Workshop

From Viral Hepatitis to Chronic Inflammation and Liver Cancer

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Abstracts
Poster Abstracts

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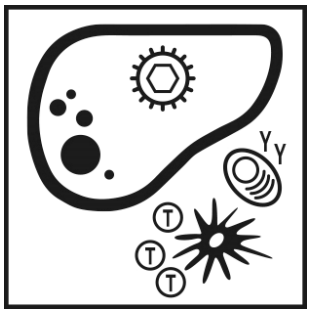
Workshop

FROM VIRAL HEPATITIS TO CHRONIC INFLAMMATION AND LIVER CANCER



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Session I

What's new in viral hepatitis?

Understanding HAV-host interactions using a novel mouse model of hepatitis A

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Hepatoviruses are unusual hepatotropic picornaviruses, distinct genetically and structurally from other members of the *Picornaviridae*. The prototypical member of the genus, hepatitis A virus (HAV), is an ancient pathogen that has likely afflicted humans since we began to live together in communities large enough to sustain chains of fecal-oral transmission, 10–12,000 years ago. Like other hepatitis viruses, the host range of HAV has long been considered restricted to humans and nonhuman primates. However, related hepatoviruses have been identified in bats and rodents with phylogenetic evidence of past shifts among these host species, and our recent work shows that human HAV replicates robustly in *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice. As in humans, HAV is highly hepatotropic in these animals, with viral RNA found only in the liver and spleen (most likely in macrophages). Infected *Ifnar1*$^{-/-}$ mice recapitulate many aspects of human hepatitis A, including fecal shedding of virus produced in the liver, acute serum alanine aminotransferase (ALT) elevations, and mixed intrahepatic inflammatory infiltrates associated with hepatocellular apoptosis. This liver injury is not reduced in mice depleted of CD8+ or CD4+ T cells, NK/NKT cells, or macrophages. In fact, T cell depletion in actively infected *Ifnar1*$^{-/-}$ mice leads to serum ALT increases while boosting viral RNA abundance within the liver. Thus, while T cells provide some measure of virus control, they are not responsible for liver injury. In contrast, no disease results from robust viral replication in *Mavs*$^{-/-}$ or *Irf3*$^{-/-}$*Irf7*$^{-/-}$ double-knockout mice, implicating IRF3 activation as the primary mediator of liver injury. As in humans, HAV replicates without cytopathic effect and is secreted from hepatocytes into biliary canaliculi as ‘quasi-enveloped’ virions (eHAV) that are stripped of their membranes by bile salts, resulting in fecal shedding of highly stable, naked virions optimized for environmental spread. Both virion types are infectious in mice.

Immune control of chronic hepatitis B: Towards curative approaches

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Hepatitis B virus (HBV) establishes a stable nuclear persistence form, the so called cccDNA, in infected hepatocytes. Unlike HCV, which can be eliminated with directly acting antivirals, the nuclear cccDNA prevents elimination of HBV. Currently used antivirally active nucleos(t)ide analogous as well as capsid assembly inhibitors or siRNAs under development efficiently control HBV replication but can't directly target its persistence via cccDNA and thus have to be applied long-term. A cure or at least a functional cure of HBV infection, however, is desired for the 260 million carriers at high risk to develop liver disease and hepatocellular carcinoma. As antiviral immune response can achieve this goal, the question is how to best activate antiviral immunity.

Immune activation can either be achieved in an antigen non-specific fashion or in an HBV-specific fashion. Interferon (IFN) α is an antiviral cytokine that is approved for therapeutic application. It can resolve HBV infection in 15–25% of chronic carriers but dosing is limited by side effects. Alternative approaches to activate innate antiviral immunity that are currently exploited include cytokines, toll-like receptor or Rig-I agonists and checkpoint inhibitors.

T cells can eliminate HBV by killing infected cells in an antigen-specific fashion, but have also been described to control HBV in a non-cytolytic fashion via secreted cytokines. Transplantation of bone marrow from HBV-immune donors has been shown to safely cure hepatitis B paving the way for HBV-directed T cell therapy. These cells show cytolytic and non-cytolytic activity targeting HBV persistence. The latter seems of particular interest since antiviral and T cell derived cytokines can purge cccDNA from infected cells without killing them. Alternatively, breaking immune tolerance against HBV via a therapeutic vaccine seems a promising approach to cure hepatitis B. Hereby, HBV-specific B- and T cell responses mimic naturally occurring cure of HBV infection. Last not least, targeting of lymphocytes using antibodies is an option. Clinical trial are of these different approaches are being initiated and we will soon learn, which approach is most promising.

Exploiting HDV entry for antiviral therapy

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Entry inhibition is a novel and interesting, potentially curative opportunity to treat viral infections. Hepatitis B (HBV) and Hepatitis D virus (HDV) are highly liver specific and exploit sodium taurocholate co-transporting polypeptide (NTCP) as a specific receptor to enter hepatocytes. The large envelope protein (L-) of HBV plays a pivotal role in this process. Synthetic lipopeptides derived from the N-terminus of the HBV L-protein have been shown to potentially interfere with HBV/HDV receptor interaction. A lead substance of such peptides (Myrcludex B/Bulevirtide) has been developed into the clinical stage. This peptidic drug opened a novel therapeutic option to treat chronically HBV/HDV infected patients either as a monotherapy or in combination with approved drugs like IFN-α or TDF. In my talk, I will discuss the current state of the art of entry inhibition for HBV/HDV and report the results of recently performed clinical trials (Myr-202 and Myr-203) B in chronically HBV/HDV infected patients. These results indicate that Myrcludex B as the first in class entry inhibitor for HBV/HDV bears curative potential.

Towards a prophylactic HCV vaccine

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Ellie Barnes is Professor of Hepatology and Experimental Medicine, and an MRC Senior Clinical Fellow at Oxford University. She has spent many years working on the immune control of HCV in natural infection has been developing T cell vaccines against HCV.

The World Health Organization has set a target to achieve elimination of HCV by 2030 – but very few countries are likely to achieve this. An effective preventative vaccine would have a major impact on HCV incidence and would represent a major advance towards global HCV control.

In this symposium, progress using simian adenoviral and MVA viral vectors encoding HCV antigens and used in prime-boost strategies, in collaboration with the industry, will be discussed. These approaches have generated durable, high-magnitude, poly-functional T cells that target multiple HCV antigens in healthy volunteers. The efficacy of the first of these vaccines is currently being assessed in a phase-II study in intra-venous drug using populations in the USA. Vaccines designed to target multiple HCV genotype are also being developed, as are genetic adjuvants designed to increase immune responses. This will be set in the context of complimentary approaches taken by others to induce HCV antibodies.

Progress in the development of new vaccine platforms means that it should be possible to generate effective HCV vaccines. The major hurdles to achieving a prophylactic vaccine are largely political and practical issues around the funding and testing of vaccine candidates.

Principles of hepatitis E virus replication, resistance and chronic persistence

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Hepatitis E virus is one of the most common causes of acute viral hepatitis worldwide with an estimated 20 million infections per year. Although the mortality rate is < 1% among the general population, pregnant woman can have a fatality rate of up to 30%. Additionally, chronic hepatitis E has increasingly become a significant clinical problem in immunocompromised patients potentially leading to liver cirrhosis and liver failure. Treatment options of fulminant infections are limited. Ribavirin remains the treatment of choice in non-self-limiting infections, however, should be carefully considered due to possible side effects and resistance development. Therefore, progress in the understanding of principles in HEV replication, resistance and persistence should contribute to improved control and treatment of HEV infections. In the last years, we have established HEV cell culture systems, which are based on the HEV strains Kernow-C1 p6 (genotype 3) and Sar55 (genotype 1). We recently used these cell culture models to assess the effect of different interferons against HEV (Todt et al. Antimicrob Agents Chemother. 2016). Furthermore, we could demonstrate for the first time in tissue culture that HEV replication is not restricted to the liver and can potentially complete the full viral life cycle in neuronal-derived tissues explaining neurological disorders during HEV infection (Drave et al. Journal Viral Hepat. 2016) as well as human placenta-derived cells (Knegendorf et al. Hepatol. Commun. 2018). In case of ribavirin treatment, we could show that the drug applies mutagenic pressure on the viral genome, which may result in viral elimination and may also lead to the selection of resistant variants in patients who do not respond. Finally, we could demonstrate that deep sequencing technology may in the future serve as a valuable tool to identify patients at risk of treatment failure (Todt et al. Gut 2016). Therefore, it may become an important component in the clinical management of patients with chronic hepatitis E and for personalized antiviral strategies in general. In the future, we aim to identify novel antiviral against HEV and to uncover host factors required for HEV replication that could be new potential drug targets. In addition, mechanisms of drug resistance and viral persistence will be elucidated.

Session II

Chronic inflammation

Lymphotoxin β receptor (LTβR) signaling-dependent control of virus clearance in chronic HBV and HCV infection

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The pro-inflammatory and homeostatic cytokines lymphotoxin (LT)α and LTβ are members of the tumor necrosis factor (TNF) superfamily. Under physiological conditions, LTs are expressed by activated T-, B-, NK-, and lymphoid tissue inducer cells and are crucial for organogenesis and maintenance of lymphoid tissues. HBV or HCV infections lead to increased hepatic LT expression *in vivo* and *in vitro*. Indeed, enhanced hepatic LTβR signaling might be of potential clinical relevance because LTβR and its ligands are drastically increased in human HBV- and HCV-induced hepatitis and HCC, compared with normal livers or non-viral, benign liver diseases. LTs can directly act on hepatocytes, which physiologically express high levels of LTβR but little LT. Pharmacological inhibition of LTβR signaling reduces pathogen- and concavalin A-induced liver injury, whereas LTβR signaling on hepatocytes appears to be beneficial during liver regeneration. We have demonstrated that sustained hepatic LT expression in mice can be injurious, causing chronic hepatitis and HCC. Thus, hepatic LT signaling might be advantageous if transiently active during liver regeneration, but detrimental if chronically triggered. Recently, we have shown that short-term LTβR activation induces APOBEC3A and B upregulation leading to efficicent cccDNA degradation. Thus, these data indicate that different kinetics of LTβR-activation lead to distinct biological effects. Here, I present novel data on the role LTβR-induced NF-kb signaling on hepatocytes in virus infection as well as how LT expression on immune cells changes their character in the context of virus clearance.

Systemic crosstalk of metabolism and inflammation in viral hepatitis

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Virus-induced tissue damage and inflammation are major causes of morbidity and mortality worldwide. Immune cell metabolism has become a vibrant area to understand the complex mechanisms of activation and regulation of the immune system. Yet, systemic crosstalk of metabolic and inflammatory processes and its potential impact on pathogen control, adaptive immunity and tissue pathology has received comparatively little attention so far. We focused on the liver as center stage for organism-wide metabolic turnover by employing a mouse model for chronic viral infection and hepatitis. Integration of longitudinal changes of transcriptomics, proteomics and metabolomics together with targeted genetic and pharmacological perturbations unveiled a novel signaling axis whereby antiviral cytokines reprogram the metabolism of hepatocytes. This in turn results in altered serum metabolite levels which impact the function of CD8 T cells and ameliorate tissue damage in the liver. These findings shed light on the complex crosstalk between metabolism, inflammation, adaptive immunity and pathology and identify a novel liver-intrinsic mechanism of immunoregulation.

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Dysfunctional T cell responses and their role in viral hepatitis

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T cell dysfunction is defined as a hypofunctional state that T cells often acquire in persistent chronic infection and cancer that is primarily triggered by persistent exposure to antigen. The concept of T cell dysfunction, also referred to as T cell exhaustion, is defined as a T cell failure to eliminate a pathogen. T cell dysfunction is associated with several significant functional and phenotypical changes. This includes a progressive loss of T cell effector functions, such as cytokine production or proliferation, altered metabolism, epigenetic changes and a unique transcriptional program compared to effector functions in memory T cells. Severe exhaustion is also characterized by the co-expression of high levels of inhibitory receptors such as PD-1. Originally, this phenotype was thought to be restricted to terminally differentiated “exhausted” cells. However, there is growing evidence that exhausted T cells consist of different heterogeneous subsets. T cell dysfunction has also been described as a hallmark of chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. Indeed, while strong and functional virus-specific $CD8^+$ T cells are clearly associated with spontaneous viral clearance, viral persistence leads to classical features of T cell dysfunction of remaining T cells. In recent years, important novel insights into T cell dysfunction in chronic viral hepatitis have emerged. For example, it has been shown that virus-specific $CD8^+$ T cells present during chronic viral hepatitis do not consist of homogenous but rather heterogeneous subsets that include severely dysfunctional cells, but also populations with a higher progenitor potential and features shared with memory cells such as expression of CD127. Importantly, there are differences in subset distribution in chronic HBV and HCV infection. For example, in HCV infection, there is a larger proportion of severely exhausted $PD\text{-}1^+$ CD127- cells, the existence of which is clearly linked to ongoing antigen-recognition. After antigen removal in the context of direct acting antiviral therapy, exhausted $CD8^+$ T cells largely disappear while the memory-like T cell subset is maintained. These memory-like cells are defined by a co-expression of TCR1, CD127 and PD-1 and show an antigen independent survival. In contrast to HCV, in chronic HBV infection a larger proportion of memory-like versus exhausted $CD8^+$ T cells is present. Interestingly, we also found significant differences in phenotype and function depending of the targeted antigen. Indeed, more core-specific $CD8^+$ T cells displayed a memory-like phenotype compared to polymerase-specific $CD8^+$ T cells, that was also in line with a more advanced differentiation and severe T cell exhaustion phenotype. Importantly, these phenotypical differences were also associated with a reduced functional capacity of polymerase- versus virus-specific $CD8^+$ T cells.

Overall, there is growing evidence that T cell dysfunction is a hallmark of chronic viral infection. T cell dysfunction/exhaustion is primarily triggered by ongoing antigen recognition as cessation of chronic antigen stimulation leads to a disappearing of highly exhausted and a maintenance of memory-like $CD8^+$ T cells. These cells do show better functions and are more easily to reinvigorate. A better understanding of the molecular features of T cell dysfunction in chronic viral hepatitis, especially chronic HBV has potential implications for the design of novel immunotherapeutic approaches in the cure of chronic viral hepatitis.

Bile acids and their therapeutic applications

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Bile acids (BA) are important regulators of BA homeostasis, metabolism as well as inflammation. BA signaling is mediated through different receptor molecules, including nuclear hormone receptors (NRs), such as the farnesoid X receptor (FXR), as well as G protein coupled membrane receptors, such as the G protein coupled bile acid receptor 1 (GPBAR1, TGR5) or the sphingosine 1 phosphate receptor 2 (S1PR2). Agonists for FXR are already approved for the treatment of primary biliary cholangitis (PBC) and are evaluated for the treatment of NAFLD in phase II and III trials. In contrast, ligands for TGR5 have only been tested in animal models, including models of liver and intestinal inflammation, such as NAFLD, lipopolysaccharide injection as well as dextran sulfate sodium induced colitis.

