)	2.8 (2.9)	1.5 (2.1)
Creatinine (mg/dl), mean (SD)	2.4 (1.3)	1.1 (0.4)	0.90 (0.30)
Bilirubin (mg/dl), mean (SD)	12.1 (12.1)	6.9 (9.1)	4.4 (5.5)
INR, mean (SD)	2.01 (0.77)	1.55 (0.43)	1.40 (0.35)

4.3.1. Components of the IFN-IRF1-IL-7 axis are detected in liver biopsies

To investigate whether GSK3 and IRF1, the main components of the Interferon-Interleukin-7 axis, were present in patients, we performed fluorescent Immunohistochemistry on patient liver biopsies. The results are shown in figure 24.

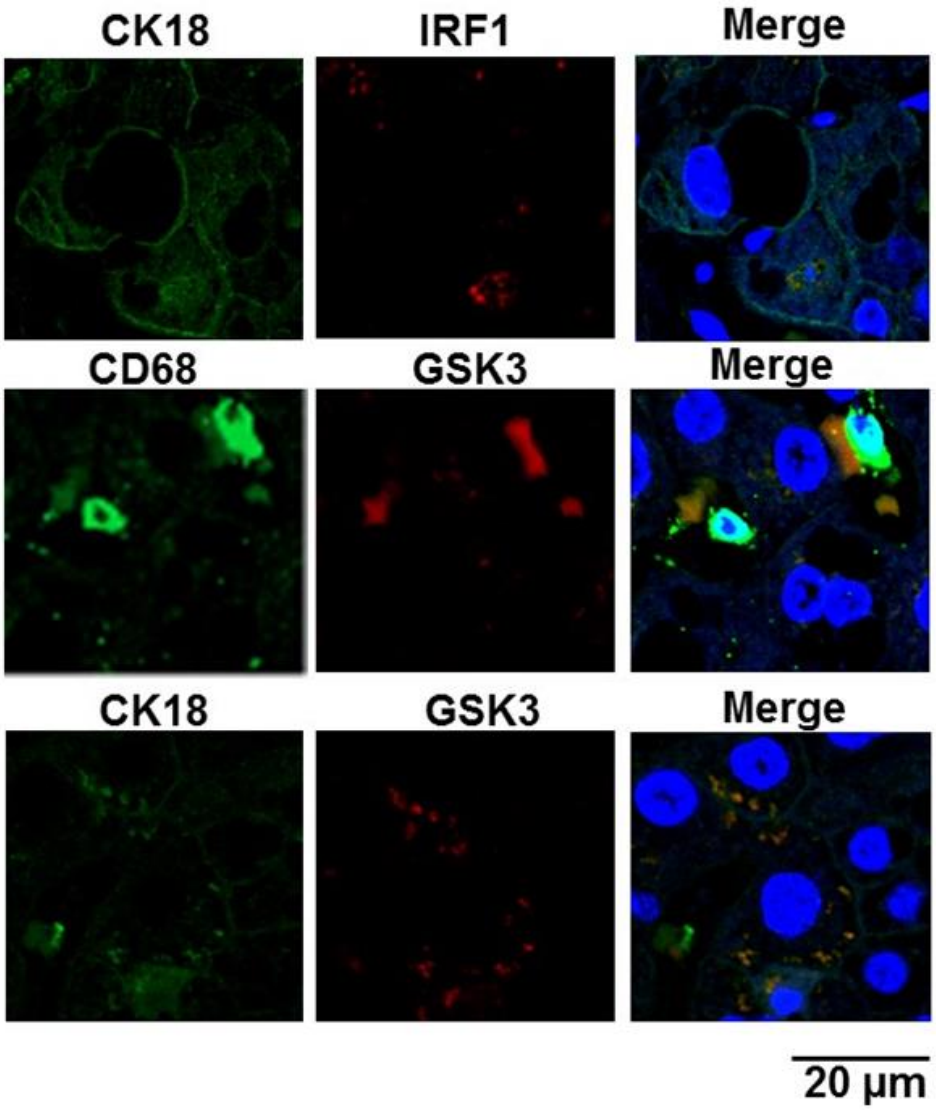

Figure 24: Components for IL-7 signal transduction are present in cirrhotic liver. Expression of IRF1, GSK3, CD68 as a marker for macrophages, and CK18 as a marker for the hepatocellular cytoskeleton in paraffin-embedded tissue of a representative patient with liver cirrhosis was assessed using immunofluorescence with Alexa Fluor® 633 (red color in middle column) and Alexa Fluor® 555 (green color in left column) and bisbenzimide H 33342 trihydrochloride as counterstaining (blue color)

