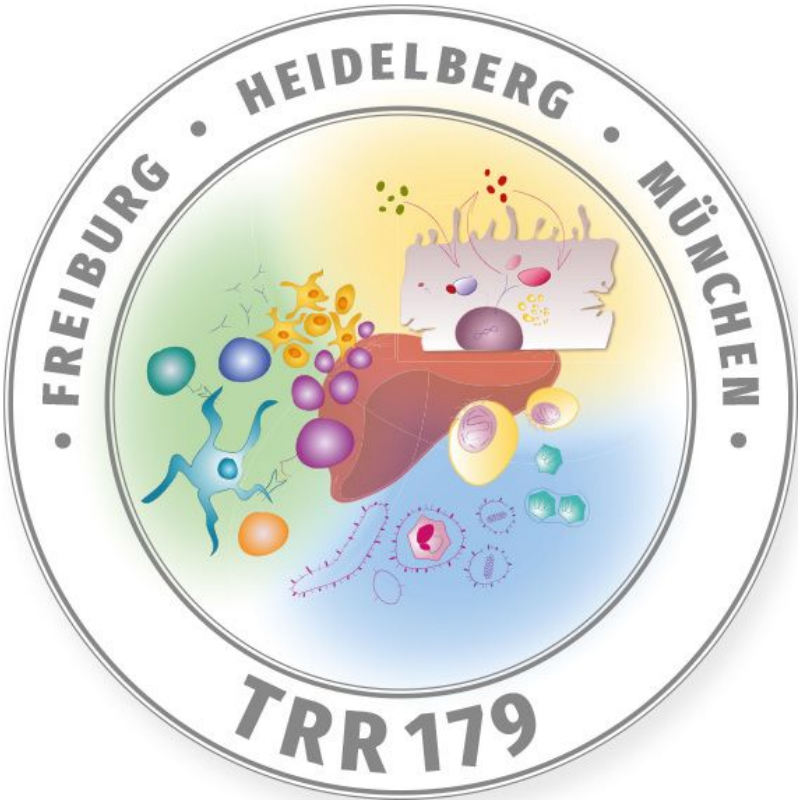


# 3RD ANNUAL IRTG PHD, MD AND POST-DOC RETREAT

## MAY 27 – 29, 2019

## ST. PETER, GERMANY

**Dr. Eva Schnober, Maike Smits, Melissa Teusel, Martin Feuerherd**

# Agenda

## May 27th

| | | |
|---|---|---|
| **Bus Departure from Freiburg, Konzerthaus** | | **11:20** |
| **Arrival and room keys** | | **12:00 – 12:30** |
| **Lunch** | | **12:30 – 13:30** |
| **Welcome note** | | **13:30 – 13:35** |

## Keynote Lecture I

**Chairs: Maike Smits, Martin Feuerherd, Melissa Teusel**

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| **Prof. Dr. sc. nat. Hanspeter Pircher** | Lessons from the LCMV infection model (30 min + 10 min discussion) | **13:35 – 14:15** |

## Session I: Adaptive Immunity 1

**Chairs: Frances Winkler, Martin Feuerherd**

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|---|---|---|
| **Katharina Wild** | OX40 stimulation and PD-L1 blockade synergistically augment HBV-specific CD4 T cells in patients with HBeAg-negative infection | **14:15 – 14:35** |
| **Benedikt Csernalabics** | CD4 T cell responses during acute Hepatitis E Virus infection | **14:35 – 14:55** |
| **Valerie Oberhardt** | Discovery of HDV-specific T cell epitopes in hepatitis Delta virus (HDV) and hepatitis B Virus (HBV) co-infection | **14:55 – 15:15** |
| **Franziska Daul** | The role of memory-like NK cells in chronically HBV/HCMV+ patients | **15:15 – 15:35** |
| **Break** | (Tea/Coffee/Snacks) | **15:35 – 16:05** |

## Session II: Adaptive Immunity 2

**Chairs: Lisa Wolff, Maike Smits**

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| **Katrin Manske** | Real-time monitoring of HBV-infection in mice revealed antigen level depended outcome of acute and chronic liver infection | **16:05 – 16:25** |
| **Kathrin Heim** | Phenotypic and functional differences of HBV core-specific versus HBV polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load | **16:25 – 16:45** |
| **Verena König** | Liver damage dampens anti-viral CD8 T cell response by inducing loss of surface T cell receptor expression | **16:45 – 17:05** |
| **Elahe Salimi Alizei** | Differential role of HLA-B*27 restricted CD8+ T cell responses and viral escape in acute and chronic Hepatitis B virus infection | **17:05 – 17:25** |
| **Sandra Kurz** | Hepatitis B virus escapes non-canonical CD8 T cell effector function | **17:25 – 17:45** |
| **Dinner** | | **18:00 – 19:00** |
| **Social event** | | **from 19:00** |

## May 28th

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| **Breakfast** | | **08:00 – 09:30** |

### Session III: Treatment

**Chairs: Elahe Salimi Alizei, Katharina Wild**

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| **Maike Smits** | Emergence of a memory follicular T helper cell signature on HCV-specific CD4 T cells after therapy of persistent infection | **09:30 – 09:50** |
| **Lisa Wolff** | Generation and characterization of human monoclonal antibodies directed against hepatitis B virus infections from single memory B cells | **09:50 – 10:10** |
| **Jakob Zillinger** | Local expression of HBV antigens in liver tissue - and not secreted antigens - are responsible for HBV-specific T cell tolerance during chronic hepatitis B | **10:10 – 10:25** |
| **Hélène Kerth** | Generation of multicistronic DNA- and mRNA-based therapeutic vaccines against chronic hepatitis B | **10:25 – 10:40** |
| **Break** | (Tea/Coffee/Snacks) | **10:40 – 11:00** |

### Session IV: T-cell Dysfunction

**Chairs: Katrin Manske, Valerie Oberhardt**

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|---|---|---|
| **Viktoria Veith** | High levels of HBs- and HBeAg are determinant for deletion or exhaustion of CD8 T cells in chronically infected patients | **11:00 – 11:15** |
| **Martin Feuerherd** | Altered HBV-specific T-cell Immunity in HBV/HIV-1 Co- versus HBV Mono-infected Patients | **11:15 – 11:35** |
| **Frances Winkler** | Metabolic programming of exhausted CD8+ T cells in chronic viral hepatitis | **11:35 – 11:55** |
| **Nina Hensel** | The exhausted fate of HCV-specific CD8+ T cells | **11:55 – 12:15** |
| **Lunch** | | **12:30 – 13:30** |