Activation of FXR and TGR5 both in the liver as well as the intestine triggers an anti-inflammatory response. While FXR is highly expressed in hepatocytes and intestinal epithelial cells, TGR5 levels in hepatocytes are negligible and high amounts of this receptor can be detected in CD14 positive monocytes as well as in organ resident macrophages, such as Kupffer cells. TGR5 downstream signaling in immune cells affects the NLRP3 inflammasome and reduces cytokine and chemokine expression and secretion as well as phagocytosis. TGR5 mRNA levels are upregulated in murine and human macrophages in vitro as well as in macrophages in murine livers in vivo following LPS or Listeria treatment through the transcription factor Klf5. Intraperitoneal injection of lipopolysaccharide or intravenous injection of Listeria monocytogenes results in elevated cytokine and chemokine levels as well as increased mortality in TGR5 knockout mice. These findings underscore the important role of BA signaling and TGR5 in inflammatory diseases.

Session III

Liver cancer

Epigenetic regulation of tumor cell plasticity

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Every cell in a multicellular organism contains exactly the same genetic information encoded on DNA. However, despite this fact a human organism maintains more than 200 specialized cells, e.g. muscle cells, skin cells or neurons, which exhibit specific genetic expression patterns in order to fulfill their function and retain their morphology. Thus, nature has evolved sophisticated systems to strictly regulate gene expression in different cell types. These systems are based on epigenetic modifications on chromatin, which restrict the accessibility of specific genetic information. Accordingly, epigenetic modifications play an important role during differentiation processes to allow tissue specific gene expression. Interestingly, it was shown that the epigenetic landscape is heavily modulated in cancer tissue and recurrent mutations in genes responsible for modifying these epigenetic marks are frequently found in almost every human cancer type, suggesting that epigenetic modifications play a crucial role in cancer development. I will summarize our recent findings on the tumor suppressive role of the MLL3/4-COMPASS like complex, one of the most frequently mutated epigenetic modifier complexes human cancer, elaborate specifically how it influences liver cancer as well as other cancer types, and demonstrate strategies to identify targets for tumors harbouring these genetic changes.

Oncolytic virotherapy-based strategies for cancer immunotherapy

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Hepatic tumors are promising targets for immunotherapies; checkpoint inhibitors are under clinical investigations with promising results. Success of checkpoint inhibition usually correlates with existing tumor infiltrating T cells suggesting that their combination with local cytolytic and immunogenic treatments could be a suitable option. Oncolytic viruses mediate tumor cell killing, activation of the tumor microenvironment and induction of tumor-directed T cell responses. Therefore, these agents may serve as immune activators for application of checkpoint inhibitors. Using oncolytic adenoviruses in various murine syngeneic tumor models, we investigated the therapeutic potential of such a viro/immunotherapy. Intratumor application of an oncolytic adenovirus alone resulted in effective tumor inflammation and interference with immunosuppressive cell subsets such as MDSCs and Tregs. In Ova-expressing tumors, adenoviral oncolysis led to effective cross presentation of SIINFEKL on MHC I of antigen presenting cells suggesting that oncolytic virotherapy works like an in-situ-vaccination for triggering antitumoral CD8 T cell responses. We investigated virotherapy and PD-1 checkpoint inhibition in triggering neoantigen-specific CD8 T cell responses according a panel based on next generation sequencing results and algorithm-based prediction. The combination of oncolysis and PD-1 blockade triggered a broad spectrum of neoepitope-specific T cell responses suggesting epitope spreading compared to monotherapies. In a transgenic model of liver cancer, resistance to PD-1-immunotherapy was abrogated by virotherapy facilitating improved elimination of lung metastasis. For optimal immune activation prior to checkpoint inhibition, we investigated whether Ad5-specific antibodies, a highly undesired byproduct of oncolytic adenovirotherapy, can be mobilized against tumors by molecular retargeting. Using bispecific adapters harboring antibody-capture domains from adenoviral hexon and a tumor ligand, we could inhibit the growth of subcu. tumors in syngeneic models (incl. B16F10 and MC38) and improved survival in a transgenic, electroporation-based model of liver cancer. Together, our observations show that the immunomodulatory properties qualify oncolytic adenoviruses as excellent tools to ‘warm up’ tumors for systemic checkpoint inhibition.

Molecular diagnostics (in liver cancer)

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Primary liver cancer includes hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), and combined hepatocellular-cholangiocarcinoma (HCC-CCA). While advanced HCC can be reliably diagnosed using dynamic imaging, the diagnosis of early HCC requires histological evaluation and frequently additional molecular characterization. Recently, morpho-molecular subtypes of human HCC have been proposed (e.g. fibrolamellar carcinoma – DNAJB1-PRKACA fusion; scirrhous HCC – TSC1/2 mutations), but current treatment algorithms for HCC follow the concept 'one-fits-all' and still lack any molecular stratification or tissue biomarker evaluation for precision oncology. Although preclinical models suggested molecular alterations that may predict either response (e.g. VEGFA amplification) or primary resistance (e.g. activation of p38 signaling) to 1st line sorafenib treatment, these have not been translated into the clinical practice. In addition, the rapidly evolving field of immune-oncology lacks biomarkers that are considered useful in HCC patients. Recent changes in clinical practice guideline opened the way for a more liberal indication for HCC biopsy, thus opening the door for prospective comprehensive molecular profiling of HCC patients.

In CCA patients, histological diagnosis is a basic principle and has opened the field of precision oncology during the last years. Molecular profiling identified several promising targets, which are currently tested in clinical trials (e.g. FGFR2 or NRTK1/2/3 gene fusions). Remarkably, the prevalence of druggable targets significantly differs between the primary locations of CCA (e.g. intra-/extrahepatic, gallbladder).

Nowadays targeted next generation sequencing (NGS) allows for the combined analyses of DNA and RNA using tiny amounts of nucleic acid isolated, thus facilitating the detection of copy number alterations, mutations, and genetic rearrangements from formalin-fixed, paraffin-embedded tumor biopsies. Applying so-called umbrella concepts, in which all patients eligible for systemic therapy receive a molecular characterization of tumor tissue, allow for biomarker identification and patient stratification both for already approved treatments and experimental therapies under clinical investigation.

Session IV

Bridging the gap

Towards an immune competent HCV mouse model

Thomas Pietschmann
TWINCORE, Hannover, Germany

Approximately 71 million people are chronically infected with hepatitis C virus (HCV) and the WHO estimates that approx. 1.75 million new infections occur annually. Although HCV infection can be cured, lack of diagnosis, and limited access to therapy impede control of HCV disease burden. Treatment-induced viral clearance does not protect from viral re- and a prophylactic HCV vaccine is not available. HCV vaccine development is hampered by lack of animals that are permissive to HCV infection.

HCV has a narrow species tropism and naturally infects only humans. Host factors governing the narrow species-tropism of HCV are incompletely defined.

We used genome-wide library screens to identify and characterize murine factors that limit HCV infection of mouse liver cells. In parallel we developed a step-wise virus adaptation procedure that led to up to 6 log increase in infectious virus production in mouse cell lines. In contrast infection of primary human hepatocytes, human stem cell-derived hepatocyte-like cells, and primary macaque hepatocytes remained unchanged, suggesting that adaptation is specific to mouse cells. Infection of primary mouse hepatocytes was inhibited by directly acting antivirals and it depended on expression of human entry factors as cells lacking human CD81 and human Occludin were not infected.

This step-wise adaptation procedure is a novel paradigm for exploring the viral determinants that govern species tropism. Ultimately, it will help to dissect risks and barriers of viral cross-species transmission. Moreover, the mouse-adapted HCV variant should facilitate development of HCV mouse models to prioritize HCV vaccine candidates and to examine important principles that govern efficacy of vaccine induced immune responses in vivo.

In vivo imaging of immune cells

Matthias Gunzer
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Migration is a key function of immune cells. In my talk I will demonstrate, how immune cells behave under normal conditions and under the impact of sterile or infectious inflammation in vivo. Thereby I will show, how they are mobilized in the bone marrow, leave the bone via special blood vessels to circulate in the blood and then enter inflamed tissues to perform their tasks. These analyses help us to understand the underlying biophysical and molecular mechanisms of immune cell traffic and function.

Linking the microbiota, chronic disease and the immune system

Dirk Haller
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The intestinal microbiome is suggested to play an essential role in the regulation of human health and disease susceptibility. Human cohort studies demonstrated changes in gut microbiota composition and function in a variety of different pathologies including inflammatory bowel diseases (IBD), colorectal cancer (CRC), obesity and type 2 diabetes. Although metagenomic resolution and bioinformatic tools considerably improved, allowing even strain level analysis, the search for microbial risk patterns in human cohorts is often confounded by environmental factors (e. g. medication) and host status (e. g. disease relapse). In addition, risk profiles partially overlap between IBD, CRC and metabolic disease, questioning the disease-specific prognostic and therapeutic value of the currently available information The gastrointestinal (GI) tract provides a compartmentalized interface with an enormous repertoire of immune and metabolic activities, where the multicellular structure of the mucosa has acquired mechanisms to sense luminal factors, such as nutrients, microbes and a variety of host-derived and microbial metabolites. The intestinal microbiome developed a highly co-evolved relationship with the host's cellular and immune system. Intestinal epithelial pattern recognition receptors (PRRs) substantially contribute to tissue homeostasis and immune surveillance. The role of bacteria-derived signals in intestinal epithelial homeostasis and repair has been addressed in mouse models deficient in PRRs and signaling adaptors. While critical for host physiology and the fortification of barrier function, the intestinal microbiota poses a considerable health challenge. Accumulating evidence indicates that dysbiotic microbial communities and/or selective pathobionts are associated with intestinal inflammation and tumorigenesis. Aberrant signal integration at the epithelial cell level contributes to development of intestinal disorders. An increased understanding of bacterial-specific structure recognition and signaling mechanisms at the intestinal epithelial interface is of great importance in the translation to future treatment strategies.

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POSTER ABSTRACTS

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Expression and function of early growth response 2 in hepatocellular carcinoma

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Introduction: Early growth response (EGR) proteins are a family of transcriptional regulators that mediate expression of multiple genes involved in cell growth and differentiation. Deregulation of EGR2 has been described in different cancer entities with in part anti- but also pro-carcinogenic effects.
The aim of this study was to investigate the expression and function of EGR2 in hepatocellular carcinoma (HCC).

Methods and Results: Quantitative RT-PCR and Western Blot analysis showed higher EGR2 expression in human HCC cell lines (Hep3B, HepG2, Huh-7, PLC) compared with primary human hepatocytes. Overexpression of EGR2 was also found in human HCC tissues compared with corresponding non-tumorous liver tissues. Using *si-RNA-pool* technique (functionally verified pool of 30 single si-RNAs against the human EGR2 mRNA), we established specific knockdown of EGR2 mRNA and protein levels in 2 different human HCC cell lines. EGR2 depletion in HCC cells resulted in reduced expression of IL-8 and ICAM-1, 2 factors known to promote HCC progression. In line with this, TCGA data analysis of 381 HCC patients using the “SurvExpress-Biomarker validation for cancer gene expression” revealed that enhanced EGR2 expression was associated with poorer overall survival.

Discussion/Conclusion: Enhanced EGR2 in HCC appears to promote cancer progression and thus, EGR2 could be a novel therapeutic target a potential candidate biomarker.

2

Sofosbuvir induces changes in cell cycle distribution by upregulation of B-MYB

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Introduction: With 80 million currently chronically infected patients, hepatitis C virus represents the second leading etiology for the development of hepatocellular carcinoma (HCC). Novel highly effective treatment with direct acting antivirals (DAAs) raised expectations of a decline in risk of HCC development. However, several groups have reported an early recurrence of HCC after DAA treatment in patients with previously cured HCC. This unexpected clinical observation has raised interest for deeper examination of underlying molecular changes after DAA treatment.

Methods: HCC cell lines were treated with DAAs daily for 4 days in a row. At day 5, specific analysis was performed. Distribution of cell cycle was analyzed by DNA staining. Expression of B-MYB mRNA was measured by qRT-PCR with a B-MYB specific probe. B-MYB and EGFR protein levels were evaluated by western blot analysis.

Results: Analysis of continuous single treatment with SOF (Sofosbuvir), DAC (Daclatasvir) and SIM (Simeprevir) on the cell cycle distribution revealed that SOF, but not DAC or SIM, treatment caused significant shift of cells from G0/G1 phase towards S and G2/M phase. We next validated that this change was due to active form of SOF. Further investigation revealed upregulation of B-MYB, which is transcription factor regulating progression of the cell cycle and its expression can be utilized as a marker of changes in cell cycle. Moreover, EGFR, upstream regulator of B-MYB, was up-regulated specifically after SOF treatment as well.

Discussion/Conclusion: SOF treatment significantly changed cell cycle distribution and upregulated B-MYB and its upstream regulator EGFR in HCC cell lines. Since both proteins are involved in cancer initiation and progression further investigation of underlying changes after SOF treatment on hepatocytes is necessary.

Immunological crosstalk between host and virus in chronic viral hepatitis

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Introduction: The immunological functions of parenchymal and non-parenchymal liver cells against hepatotropic viruses are not well understood. Therefore, aim this project is to characterize the toll-like receptor (TLR) signaling, antiviral capacity and virus – host interaction in primary human parenchymal and non-parenchymal liver cells.

Methods: Primary human hepatocytes (PHH), Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC) and hepatic stellate cells (HSC) were isolated from liver tissues by collagenase perfusion, density gradient centrifugation and magnetic-activated cell sorting. Cells were stimulated with TLR ligands, infected with/exposed to cell culture-derived hepatotrophic viruses (HepG2.117, JFH1/J6 HCVcc) and analyzed by gene array, quantitative RT-PCR, western blot and ELISA.

Results: Parenchymal and non-parenchymal liver cells expressed and secreted inflammatory cytokines in response to toll-like receptor stimulation. However, only poly (I:C)-activated PHH, KC, LSEC and HSC mediated an antiviral activity against hepatitis C virus. *In vitro*, HCVcc infection in PHH was cleared within few days, due to a strong interferon response. Interestingly, responsiveness to poly(I:C) was enhanced in PHH and LSEC from hepatitis C virus-positive donors, represented by significantly elevated gene expression levels of *IFNB1 and IFNL1-3* compared to uninfected controls. In contrast to HCVcc, PHH could be efficiently infected with cell culture-derived HBV particles, indicated by the secretion of viral antigens and expression of viral mRNA transcripts. In the initial phase of infection PHH were able to sense HBV particles via TLR2, which led to an interferon-independent antiviral effect that however failed to clear the infection. Again, PHH isolated from chronic HBV-infected livers exhibit a higher sensitivity to TLR2 activation.

Discussion/Conclusion: The hepatic immune system is involved in detecting and defending hepatitis B and hepatitis C viruses. Chronic infection seems to specifically sensitize the immune system without eliminating the virus but facilitating inflammation and tumor progression.