IRF-1 and GSK3 could both be shown in liver biopsies. IRF1 co-localized with cytokeratin-18, a marker for hepatocytes. GSK3 on the other hand could also be located in macrophages, as measured by co-localization with CD68. These findings suggest an intact Interferon and Interleukin-7 signaling system in chronic liver disease.

4.3.2. Cirrhosis patients display decreased Interleukin-7 serum levels

As a next stop, we aimed to quantify serum Interleukin-7 levels of patients with chronic liver diseases compared to healthy controls. ELISA measurements were performed as shown in figure 25.

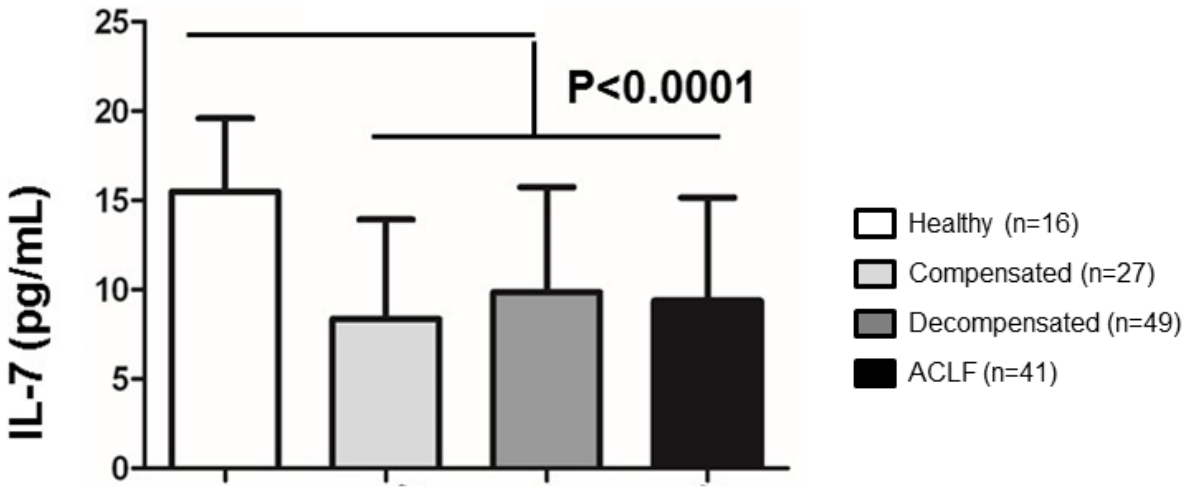

Figure 25: Serum Interleukin-7 is reduced in cirrhosis patients. Serum concentrations of IL-7 in patients with compensated liver cirrhosis, decompensated liver cirrhosis, or acute-on-chronic liver failure (ACLF), as well as in healthy controls were determined by ELISA.

In contrast to the liver biopsies, Interleukin-7 was significantly reduced in patients' serum compared to healthy controls. However, there was no correlation with the severity of the disease. This reduction in Interleukin-7 could be an explanation for higher monocyte frequencies and LPS tolerance observed in chronic liver disease (Albillos et al. 2014).

4.3.3. Immune cell populations are slightly altered in cirrhosis patients

In Hepatology and Gastroenterology, CD127 positive immune cells show correlations with disease severity. In case of chronic Hepatitis C virus infection, intrahepatic T cells have been demonstrated to be CD127low (Radziewicz et al. 2007). In addition, the number CD127 positive T cells were shown to be of prognostic relevance for multi-organ failure from acute pancreatitis (Wang et al. 2017). Therefore, we aimed to assess, how liver cirrhosis affects immune cell populations and especially CD127 positive immune cells. Multicolor flow cytometry was performed on PBMCs from patients and healthy controls and the results are displayed in figure 26.