### Keynote Lecture II

**Chairs: Maike Smits, Martin Feuerherd, Melissa Teusel**

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|---|---|---|
| **Prof. Dr. med. Hartmut Hengel** | Good scientific practice and beyond (120 min) | **13:30 – 15:30** |
| **Break** | (Tea/Coffee/Snacks) | **15:30 – 16:00** |
| **Networking and Recreation: How to tackle major obstacles during the phd/md thesis** | | **16:00 – 18:00** |
| **Dinner** | | **18:00 – 19:00** |

## May 28th

### Session V: Innate Immunity

**Chairs: Hendrik Welsch, Melissa Teusel**

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| **Marcus Rosenblatt** | Disentangling the molecular mechanisms regulating the sensitization of interferon alpha signal transduction | **19:00 – 19:20** |
| **Ombretta Colasanti** | Quantitative analysis of of TRIF cleavage by Hepatitis C and Hepatitis A virus proteases and its impact on innate immunity | **19:20 – 19:40** |
| **Nadine Gillich** | Mode of action of the RIG-I like receptor LGP2 in the interferon response triggered by viral infections | **19:40 – 20:00** |
| **Arthur Lang** | Functional analysis of Toll-like-receptor 3 response in liver cells | **20:00 – 20:20** |
| **Sebastian Altstetter** | Immune control of hepatitis delta virus infection in vitro and in vivo | **20:20 – 20:40** |
| **Qutaiba Mustafa** | Photoacoustic detection of fluorescent protein via temporal analysis of light absorption variation | **20:40 – 21:00** |

## May 29th

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|---|---|---|
| **Breakfast** | | **08:00 – 09:15** |

## Session VI: System Devlopment

**Chairs: Nadine Gillich, Samuel Hofmann**

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|---|---|---|
| **Hendrik Welsch** | Reshaping of the cellular signaling landscape under continuous stimulation of innate antiviral responses | **09:15 – 09:35** |
| **David Zander** | Effect of antiviral signaling on cell death, proliferation and tumorigenesis | **09:35 – 09:50** |
| **Carolin Schmelas** | Combinatorial knock-down / knock-out strategies to reconstitute anti-hepatitis B virus immune response and to eliminate persisting hepatitis B virus cccDNA | **09:50 – 10:10** |
| **Andreas Oswald** | Skipping transcription: a mRNA based CRISPR/Cas9 approach to target viral infection | **10:10 – 10:30** |
| **Break** | (Tea/Coffee/Snacks) | **10:30 – 10:50** |

## Session VII: HBV Virology

**Chairs: Andreas Oswald, Carolin Schmelas**

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|---|---|---|
| **Peter Zimmermann** | High-throughput compatible, minimally trans-complementation dependent hepatitis B virus reporter vectors | **10:50 – 11:10** |
| **Melissa Teusel** | Harnessing an effective antiviral response targeting hepatitis B virus (HBV) | **11:10 – 11:30** |
| **Verena Plank** | Hepatitis B virus X (HBx) SUMOylation by the host cell regulates cccDNA establishment | **11:30 – 11:50** |
| **Samuel Hofmann** | HBV Core protein SUMOylation promotes PML association and chronic infection | **11:50 – 12:10** |
| **Bingqian Qu** | Hepatitis B Virus X protein-mediated transcription of covalently closed circular DNA and its inhibition by a neddylation inhibitor | **12:10 – 12:30** |
| **Lunch** | | **12:30 – 13:30** |
| **Bus Departure to Freiburg** | | **14:00** |

**OX40 stimulation and PD-L1 blockade synergistically augment HBV-specific CD4 T cells in patients with HBeAg-negative infection**

Katharina Wild[1], Felix Jacobi[1], Maike Smits[1], Katharina Zoldan[1], Tobias Flecken[1], Julia Lang[1], Philipp Ehrenmann[1], Florian Emmerich[2], Maike Hofmann[1], Christoph Neumann-Haefelin[1], Robert Thimme[1], and Tobias Boettler[1]

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Keywords: HBV; Adaptive Immunology; CD4; Th1 cells, T follicular helper cells

Chronic hepatitis B virus (cHBV) infection is a common cause of progressive liver disease that eventually leads to hepatocellular carcinoma. Antiviral therapeutic options are still limited and cannot achieve the elimination of persistent HBV infection. HBV-specific T cells are crucial for HBV control and act in a protective manner after discontinuation of antiviral therapy but appear functionally impaired during cHBV infection. Thus, T-cell based immunotherapy appears to be a promising therapeutic approach. Aim of the study was to characterize the *ex vivo* phenotype of HBV-specific CD4 T cells, the identification of new HBV-specific CD4 T cell epitopes and to analyze whether the functionality of CD4 T cells can be enhancement by targeting different immunological pathways. The expression of relevant costimulatory and coinhibitory molecules (e.g. CD127, OX40 and PD-1) on HBV- and Influenza (Flu)-specific CD4 T cells was analyzed using MHC class II-tetramers in 11 cHBV patients and 11 healthy volunteers. 66 patients (cHBV, genotype D) were screened for HBV-specific CD4 T cell responses after stimulation with overlapping peptides (OLPs) spanning the entire HBV-polyprotein. Stimulation with recombinant IL-7, an agonistic OX40-antibody or blockade of PD-L1 was performed in antigen-specific *in vitro* cultures. Cytokine secretion and expression of transcription factors were analyzed by flow cytometry. Responses targeting Influenza, Epstein-Barr virus and tetanus toxoid served as controls. Tetramer-staining revealed that the IL-7 receptor-alpha (CD127), OX40 and PD-1 constitute possible therapeutic targets as they were all strongly expressed on HBV-specific CD4 T cells *ex vivo*. The HBV-specific CD4 T cell responses identified by OLP-screening targeted predominantly the HBV-polymerase and core proteins. Combined OX40 stimulation and PD-L1 blockade significantly augmented IFN-gamma and IL-21 producing HBV-specific CD4 T cells *in vitro,* suggesting active Th1 and follicular T helper cell programs. Indeed, transcription factors T-bet and Bcl6 were strongly expressed in cytokine producing cells.

Collectively, our observations demonstrate that synergistic effects of combined OX40 stimulation and PD-L1 blockade augment the secretion of Th1 and Tfh signature cytokines IFN-g and IL-21, suggesting that these pathways are promising candidates for immunotherapeutic interventions for cHBV infection.