4

Induction and interference of toll-like receptor 3 signaling by hepatitis A and hepatitis C virus

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Introduction: Hepatitis A (HAV) and hepatitis C virus (HCV) both have been shown to potentially interfere with toll-like receptor 3 (TLR3) signaling by proteolytic cleavage of the TLR3 adaptor TRIF. However, the functional significance of TRIF cleavage for HCV and HAV replication has not been clarified yet. We therefore aimed to assess the level of TLR3 activation induced by HCV and HAV, quantify the efficiency of TRIF cleavage by HCV NS3/4A and HAV 3CD protease and analyze the importance of interference with TLR3 activation for replication of both viruses.

Methods: Huh7 cells were used as a model for hepatic TLR3 activation, since they support replication of both viruses. This cell line, in contrast to human hepatocytes, does not express endogenous TLR3; TLR3 expression and signaling was therefore reconstituted by lentiviral transduction. We further generated Huh7-TLR3 cell clones with knockout of endogenous TRIF, allowing reconstitution with protease resistant variants.

Results: HCV replication induced a strong TLR3 response in Huh7 cells, mediated by viral replication intermediates (dsRNA) reaching the endosomal compartment. In case of HAV, we found no induction of TLR3, indicating that either viral dsRNA did not reach the endosome, or that the TLR3 pathway is efficiently blocked. To quantitatively assess TRIF cleavage and interference with TLR3 signaling we are currently establishing a transient expression system using tagged viral proteases (active- and inactive form) and TRIF (cleavable and cleavage-resistant). We are furthermore reconstituting TRIF expression in TRIF-knockout cells, using cleavable and cleavage resistant TRIF variants to unravel the importance of TRIF cleavage for HAV and HCV replication.

Discussion/Conclusion: Our results indicate that HAV and HCV have very different strategies to cope with TLR3 responses. We will further evaluate how these differences contribute to clearance and persistence of hepatotropic RNA viruses.

Differences in UDCA treatment response among primary biliary cholangitis patients with and without cirrhosis

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Introduction: Primary biliary cholangitis (PBC) is a rare cholestatic multifactorial liver condition associated with positive AMA's, alkaline phosphatase (AP) elevation, and risk of progression to cirrhosis if not diagnosed at early stages. The elected treatment is oral ursodeoxycholic acid (UDCA), but differences in treatment response among PBC patients with and without cirrhosis has not been previously described.

Objective: To determine differences in UDCA treatment response among PBC patients with and without cirrhosis.

Methods: We realized a retrospective, observational, analytic, transversal study from January 2012 to December 2018 in which we included all patients with confirmed PBC diagnosis from two centers, with a minimum of six months receiving UDCA treatment at stablished doses (13–15mg/kg/day). We analyzed percentage of reduction in AP levels, changes in severity calculated with the Barcelona system and then we compared the percentages of response between different age stages, and then response between patients with and without cirrhosis was compared.

Results: A total of 71 female patients with confirmed PBC diagnosis were initially selected. From those, 5 patients were excluded because lost of follow-up, 8 because lack of recent lab results, and 2 because of decompensation associated with other causes (DILI, infections). At the end, 56 female PBC patients (100%) were included for the final analysis, with a media of age of 56.6 years, from which 29 (51.78%) were non-cirrhotic and 27 (48.27%) were cirrhotic: 13 patients with Child Pugh A (23.2%) and 14 (25%) with Child Pugh B, with non-significant differences in demographic characteristics between groups. The media in AP before treatment was 472, and the media after 6 month-UDCA treatment was 266, with a media of AP reduction of 202, and an average percentage of response of 42.8%. Taking into account only patients without cirrhosis, media of AP reduction was 265, and an average percentage of response of 47.8%. As for PBC patients with cirrhosis, media of AP reduction was 141.3, and an average percentage of response of 36.8%. We found a delta of 11% in treatment response favoring the non-cirrhosis group, with an OR 1.9 of having a good treatment response after six months compared with the cirrhotic patients group (CI 1.08–3.47, $p = 0.01$).

Conclusions: In PBC, we found a difference in UDCA treatment response favoring non-cirrhotic patients compared with those with cirrhosis. This highlights the importance of early diagnosis and detection in order to start treatment and stop progression of disease.

6

NK-cell responses are biased towards CD16-mediated effector functions in chronic hepatitis B virus infection

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Introduction: Phenotypical and functional NK-cell alterations are well described in chronic hepatitis B virus (cHBV) infection. However, it is largely unknown whether these alterations result from general effects on the overall NK-cell population or the emergence of distinct NK-cell subsets. Memory-like/adaptive NK cells, for example, emerge in association with human cytomegalovirus (HCMV) infection, that is common in cHBV infection. Phenotypically and functionally, these memory-like/adaptive NK-cells differ from conventional NK cells. Especially the absence of Fc<epsilon>RI<gamma> marks memory-like/adaptive NK cells, including superior CD16-mediated effector functions.

Methods: To assess the impact of memory-like/adaptive NK cells on phenotypic and functional alterations in cHBV infection we performed in-depth analyses of circulating NK cells from patients with HBV (n = 52), or chronic Hepatitis C infection (HCV) (n = 45) and age- and sex- matched healthy donors (HD) (n = 50), with respect to their HCMV serostatus.

Results: In cHBV/HCMV+ patients, Fc<epsilon>RI<gamma>- CD56dim memory-like/adaptive NK cells were present in higher frequencies and with a higher prevalence compared to HD/HCMV+. This pronounced HCMV-associated memory-like/adaptive NK-cell expansion could be identified as key determinant of the NK-cell response in cHBV infection. Furthermore, we observed that memory-like/adaptive NK cells consist of epigenetically distinct subsets and exhibit metabolic key characteristics of long-living cells. Despite ongoing chronic infection, the phenotype of memory-like/adaptive NK cells was conserved in cHBV/HCMV+ patients. Functional characteristics of memory-like/adaptive NK cells also remained largely unaffected by cHBV infection with the exception of an increased degranulation capacity in response to CD16 stimulation.

Hence, NK-cell responses are biased toward CD16-mediated effector function in cHBV infection.

Discussion/Conclusion: The emergence of HCMV-associated memory-like/adaptive NK cells shapes the overall NK-cell response in cHBV infection and contributes to a general shift towards CD16-mediated effector functions. HCMV coinfection therefore needs to be considered with respect to the design and application of new immuno-therapies in HBV cure targeting NK cells.

7

Expression and function of histone deacetylases in liver fibrosis and hepatic stellate cells

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Introduction: Histone deacetylases (HDAC) comprise in humans 18 members divided in 4 classes. Recent evidence has highlighted a pathological imbalance in hepatic fibrosis between the acetylation and deacetylation of histone proteins regulated by histone deacetylases. Application of HDAC inhibitors (HDACi) appears as promising therapeutic strategy in different types of cancer. However, detailed information about HDAC expression and functional mechanisms of HDACi-action in liver fibrosis are lacking.
The aim of this study was to describe the expression pattern of the class I, II and IV HDACs in hepatic fibrosis and HDACi effects on hepatic stellate cells (HSC).

Methods and Results: Quantitative RT-PCR analysis revealed significantly increased expression of (i) HDAC 1/2/3/8 (class I); (ii) HDAC 4/5/6/9 (class IIa); (iii) HDAC 6/10 (class IIb) and (iiii) HDAC 11 (class IV) in different murine liver fibrosis models (TAA treatment, methionine-choline deficient diet, Western type diet and bile duct ligation. Analysis of human fibrotic liver tissues revealed strong correlation of HDAC2/8/4/5/7/9 and 10 with alpha smooth muscle actin, a marker of activated HSC. These HDACs were also strongly expressed by activated HSCs from different human donors, and HDAC9 and 11 expression was found to increase during HSC activation in vitro. Next, we analyzed the toxic dose range of different HDACi (suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA) and trapoxin (TPX)) on LX2 cells, a human HSC line and primary murine HSC *in vitro*. All 3 HDACi caused dose-dependent toxicity in HSC, while the same doses did not induce any toxicity in primary human hepatocytes. The maximally tolerated, non-toxic doses (500nM (SAHA), 50nM (TSA) and 1nM (TPX)) were applied to HSC to analyze functional effects. All 3 HDACi caused a dose-dependent reduction of proliferation as assessed by impedance based real time proliferation assays.

Discussion/Conclusion: During liver fibrosis HDAC expression levels appear to generally increase and to correlate with the stage of fibrosis and the activation of hepatic stellate cells. Different HDACi reduce the viability of activated HSC or reduce their proliferation in subtoxic doses, respectively. Our findings indicate the potential of HDAC inhibition as anti-fibrogenic therapeutic strategy in chronic liver disease.

A novel Krueppel-like factor 12/Interleukin-6 signal transducer – microRNA-188-5p interaction reveals therapeutic targets for hepatocellular carcinoma

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Introduction: MicroRNAs (miRs) decisively shape the molecular landscape underlying hepatocellular carcinoma (HCC). Our previous finding that microRNA-188-5p down-regulation contributes to the aggressive phenotype of activated synovial fibroblasts in rheumatoid arthritis (Ruedel et al., 2015) prompted us to hypothesize that miR-188-5p could affect cancer cells including HCC.

Methods: Human HCC cell lines (PLC, Hep3B, HepG2, and Huh-7) and primary human hepatocytes (PHH) were used for expression and functional analyses. *In vivo* gene expression was assessed in patient-derived HCC tissue samples and corresponding non-tumorous liver tissues. RNA expression arrays (Affymetrix) were used to identify novel microRNA target genes. Functional experiments including Boyden chamber migration assays, clonogenicity assays, and real-time cell proliferation analysis were performed after overexpression of miR-188-5p-mimics or siRNA-Pool-mediated silencing of target genes.

Results: MiR-188-5p was strongly downregulated in HCC and re-expression of miR-188-5p revealed strong tumor suppressive effects on HCC cell lines. Using RNA expression arrays, we identified a list of potential novel target genes of miR-188-5p in HCC including Krueppel-like factor 12 (KLF12), Interleukin-6 signal transducer (IL6ST) and Discs large MAGUK scaffold protein 5 (DLG5). MiR-188-5p-mediated regulation of these potential targets was confirmed *in vitro*. Additionally, KLF12, IL6ST, and DLG5 were found to be elevated in patient-derived HCC samples as compared to matched non-tumorous tissues. Functional analyses following siRNA-Pool-mediated knock-down identified IL6ST and KLF12 as potent regulators of HCC cell migration and clonogenicity, respectively.

Discussion/Conclusion: KLF12 and IL6ST were found to be novel target genes of the tumor suppressive microRNA-188-5p in HCC. Moreover, these miR-targets were identified to be potential novel therapeutic targets in liver cancer.

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Deciphering the role of bile acid signaling in intrahepatic cholangiocarcinoma (iCC)

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Introduction: Cholangiocarcinoma (CC) it is a heterogeneous group of liver malignancies with features of biliary tract differentiation, it is the second most frequent primary liver malignancy after hepatocellular carcinoma (HCC). Taking in account the late diagnosis of CC, its bad prognosis and the scarcity of therapies available, there is an urgent need to develop novel therapeutic strategies. Primary sclerosing cholangitis, infections with liver flukes, liver cirrhosis or HBV/HCV virus infections are the main known risks for CC. All these risk factors share, as a putative pathogenic mechanism, chronic inflammation involving the biliary tract, in which Kupffer cell-triggered TNF/JNK signaling plays an important role. Intrahepatic accumulation of bile acids may favour this carcinogenic process, by triggering an inflammatory response and cholangiocyte proliferation. It has been demonstrated that bile acids activate the MAPK signaling pathways including extracellular signal-regulated kinases 1/2 (ERK1/2) and JNK, which may be involved in bile acid induction of cytokines, chemokines, and adhesion molecules that mediate the inflammatory response. However, the individual role of each individual bile acid receptor has not been fully clarified because different types of bile acids have been shown to have different effects on intracellular signals, and the effect depends on the type of bile acid, and on the context (fasting, over-nutrition etc.). In the context of intrahepatic CC mouse models (iCC), the manipulation of the signaling of individual or combined bile acid receptors by genetic or chemical means has not yet been studied.

Methods: We used hydrodynamic tail vein injection to deliver iCC-inducing oncogenes in the mouse liver, resulting in iCC development.

Results: Interestingly, the treatment of iCC-bearing mice with a bile acid-supplemented diet resulted in increased liver damage but in a consistent reduction of tumor initiation and significantly increased mouse survival. In contrast, whole-body overexpression of an individual bile acid receptor resulted in decreased survival.

Discussion/Conclusion: We hypothesize that TNF/JNK axis and bile acid signaling cooperatively promote the hepatic pro-inflammatory environment and cholangiocyte transformation/proliferation that leads to iCC, and therefore could be used to develop new therapies for iCC.

Novel feedback inhibition in hepatocellular carcinoma of Lin 28 homolog A by tumorsuppressor miR-622

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Introduction: Hallmarks of cancer progression and emergence of therapy resistance are associated with embryonic and/or stem cell characteristics. Lin 28 homolog A (LIN28A) is a proto-oncogene and RNA binding protein, which represses the let-7 family of miRNAs in embryonic stem cells. The microRNA (miR) let-7 family comprises potent tumorsuppressive microRNAs. However, the exact role of the embryonic stem cell marker LIN28A is uncertain in hepatocellular carcinoma (HCC) development. Moreover, the specific interaction and regulation between LIN28A and the miRs is still not identified.

Methods: Primary human hepatocytes and several human HCC cell lines (PLC, Hep3B, HepG2 and Huh 7) were used for expression and functional analysis. *In vivo* expression analysis was performed using paired tumor and corresponding non-tumor liver tissues from HCC patients. Gene expression and cellular localization was analyzed qRT-PCR, Western blot, and Fluorescence microscopy. MicroRNA mimics and inhibitors for miR-622 were used for functional analysis.

Results: LIN28A expression was increased in HCC cell lines as compared to primary human hepatocytes. Tissue micro array analysis revealed enhanced LIN28A protein expression in HCC *in vivo*. Fluorescence-based microscopic analysis showed that LIN28A can be identified in the nucleus of HCC cells and in patient tissues. Furthermore, Clonogenicity assays revealed that LIN28A induced stem cell properties in HCC cells. Multi-step based in silico and experimental analysis showed a novel feedback regulation of LIN28A expression in HCC mediated by the tumorsuppressive microRNA-622.

Conclusions: We found that the embryonic stem cell marker LIN28A is overexpressed in HCC *in vitro* and *in vivo* and correlated with tumor stages. LIN28A was also sufficient to induce stem cell properties. Further, we detected a novel feedback inhibition of LIN28A expression which was mediated by the tumorsuppressive microRNA-622 in hepatocellular carcinoma.