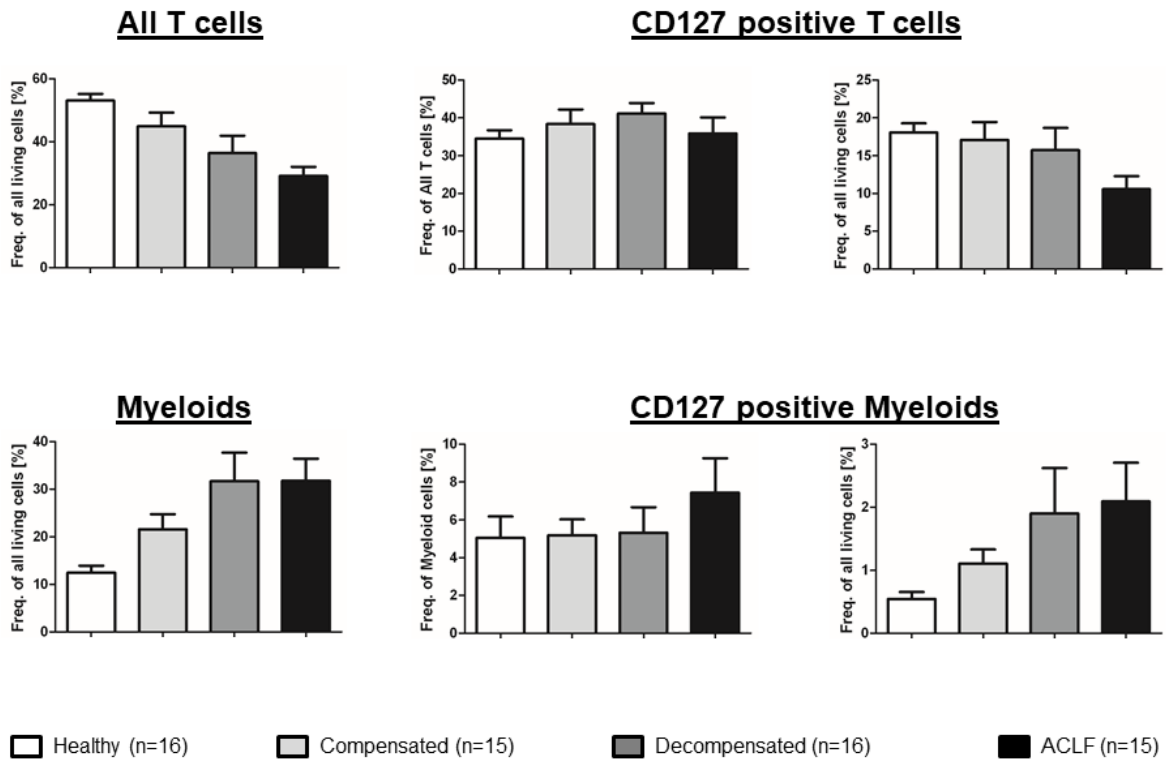

Figure 26: Frequencies of CD127 positive immune cells are altered in cirrhosis patients. Frequencies of immune cells were measured by Multicolour Flow Cytometry and analyzed with FlowJo according to the gating strategy in figure 2. Middle column shows CD127 positive cells as the frequency of their respective parent population while the right column shows these cells as the frequency of all living cells.

We could observe lower frequencies of T cells and higher frequencies of myeloid cells, which mainly include monocytes. The frequency of CD127 positive cells on the parent generation remained unaltered. Yet, due to the general changes in T cells and myeloids, CD127 positive T cells were reduced and CD127 positive myeloids were enhanced in cirrhosis patients. Therefore, the severity of liver chronic liver disease does not lead to a selective decay of CD127 immune cells. In addition, the ability of LPS to induce CD127 is intact in the healthy and diseased liver.

Taken together, part III displays a relevance of the Interleukin-7 pathway in cirrhosis patients. Even though the concentrations of Interleukin-7 were strongly reduced, we could detect main components and regulatory pathways from *in vitro* approaches also in patients, which influence possible therapeutic applications of Interleukin-7.

5. Discussion

This thesis provides evidence about the regulation of hepatic Interleukin-7 expression upon Interferon-signaling. In addition, it displays a pro-inflammatory impact on human macrophages. Figure 27 summarizes the most important aspects of hepatic immunoregulation of Interleukin-7 with respect to cirrhosis patients.