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## CD4 T cell responses during acute Hepatitis E Virus infection

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Keywords: hepatitis E, CD4 T cell subsets, follicular helper T cells, longitudinal study, viral hepatitis, Adaptive Immunology, Virology, Epitope mapping

Clearance of many viral infections is associated with a strong antigen-specific T cell response, which is crucial for viral control as well as its elimination. The role of CD4 T cells during spontaneous resolution of acute viral infection in humans remains largely unknown, due to a lack of appropriate human disease models. Here, the hepatitis E virus (HEV) infection provides an evolving research platform.

In most cases, HEV infections remain asymptomatic. When a clinical hepatitis is developed, the symptoms are usually self-limiting and resolve within a few weeks. Previous studies revealed that acute HEV infection is associated with a strong, antigen-specific CD8 T cell response. Also, it has been shown that specific HEV-IgG antibodies are generated during the course of infection, suggesting an effective B cell response induced by T follicular helper (Tfh) cells. Nevertheless, not much is known about CD4 T cells in this setting.

Therefore, we aimed to characterize the CD4 T cell response during acute HEV infection with focus on CD4 T cells and their subsets.

Different CD4 T cell subsets (Th1, Th2, Th17, Treg, Tfh) will be analyzed longitudinally by flow cytometry. Furthermore, we will analyze activation induced markers and relevant costimulatory and coinhibitory molecules to elucidate the involvement of the different T cell subsets in the resolution of the infection. In addition, we aim to identify specific CD4 T cell epitopes within the viral proteins in order to be able to characterize HEV-specific CD4 T cell responses in selected patients.

The generated information about the role of CD4 T cell subsets in the development of HEV immunity will be of great value for vaccine development against human viral infections.

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**Discovery of HDV-specific T cell epitopes in hepatitis Delta virus (HDV) and hepatitis B Virus (HBV) co-infection**

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Keywords: HDV, HBV, Adaptive Immunology, CD8, Epitope Mapping

Hepatitis D virus (HDV) super-infection of hepatitis B virus (HBV)-infected patients is associated with rapid progression to liver cirrhosis and hepatocellular carcinoma. The virus-specific CD8 T-cell response is thought to have a major impact on the outcome of HDV infection. However, the HDV-specific T-cell epitope repertoire is only poorly characterized and mechanisms contributing to T-cell failure during chronic infection are not yet fully understood. Previously, we identified HDV-specific CD8 T-cell epitopes in regions of the large HD antigen (L-HDAg) in which the HDV tolerates mutations to escape immune recognition. In this study, we aimed to discover T-cell epitopes that are located in conserved regions, in order to characterize the influence of these T cells on infection outcome.

By using the consensus sequence of the L-HDAg from 171 HDV genotype-1 sequences an overlapping peptide (OLP) library was designed. PBMC from chronic and resolved HDV/HBV co-infected patients were stimulated with a pool of all peptides and then expanded for 10 days. Subsequently, cells were restimulated with peptide pools (12 pools) and promising individual OLP candidates were analysed by cytokine secretion assay.

Of the screened patients (n=16) almost 60% responded to one or more OLP pool, including 57% of resolved and 55% of chronic patients. OLP-specific CD8 T cells predominantly co-expressed TNF-□□□and IFN□. Interestingly, L-HDAg$_{37-51}$ epitope-specific CD8 T cells were detected in 25% of all patients. HLA allele restriction analyses revealed that this epitope is presented by the HLA-A*2402 allele.

In conclusion, we were able to detect novel HDV-restricted CD8 T-cell epitopes in chronic and resolved HDV/HBV co-infected patients. OLP-specific T cells proliferated and secreted cytokines upon peptide-specific restimulation, indicating that these cells remained partially functional in chronic HDV infection. These findings will enable further phenotypical analyses of HDV-specific CD8 T cells specific for epitopes that are not affected by viral escape.

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## The role of memory-like NK cells in chronically HBV/HCMV$^{+}$ patients

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Keywords: HBV, HCMV, memory-like NK cells

Phenotypical and functional NK-cell alterations are well described in patients with chronic Hepatitis B virus (cHBV) infections. In particular, in cHBV patients who are co-infected with human cytomegalovirus (HCMV) increased frequencies of FcεRIγ$^{-}$CD56$^{dim}$ memory-like NK cells are detectable and shape the NK cell population compared to healthy donors (HD)/HCMV$^{+}$ and chronic Hepatitis C virus (cHCV)/HCMV$^{+}$ infected patients. Phenotypically and functionally, these FcεRIγ$^{-}$CD56$^{dim}$ memory-like NK cells differ from conventional FcεRIγ$^{+}$CD56$^{dim}$ NK cells. Fc□RIγ$^{-}$CD56$^{dim}$ memory-like NK cells are characterized by reduced cytokine responsiveness and functionality mediated by missing-self recognition but higher CD16-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). However, the exact role of memory-like NK cells is not fully understood. To gain further insights into memory-like NK-cell responses in cHBV/HCMV+ patients we first want to determine if anti-HBV or/and anti-HCMV antibodies are responsible for the memory-like NK cell expansion. Second, we aim to characterize unconventional effector functions of memory-like NK cells, e.g. tissue protection or B-cell help. Third, we will analyze the effects of type I interferon (IFN) on the effector function of memory-like NK cells. With these experimental approaches we will broaden our knowledge about memory-like NK cells in general and about their role in cHBV infection and their potential implication in new immunotherapeutic approaches in HBV cure.