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Mode of action of the RIG-I like receptor LGP2 in the interferon response triggered by viral infections

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Introduction: The cytoplasmic RIG-I like receptors (RLRs) RIG-I, MDA5 and LGP2 bind to viral RNA and mount a powerful first line of defense in vertebrate cells. Binding of RLRs to RNA triggers a signaling cascade resulting in the activation of type I and III interferons (IFNs) and IFN stimulated genes (ISGs). RIG-I and MDA5 initiate this event through direct activation of the downstream adaptor mitochondrial antiviral-signaling protein (MAVS). Given that LGP2 lacks the MAVS interaction domain it cannot initiate this innate response. Instead, it plays a regulatory role by influencing the magnitude of the IFN response induced by RIG-I and MDA5. LGP2 enhances the IFN response triggered by MDA5, but has a negative effect on the IFN response in the case of RIG-I. Here we studied how LGP2 might switch between these disparate modulatory functions with the aim to better understand the regulation of the IFN system.

Methods: To characterize LGP2's regulatory role we aim to identify post translational modifications and plan to determine the influence of RLR protein levels on LGP2 modulated IFN induction. The experiments are conducted in different cell culture systems and include knockout and overexpression approaches. As read-out we use activation of the IFN system by measuring mRNA and protein levels of ISGs.

Results: In Huh7 and A549 cells stably overexpressing LGP2 we reproducibly observe an enhancement of the MDA5 mediated IFN response and a suppression of RIG-I mediated signaling. These effects correlate with LGP2 protein abundance and were found with different viruses (a Mengovirus mutant called Mengo Zn virus, Sendai virus, hepatitis C virus). Mass spectrometry revealed possible phosphorylation sites in LGP2 that are currently validated by using functional assays.

Discussion/Conclusion: Our overall goal is to shed more light on how LGP2 regulates the IFN response in cells and how it influences viral replication.

The exhausted fate of memory-like HCV-specific CD8+ T cells

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Introduction: In chronic HCV infection, T cell exhaustion is described as a functional impairment of virus-specific T cells. We have previously reported that exhausted HCV-specific CD8+ T cells are comprised of terminally exhausted CD127-PD1hi and memory-like CD127+PD1+ subsets. However, to what extent memory-like HCV-specific CD8+ T cells resemble conventional memory or exhausted cells and which impact viral antigen recognition and successful therapy has on the phenotype of these cells remain unclear.

Methods: In order to define the molecular determinants of memory-like subsets, we conducted low-input RNAseq analyses of CD127/PD1-based HCV-specific CD8+ T cell subsets obtained during and after chronic HCV infection targeting consensus and escaped epitopes (n = 5 patients) and after spontaneous resolution of acute HCV infection (n = 3 patients). Via unsupervised clustering, DESeq2 analyses and WGCNA, we investigated the similarities and differences among the different subsets in distinct clinical conditions.

Results: Although in chronic HCV infection memory-like HCV-specific CD8+ T cells exhibit characteristics of memory T cells, on a transcriptional level, however, an exhausted signature is dominant even after DAA-mediated viral clearance. This suggests an imprinted exhausted T cell fate. Thus, memory-like HCV-specific CD8+ T cells are distinct from conventional memory T cells and rather resemble exhausted T cells. Furthermore, HCV-specific CD8+ T cells targeting escaped epitopes also showed a clear exhausted profile. Thus, chronic HCV infection is accompanied by a HCV-specific CD8+ T cell differentiation program, namely exhaustion, irrespective of viral escape. Additionally, regulatory networks of transcription factors important in CD8+ T cell differentiation differed in chronic compared to resolved HCV infection.

Discussion/Conclusion: Thus, chronic HCV infection is strictly linked to an “exhausted” T cell differentiation that is not simply reverted by removal of viral antigen or loss of antigen recognition. This has potential implications for the control of re-infection and therapeutic vaccines.

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Characterization of naturally occurring single nucleotide polymorphisms in the toll-like receptor 3 gene

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Introduction: Several single nucleotide polymorphisms (SNPs) in the toll-like receptor 3 (TLR3) gene have been reported to be associated with human diseases and the outcome of viral infections. We therefore selected and analyzed 25 SNPs affecting the TLR3 amino acid sequence according to their clinical relevance, their position in structurally important loci and their frequency in the population, in order to gain a better understanding of the functionality of TLR3 and its role in innate immunity. We focused on responses to viral infections in hepatocytes, showing high endogenous TLR3 expression.

Methods: Human hepatoma cell lines (Huh7) lacking endogenous TLR3 expression were stably transduced with lentiviral vectors encoding TLR3 variants. TLR3 induction was assessed by poly (I:C) stimulation and RT-qPCR based measurement of interferon stimulated genes (ISG). Different viral model systems will be used to test the effect of TLR3 on viral load, cell survival and ISGs induction.

Results: We first confirmed equal TLR3 expression on both mRNA and protein level in all cell lines ectopically expressing TLR3 variants. After poly (I:C) stimulation we quantified ISG56, CxCL10 and IL29 expression by RT-qPCR. Interestingly, we identified several SNPs unable to mount an ISG response upon poly (I:C) stimulation. However, all SNPs with complete lack of function have a low frequency in the human population. Several other SNPs led to an apparently increased ISG induction compared to wild type TLR3. SNPs with clinical relevance did not show major pathway deficiencies upon primary investigation. In depth analysis of TLR3 responses using different viral models and more focused mechanistic studies will unravel more subtle discrepancies.

Discussion/Conclusion: SNPs can have a major impact on TLR3 induction and hereby influence the outcome of viral infections. Further investigation will be performed to better understand the molecular impact of mutations in the TLR3 gene on receptor functionality.

Knockout of type I interferon-response leads to reduced induction of autophagy in HBs-transgenic mice

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Introduction: Hepatitis B is one of the most common infectious diseases. Since chronically infected patients have low chance of curation, new therapeutic strategies are needed. Autophagy is essential for cell-survival during starvation, for protein turnover and cellular immunity. The proteins HBs and HBx can modulate autophagy. Our goal was to characterize the role of the interferon signaling on autophagy in HBs-transgenic mice.

Methods: We used HBs-transgenic IRF3/7-knockout mice on BALB/c background. The livers of 8- and 12-week-old male mice were analyzed via western blot, immunofluorescence, immunohistochemistry, and RT-PCR. Markers of autophagy (p62, LC3 and ubiquitin), markers of ER Stress (ATF3, CHOP) and interferon-modulated genes (Oas1a, ISG-15 und Ifit1) as well as intrahepatic fat and glycogen accumulation were assessed.

Results: Accumulation of p62 and LC3-II was shown in HBs-transgenic mice via western blot. p62 and LC3 were not regulated on transcriptional level. IRF3/7-KO mice accumulate less LC3-II while maintaining the same amount of p62 compared to non IRF3/7-KO. The expression of CHOP and ATF3 was increased in HBs-transgenic and IRF3/7-KO HBs mice. Accumulation of ubiquitin, fat, and glycogen in aggregates was observed histologically. Colocalization of ubiquitin, HBs, p62, and PLIN2 a marker for lipid droplets was shown. Activation of Ifit-1, Oas1a, and ISG-15 was exclusively prevalent in HBs-transgenic mice.

Discussion/Conclusion: Indicators for activation and late inhibition of autophagy were found in HBs-transgenic IRF3/7-KO mice. The knockout of the interferon response caused a reduced activation of autophagy while not changing the amount of degraded protein (Flux). The formation of aggregates indicates an inhibition of autophagy and could be a cause for the ER stress and interferon response occurring, that then could also modulate autophagy. Our results regarding the regulation of cell stress and autophagy via type I interferon-signaling could be the basis for the development of new diagnostic and therapeutic approaches.

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CB1 knockout alleviates hepatic steatosis via lipophagy and lipolysis in HBs transgenic mice.

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Introduction: The endocannabinoid (EC) system has been implicated in the pathogenesis of several metabolic diseases, including nonalcoholic fatty liver diseases (NAFLD). The present study aims to describe the effect of selective CB1 knockout on the hepatic lipid metabolism in a HBs transgenic mouse model.

Methods: Hybrids of HBs transgenic mice and CB1-knockout mice were bred on genetic backgrounds of B6. The intracellular lipids were assessed using Oil-Red-O staining, biochemical triglyceride (TGs) assay and thin layer chromatography (TLC). The expression of key proteins regulating autophagy and lipid metabolism were analyzed by IHC, IF and Western blot.

Results: CB1 knockout mice showed a reduction in body weight and LDs sizes and TGs in HBs/CB1$^{-/-}$ mice as compared to HBs transgenic mice. CB1 KO mediates metabolic disturbances as shown by increased GLUT1 expression on the surface of hepatocytes and activation of transcription factors CREB and FOXO1. Metabolic disturbances induces autophagy flux progression as shown by decreased LC3B-II and LAMP1 by WB and IF HBs/CB1$^{-/-}$compared to HBs. LAMP1 and lipid droplets binding protein PLIN2 are co-localized in HBs/CB1$^{-/-}$ indicating autophagy of LDs i.e. lipophagy. Autophagy mediated decrease of PLIN2 protein level was also associated with elevated expression of lysosomal acid lipase (LAL), cytoplasmic lipases, hepatic lipase (LIPC) and adipose triglyceride lipase (ATGL). ATGL and PLIN2 co-localization further indicated enhanced ATGL interaction with LDs promoting the lipophagy.

Discussion/Conclusion: In conclusion, the present study shows that CB1 receptor knockout decreases hepatic steatosis via lipophagy and cytoplasmic lipolysis in HBs transgenic mice.

Intracellular but not extracellular IFNL4 is a stronger inducer of STAT1 and ISGs in liver cell lines than IFNL3

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Introduction: Interferons lambda (IFNL) 1 to 4 represent the type III interferon family. Whereas IFNL1–3 mediate antiviral immunity by inducing interferon sensitive genes (ISGs) in epithelial tissues through the JAK-STAT signaling pathway, a minor allele rs368234815 ΔG creating the functional gene from *IFNL4* pseudogene is associated with impaired clearance of hepatitis C virus (HCV) despite of higher liver expression of ISGs in untreated HCV patients. High expression of ISGs contradicts poor secretion of IFNL4. We aimed to explain these discrepancies.

Methods: Expression vectors containing *IFNL4* open reading frame, *IFNL4* pseudogene and *IFNL3* were used for transfections of wild type and IFNLR1-KO HuH7 cells generated by CRISPR/Cas9. Expression profiling was performed using the Illumina HumanHT-12 v4 chip and validated by RT-PCR. STAT1 and pSTAT1 levels were detected by western blot. Next, we performed confocal microscopy of IFNL3 and IFNL4 transfected wild type and IFNLR1-KO cells in order to assess their subcellular localization. Major findings were validated in HepG2 cells.

Results: Neither IFNL3 nor IFNL4 induced STAT1, ISGs and STAT1 phosphorylation in *IFNL3* or *IFNL4* gene-transfected or protein-stimulated HepG2 and Huh7 cells lacking the IFNLR1 receptor subunit. Control cells transfected with IFNL4 showed higher increase of STAT1 and ISGs expression and STAT1 phosphorylation than IFNL3 transfected cells but no such difference was observed between the protein-stimulated cells. Control cells transfected with *IFNL3* and *IFNL4* lacking the signal peptides accumulated the interferons in cytosol but JAK-STAT signaling was not triggered and ISGs expression remained unchanged. JAK-STAT signaling activation triggered by extracellular interferons was intact as well.

Discussion/Conclusion: The results confirm that upregulation of ISGs stimulated by IFNL3 and IFNL4 is mediated exclusively via the IFNLR1/IL10R2 receptor. The canonical JAK-STAT signaling is not affected by overexpression of type III interferons in cytosol. Potential role of other cell compartments is under investigation.

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Linking gut dysbiosis in hepatitis C and interleukin 10 (rs1800872) and heat shock protein 70-2 (rs1061581) genes polymorphisms

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Introduction: Multiple immune evasion strategies employed by hepatitis C virus (HCV) include manipulation of cytokine responses. IL-10 as anti-inflammatory is capable of inhibiting synthesis of pro-inflammatory cytokines such as IL-2, IL-3, IFN-γ, TNFα, etc. It also has a potent ability to suppress the antigen-presentation capacity of antigen presenting cells; it may also be stimulatory towards Th2-cells. Recent studies display IL-10 associations with effectiveness of chronic hepatitis C (CHC) treatment. The heat shock protein 70 (HSP70) acts as both potent cell protector and HCV assembly blocker. Furthermore, both cytokines may bridge gut microbiota, inflammation and chronic HCV infection with immune mechanisms and fibrosis. The aim of the study is to clarify the association of IL-10 (rs1800872) and HSP70-2 (rs1061581) genetic polymorphisms (SNP) in dysbiosis development under CHC.

Methods: The study involved 25 individuals (mean age 38.66 ± 3.11) with CHC and microbiologically confirmed dysbiosis. Control formed by 30 practically healthy individuals. RT-PCR was used to study the HSP70-2 (A1267G, rs1061581) and IL-10 (C-592A, rs1800872) genes' SNPs. Data analyzed in SnapGene and Vitran software packages.

Results: No statistically reliable differences in genotypes and alleles of both HSP70-2 (A1267G) and IL-10 (C-592A) genes' SNPs relative frequencies between study and control groups were observed. HSP70-2 (rs1061581) gene's G-allele, and IL-10 (rs1800872) gene's C-allele dominated ($p < 0.001$) in both groups. Distribution of HSP70-2 gene's 1267A→G and IL-10 gene's C-592A SNPs in studied cohort meets the Hardy-Weinberg equilibrium ($p > 0.05$) with a slight excess of heterozygosity, which generally does not disturb the population balance. Polymorphic variants of HSP70-2 gene may not be additional risk or protection factors for dysbiosis in observed group; IL-10 gene's (rs1800872) minor A-allele associates with double increased dysbiosis risk [OR = 1.95; 95%CI: OR = 0.9–4.12; p = 0.046] accompanied by marginal protection role of C-allele [OR = 0.51; 95%CI: OR = 0.25–0.98; p = 0.057].

Discussion/Conclusion: Mutant A-allele of the IL-10 (rs1800872) gene associates with almost twice-increased dysbiosis risk, with protective role of C-allele. Polymorphic variants and alleles of the HSP70-2 (rs1061581) gene are not additional risk factors in the studied population.

PNPLA3 (adiponutrin) p.I148M risk allele carriers might be at-risk of chemotherapy-associated steatohepatitis (CASH)

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Introduction: Patients receiving systemic chemotherapy may develop liver steatosis, which increases the perioperative risks of liver resection. The pathogenesis of chemotherapy-associated steatohepatitis (CASH) is poorly understood. As shown previously, three critical gene variants, i.e. *PNPLA3* p.I148M, *TM6SF2* p.E167K and *MBOAT7* rs641738 are associated with higher hepatic fat contents. Here we investigate the association of these variants with CASH.

Methods: Prospectively we recruited 60 patients (age 31–81 years) scheduled for systemic 5-fluorouracil- or gemcitabine-based chemotherapy (68% palliative) for gastrointestinal cancer. Hepatic fat (controlled attenuation parameter, CAP) and liver stiffness (LSM) were measured by elastography before initiation of chemotherapy (CAP0) and after at least two and four cycles (CAP1 and CAP2). Patients with right lobe liver metastases were excluded. *PNPLA3*, *TM6SF2* and *MBOAT7* variants were genotyped using TaqMan assays.