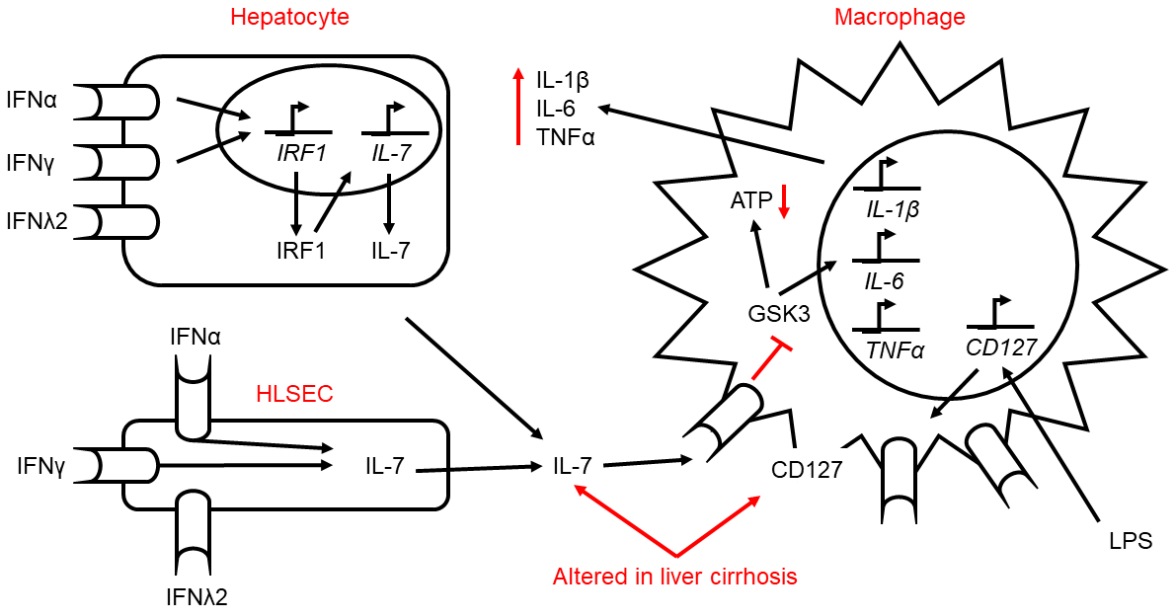

Figure 27: Graphical summary of the main results. Interleukin-7 is produced in IFN stimulated hepatocytes and HLSEC and restricted by IRF1. Interleukin-7 impacts proinflammatory on MDM metabolic and activation state. Cirrhosis patients show decreased serum Interleukin-7 but intact signal transduction and regulatory circuits.

Our study suggests the existence of a pro-inflammatory cascade in which type I and II IFNs induce hepatic Interleukin-7 in an IRF-1-restricted manner. This secretion of Interleukin-7 affects innate immune cells regarding pro-inflammatory activity and survival. Patients display components of this cascade but show altered levels of serum Interleukin-7 and CD127 positive immune cells compared to healthy controls.

Given the in vitro data of Part I and II and the patients' data of Part III, this section discusses whether Interleukin-7 can be described either as beneficial or adversely in chronic liver disease. Plus, it deals with the question if the Interleukin-7 signaling cascade can be considered a therapeutic option in chronic liver disease. Finally, this section states limitations of this thesis and provides an outlook about possible experimental approaches in the future.

5.1. Interleukin-7: Friend or foe in hepatic immunoregulation?

Our experimental data suggest a pro-inflammatory role for Interleukin-7 as it was induced by IFNα and IFNγ, but not IFNλ2. As it was also induced by LPS this proves the need for Interleukin-7 not only for simple maintenance of immune cells but also for acute danger signals. IFNs are key players of early innate immune responses of the liver. Viral infections, bacterial infections or intestinal translocation of bacterial products like LPS can induce type I, II and III IFN in hepatocytes (type I and III IFN), or in LSECs, macrophages and other innate immune cells (type I and II IFN) via TLR signaling (Heim et al. 2013, Wack et al. 2015 and Schindler et al. 2008). IFN signaling results in the induction of numerous ISGs. ISGs induced by type I and III IFNs show significant overlap because the formation of pStat1-pStat2 heterodimers is considered to be the predominant event downstream of both the IFNα and IFNλ receptor (Heim et al. 2013, Wack et al. 2015 and Schindler et al. 2008). In contrast, IFN-γ signaling predominantly results in the formation of pStat1-pStat1 homodimers, and ISGs induced by IFN-γ are partially distinct from that of type I and III IFN signaling (Dill et al. 2012). The fact that the induction takes places in hepatocytes and endothelial cells proves the importance of non-immune cells in immunology and especially of the endothelial cells as they are and the foremost cellular barrier to pathogens or circulating immune cells entering the liver (Knolle et al. 2014).