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**Real-time monitoring of HBV-infection in mice revealed antigen level depended outcome of acute and chronic liver infection**

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Keywords: HBV, Adaptive Immunology, CD8

Infections by Hepatitis B virus (HBV) lead to self-limited disease in most cases. However, 5% of patients develop chronic hepatitis accounting for 240 million chronic hepatitis B cases worldwide. The decisive factors ruling between acute or chronic infections are so far ill understood. Furthermore, analysis of time-resolved CD8 T-cell response and virus elimination in humans are hindered by heterogeneous course of disease and lack of biomarkers alongside serum alanine aminotransferase (sALT) levels and HBs/HBe antigens. We established a recombinant adenovirus delivering a HBV1.3 over-length genome combined with the reporter gene luciferase (Ad-HBV-Luc) into hepatocytes. We infected mice with 10e5 to 10e9 pfu Ad-HBV-Luc and monitored virus elimination by in-vivo-bioluminescence measurement, HBs/HBe antigens and sALT. We characterized the dynamics of anti-viral CD8 T-cells by flow-cytometry and re-stimulation assay. Infection of mice by Ad-HBV-Luc leads to infection of the liver. Dose-dependent infection revealed elimination of Ad-HBV-Luc at an infectious dose of 10e7 pfu. Higher infectious doses resulted in virus persistence. During acute disease outcome, CD8 T-cells could be re-stimulated by virus-specific peptides leading to IFNg and TNF secretion, whereas re-stimulation capacity of CD8 T-cells during persistent infection was absent. During persistent Ad-HBV-Luc infection, CD8 T-cells co-expressed PD-I, Lag-3, Tim-3 on antigen-specific CD8 T-cells early after infection. In contrast, during self-limited infection, CD8 T-cells co-expressed PD-I, Lag-3, Tim-3 exclusively during virus elimination. Real-time monitoring of viral clearance by bioluminescence unraveled heterogeneous elimination kinetics in individual mice comparable to HBV clearance in humans. We developed a murine HBV infection model system where virus elimination can be monitored in real-time in any individual mouse. Following the HBV elimination kinetics gives insides into time-resolved CD8 T-cell immunity dependent on the course of disease.

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**Phenotypic and functional differences of HBV core-specific versus HBV polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load**

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Keywords: HBV, Adaptive Immunity, CD8+ T cells

T-cell exhaustion is considered as a major mechanism underlying HBV-specific CD8+ T-cell failure. However, due to limited number of detectable HBV-specific CD8+ T cells in chronically HBV-infected patients, it is still not known whether HBV-specific CD8+ T cells targeting different epitopes are similarly impaired and share molecular profiles indicative of T-cell exhaustion.

By applying pMHCI tetramer-based enrichment method, the detection rates of circulating HBV-specific CD8+ T cells specific for HBV core- and polymerase–derived epitopes were improved. Subsequently, phenotypic and functional in-depth analyses were performed using multicolor flow cytometry.

We were able to detect HBV-specific CD8+ T cells *ex vivo* in the majority (>80%) of tested patients. Specifically, core- and polymerase, but not envelope-specific CD8+ T cells were frequently found. Interestingly, HBV-specific CD8+ T cells in cHBV infection predominated in the less differentiated memory-like CD127+PD1+ subset, showed low TOX expression and lacked signs of terminal exhaustion. Importantly, antigen specificity clearly impacted subset distribution and thus the phenotype and function of core- versus polymerase-specific CD8+ T cells. More precisely, core-specific CD8+ T cells showed a higher porportion of memory-like T cells and a decreased expression of KLRG1 and Eomes suggesting the lack of terminal exhaustion. In line with this, core-specific CD8+ T cells displayed a surperior expansion capacity compared to polymerase-specific CD8+ T-cell populations. This was not primarily due to a differential proliferative capacity but rather to different survival characteristics reflected by an increased expression of BCL2 in core-specific CD8+ T cells compared to polymerase-specific CD8+ T cells. These differences were only detectable in HBV-specific CD8+ T cells obtained from chronically infected patients and not from patients who resolved HBV.

Overall, the molecular mechanisms underlying defective T-cell responses differ with respect to the targeted HBV antigens. These findings may have potential implications for the design of immunotherapeutic interventions in HBV cure.

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# Liver damage dampens anti-viral CD8 T cell response by inducing loss of surface T cell receptor expression

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Keywords: HBV, Adaptive Immunology, CD8 T cells

Chronic HBV infection is associated with high antigen levels and chronic liver damage. Virus-specific CD8 T cells have been shown to be dysfunctional and scarce during chronic HBV infection. Here, we investigate the impact of liver damage on virus-specific CD8 T cell responses and consequently on the outcome of the immune response against viral infection of the liver. To study the dynamics of interaction between virus, hepatocytes and immune cells in the liver, we exploit an *in vivo* liver infection model consisting of a hepatotropic adenovirus. We observed that low infectious doses of Ad-CMV-GOL were cleared within 10 to 14 days, whereas high infectious doses led to persistent liver infection. Clearance of low-dose Ad-CMV-GOL infection was associated with CD8 T cell-mediated ALT-increase between day 7-10 p.i. In contrast, during high-dose Ad-CMV-GOL infection liver damage occurred already from day 3-5 p.i. and no further CD8 T cell-mediated ALT-peak was observed at day 7-10 p.i. Early liver damage was CD8 T cell independent indicating involvement of innate immune effector mechanisms, and suggesting that early liver damage abrogated CD8 T cell immunity. We confirmed this notion by using a low-dose recombinant adenovirus coding for DTR (Ad-CMV-DTR) that did not cause rapid liver damage by itself. Liver damage induction upon DT application, however, abrogated CD8 T cell-mediated immunity against low-dose Ad-CMV-GOL that was coinfected with Ad-CMV-DTR. At the cellular level, we found that adoptively transferred ovalbumin-specific CD8 T cells (OT-1), identified by a congenic marker, almost completely lost cell surface expression of the Valpha2/beta5 TCR. Our data suggest that liver damage shuts down anti-viral CD8 T cell immunity locally in the liver. Since protective CD8 T cell immunity clearing virally infected hepatocytes from the liver is necessarily associated with liver damage, our findings may reveal a negative regulatory feed-back loop with tissue-protective properties where CD8 T cell-induced liver damage impairs further antiviral CD8 T cell immunity – which in consequence would result in persistent infection and chronic liver damage.

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**Differential role of HLA-B*27 restricted CD8+ T cell responses and viral escape in acute and chronic Hepatitis B virus infection**

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Keywords: HBV, adaptive immunity, CD8+ T cell, viral escape, HLA-B*27

Virus-specific CD8+ T cells play an important role in viral clearance. Protection against viral escape following HIV and HCV infection has been linked to HLA-B*27 presentation of viral epitopes to CD8+ T cells. In contrast, viral escape is considered to play a minor role in HBV-specific CD8+ T cell failure. However, these findings are mainly based on studies on HLA-A*02 restricted epitopes. Therefore, we aimed to define the role of HLA-B*27 restricted CD8+ T cell responses in HBV infection.

124 patients with chronic HBV (cHBV) genotype D infection were analyzed for HLA-B*27 associated viral sequence polymorphisms. In a second approach epitopes were predicted by unbiased epitope identification in acute-resolved and chronic infection. The predicted epitopes were then confirmed in patients with acute-resolved or chronic HBV infection by IFNγ- and tetramer staining.