Results: Included patients displayed following CAP values: CAP0-215.0 ± 55.73 dB/m, CAP1-223.3 ± 53.6 dB/m, CAP2-223.4 ± 56.7 dB/m, consistent with mild steatosis. Initial CAP correlated with BMI ($p < 0.001$) and serum triglycerides ($p = 0.026$). Before initiation of chemotherapy, none of the gene variants was associated with liver steatosis or LSM. Interestingly, at CAP1, carriers of the prosteatotic *PNPLA3* allele [148M] showed significantly ($P = 0.008$) higher CAP (248.9 ± 46.5 dB/m) as compared to patients with wild-type genotypes (209.8 ± 52.2 dB/m). On average, in carriers of the [148M] risk allele liver fat increased by 14.7 ± 23.1% between CAP0 and CAP1, whereas in carriers of the wild-type genotype the increment of liver fat was significantly ($p = 0.034$) lower (2.1 ± 22.6%). This difference disappeared at CAP2. Neither *TM6SF2* nor *MBOAT7* polymorphisms affected liver fat contents.

Discussion/Conclusion: Individuals carrying the *PNPLA3* p.I148M risk allele might be prone to hepatic fat accumulation during chemotherapy. Our results imply that variant *PNPLA3* might confer more rapid remodeling of hepatic lipids under metabolic stress in this setting. The effects of liver fat deposition during chemotherapy should be investigated further in clinical trials.

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Hepatocellular c-Jun activation via S. mansoni infection in a hamster model and primary human hepatocytes

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Introduction: Schistosomiasis is an infectious disease affecting more than 200 million people worldwide. S. mansoni eggs mostly get trapped in the liver, leading to severe complications like portal hypertension and fibrosis. Clinical studies have shown that a S. mansoni infection can lead to an increased incidence of HCC in combination with HBV- or HCV-infection. This study aimed to identify signaling-pathways that may contribute to S. mansoni-promoted carcinogenesis.

Methods: Primary human hepatocytes (hiPS-HEP-cells) were stimulated with proteins secreted by the S. mansoni eggs and oncogenic pathways were analyzed. In addition, inhibition experiments were performed to investigate the activation mechanism of cellular factors like c-Jun. Furthermore, liver tissue of bisex/singlesex S. mansoni infected hamsters was examined by Western blots, Proteome Profiler, and immunohistochemistry.

Results: In human hepatocytes the activation of JNK, the upstream-kinase of c-Jun and its activator MKK4 was detected by stimulating the cells with SEA (soluble egg antigen) or IPSE (the major component of SEA). The phosphorylation of c-Jun via JNK was verified by inhibition experiments. Downstream factors of c-Jun like MCM2 and cleaved Caspase 3 were induced in cells stimulated with SEA/IPSE, while inhibition of JNK prevented this effect. Immunhistochemical analyses showed the co-localization of c-Jun and γ-H2AX in hepatocytes of livers of bisex infected hamsters.

Discussion/Conclusion: The JNK-dependent activation of c-Jun leads to a higher expression of proliferation markers like MCM2 in hepatocytes and in whole liver tissue of schistosome-infected hamsters. Simultaneously, with γ-H2AX it was possible to detect DNA-damage in c-Jun positive hepatocytes. Both results lead to the conclusion that soluble factors of S. mansoni eggs induced replicative stress which may be associated with the induction of carcinogenesis. Our work identified first target structures for the increased HCC incidence after S. mansoni infection in combination with HBV and HCV, which could lead to follow-up studies investigating diagnostic- and therapeutic approaches.

Ultrastructural characteristics of liver sinusoidal endothelial cells in morphogenesis of pediatric autoimmune hepatitis: the first electron microscopic report

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Introduction: The pathological mechanisms of autoimmune hepatitis (AIH) – a chronic immune-mediated, autodestructive liver disease – are not fully understood. Although AIH in childhood is rare, it leads to cirrhosis more often than in adults. Recently, in the disease morphogenesis an increasing role has been ascribed to non-parenchymal hepatic cells (NPCs), particularly Kupffer cells/macrophages (KCs/MPs) and liver sinusoidal endothelial cells (LSECs). Unfortunately, the research has been limited mainly to adult patients. The involvement of LSECs, accounting for approximately 40% of the NPC population, in the pathogenesis and progression of AIH is extremely interesting, yet still not fully known. The current study objective was electron-microscopic analysis of LSECs in pediatric AIH.

Methods: Ultrastructural investigations of LSECs were performed on pretreatment biopsies obtained from 19 children, aged 4–17 years (5 boys and 14 girls), with clinically and histologically diagnosed AIH. Fresh small liver blocks (1 mm^3 volume) were fixed in solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 and routinely processed for TEM analysis.

Results: Our study is the first to describe alterations in LSECs, from swelling to necrosis, demonstrating their important role in the morphogenesis and progression of pediatric AIH. Swollen endothelial cells relatively frequently showed features of defenestration, i.e. contained a smaller number of oval fenestrae characteristic of normal LSECs. Frequently damage to LSECs coexisted with significantly activated KCs/MPs, fibrogenesis and fibrosis, but not cirrhosis, accompanied by the appearance of transitional form of hepatic stellate cells (T-HSCs). Collagen fibers, especially in the perisinusoidal spaces of Disse, adhering directly to T-HSCs were observed. Interestingly, even though in half of the AIH children the sinusoidal vessels were found to undergo transformation of discontinuous into continuous endothelium showing features of defenestration, the true basement membrane did not form underneath. Thus, we failed to notice distinct morphological transformation of LSECs into vascular-type endothelial cells.

Discussion/Conclusion: Our results show that severe damage to LSECs, including necrosis, and damage to other NPCs, contributes substantially to the morphogenesis of pediatric AIH. The fact that the basement membrane is not formed underneath the endothelium, even when LSECs are markedly damaged, may indicate that regenerative capacities of these cells are still preserved and there is a chance for the lesions to retreat, which may significantly contribute to therapeutic effects of pediatric AIH.

In hepatitis B virus surface antigen transgenic mice hepato-carcinogenesis is associated with inflammation

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Introduction: Chronic hepatitis B virus (HBV) infection is a key risk factor for the development of hepatocellular carcinoma (HCC). Previous studies have shown that HCC develop more frequently in hepatitis B virus surface antigen (HBsAg)-transgenic mice (HBsAg-tg) than in wild-type (WT) mice. However, the mechanism of this HCC model has not been well documented. Toll-like receptor (TLR) signaling probably links innate immunity and HCC progression. This study was designed to investigate the particular mechanism of innate immunity in hepatocarcinogenesis in HBsAg-tg mice.

Methods: Immunohistochemistry was performed to analyze oncogene expression in HCC mice. Quantitative RT-PCR and western blot were performed to analyze gene expression and activation of signaling kinases, respectively, in vivo and in vitro. GEO data were reanalyzed to search for eligible signaling pathways associated with HBV infection.

Results: Images of transgenic mice liver illustrated that HBsAg expression induced hepatocarcinogenesis. Quantitative PCR results showed that TLR signaling as well as oncogene expression were activated in transgenic mice liver. Furthermore, immuno-histochemistry of HBsAg-transgenic mice liver showed that oncogenes (BMI1, β-catenin, AFP) and proliferation maker (Ki-67) were age-dependently upregulated. As prove of concept, LPS treatment of Hepa1-6 cell line not only induced NFκB P65 nucleus translocation, but also induced the upregulation of BMI1. This indicates that TLR signaling may link inflammation and tumor progression during chronic HBV infection. Gene set enrichment analysis results showed that HIPPO signaling and WNT/CTNNB1 pathway may play a key roles in TLR-mediated innate immunity and subsequent in hepatocarcinogenesis.

Conclusion: These findings directly links inflammation and hepatocarcinogenesis in Hepatitis B virus surface antigen transgenic mice, a context that might explain tumor progression in non-cirrhotic HBV-infected patients.

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Synergistic effects of iso-alpha-acids and xanthohumol in in vitro models of hepatic steatosis and fibrosis

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Introduction: Xanthohumol (XN) is the principal prenylated chalconoid of the female inflorescences of the hop plant. XN has shown various beneficial effects including anti-inflammatory, antioxidant, hypoglycemic activities, and anticancer effects. Iso-alpha-acids (IAA), hop-derived bitter compounds in beer, have been shown to beneficially affect different components of the metabolic syndrome such as insulin resistance, dys-lipidemia and fatty liver.
The aim of this study was to analyze the combined effects of XN and IAA on fatty acids-induced hepatic steatosis as well as on activated hepatic stellate cells in *in vitro* models.

Methods and Results: Primary human hepatocytes (PHH) were pre-incubated with serial concentrations of XN (up to 5 µM), IAA (up to 5 µg/ml) or their combinations for 1 h. Subsequently, cells were exposed to 0.4 mM fatty acids (FA) for 24 h. Under these conditions, neither XN nor IAA or their combinations showed toxic effects as indicated by analysis of the levels of transaminases in the supernatant and microscopic documentation. FA treatment significantly induced the hepatocellular triglyceride accumulation and the gene expression of pro-inflammatory cytokines (IL-8 and ICAM-1). These oleate-induced effects were slightly inhibited by XN or IAA pre-incubation in dose-dependent manner. However, combined XN and IAA treatment had a very strong inhibitory effect on the FA-induced hepatocellular triglyceride and pro-inflammatory gene expressions. Next, we analyzed the combined effects of XN and IAA on activated hepatic stellate cells, the central mediator of hepatic fibrosis. Also in these cells, XN and IAA synergistically reduced the gene expression of pro-fibrogenic factors (Coll-I and TGFß1) and the pro-inflammatory factors (MCP1 and IL-8) as well as the proliferation rate of HSCs in a dose dependent manner.

Conclusion: XN and IAA exhibit synergistic beneficial effects in an *in vitro* model of fatty acids-induced hepatocellular steatosis and inflammation in hepatocytes and anti-fibrogenic and anti-inflammatory properties in hepatic stellate cells. Together with previous studies showing the safety of XN and IAA applications in humans our data suggest XN/IAA-combination as promising therapeutic strategy for the prevention and treatment of (non-alcoholic) fatty liver disease.

Functional microRNA screening to improve hepatocyte formation via direct reprogramming

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Introduction: Liver failure is one of the leading causes of death worldwide. Due to a shortage of organ donors, it is of utmost importance to pursue readily available sources of hepatocytes. We have previously demonstrated that overexpression of the transcription factors FOXA3, GATA4, HNF1A and HNF4A is able to reprogram hepatic myofibroblasts into induced hepatocytes. However, direct reprogramming protocols require substantial improvements as the resulting in vitro generated hepatocyte-like cells (iHeps) do not match therapeutic potential for cell-based therapies. Recently, microRNAs (miRNAs) have been suggested as important regulators of hepatocyte differentiation. However, specific hepatogenic miRNAs that are able to improve the iHep differentiation remains to be identified. Hence, to improve the reprogramming, we performed functional miRNA screening during cell fate conversion of murine embryonic fibroblasts into hepatocytes.

Methods: First, murine embryonic fibroblasts were transduced with a lentiviral vector expressing FOXA3, GATA4, HNF1A and HNF4A transcription factors. We transfected 302 miRNA mimics during iHep formation via direct reprogramming. The iHeps were characterized by real-time RT-PCR analysis for hepatocyte-specific markers and immunocytochemistry. Furthermore, hepatocyte functions were evaluated by measuring albumin and alpha-1-antitrypsin secretion and CYP3A activity.

Results: Our miRNA mimic library screen revealed that 20 miRNAs, which are conserved between mouse and human, are able to increase albumin secretion from in vitro generated iHeps significantly. Of those 20 miRNAs, we identified 6 miRNAs that are capable of enhancing direct reprogramming of murine embryonic fibroblasts into iHeps.

Discussion/Conclusion: We demonstrated that 6 miRNAs are able to improve reprogramming of murine fibroblasts into iHeps. Currently, we will investigate the effect of these miRNAs on the direct reprogramming of human fibroblasts into human iHeps. Taken together, our results suggest microRNA modulation is potential promising approach to improve hepatocyte formation via direct reprogramming.

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Effect of long-term IFN exposure on CD8 T cell functionality

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Introduction: Infection with the hepatitis C virus (HCV) is a major risk factor for serious liver diseases and accounts for a total 150 million chronic infections worldwide. A hallmark of HCV is the high propensity to establish persistence, which occurs in around 80% of infected individuals. This is achieved by active suppression of both innate and adaptive immune response. Innate immune response, most notably induction of interferons (IFNs), is the first line defense limiting viral replication and critically determining the outcome of an infection. However, it has been shown that sustained activation of IFN system in case of persistent *lymphocytic choriomeningitis virus* (LCMV) infection has detrimental impact on T cell response. A sustained IFN signature has been seen in patients with chronic hepatitis C and we hypothesize that this might have detrimental impact on T cell responses, thus contributing to persistence.

Methods: In this project we would like to investigate effect of long-term IFN exposure on HCV-specific CD8 T cell function. For this purpose, we will culture those T cells in the absence or presence of different concentrations of IFN alpha. In order to assess their anti-viral efficacy, we will use cell-culture model based on a subgenomic HCV replicon-containing cell line, which was stably transduced with the HLA-A2 gene.

Results: Long-term IFN alpha treatment did not suppressed responsiveness of HCV-specific CD8 T cells to their target cells.

Discussion/Conclusion: Type I IFNs exerts either stimulatory or inhibitory actions on antigen-experienced CD8 T cells. Furthermore, IFNs can act indirectly on T cells by interfering function of other immune cells (i.e. dendritic cells). In our study, direct IFN signaling did not inhibit anti-viral function of HCV-specific CD8 T cells. Therefore, we assume that elevated levels of IFNs influence function of other immune cells (i.e. antigen presenting cells) and act indirectly on CD8 T cells.

HBV bypasses the innate immune system and does not protect HCV against the antiviral effect of IFN

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Introduction: Hepatitis B and C virus (HBV, HCV) are a global health problem. While HCV is inducing an interferon (IFN) response and is highly sensitive towards IFN treatment, the impact of the IFN system on the HBV life cycle is discussed controversially. Although HBV is regarded as "stealth" virus avoiding the activation of the IFN response, several studies reported an active suppression of the IFN-induced antiviral state. Here we studied possible interactions of HBV with the IFN system in immune-competent infection systems and analyzed the impact of HBV on HCV infection.

Methods: Differentiated HepaRG cells overexpressing the HBV receptor sodium taurocholate co-transporting polypeptide (dHepaRGNTCP) and primary human hepatocytes (PHH) were used for infection experiments. HBV/HCV co-replication and IFN cross-protection was analyzed by transfecting in vitro generated HBV genomes, mimicking covalently closed circular DNA, into Huh7 cells that were subsequently infected with HCV. Single cell analyses were performed, complemented by RT-qPCR and ELISA.