In our experiments, we identified IRF1 as the protein mainly contributing to Interleukin-7 expression in hepatocytes. This seems logical as IRF1 is known to bind and induce Interleukin-7 gene expression in intestinal epithelial cells (Oshima

et al. 2004). In addition, we display an unconventional mechanism of ISG induction, i.e. de novo translation and parallel transcription of IRF-1 RNA and protein in response to type I and II, but not type III IFN. IRF-1 translation and transcription, therefore, appears to restrict expression of Interleukin-7 to the otherwise rather distinct type I and II IFN system. Furthermore, parallel induction of IRF-1 protein translation and mRNA transcription provides a mechanism of longer-lasting ISG-expression and thereby a possible way to overcome IFN-refractoriness for selected ISGs.

Regarding potential recipient cells of Interleukin-7, we could show that many cell types express the receptor components for Interleukin-7 signaling. While the dependence of CD4+ T cells, CD8+ cells, or mucosal-associated invariant T cells (MAIT) on IL-7-induced Jak-Stat5 signaling is well known, only a few reports have described a role for IL-7 signaling in macrophages. Yet, Interleukin-7 production of LSECs provides signals to circulating lymphocytes or macrophages recruited to the liver (Knolle et al. 2014). Our finding that Interleukin-7 induced phosphorylation (i.e. inhibition) of GSK3, but not of Stat5, in macrophages adds further knowledge to the understanding of the role of Interleukin-7 in the regulation of macrophages. In our study, inhibition of GSK3 by Interleukin-7 in LPS-primed macrophages resulted in augmented cytokine secretion and blunted LPS tolerance of macrophages, which, however, appears to be burdened with fitness costs in these cells as we have observed decreased intracellular ATP concentration and macrophage survival in response to Interleukin-7. These data confirm a previous report showing that TNF-α-induced activation of GSK3 (in contrast to GSK3-inhibition by Interleukin-7) confers endotoxin tolerance and survival in macrophages (Park et al. 2011). The reduced survival of innate immune cells could be of importance in patients as monocytes which high-inflammatory activity are a known problem in liver diseases (Liaskou et al. 2013). Therefore, targeting their fitness by Interleukin-7 seems to be a beneficial characteristic. The elevated cytokines are hard to judge as they might increase responses to pathogens but also local tissue damage. IL-6 and TNFα have already been demonstrated to be closely connected of ACLF severity (Clària et al. 2016). However, Interleukin-7

itself was very low in our cirrhosis and ACLF patients which might indicate a rather beneficial role if it was applied as a therapeutic option.

To conclude, Interleukin-7 shows an ambiguous role in patients with cirrhosis and ACLF. As it triggers proinflammatory cytokines in macrophages their ability to fight pathogens, which is a trigger of acute decompensation, this might improve patient survival. However, IL-1β, IL-6, and TNFα might increase local tissue damage and have partially already been correlated with disease severity of ACLF. On the other hand, Interleukin-7 reduces macrophage survival and might be a mechanism to control overshoot of innate immune cells in the damaged liver. Therefore, more experiments with complex cell culture techniques and animal models should be applied to examine Interleukin-7 for therapeutic purposes.