12 novel HLA-B*27-restricted HBV-specific CD8+ T cell epitopes were identified. 5 of these epitopes were dominantly targeted in acute-resolved and chronic infection without evidence for viral escape. In contrast, 7 epitopes were preferentially targeted in chronic infection and were associated with viral evolution. Importantly, viral variant peptides restricted by HLA-B*27 were only partially cross-recognized by HBV-specific CD8+ T cells, indicating viral escape. To analyze why epitopes with immune escape are only targeted in chronic HBV-infected patients, we are planning to compare the avidity and the naïve precursor frequencies of CD8+ T cells targeting epitopes with and without evidence for immune escape. Moreover, as reported previously, HLA-A*02 was not strongly associated with viral escape.

In conclusion, viral escape may play an important role in HBV infection, is linked to HLA-class I restriction and may specifically affect CD8+ T cell epitopes that are targeted during persistent infection only. On the long run, these results will provide the basis for the development of novel immunotherapeutic strategies targeting non-escaping epitopes.

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## Hepatitis B virus escapes non-canonical CD8 T cell effector function

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Keywords: HBV, Adaptive Immunology, Virus sensing, Viral escape

The non-canonical CD8 T cell effector function relies on the stimulation of cytotoxic CD8 T cells by non-infected liver sinusoidal endothelial cells (LSECs) cross-presenting viral antigens leading to secretion of tumor necrosis factor (TNF). Secreted TNF induces apoptosis specifically in adenovirus- or lymphocytic choriomeningitis virus-infected hepatocytes but not in non-infected hepatocytes. Therefore, viral infection sensitizes virus-infected hepatocytes towards TNF-mediated death-inducing signaling processes. Mitochondria serve as central integrators of death signaling in hepatocytes. Therefore, alterations in mitochondrial functionality and characteristics largely influence TNF-induced signaling that can either promote cell death or survival. We found that adenoviral infection influences hepatocyte mitochondria function as it increases the stress vulnerability of mitochondria. This increased stress vulnerability is responsible for shifting the outcome of TNF-signaling towards apoptosis and thereby contribute to TNF-mediated control of viral infection in the liver. Importantly, we found that Hepatitis B virus bypasses this TNF-induced death signaling pathway in hepatocytes. Injection of TNF in mice infected with an adenovirus bearing the 1.3-overlengh HBV genome (Ad-HBV) did not cause hepatitis measured by elevation of serum Alaninaminotransferase levels. Moreover, the stress vulnerability of mitochondria from Ad-HBV-infected hepatocytes was largely decreased compared to mitochondria from hepatocytes infected with recombinant adenoviruses coding for non-HBV antigens. Our observations for the first time identify an immune escape of HBV in infected hepatocytes that circumvents the non-canonical CD8 T cell effector function at the level of mitochondrial functionality. Since antiviral activity of virus-specific CD8 T cells depends to at least 50% on the non-canonical effector function, the HBV immune escape described here is likely to be a major factor for the persistence of HBV infection.

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**Emergence of a memory follicular T helper cell signature on HCV-specific CD4 T cells after therapy of persistent infection**

Maike Smits[1,2], Katharina Zoldan[1], Naveed Ishaque[3], Zuguang Gu[3], Katharina Jechow[3], Christian Conrad[3], Catherine Fauvelle[4], Thomas Baumert[4], Florian Emmerich[5], Christoph Neumann-Haefelin[1], Maike Hofmann[1], Robert Thimme[1], and Tobias Boettler[1]

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Keywords: HCV, Direct Acting Antivirals (DAA), T cells, T follicular helper (Tfh) cells

Hepatitis C Virus (HCV) infection leads in 80 % of all cases to a chronic infection, which can cause progressive liver disease. Although CD4 T cells have been shown to be key regulators of the antiviral immune response, chronic HCV (cHCV) infection is characterized by an impairment of the HCV-specific CD4 T cell help. T follicular helper (Tfh) cells are required for an effective immune response and for B cell help and therefore for a successful clearing of pathogens in mice and humans. During acute HCV infection HCV-specific Tfh cell frequencies in the blood decrease over time. However, the fate of HCV-specific CD4 T cells during cHCV infection in humans is largely unknown. Moreover, nothing is known about HCV-specific CD4 T cells after elimination of the virus by direct acting antiviral (DAA) therapy. With the help of MHC class II tetramers, we were able to analyze HCV-specific CD4 T cells in chronic infection, during and after therapy. Relevant surface markers, costimulatory and coinhibitory markers, as well as cytokine secretion were determined by flow cytometry. By RNA Sequencing, 3 patients were transcriptional profiled at selected time points. We could show that two weeks after initiation of the therapy the frequency of the HCV-specific cells increased, which correlated with the decrease of hepatic inflammation. Moreover, surface markers indicating exhaustion and activation decreased while memory markers increased. The HCV-specific CD4 T cells acquired a Tfh-like phenotype, which we could also show by transcriptional profiling by RNA sequencing. Furthermore, we observed a decrease in HCV-specific neutralizing antibodies and CXCL13 levels, which is an indicator for the germinal center activity. Summarizing, our data show therapy induced changes in the HCV-specific CD4 T cell population. After elimination of the antigen, activation and inhibition markers decline, while a memory Tfh phenotype is formed.

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**Generation and characterization of human monoclonal antibodies directed against hepatitis B virus infections from single memory B cells**

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Keywords: HBV, Adaptive Immunology, B cells, Antibodies

Despite the availability of an effective vaccine, 257 million people worldwide are chronically infected with the hepatitis B virus (HBV) which leads to an increased risk for the development of liver cirrhosis and hepatocellular carcinoma. Current standard therapies only suppress viral replication, but mostly fail to eradicate the infection.

Since serum antibodies against hepatitis B surface antigen (HBsAg) are associated with clinical cure of HBV, the application of monoclonal HBV-specific antibodies (mAbs) represent a promising and novel immunotherapeutic approach. These antibodies should recognize viral antigens on the surface of infected hepatocytes, activate the host immune system to kill the infected cells and thereby achieve viral clearance.