Results: Neither IFN nor IFN-stimulated genes (ISGs) were upregulated upon infection of dHepaRGNTCP or PHHs with HBV. Interestingly, RIG-I, MDA5 and TLR3 signaling could still be activated in HBV-infected cells indicating a passive escape of HBV from the innate immune system. Further, treatment of HBV-infected cells with IFN-α2 had only little impact on viral replication although HBV did not block the JAK/STAT signaling pathway in infected cells.

Consistently, only HCV but not HBV was diminished by type I and type III IFN treatment in a HBV/HCV co-replication system. Similar results were obtained after treatment with direct-acting antivirals (DAA) against HCV supporting the notion that DAAs are of clinical benefit both in HCV mono- and HBV/HCV co-infected individuals.

Discussion/Conclusion: Our results validate the concept of HBV passively bypassing the IFN system. Further, HBV cannot protect HCV against the antiviral activity of IFN or DAAs.

Generation of proliferating mouse hepatocytes (upcyte® mouse hepatocytes)

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Introduction: The concern about the use of laboratory animals is increasing and leads to the support of alternative methods. Laboratory mice are frequently used for gene knockout studies *in vivo*. Additionally, isolated mouse cells are an appropriate tool for gene knockout studies on a cellular level. However, the use of primary mouse cells is hampered by e.g. short culture longevity, the limited quantity of cells that can be isolated from one mouse and the lack of proliferation capacity.

Since we have successfully generated several human upcyte® cells (e.g. upcyte® hepatocytes), the feasibility of the upcyte® technology on other species is of interest. Here, we show for the first time that the transduction of proliferation-inducing genes could extend the lifespan of primary mouse hepatocytes without losing their primary characteristics. For this purpose, primary mouse hepatocytes from three wildtype (WT) and three knockout (KO) C57BL/6 mice were isolated and subsequently transduced with upcyte® proliferation genes.

Methods: Murine hepatocytes were isolated from three wildtype (mouse 16, 21 and 22) and three knockout (mouse 17, 23 and 24) C57/BL6 mice using a two-step collagenase perfusion technique. Primary cells were transduced and cells were monitored for proliferating spots of hepatocytes.

Results: After 13 days proliferating cells were visible, whereas only senescent cells where found in untransduced control wells. For all six mice proliferating upcyte® cells were found. All six upcyte® mouse hepatocytes were analyzed for their morphology and for the expression of mouse hepatocyte marker proteins.

Conclusion: In conclusion, the upcyte® technology can be used to generate proliferating mouse hepatocytes from wildtype and knockout mice, while retaining their phenotype. The resulting cells called “upcyte® mouse hepatocytes” express hepatocyte markers such as CK8, CK18 and MSA. Thus, the upcyte® technology can contribute to the 3Rs concept and provide a suitable tool for knockout studies on a cellular level.

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Hepatitis B virus transcription from cccDNA is inhibited by Am80 (tamibarotene), an agonist of the retinoic acid receptor alpha

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Chronic hepatitis B virus (HBV) infection affects more than 250 million people worldwide and can lead to liver cirrhosis and hepatocellular carcinoma. Since cure can only rarely be achieved in chronically infected patients, new therapeutic options are needed. Using a medium-content screening approach, we identified retinoic acid receptor (RAR) agonists as potent inhibitors of HBV replication in different cell culture systems. Am80, a specific agonist for RARα, which is approved in Japan for treatment of promyelocytic leukemia, was particularly potent in inhibiting HBV. We here investigated the mode of action of Am80 on HBV infection using NTCP-expressing in vitro infection systems. We found that Am80 treatment was able to potently interfere with an already established HBV infection at IC50s <10 nM in differentiated HepaRG-NTCP cells and primary human hepatocytes. The effect slowly augmented over time and persisted even after removal of the drug for at least 12 days. Am80 reduced all viral RNAs without affecting the levels of covalently closed circular DNA (cccDNA), indicating interference with viral transcription. As Am80 neither decreased viral markers secretion of transfected cells nor of a cell line carrying integrated HBV genome, we concluded that it acts specifically on cccDNA. Since the viral HBx protein is important for cccDNA-mediated transcription, we tested the interplay of Am80 with HBx. Chromatin immunoprecipitation revealed specific binding of RARα to HBV DNA. In conclusion, the RARα-specific agonist Am80 strongly and persistently inhibits HBV infection. This effect is probably mediated by binding of RARα and interference with transcription from transcriptionally active cccDNA. Clinically approved RAR agonists are available, which may allow for a fast translation for testing in HBV patients.

Hepatocellular activation of oncogenic components of the Wnt-pathway in Schistosoma mansoni infected hamsters

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Introduction: Schistosomiasis is one of the most important parasitic infections worldwide and caused by trematodes of the genus Schistosoma. At least 200 million people required preventive treatment in 2018 (WHO 2018). Eggs of S. mansoni trapped in the liver sinusoids induce an inflammatory reaction with granuloma formation. Clinical data suggested that schistosomiasis promotes hepatocellular carcinogenesis, e.g. during coinfection with HBV or HCV. We could recently show permanent activation of hepatocellular carcinoma-associated proto-oncogenes such as c-Jun and associated transcription factors including STAT3 by soluble substances released from tissue-trapped schistosome eggs. The goal of the current study was the identification of additional early mechanisms that might potentially contribute to the promotion of hepatic carcinogenesis by S. mansoni-infection.

Methods: The hepatic expression and localization of β-Catenin, Survivin, Oct4, and Cyclin D1 was analyzed by Western blotting and immunohistochemistry in a hamster model during patent infection with S. mansoni. Electrophoretic Mobility Shift Assay (EMSA) was performed to analyze the regulation of the survivin gene by Stat3.

Results: The hepatic expression of β-Catenin, Survivin, Oct4, and Cyclin D1 was induced during patent infection with S. mansoni. Immunohistochemical analysis demonstrated a nuclear accumulation of survivin in hepatocytes around periovular granuloma. EMSA assays demonstrated the enhanced Stat3 binding to the labelled consensus oligonucleotide of the survivin promotor using hepatic nuclear protein extracts of S. mansoni-infected hamsters in comparison to controls.

Discussion/Conclusion: The obtained results suggest the activation of oncogenic effectors of the Wnt-signal pathway in hepatocytes during patent infection with S. mansoni. Furthermore, EMSA data indicate the induction of survivin by Stat3. Survivin, a known apoptosis inhibitor, might function as a potential target of oncological HCC therapy as well as HCC immunotherapy.

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The influence of hepatitis C virus on the induction of CXC chemokine expression in response to the inflammatory cytokines TNFα and Il1β

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Introduction: The hepatitis C virus (HCV) intervenes in several cellular processes to accomplish persistence. HCV influences the immune response of the cell by interaction of viral proteins with cellular signaling intermediates. We could recently demonstrate that HCV enhances basal and EGF-dependent expression of the chemokines CXCL1, 2, 3 and 8 (Groepper et al., 2018). In this study we tried to elucidate the effects of Il1β and TNFα on the regulation of CXCR2 chemokine expression.

Methods: The activity of proteins downstream the PI3K signaling pathway was determined in cells harbouring the subgenomic replicon of HCV after stimulation with TNFα and Il1β. Additionally, chemokine expression was measured in the cells infected with the HCVcc strain JC1 and in cells harbouring the subgenomic replicon. Chemokine expression was also quantified after inhibition of key enzymes by addition of specific inhibitors and after RNAi knockdown.

Results: The results indicate that HCV activates key enzymes of the PI3K/Akt and MAPK pathway after TNFα and Il1β stimulation of cells harbouring the HCV subgenomic replicon. Furthermore, TNFα and Il1β stimulation results in an enhanced CXCR2 ligand expression. Hence, knock down of EGF expression by siRNA does not lead to any impairment of CXCR2 ligand expression after stimulation with Il1β. Akt inhibitor (Triciribine), inhibitor of NfκB (IKK Inhibitor II) and p38 inhibitor (SB203580) were also tested with regards to CXCR2 ligand gene expression induction after TNFα and Il1β stimulation.

Discussion/Conclusion: The present study indicates that TNFα and Il1β have got an effect on the upregulation of CXCR2 ligand expression. This enhancement is still higher in cells harbouring the subgenomic replicon and in cells infected with the HCVcc strain JC1. In this study we additionally analyze the upregulation of CXCR2 ligand expression after stimulation with TNFα and Il1β in response to different inhibitors.

Immunodominant HLA-B*35:01 restricted CD8+ T cell epitope is associated with clustered viral evolution in HBV polymerase

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Introduction: CD8+ T cell exhaustion and viral escape are considered as the main mechanisms in chronic HBV infection; however, the relative role of viral escape herein is not well defined. Until now, viral escape has been mainly described for the core protein. In this study, we aimed to address the role of viral escape from CD8+ T cell responses targeting HBV polymerase.

Methods: 114 patients chronically infected with HBV genotype D were analyzed by HLA class I typing and HBV full-genome sequencing. HLA class I associated HBV polymorphisms were determined. The optimal epitope peptide corresponding to a cluster of three HLA-B*35:01 associated HBV polymerase sequence mutations was defined. Tetramer-based enrichment of CD8+ T cells specific for the novel HLA-B35:01 polymerase- epitope as well as two previously described HLA-A01:01 and HLA-A11:01 restricted core-epitopes was performed. A cohort of 51 patients with chronic HBV genotype D infection was screened.

Results: Several HLA class I associated sequence polymorphisms were observed in the polymerase protein, including a striking cluster of three HLA-B35:01 associated polymorphisms (pol174, 180 and 181) located within a single predicted HLA-B35:01 restricted CD8+ T cell epitope. The epitope was confirmed after peptide-specific expansion followed by intracellular interferon-gamma staining in 3 HLA-B*35:01+ patients. By using a sensitive ex vivo tetramer-based enrichment protocol, we were able to detect polymerase-specific CD8+ T cells responses in 4/20 HLA-B35:01+ patients. The frequency was similar to the detection rate of CD8+ T cells targeting a previously described immunodominant HLA-A01:01 and a HLA-A11:01 epitope with 2/9 HLA-A01:01+ and 3/22 HLA-A11:01+ patients, respectively. Phenotypical analysis of the CD8+ T cells targeting the polymerase- specific HLA-B*35 restricted epitope differs from CD8+ T cells targeting the core-specific HLA-A11 restricted epitope. HLA B35:01 polymerase-specific CD8+ T cells expressed higher levels of KLRG1 and Eomes and lower levels of T-bet. Furthermore, less HLA B35:01 polymerase-specific CD8+ T cells revealed the CD127+PD1+ memory-like phenotype in comparison to HLA-A11:01 core-specific CD8+ T cells.

Discussion/Conclusion: We identified a novel HLA-B35:01 restricted HBV-specific CD8+ T cell polymerase epitope. This epitope was associated with viral escape mutations at several positions, indicating that viral escape in polymerase may be more constrained compared to viral escape in the core protein. In conclusion, these results could provide the basis for the development of novel therapeutic vaccination strategies targeting non-escaping epitopes and immunomodulatory therapies restoring the function of HBV-specific CD8+ T cells with the aim of virus elimination.

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Metabolic changes upon inhibition of the lysine-specific-demethylase-1 (LSD1) in hepatocellular carcinoma

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Introduction: Current research indicates that epigenetic alterations play an important role in the most malignant entities and might be targets for novel therapeutic approaches. The epigenetic modifier LSD1 was shown to be overexpressed in many cancer types, including hepatocellular carcinoma (HCC). LSD1 demethylates histone 3 at lysine 4 and 9 (H3K4/9) and causes either transcriptional repression or activation. In the present study the impact of the novel LSD1 inhibitor HCI-2509 was tested on HCC cells.

Methods: Ultra-deep RNA-sequencing of LSD1 inhibited and non-inhibited hepatoma cells was performed and analyzed. According to the results the mitochondrial function was investigated further by confocal microscopy, flow cytometric quantification of the mitochondrial membrane potential and an assessment of the respiratory function, using a metabolic analyzer.

Results: Upon pharmaceutical LSD1 inhibition of HCC cells, gene expression profiling followed by pathway analysis revealed a downregulation of genes involved in cell cycle control and metabolism, but also of genes encoding subunits of the mitochondrial respiratory complex I. Furthermore, morphology of mitochondria altered after LSD1 inhibition. Flow cytometry proved a distinct decrease of the mitochondrial membrane potential in response to HCI-2509 treatment. Metabolic analysis of various hepatoma cell types confirmed the impairment of mitochondrial respiration, showing a reduced baseline in the oxygen consumption rate, as well as a lower ATP production and respiratory capacity after pharmacological LSD1 inhibition.

Discussion/Conclusion: Our findings show that LSD1 inhibition leads to an impaired cellular respiration caused by a dysregulation of the electron transport chain complex I. Hence, the cancer cell metabolism is influenced in a disadvantageous way and the malignant cell is not able to serve its energetic demands anymore. This data emphasizes the value of pharmaceutical LSD1 inhibition as an antineoplastic therapeutic approach, affecting HCC cell proliferation not only by cell cycle interruption, but also by energetic restriction.

Cancer (HCC and MM) in Gaucher disease: A multicentric analysis from 4 German centers

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Introduction: Gaucher disease (GD) is a rare monogenic autosomal-recessive disease caused by deficient β-glucocerebrosidase. Due to intralysosomal accumulation of glucocerebroside, chronic macrophagocytic challenge results with inflammatory transformation. As shown in previous own work (1–3), total cancer incidence among GD patients is increased 2.5-fold and 12.7-fold for hematological tumors as compared to the general population (1). Among these neoplasms, multiple myeloma and liver cancer are highly increased, with splenectomized patients being at highest risk.

Methods: In a cohort of approximately 250 patients from 4 German centers, cases of HCC and MM were analyzed. Their relationship to preexistence of cirrhosis and the presence of MGUS was investigated.

Results: In approx. 250 GD patients, 4 cases of liver cancer and 3 cases of multiple myeloma were found. In most cases, cirrhosis was not present prior to advent of HCC. Rapid HCC recurrence upon therapy and a lethal course were observed in ¾ HCC cases. All cases of multiple myeloma developed on the basis of ongoing MGUS, with a rise in chitotriosidase activity preceding MM diagnosis.

Discussion/Conclusion: The role of sphingolipids and immune dysregulation related to tumorigenesis in GD remains to be elucidated. Practical recommendations are: Splenectomized GD patients should undergo hepatologic surveillance with regular AFP determination, ultrasound and elastography measurements. Multiple myeloma should be considered in all cases with paraprotein increase and patients who exhibit a sudden rise in plasma chitotriosidase activity despite efficient therapy. Glucosylceramide-laden, chronically activated macrophages are known to release immunologic factors, e.g. interleukins, and accumulation of sphingolipids might contribute to tumorigenesis under these conditions.