5.2. Possible use of the Interleukin-7 cascade in immunotherapy

Interleukin-7 is a key cytokine required for the maintenance and proliferation of adaptive immune cells and therefore, several connections to different pathologies have already been discovered. Our data raise the question if Interleukin-7 levels should be raised in cirrhosis and ACLF either directly or via the Interferon signaling. The finding that IFNλ2 is not sufficient to induce IRF-1 translation/transcription and subsequent Interleukin-7 production by hepatocytes and/or endothelial cells is of potential clinical importance. IFN-λ is under clinical evaluation for the treatment of chronic hepatitis B (Chan et al. 2016). The major advantage of IFN-λ over the approved therapies with pegylated IFNα is its better tolerability, which is explained by the restricted expression of the IFNλ receptor on hepatocytes and certain epithelial cells. However, the establishment of effective adaptive immune responses is key to clear HBV, and the lacking induction of hepatic Interleukin-7 by IFN-λ may result in less efficient T cell responses compared to conventional therapies with IFN-α. Beyond their antiviral properties, IFNs display important antiproliferative and anti-tumor properties (Takaoka et al. 2003). IRF-1 is an important suppressor of certain malignancies (Bouker et al. 2005). In view of our findings, one may, therefore, speculate that IFNλ is a less

powerful antitumor agent compared to IFNα or IFNγ. Plus, Interleukin-7 is in use against cancer which is useful as it prolongs survival of adaptive immune cells and increases the inflammatory capacity of innate immune cells (Capitini et al. 2009). As IFNα and IFNγ induce other distinct cytokines in combination with Interleukin-7 it might be an interesting experimental approach to analyze potential cytokine combinations.

Our patients show decreased levels of T cells as a response to decreased serum Interleukin-7. For this, Interleukin-7 might be a useful option as in an animal model of multiple sclerosis, inducible hepatic Interleukin-7 upon toll-like receptor (TLR) signaling has been identified as a relevant source of Interleukin-7 to promote survival of CD4+ and CD8+ T cells (Sawa et al. 2009). As Interleukin-7 was demonstrated to enhance also pathogen-specific T cells this might provide a way to fight infections in ACLF (Terrazzini et al. 2018). It also impacts on innate lymphoid cells (Yang et al. 2018), which are an important source for Interleukin-22 in the liver (Björkström et al. 2013). In addition, Interleukin-7 is in trials for sepsis which shows comparable dysregulations of immune responses to ACLF (Clària et al. 2016, Francois et al. 2018 and Kulkarni et al. 2018). Our finding that patients with liver cirrhosis including patients with ACLF have lower serum concentrations of IL-7 compared to healthy controls may therefore indicate that Interleukin-7 could be a therapeutic agent in selected patients with liver cirrhosis in order to overcome the high vulnerability to infections of these patients due to e.g. high numbers of suppressive innate immune cells (Albilos et al. 2014 and Bernsmeier et al. 2015). A therapeutic intervention is indeed possible as our immunophenotyping showed comparably robust frequencies of CD127 positive immune cells at least in peripheral blood. To sum up, a therapeutic use of IL-7 in viral and bacterial infections is under detailed investigation (Pellegrini et al. 2011 and Unsinger et al. 2010) and its role in immunomodulation may lead to further target pathologies. Besides, immunotherapy of ACLF is already an emerging field with G-CSF as a promising clinical candidate (Chavez-Tapia et al. 2015).

5.3. Technical limitations of the thesis and troubleshooting

For our experiments, we focused on *in vitro* cell culture approaches. Of course, this only partially reflects the complex communication situation found in an organ such as the liver. In addition, even though MDMs are primary cells they might show alterations e.g. in metabolic activity compared to cells which were not maturated with growth factors such as M-CSF for several days. Yet, it is an established model (Murray et al. 2014) and due to the maturation process, they provide higher cell counts for downstream experiments.

For our MDMs, we experienced variations in the expression of CD127 and CD132 between the different donors as also displayed in figures 13 and 16. This can be seen as a cause for high variations also in Interleukin-7 signal transduction. As we have not checked every batch of MDMs for receptor expression this might only explain variations between different donors. In addition, we did not quantify our protein bands as we worked with an HRP detection approach for better sensitivity.

Our T cell data showed a decrease in phosphorylated GSK3 due to high doses of Interleukin-7. Even though it could be an explanation for the contrasting survival outcomes of innate and adaptive immune cells after Interleukin-7 stimulation, there is evidence in the literature that GSK3 is also phosphorylated in T cells after Interleukin-7 stimulation (Barata et al. 2004). Therefore, this decrease reflects more likely technical problems in the Immunoblotting process.

Regarding our metabolic data, we saw a contradiction between the observable changes in intracellular ATP levels but steady levels of metabolites such as glucose or lactate. This might be due to excess glucose in the culture medium so that changes can hardly be observed. For a detailed characterization of the metabolic status, one could compare the activity of enzymes participating e.g. in glycolysis.