For the generation of HBV-specific mAbs, single B cell antibody technology was used. B cells from patients having cleared an acute HBV-infection were isolated from peripheral blood. HBV-specific memory B cells were then isolated by incubation with biotinylated HBsAg, followed by single cell FACS sorting of CD19+ IgG+ HBsAg+ cells. Amplification of immunoglobulin genes from single memory B cells and sequencing yielded sequence information about variable heavy and light chain genes. After cloning the corresponding immunoglobulin chains into IgG expression vectors, antibodies were expressed in HEK293 cells. Two mAbs, HuMab6 and HuMab8 were found to be highly specific for HBsAg by ELISA with EC50 values in a nanomolar range. Additionally, both mAbs showed distinct neutralization capacity using HepG2 NTCP cells infected with HBV, were able to prevent HBV uptake in vitro and could detect surface bound-HBsAg from different HBV genotypes. Furthermore, HuMab6 and HuMab8 activated Fc□RIII-expressing cells to secrete cytokines upon recognition of HBsAg.

In conclusion, high-affinity human HBV-specific monoclonal antibodies can be generated from human memory B cells from peripheral blood and may be used for immunotherapeutic applications in future works.

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**Local expression of HBV antigens in liver tissue – and not secreted antigens – are responsible for HBV-specific T cell tolerance during chronic hepatitis B**

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Keywords: HBV, Adaptive Immunology, CD8 T cells, shRNA

Viral hepatitis is the 7$^{th}$ most frequent cause of death world-wide with Hepatitis B Virus (HBV) alone causing an estimated 880.000 fatalities each year. This is at least in part attributable to a lack of curative treatments which leaves chronically infected individuals at risk to develop fibrosis, cirrhosis or Hepatocellular Carcinoma (HCC). HBV persistence strictly correlates with a dysfunctional antiviral immunity. CD8 T cells have been identified as key mediators of virus control but fail to clear the virus from chronically infected hosts. In a previous project, it was shown that high loads of HBV antigens mediate HBV immune tolerance. For this, HBV-transgenic mice were pre-treated an hepatotropic Adeno-Associated-Virus (AAV) vector expressing an HBV shRNA (shHBV) to lower HBV antigen expression. Eight weeks later, mice received a therapeutic hepatitis B vaccine (*TherVacB*) consisting of a protein prime / Modified Vaccinia Ankara (MVA) boost vaccination or remained unvaccinated. shHBV-treatment suppressed HBV antigens stably by 1-2 $\log_{10}$ scales, however no spontaneous induction of B- or T cell responses could be detected following antigen suppression alone. Therapeutic vaccination of control shRNA-treated HBVtg mice with high antigen titers was also not successful in inducing HBV-specific CD8 T cell responses. In contrast, shHBV-pretreated HBVtg mice with suppressed HBV antigen titers responded to vaccination with the induction of strong HBs- as well as HBc-specific CD8 T cell responses. This went along with suppression of HBV-replication to below the detection limit, which was not the case in any other treatment group. This data shows that high HBV antigens levels during chronic hepatitis B inhibit the induction of curative CD8 T cell responses. The underlying mechanism, however, remains unclear. Several mechanisms have been proposed by which high viral antigen loads induce T cell tolerance, including clonal depletion of T cells or driving T cells into an exhausted phenotype. To further shed light on the relevant mechanisms, I plan to examine if high HBV antigen loads in the serum, or local antigen expression in the liver are responsible for HBV immune tolerance. This could not only give hints regarding the mechanism of HBV immune tolerance, but also help to further improve immune therapies for chronic hepatitis B.

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## Generation of multicistronic DNA- and mRNA-based therapeutic vaccines against chronic hepatitis B

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Keywords: HBV, adaptive immunology, CD8 T cells, DNA and mRNA vaccines

According to the WHO Viral Hepatitis represents the eighth most frequent cause of mortality worldwide. Available treatment of chronic hepatitis B rarely leads to viral clearance; thereby new therapeutic approaches are urgently needed. Our lab previously developed a therapeutic Modified Vaccinia virus Ankara vector (MVA-HBVac) based hepatitis B vaccine (*TherVacB*), based on two protein immunizations containing recombinant surface and core antigens (HBsAg, HBcAg) and a MVA boost expressing HBV antigens. *TherVacB* induces strong humoral and cellular immune response leading to long term control of HBV. We aimed to employ genetically engineered DNA and mRNA vaccines covering the most common HBV genotypes and serotypes to broaden and improve B- and T-cell immune responses elicited by TherVacB regimen.

We generated therapeutic DNA and mRNA-based vaccines (DNA-HBVac, RNA-HBVac), encoding HBV small and large envelope proteins of genotypes A and B/C, core protein of genotypes B/C and D and consensus sequence of viral polymerase's reverse transcriptase domain. *In vitro* expression, integrity and stability of all encoded proteins were confirmed by Western blotting and ELISA. In order to increase the half-life of mRNA-HBVac, two different sets of chemical modifications were implemented to mRNA backbone. After transfection of HEK293 cells and HepG2-NTCP K7 hepatoma cell line, both of our mRNA vaccine constructs showed improved cell viability and HBV protein expression both in cell lysate and supernatant. Currently, we are testing the immunogenicity of our new optimized vaccines *in vitro* and *in vivo* in C57BL/6 mice. Our preliminary data suggest that genetically engineered DNA and mRNA therapeutic vaccines may be promising candidates to optimize our *TherVacB* regimen, without the need for labor intensive and expensive recombinant protein generation and purification.

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**High levels of HBs- and HBeAg are determinant for deletion or exhaustion of CD8 T cells in chronically infected patients**

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Keywords: HBV, Adaptive Immunology, T-cell Exhaustion, CD8 T cells

Hepatitis B virus (HBV) infection continues to be a serious health problem worldwide. HBV-infected adults usually develop self-limited acute infection, whereas most neonates acquiring HBV infection perinatally develop persistent infection. More than 250 million people with chronic HBV infection are at high risk of developing end-stage liver diseases and liver cancer. HBV is a non-cytopathic enveloped virus that forms covalently closed circular DNA (cccDNA) in infected hepatocytes that serve as a template for viral replication. cccDNA can reside in the nucleus for decades and is a key of HBV persistence. The adaptive immune responses play a major role in the defense against infection. A vigorous polyclonal virus-specific CD8 T cell response is detected in peripheral blood in patients with acute hepatitis B. In contrast, in chronically infected patients the virus-specific CD8 T cells are either absent or functionally exhausted and are not able perform effector functions. The exhaustion can be caused by high viral load and high antigen levels, sustained expression of multiple inhibitory molecules and immunosuppressive microenvironment of the liver. Virological factors that can influence persistence include the continuous production of HBV surface antigen (HBsAg) that may act as a decoy for HBV-specific humoral response or may promote CD8 T cell exhaustion or deletion. Apart from presence of HBsAg, HBV e antigen (HBeAg) can be found in serum of chronic patients, indicating their immunomodulatory role in HBV persistence.