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Xanthohumol, a prenylated chalcone derived from hops, inhibits liver metastasis

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Introduction: In many cancer entities, including melanoma, hepatic metastasis is the critical factor determining tumor associated mortality. Xanthohumol, a prenylated chalcone derived from hop cones, is known to possess chemopreventive and anti-cancer activities. However, its effect on (liver) metastasis has not yet been analyzed.
The aim of this study was to analyze the effect of Xanthohumol on (hepatic) metastasis of melanoma cells.

Methods: Functional effects of XN on viability, proliferation, migration and colony formation of human melanoma cell lines were analyzed *in vitro*. Furthermore, we tested XN effects in a syngeneic murine model of hepatic metastasis (splenic injection of B16 melanoma cells in C57/BL6 mice). XN was applied with a daily dose of 10 mg/kg body weight using implanted pellets (Innovative Research of America). Control mice were administered placebo pellets.
In addition to liver weight and histological analysis of the livers, expression of MIA, a gene specifically expressed in melanoma cells, was analyzed to measure hepatic metastasis.

Results: XN induced cytotoxic effects in melanoma cells beginning in the dose-range of 40–60 µM. Notably, 10-fold higher XN concentrations did not impair the viability of primary human hepatocytes. Incubation with XN in the subtoxic range dose-dependently decreased proliferation, migration and colony formation of melanoma cells. In the *in vivo* model, liver weight and hepatic MIA expression were significantly lower in XN treated mice. Furthermore, tumors formed in the liver of Xanthohumol treated mice revealed significantly larger areas of central necrosis and lower Ki67 expression scores compared to control mice.

Discussion/Conclusion: Xanthohumol inhibits tumorigenicity of cancer cells *in vitro* and significantly reduced hepatic metastasis in mice. These data, in conjunction with an excellent safety profile which has been confirmed in previous studies, point XN out as a promising agent for the treatment of hepatic (melanoma) metastasis.

Effect of chain length and saturation status of fatty acids on viability and tumorigenicity of hepatocellular carcinoma cells

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Introduction: Hepatocellular carcinoma (HCC) is one of the most common cancer types worldwide, and often has a poor prognosis. A well-known risk factor for the development of HCC is obesity, and in obese people, levels of circulating fatty acids (FA) are elevated. However, systematic analyses of the effects of different dietary FA on HCC cells are missing.
The aim of this study was to systematically examine the effects exerted by dietary FA with different chain length and saturation status on HCC cells *in vitro* with regard to cytotoxicity, triglycerides (TG) accumulation and pro-inflammatory and pro-angiogenic gene expression.

Methods & Results: The human HCC cell lines Hep3B and PLC were treated with the following FA (complexed to albumin): palmitate (C16:0), stearate (C18:0), palmitoleate (C16:1) and oleate (C18:1). Saturated FA caused significant cytotoxicity already at doses as low as 0.2 mM, while doses of unsaturated FA as high as 0.8 mM did not affect viability of Hep3B and PLC cells. Furthermore, C18 FA were more cytotoxic compared to C16 FA. In subsequent experiments with subtoxic doses, all FA species induced a dose-dependent intracellular lipid accumulation. Lipid accumulation was significantly higher for C16:1 palmitoleate compared to C18:1 oleate in both cell lines, while there was no difference with regard to chain length for saturated FA. In Hep3B cells, induction of pro-inflammatory and pro-angiogenic gene expression was significantly higher for saturated compared to unsaturated FA. Additionally, induction of pro-angiogenic gene expression was significantly higher for C16 FA compared to C18 FA. In contrast, in PLC cells, gene expression was exclusively and only moderately induced by C16:0 palmitate.

Discussion/Conclusion: Our study reveals significant differences in the effects caused by different types of FA with regard to induction of toxicity, lipid metabolism and pro-tumorigenic gene expression in HCC cells. Potentially, these findings could lead to dietary recommendations for patients with (risk for) HCC.

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Hepatocyte-derived calcineurin regulates the development of hepatocellular carcinoma independent of hepatic steato-hepatitis

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Introduction: Hepatocellular carcinoma (HCC) is caused by chronic damage to the liver. Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation. A subset of patients further develops hepatic inflammation resulting in non-alcoholic steatohepatitis (NASH), which can in a subset of patients lead to HCC development. Within NASH and HCC, the role of common inflammatory signaling pathways such as NfkB and STAT3 have been extensively investigated. Here we aimed to investigate the roles of calcineurin (Cn) and its downstream mediators of the nuclear factor of activated T cell family (NFAT) in NASH-associated HCC.

Methods: To study the role of Cn, we used male mice with hepatocyte-specific deletion of calcineurin (AlbCre$^{tg/wt}$ *Cnb1*$^{fl/fl}$ or *Cnb1*$^{\wedge Hep}$) which we treated with diethylnitrosamine (DEN) at 2 weeks of age and fed a high-fat diet deficient for choline (CD-HFD) for 32 weeks.

Results: Mice deficient for Cn showed a reduction in tumor size and tumor load compared to wildtype (WT) mice (Cre-negative *Cnb1*$^{fl/fl}$ littermates), which underwent the same treatment. Additionally, hepatic tumors from WT litters presented with an increase in TNF-α and Il1β mRNA expression compared to *Cnb1*$^{\wedge Hep}$ mice. Neither a short-term treatment with CD-HFD plus DEN nor a long-term treatment with CD-HFD without DEN showed differences in hepatic or systemic inflammation between *Cnb1*$^{\wedge Hep}$ -mice and WT littermates, suggesting that hepatocyte-derived calcineurin does not control HCC development through regulation of hepatic inflammation. Inhibiting the binding of Cn to NFATs by intravenous application of VIVIT, a peptide known to disrupt Cn-NFAT-associations, also resulted in decreased tumor multiplicity and size.

Conclusion: In summary, these data indicate that the Cn-NFAT axis has a pro-oncogenic role in HCC without affecting the severity of NASH. Further research will focus on the mechanisms of how hepatic calcineurin regulates HCC development and its potential use in the prevention and treatment of HCC.

Possible genetic background for systemic inflammation, liver fibrosis, carcinogenesis and endothelium vascular injury in chronic hepatitis C patients

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Introduction: Chronic Hepatitis C (CHC) is considered to be the major cause of hepatic carcinogenesis and to some extend not a genetic disease. However, some genome-wide association studies demonstrated association between interleukin-28B polymorphism and peginterferon-α and ribavirin treatment response for genotype 1 HCV, as well as spontaneous clearance of acute HCV infection, and the association between inosine triphosphatase variation and RBV-induced hemolytic anemia. Although the role of proangiogenic and proinflammatory factors is well established in hepatic carcinogenesis, the associations of endothelial nitric oxide synthase (eNOS) gene's T894G single nucleotide polymorphism (SNP) with cytokines profile, endothelial injury in patients with hepatic carcinoma due to CHC is not studied yet.

Methods: Study group included 9 patients with liver malignancies due to CHC cirrhosis (4 female, 5 male, mean age 67.2 ± 7.31); control group included 12 healthy volunteers. All patients had confirmed vascular endothelial injury in a form of concomitant arterial hypertension and chronic heart failure. Liver function markers, set of cytokines, including IL-4, TNF-α, TGF-β1, and pro-Atrial Natriuretic Peptide (pro-ANP) plasma concentrations were defined in ELISA; eNOS gene's T894G SNP (rs1799983) was assessed in RT-PCR.

Results: Difference in genotypes distribution between groups was not significant. Presence of T-allele in patients with liver cirrhosis was associated with significant increase of AST activity (27.4%, $p < .05$), urea concentration (33.3%, $p < 0.05$), creatinine (22.2%, $p < 0.05$) compared to GG-carriers. In T-allele carriers concentration of pro-ANP was higher (89.2%, $p < 0.001$), than in patients with GG-genotype. IL-4, TNF-α and TGF-β1 did not differ reliably between genotypes, but TNF-α was significantly higher in research group patients ($p < 0.001$). In research group males T-allele also combined with the increased LVMI (by 12.2%, $p<0.05$) compared to GG-genotype patients.

Discussion/Conclusion: eNOS highest values in humans are observed in spleen marking its possible role in hepato-splenic syndrome. In addition, it is tightly connected with hepatic circulation via changes of both endothelial and metabolic functions; inflammation reflects widespread vascular damage as proved by observed cardiovascular changes. We hypothesize that eNOS gene's T894G SNP under Hep C infection and fibrosis oxidation, systemic inflammatory reaction and unregulated cellular proliferation may associate with observed changes. eNOS gene's T-allele associates with increased cytolysis, indirect fibrotic liver changes, cardiovascular failure (pro-ANP) and may be a risk factor for liver cancer in CHC patients.

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Changes of gut microbiota in chronic HCV infection compared to NAFLD

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Introduction: Multiple studies showed that imbalances of the intestinal flora easily lead to disorders of the intestinal immune system and cause a variety of diseases such as IBD, autoimmune liver disease, etc. In addition, gut microbiota is involved in NAFLD and associated with the progression of NAFLD to NASH, cirrhosis, or HCC. Liver can be greatly affected by changes in gut microbiota due to increased intestinal permeability with passage of microbial antigens and LPS into the liver. While chronic hepatitis C (CHC) progress to severe liver failure, liver fibrosis, cirrhosis and hepatocellular carcinoma there are "white spots" in tight relationship between gut microbiota, immunity, viral infection and hepatic dysfunction. Following idea that gut dysbiosis is associated with severe liver disease we hypothesized that changes of gut microbiota are somehow universal and in chronic HCV infection may be similar compared to NAFLD.

Methods: Study included 27 patients with lab/clinically proven CHC (seven [25.9%] with minor or absent fibrosis, 20 [74.1%] with cirrhosis), mean age – 49.03 ± 7.29. Sixty patients with verified NAFLD participated in the study (mean age 57.9 ± 9.8). Twenty-five practically healthy volunteers of respective age and gender formed control group. Colonic lumen microbiota studied in not less than three fecal samples taken with interval of not less than two weeks to avoid interference with diet or other factors. All patients received no antibiotics or pre-/probiotic treatment for not less than month before the study.

Results: Compared to control, colonic microbiota in CHC patients differed significantly with respect to hepatic dysfunction: significant ($P < 0.01–0.05$) decrease or elimination of anaerobic autochthonic microorganisms (both Lacto- and Bifidobacteria) and increased population levels of conditionally pathogenic Enterobacteriacea family: E. coli, including Hly+ reached 9.47 ± 0.58 lg CFU/g against 7.39 ± 0.56 lg CFU/g in control; Klebsiellae – 5.26 ± 0.39 lg CFU/g against 3.48 ± 0.49 lg CFU/g in control, Proteus – 6.35 ± 0.31 lg CFU/g, and Serratia – 4.86 ± 0.61 lg CFU/g (not found in control). No differences between different HCV genotypes were observed. Compared to NAFLD reliable worse dysbiosis grade were observed only in CHC patients with decompensated cirrhosis.

Discussion/Conclusion: Both CHC and NAFLD patients demonstrate impaired intestinal microbiota diversity, excessive growth of conditionally pathogenic Enterobacteriacea and decrease of autochtonic strains. In contrast to some studies, our findings show possible interdependence of hepatic dysfunction and gut microbiota with lesser significance of hepatic dysfunction etiology.

Hepatitis B virus (HBV) DNA integration is not driven by viral proteins

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Introduction: Integration of viral DNA into host cell genome occurs early in HBV infection. HBV DNA integrations arise when HBV double-stranded linear (dsl)DNA acts as a substrate for the repair of cellular DNA breaks. Intriguingly, HBV DNA integration has been detected in most patients with HBV infection, despite this form not being necessary for new virion production. Of clinical importance, HBV DNA integration may drive cancer and viral persistence (e.g. as a source of HBV immunomodulatory surface antigen). It remains unknown if HBV actively drives its integration or if it occurs passively through cellular DNA repair factors. Thus, we explored HBV DNA integration and its dependence on various viral factors.

Methods: First, Huh7-NTCP cells were infected with either wild-type or a core antigen-knockout HBV mutant. HBV DNA Integration was detected using inverse nested PCR. To find viral factors associated with HBV DNA integration, we compared integration resulting from 1) HBV infection, 2) transfection of DNA extracted from cell culture-derived HBV, and 3) transfection of a linearized plasmid-derived 1-mer of HBV DNA. A peptide nucleic acid (PNA)-mediated quantitative real-time PCR (qPCR) clamping assay that amplifies only dslDNA was used to quantify dslDNA levels in the sera of HBeAg-positive (n = 30) and HBeAg-negative (n = 32) patients with CHB (n = 16) or LC and LC-developed HCC (n = 17).

Results: Both wild-type and replication-deficient HBV mutants integrated at the same rate, indicating that HBV DNA integration can occur without formation of de novo reverse-transcribed virus genomes. HBV dslDNA no matter its source (either from an infection or transfection with purified DNA) integrated at similar frequencies, suggesting that HBV integration does not depend on viral proteins or RNA in the input virus but only needs the presence of dslDNA. Supporting this, we found that HBV integration by serum-derived HBV containing ~6% dslDNA was ~5-fold lower compared to cell culture-derived HBV at ~30% dslDNA. Interestingly, we found that circulating dslDNA increases from HBeAg-positive (average = ~10%) to HBeAg-negative (~25%) phase in patient serum with the greatest levels observed in HCC patients (> 50%). Finally, we used a novel RT inverse nested PCR assay to show that HBV DNA integrations are transcriptionally-active in primary tumor and surrounding

non-tumor tissues in HBV-infected patients (n = 12). Thus, HBV DNA integrations can indeed contribute to the expression of HBsAg in a true infection.

Discussion/Conclusion: In summary, HBV dslDNA integrates into the cellular genome without the help of specific viral protein factors. Rather, it is likely to occur through cellular DNA repair mechanisms. As HBV integrations (unlike the replicative episomal viral DNA form) are not lost during regenerative cell mitosis, they form a stable reservoir for HBV antigen expression in an infected liver. Our studies are now being extended using HBV inocula with specific mutations in circularisation signals to see if higher dslDNA levels lead to higher integration rates. If HBV integration does indeed drive persistence and resistance to clearance by the immune response as reported, then prevention of ongoing integration (e.g. by inhibiting virus entry) should be considered.

Reshaping of the cellular signaling landscape under continuous stimulation of innate antiviral responses

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Introduction: The interplay of viral factors and antiviral signaling that usually leads to viral clearance or induction of cell death has been studied extensively in acute infection. However, certain viruses such as Hepatitis C virus (HCV) are able to establish persistent infection, characterized by long-lived, low-level replication and particle formation, despite continuous antiviral signaling in the infected tissue. We hypothesize that this seemingly paradoxical coexistence is the result of complex adaptations of the cellular signaling landscape, leading to a new equilibrium that we term the infected-state homeostasis. We aim to identify and characterize the changes imposed onto homeostatic signaling networks in cells experiencing continuous stimulation of antiviral pathways, as well as how these cells react to external stimuli such as growth factors or death ligands.

Methods: We established a cell culture model that mimics virus infection by utilizing HCV RNA polymerase NS5B's ability to produce virus-like RNA products, which in turn trigger the RIG-I antiviral signaling pathway.