Finally, most patient material reflects only the systemic situation in peripheral blood. Liver material of cirrhosis and especially ACLF patients is hard to acquire as patients usually arrive at the clinic in bad general health condition which also

worsens rapidly in case of ACLF. In addition, many patients undergo anti-coagulant therapy which reflects a contraindication for liver biopsy. Also, many liver pathologies such as fibrosis or steatosis can be examined by non-invasive methods such as Controlled Attenuation Parameter (CAP) nowadays (Kennedy et al. 2018 and Wiegand et al. 2013).

5.4. Future experimental approach

Our experimental designs were based on in vitro approaches with only one cell type at a time. As we only focused on innate immune cells, we have not worked with co-culture experiments yet. Nevertheless, culturing and treating two types of cells simultaneously in a single cell culture dish with recombinant Interleukin-7 would give a deeper insight of direct and indirect effect of Interleukin-7 stimulation.

Besides recombinant Interleukin-7, the use of conditioned medium, for example from IFN stimulated Huh7.5 would provide evidence about the role of Interleukin-7 in comparison to the other induced mediators and cytokines mentioned in figure 6. As a first step, co-stimulation of cells with recombinant Interleukin-7 and a recombinant protein of one of the cytokines in figure 6 could be applied. DPP4, for example, has already been found to be linked to metabolic problems (Ghorpade et al. 2018). Alongside, our data suggest differences in the expression kinetics of Interleukin-7 in response to different Interferons. This should be studied in more detail as it might provide more information about other regulatory mechanisms.

In addition, there have been more signaling mediators of Interleukin-7 identified (Aiello et al. 2018). Studying these new targets, which undergo phosphorylation, in adaptive and innate immune cells might provide a better understanding of functional consequences within these cell types. For example, Interleukin-7 has recently been found to be linked to autophagy in mice (Zhu et al. 2018).

Regarding the analysis of patient material, a lot more experimental designs are imaginable. Like Interleukin-7, the amount of soluble CD127 could be quantified by ELISA. As soluble CD127 influences Interleukin-7 signaling it is worth to study whether cirrhosis patients show altered levels of soluble CD127. This would

manipulate efficient Interleukin-7 signaling in addition to the reduced serum levels of Interleukin-7. The ability of Interleukin-7 to enhance production of Interleukin-1β raises the question whether Interleukin-7 acts on the inflammasome of innate immune cells. To address this question a complete set of genes termed “Inflammasome gene modules” could be analyzed. This would give more insight for judging Interleukin-7 as beneficial or adversely.

As cirrhosis and ACLF are closely related to systemic inflammation as seen in multiplex cytokine analyses in literature, a complex immunophenotyping of all patient immune cells, comparable to Maecker et al. 2012 but also including rare populations such as innate lymphoid cells, would be a logical next step, as they are important cells in liver health and disease (Forkel et al. 2017). This could be performed in peripheral blood and in portal venous blood. As cirrhosis is often linked to dysfunctions in the gut, alterations of immune cells in the gut are very likely. Because these cells might then drain into the liver through the portal vein and shape inflammatory processes there, immunophenotyping of portal venous PBMC can be useful, even though blood collection in healthy controls is not possible.

As indicated in figure 26 there are shifts detectable in immune cell population frequencies. Most interesting would be to look for certain subpopulations, for example of T cells. This could characterize the dysregulation of inflammatory processes in more details, whether e.g. activation processes are enhanced or increased or whether immune cell development is disturbed. Regarding immune cell function, it would be important to check whether the abrogation of endotoxin tolerance in healthy donor MDMs can also be observed in MDMs from cirrhosis or ACLF patients (Weiss et al. 2017).

As mentioned before, acquisition of liver biopsies is very limited. Therefore, co-culture systems and especially liver organoids might be a suitable way to study effects of Interleukin-7 in a multicellular system. Organoids of liver cancer have already been described (Broutier et al. 2017). Of course, animal studies in mice or rats would also feasible. As ACLF impacts on several different organs, animal

studies would provide a way for global monitoring. However, there is no good model for ACLF which sticks closely to disease progression and transferring immunological information from rodents to humans has been problematic in many cases.

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Appendix

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