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**Altered HBV-specific T-cell Immunity in HBV/HIV-1 Co- versus HBV Mono-infected Patients**

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Keywords: HBV, HIV, Adaptive Immunology, T cells, T-cell Exhaustion, Treg

Hepatitis B virus (HBV)-specific T-cell immunity is key to control HBV infection. Current evidence suggests that HIV-1 co-infection has an adverse impact on HBV-related liver disease progression. The aim of our study is to investigate how HIV-1 co-infection influences HBV-specific immunity. Thus, we compared CD4 and CD8 T cells in HBV/HIV-1 co-infected versus HBV mono-infected patients and studied the influence of host-specific factors.

After recruitment of 33 and 30 patients respectively, peripheral blood mononuclear cells (PBMC) were isolated. For the analysis of T-cell responses, PBMC were restimulated with overlapping peptide pools derived from HBV core, envelope and polymerase sequences. Frequencies and cytokine expression patterns of HBV-specific CD4 and CD8 T-cell responses, regulatory T-cell (Treg) populations and the phenotype of exhausted CD8 T cells were analyzed by flow cytometry. T-cell responses, regulatory T cells and T-cell exhaustion were correlated with clinical data and demographic characteristics of the patients.

Analysis showed that PBMC of 36 % of HBV/HIV-1 co-infected patients did not respond with a CD4 T-cell response, whereas those that did respond, showed higher HBV core- and polymerase-specific CD4 T-cell responses ($p < 0.05$) in comparison to HBV mono-infected patients whose PBMC did not respond in only 10 %. HBV surface- ($p < 0.05$) and polymerase-specific ($p < 0.001$) CD8 T-cell responses were lower in HBV/HIV-1 co-infected versus HBV mono-infected patients. Treg subpopulation levels and expression of T-cell exhaustion markers were not significantly different between both cohorts, but HBV/HIV-1 co-infected patients showed higher levels of the transcription factor Tbet ($p < 0.0001$) in CD8 T cells indicating a more profound effector and memory phenotype.

Our data indicate that HBV/HIV-1 co-infection is associated with significant alterations of HBV-specific CD4 and CD8 T cells. To elucidate the mechanisms behind induced CD4 and impaired CD8 T-cell responses, we investigated the role of regulatory T cells and T-cell exhaustion revealing both to not be the imminent cause for the changes. Therefore we are planning the identification of the phenotypes of T-cell subtypes by tSNE and RNAseq analyses.

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## Metabolic programming of exhausted CD8$^+$ T cells in chronic viral hepatitis

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Keywords: Hepatitis B, Hepatitis C, T cell exhaustion, Metabolism

Accumulation of exhausted T cells ($T_{EX}$) is a major concern in patients with chronic hepatitis B and -C virus (HBV/HCV) infection. These $T_{EX}$ are characterized by the co-expression of inhibitory receptors, poor effector function and substantial changes in their transcriptional profile compared to functional T cells. Recent evidence points to a central role for metabolic regulation in the development of $T_{EX}$.

This study aims to identify the metabolic pathways underlying the differentiation of CD127$^+$ PD-1$^+$ $T_{EX}$ with homeostatic precursor potential and more terminally exhausted CD127$^-$ PD-1$^+$ $T_{EX}$.

Glycolysis has been shown to be critical for T cell effector function whereas mitochondrial oxidative phosphorylation is crucial for long-term persistence of memory T cells. Mitochondrial and glycolytic properties were analyzed by flow cytometry. Metabolic pathways which were differentially regulated were further investigated following transcriptome profiling by gene-set enrichment analysis.

Interestingly, we observed that CD127$^+$ PD-1$^+$ $T_{EX}$ upregulated genes involved in glycolysis in contrast to the CD127$^-$ PD-1$^+$ subset. This is in line with suppression of glycolysis mediated by inhibitory receptor signaling in more severe exhaustion. Functional testing revealed elevated glucose uptake in CD127$^+$ PD-1$^+$ $T_{EX}$ compared to CD127$^-$ PD-1$^+$ cells, again suggesting repression of glycolysis in more severe exhaustion. We then explored alternate nutrient sources for $T_{EX}$ that can provide TCA intermediates in the setting of glucose restriction for their ability to revitalize Tex. We found that acetate which can enhance acetyl-CoA levels, improved T cell function in PD-1$^{hi}$ $T_{EX}$ isolated from HCV patients.

These results suggest that differential metabolic programming underlies the differentiation of diverse subtypes of virus-specific $T_{EX}$ in chronic infection. We are currently exploring the molecular mechanisms responsible for the regulation of $T_{EX}$ metabolism. Together, these data provide novel insights into the metabolic determinants of T cell exhaustion in human chronic viral infection and highlight pathways with therapeutic potential.

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## The exhausted fate of HCV-specific CD8+ T cells

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Keywords: HCV, CD8, adaptive immunology

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. To what extent memory-like HCV-specific CD8+ T cells resemble conventional memory or exhausted cells and which impact viral antigen recognition has on the phenotype of these cells remain unclear. 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) and after spontaneous resolution of acute HCV infection (n=3). Via unsupervised clustering, DESeq2 analyses and WGCNA, we investigated the similarities and differences among the different subsets and clinical conditions. To further investigate the heterogeneity of HCV-specific CD8+ T cells, as well as their differentiation trajectory, we performed single-cell RNAseq analyses of exhausted HCV-specific CD8+ T cells from cHCV-infected patients before (n=6) and after (n=2) DAA-mediated therapy. 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 and regulation of gene expression 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, rather resemble exhausted T cells and are a potential progenitor subset of the terminally exhausted HCV-specific CD8+ T cells. Furthermore, HCV-specific CD8+ T cells targeting escaped epitopes also show 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. Thus, chronic HCV infection is strictly linked to an “exhaustive” T-cell differentiation that is not simply reverted by removal of viral antigen or loss of antigen recognition.