Results: NS5B expression in A549 cells induced a sharp increase in ISG expression levels. However, this was followed by a gradual decrease over the course of three weeks, which was accompanied by a decrease in NS5B levels. More detailed analysis showed that NS5B high expressing cells exhibited a general growth disadvantage and were gradually lost over the culture period.

Discussion/Conclusion: We conclude that a small proportion of cells expressed high enough NS5B levels to induce an IFN production. Cytokines secreted from these cells continuously triggered antiviral responses also in NS5B low expressing cells, thereby closely mimicking the situation in an infected organ. Interestingly, IFN producing cells had a significant growth-disadvantage and disappeared from the culture over the course of two to three weeks. This might highlight the importance for HCV to maintain virion production and continuously re-infect new cells in order to sustain persistence.

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Outcome in the German HCV (1b)-anti-D cohort over a period of 40 years

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Introduction: Studies of HCV infections showing a high rate of cirrhosis are faced with those of a slow progression of fibrosis. So far, there are only few long-term studies with a known date of infection which include all those infected persons without bias as it is the case in the German anti-D cohort. An additional issue is to find out to what extent the prognosis after four decades has been influenced by the anti-HCV therapy.

Methods: The anti-D immunoglobulin batches administered in 1978/79 to 2867 East German women were unknown contaminated with HCV. This cohort is of particular interest because it allows precise statements about the spontaneous course. The Leipzig anti-D cohort as part of the total cohort includes 356 women, of whom now 181 were followed up after 40 years. In the last 3 years 14 women were treated with IFN-free regimen.

Results: After 40 years, 85% of the 181 women in the HCV ELISA were positive. 33% were viremic (HCV PCR positive). Only 11 (8.3%) of viremic women had liver cirrhosis, 9 (5.4%) had advanced fibrosis. In the last 15 years a continuous, but slow rise of advanced fibrosis score was observed. HCC has not been diagnosed. 48 women with an IFN-based therapy and 14 women with an IFN-free regimen were treated successfully (SVR). Since 1978 six HCV RNA-positive women of the Leipzig cohort died; eight women died after viral clearance. The overall mortality in the therapeutic SVR group was lower than in the treatment-naïve group.

Discussion/Conclusion: Young women without comorbidity eliminate HCV (1b) infection spontaneously in approximately half of the infected cases. However, after 40 years, a continuous but relatively low progression with regard to final states could be confirmed in this cohort. These results are similar to the data published in 2017 from the Ireland cohort. IFN-free therapies should be recommended because all 14 cases responded and the overall mortality after successful therapy (SVR) was lower too.

The long non-coding RNAs *lncRNA-ADM-2* and *lncRNA-NBPF3-9* outline potential novel targets for hepatocellular carcinoma

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Introduction: Long non-coding RNAs (lncRNAs) represent a constantly growing class of > 200 nucleotide-long RNA molecules. They operate on an epigenetic, transcriptional and post-transcriptional level to regulate multiple cellular mechanisms including proliferation, pluripotency and apoptosis, thereby affecting hallmarks of cancer. However, the role of the majority of lncRNAs in HCC is completely unknown. The aim of this study was to identify and functionally verify novel long non-coding RNAs in HCC.

Methods: A qRT-PCR-based lncRNA expression array was used to analyze 87 potentially cancer-associated lncRNAs. LncRNA-expression profiles of two HCC cell lines (Hep3B, PLC) were compared to primary human hepatocytes (PHH) and activated hepatic stellate cells (aHSC). Sanger sequencing and DNA gel electrophoresis served for confirmation of identified differentially expressed lncRNAs. LncRNA silencing using specifically pre-designed si-RNA-pools was used to study lncRNA function. Boyden chamber and anchorage-dependent clonogenicity assays were performed to study lncRNA-mediated effects on migration, invasion and stem cell properties.

Results: Eight lncRNAs were found to be differentially regulated in HCC cells as compared to PHHs or aHSCs. However, only two lncRNAs (lncADM-2, lncNBPF3-9) were specifically and strongly overexpressed in HCC cells as compared to both PHHs and aHSCs, pointing to cancer(-subtype)-specific de-regulation. DNA gel electrophoresis and sanger sequencing confirmed strong expression of the predicted lncADM-2 transcript and revealed that a predicted intron of lncNBPF3-9 is not spliced in HCC cells. First experiments exploring the function of lncADM-2 showed decreased migration and clonogenicity of cancer cells after si-RNA-pool-mediated knockdown.

Discussion/Conclusion: Our study revealed overexpression of two novel lncRNAs (lncADM-2, lncNBPF3-9) in HCC. Furthermore, a predicted exon of lncNBPF3-9 is not spliced in HCC cells resulting in a longer, potentially functional novel transcript variant that will be further evaluated. Moreover, a specifically designed si-RNA-pool targeting lncADM-2 revealed this lncRNA to functionally promote migration and clonogenicity, outlining a potential novel therapeutic target in HCC.

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The relation of serum galectin-3 and hyaluronic acid with the severity of liver fibrosis in patients with chronic hepatitis B infection

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İntroduction and Purpose: Liver fibrosis is the major cause of morbidity and mortality in chronic liver disease. İn this study, we aimed to analyze the relation of hyaluronic acid (HA) and galectin-3 (Gal-3) with the degree of liver fibrosis in patients with newly diagnosed chronic hepatitis B.

Materials and Methods: A total of 35 patients with newly diagnosed chronic hepatitis B and 35 healthy controls were enrolled to the study. Liver biopsy results were evaluated accordıng to Ishak modified histological activity index. Serum HA, Gal-3 levels, liver function tests, body mass index (BMI), waist circumference, alpha fetoprotein and HBV DNA levels were compared in both groups.

Results: Patients and control groups have similar serum HA and Gal-3 levels. The relation of HA and Gal-3 levels with fibrosis levels was statistically significant (HA are $r = -0.382$, $p < 0.05$, Gal-3 are: $r = -0.424$, $p < 0.05$). In the patient group, a significant relation was found between HA and Gal-3 levels with BMI (HA are $r = 0.342$, $p < 0.05$, Gal-3 are $r = 0.353$, $p < 0.05$). HA levels of female patients were non-significantly higher in patients with high BMI ($r = 0.397$, $p = 0.061$). The relation of Gal-3 levels with BMI was statistically significant in female patient group ($r = 0.417$, $p < 0.05$).

Conclusion: Negative association between severity of liver fibrosis with serum HA and Gal-3 in patients with mild fibrosis suggest the idea that these markers should be used in patients with severe liver fibrosis.

Key words: galectin-3, hyaluronic acid, liver fibrosis

The decrease of intracellular tumor suppressor miR-198 is correlated with vesicle release from hepatocellular carcinoma cells

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Background and Aim: miR-198 has been shown as a tumor suppressor in different cancer types, inhibiting cell growth and proliferation. Previous studies have shown that miR-198 is one of the most downregulated miRNAs with the progression of liver diseases. Therefore we aimed to study the regulation of miR-198 in liver cancer cells.

Methods: miR-198 expression cassette was cloned under doxycycline induced tet-on promoter and stably transfected into liver cancer cells. RNA was isolated and real time PCR was performed to analyze miR-198 expression level. As well, the supernatants were collected and after serial centrifugation the exosomes were precipitated. The isolated exosomes were further characterized by Western Blotting and real time PCR. Immunoprecipitation method was used to identify miR-198 associated proteins. Furthermore, cell viability assay was performed to study the effect of conditioned media collected from miR-198 overexpressing cells.

Results: Intracellular miR-198 levels were at first upregulated, which were followed by a significant decrease. Interestingly, the increase of extracellular miR-198 was strongly associated with intracellular miR-198 decrease. Exosomes, isolated from supernatant of miR-198 overexpressing cells, were characterized by nanoparticle tracking method and validated by exosome markers, CD63 and HSP70. Exosomal miR-198 level also corresponded with that in the supernatant. Conditioned media, containing exosomes from miR-198 overexpressing cells, strikingly inhibited proliferation of cell types which have no endogenous miR-198.

Conclusion: In liver cancer cells, intracellular miR-198 is tightly controlled and is preferably sequestered in vesicles and secreted via exosomes, which provides novel insight into decrease of tumor suppressor microRNAs in liver cancer. Further experiments would rely on miR-198 anti-sense conjugated nanoparticles to unravel the miRNA sorting mechanisms.

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Towards a novel RNAi and AAV vector-based gene therapy against hepatitis E virus

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Introduction: Hepatitis E virus (HEV) is a positive strand RNA virus, whose genome encodes three partially overlapping open reading frames (ORF1-3). Infections with HEV are among the most frequent causes of acute hepatitis in the world. Tropical HEV infections may lead to acute liver failure, especially in pregnant women, while autochthonous HEV infections may lead to acute-on-chronic liver failure in patients with underlying liver diseases. Current therapeutic options are limited to off-label ribavirin (RBV) and pegylated interferon-α. Thus, alternative treatment options are urgently needed. Over the past three decades, Adeno-associated viruses (AAVs) have been established as a very promising, safe and powerful vector for therapeutic gene delivery in humans. Here, we engineered AAV vectors of serotype 6 (AAV6) to co-express short hairpin (sh)RNAs against HEV transcripts, as a relevant model for future clinical treatment of HEV infections by means of liver-directed AAV/RNAi gene therapies.

Methods: Twenty different shRNAs targeting the HEV genome were designed, expressed from recombinant AAV6 viruses and screened for their potency to inhibit HEV replication. The effect on HEV replication was assessed by AAV transduction of a hepatoma cell line harboring a selectable subgenomic HEV gt3 replicon (Kernow-C1 p6 strain) with a Gaussia luciferase (GLuc) reporter. The three most potent shRNAs were, either alone or in multiplexed form, further validated with full-length HEV p6 infection. HEV infection was assessed by immunofluorescence staining of HEV capsid protein ORF2 or quantification of viral genomes by real-time qPCR.

Results: We have identified multiple shRNAs that efficiently downregulate HEV replication in both HEV systems. Upon transduction, GLuc levels secreted by the replicon cells were reduced up to 95%. In infected cells, shRNA expression led to a reduction of ORF2-positive cells and viral copy numbers of up to 90%. The identified inhibitory shRNAs are targeted against the Methyltransferase domain in ORF1, the junction region between the ORFs, and the 3´cis-acting element. By targeting all three regions at the same time through multiplexing of the shRNAs, the inhibitory potency was further enhanced.

Discussion/Conclusion: Our data show that the approach of combining RNA interference and AAV vector-based gene therapy has great potential to suppress HEV replication. We will now study a potential synergism of co-treating cells with RBV and

shRNAs on other HEV genotypes. Concurrently, we will validate the shRNA-expressing AAVs in induced pluripotent stem cells derived hepatocyte-like cells, a more physiologically relevant system. Considering the widely documented safety of AAV vector-based gene therapies, our approach is, in principle, amenable to clinical translation. In particular, we hope that a synergistic effect from co-application with RBV will lead to viral clearance in chronically infected HEV patients, and that our strategy to target the viral RNA with multiplexed shRNAs will counteract viral escape through mutation.

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IFN response suppresses HDV persistence during hepatocyte proliferation in vitro

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Introduction: Hepatitis B virus (HBV) and D virus (HDV) co-infections cause the most severe form of viral hepatitis. Extracellular spreading pathways are important for persistence of both viruses and can be blocked by the entry inhibitor Myrcludex B. However, HDV can also propagate through cell division. We recently found that HDV replication induces profound IFN response via MDA5. The aim of this study is to evaluate the effect of the IFN response on HDV persistence during hepatocyte proliferation.

Methods: Susceptible innate immune competent and immune deficient cell lines were infected with HDV and splitted (1:6) at day 5 post infection and further splitted every 5 days. Different IFNs were applied during cell proliferation. HDV infected cells and viral markers (HDV RNA and antigens) were quantified using immunofluorescence and RT-qPCR.

Results: Over 6 passages following HDV infection, HDV-specific markers were efficiently amplified in HuH7-NTCP cells which are defective to produce IFNs, but profoundly lost in HepaRG-NTCP cells which are innate immune competent. BrdU labeling of newly synthesized DNA indicated that HDV replication does not impair HepaRG-NTCP cell division. Blocking of the endogenous IFN response by MDA5 depletion and inhibitors targeting the IFN signaling pathway profoundly promoted HDV amplification by cell division, indicating endogenous restriction of HDV amplification by the “self-induced” IFN response. This finding was in line with the significant suppression of HDV persistence by exogenous IFN-alpha, -beta and -lambda1 treatment in HuH7-NTCP cells.

Discussion/Conclusion: Both exogenously and endogenously induced IFN responses restrict HDV persistence during hepatocyte proliferation. This finding helps to understand the clinical observation of the Myr-203 study demonstrating a strong synergism of combining IFN and the entry inhibitor Myrcludex B. The system also provides a cell culture model for the identification of other synergistically acting immune modulators for future clinical combination therapies.

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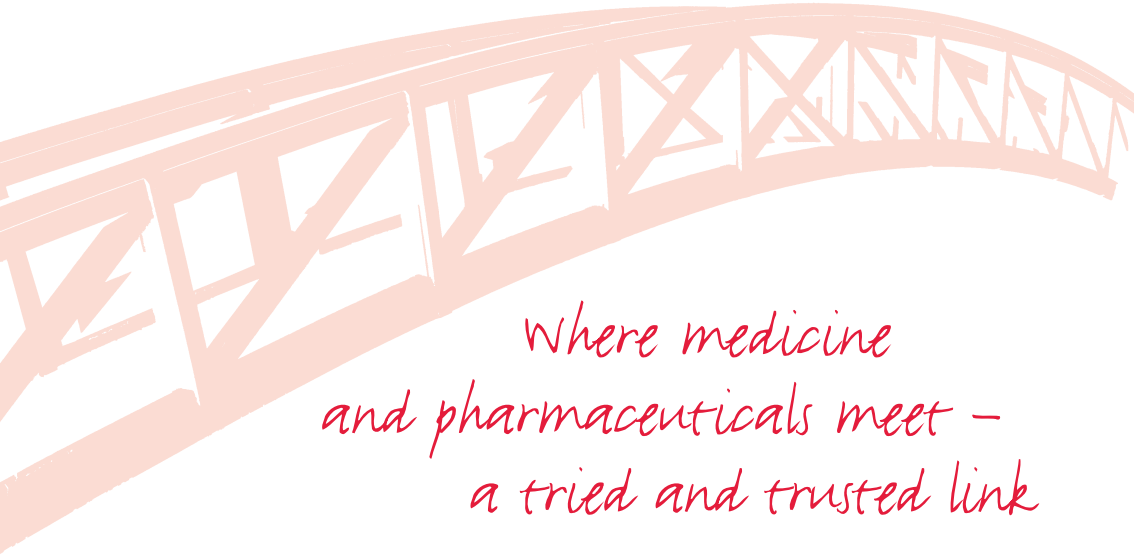

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