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**Disentangling the molecular mechanisms regulating the sensitization of interferon alpha signal transduction**

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Keywords: Mathematical Modeling, IFNα Signal Transduction, Pathway Sensitization

Tightly interlinked feedback regulators control the extent of intracellular responses elicited by the activation of signal transduction pathways. Interferon alpha (IFNα) orchestrates antiviral responses in hepatocytes, yet mechanisms that define the sensitization of the signaling pathway in response to prestimulation with a low or a high dose of IFNα remained to be resolved. We established based on quantitative time-resolved measurements obtained in the hepatocoma cell line Huh7.5 an ordinary differential equation model for IFNα signal transduction that comprises the seven different feedback regulators STAT1, STAT2, IRF9, USP18, SOCS1, SOCS3 and IRF2 and covers a time-span of 32 hours including multiple treatments. The model-based analysis showed that mediated by the signaling proteins STAT2 and IRF9 pretreatment with a low IFNα dose hypersensitizes the pathway, while pretreatment with a high pretreatment dose of IFNα leads to a gradual dose-dependent desensitization mediated by the negative regulators USP18 and SOCS1 that act at the receptor. The analysis of basal protein abundance in primary human hepatocytes revealed patient-specific amounts of STAT1, STAT2, IRF9 and USP18. Based on our mathematical model we revealed that the basal amount of USP18 determines patient-specific pathway desensitization, while the in vivo abundance of STAT2 predicts the patient-specific IFNα signal response.

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**Quantitative analysis of of TRIF cleavage by Hepatitis C and Hepatitis A virus proteases and its impact on innate immunity**

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Keywords: positive strand RNA viruses, HAV, HCV, innate immunity, TLR3 sensing

Despite strong similarities in terms of tropism and biology, Hepatitis A (HAV) and Hepatitis C virus (HCV) cause opposing infection outcomes, being HAV always cleared and HCV establishing persistence. Both viruses generate dsRNA, potentially sensed by Toll-like-receptor 3 (TLR3), and have been reported to interfere with its signaling by proteolytic cleavage of the TLR3 adaptor TRIF. We aimed at analyzing the importance of this specific interference, in order to understand its role in persistence or clearance of HCV and HAV infections.

To analyze TLR3 activation, Huh7 cells were used as a model, since they support replication and infection of both viruses. TLR3 and TRIF expression and signaling were reconstituted by lentiviral transduction. HCV replication strongly induced TLR3 response, in contrast to HAV, which did not significantly stimulate the pathway. To unravel the functional importance of TRIF cleavage in viral interference to TLR3, we generated stable cell lines expressing TRIF and viral proteases. Upon exogenous stimulation of TLR3 by poly(I:C) we detected only a moderate interference, indicating that TRIF cleavage efficiency is not sufficient to shut down TLR3 response. Lastly, to quantitatively assess TRIF cleavage, we established a transient expression system, using tagged viral proteases and TRIF. Here, we found only a moderate level of TRIF cleavage in case of HAV 3CD, whereas no detectable TRIF cleavage was found for HCV NS3-4A.

Our results indicate that HAV and HCV have very different strategies to cope with TLR3. The absence of TLR3 activation by HAV seems to rely on a lack of induction, since the HAV protease 3CD only partially cleaves TRIF, but not to a level to block TLR3 response. In contrast, HCV activates TLR3 but its protease is not capable to cleave TRIF. Here, TLR3 response is rather modulated by partial exosomal secretion of dsRNA, as we have shown previously.

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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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Keywords: innate immunity, RIG-I like receptors, antiviral response, virus infection, phosphorylation

Cytoplasmic RIG-I like receptors (RLRs) RIG-I, MDA5 and LGP2 bind to RNA of incoming viruses or replicated RNA and mount a powerful first defense system in vertebrate cells. Binding of RLRs to these RNAs 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 the innate response. Instead, it plays a regulatory role by influencing the magnitude of the IFN response induced by RIG-I and MDA5. Several studies revealed an enhancing IFN effect of LGP2 on MDA5 but a negative IFN effect on RIG-I. We were wondering how LGP2 might switch between these disparate regulatory functions and whether the different IFN outcomes are two sides of the same coin.

To characterize LGP2's regulatory role we aim to identify post translational modifications by mass spectrometry and interaction partners by using an RNA interference-based screen. Moreover we want to elucidate the influence of RLR protein levels on IFN induction. The experiments are conducted in different cell culture systems and complemented by knockout and overexpression approaches.

Huh7 and A549 cells stably overexpressing LGP2 reproducibly enhance the MDA5 pathway and suppress RIG-I mediated signaling. These effects were found with different viruses (Sendai virus, Rift valley fever virus, Mengo Zn virus, hepatitis C virus). Moreover, we identified three specific phosphorylation sites of LGP2 which are partially regulated after immune stimulation. Single and combined phosphomutants of LGP2 were generated and expressed in A549 and HepaRG cells but did not show any clear-cut phenotpyic effect so far. Further experiments are ongoing to shed more light on how LGP2 regulates the IFN response in cells and how it influences viral replication.

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## Functional analysis of Toll-like-receptor 3 response in liver cells

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Keywords: HCV, Innate Immunology, Virus Sensing

Toll-like receptor 3, which belongs to the well-known family of Toll-like Receptors, acts as a pattern recognition receptor (PRR) and is primarily localized in the membrane of the endosomal compartment. Upon viral infection, it can bind to and sense double-stranded RNA, a common intermediate in the replication cycle of positive-sense RNA-viruses like Hepatitis C Virus (HCV). After recognition of dsRNA, TLR3 activation leads to phosphorylation and nuclear translocation of different transcription factors which eventually induce expression of antiviral type I and III Interferons, Interferon-stimulated genes (ISGs) and other proinflammatory cytokines. However, the specific contribution of the TLR3 pathway to the innate immune response in hepatocytes, possibly relevant to the understanding of mechanisms of viral persistence, remains not fully understood.

Previously, our group performed a genome-wide CRISPR/Cas9 knockout screen to identify host genes involved in TLR3 signaling. A pool of single guide RNAs (sgRNAs) was transduced into two different hepatoma cell lines, Huh7 Lunet and PH5CH8, leading to a targeted knockout of different host genes followed by a negative selection of only TLR3-pathway-deficient cells. Through deep sequencing we identified a list of candidate genes involved in the TLR3 pathway. A first validation of these candidates via targeted gene knockdown by small interfering RNAs (siRNAs) and subsequent TLR3-specific stimulation with synthetic dsRNA analog poly(I:C) showed a strong reduction of IFIT1 mRNA expression in qPCR, an ISG used to quantify TLR3 signaling, particularly for three interesting genes: Receptor-type tyrosine-protein phosphatase T (PTPRT), Lysine-specific demethylase 2A (KDM2A), and RNA-binding protein 39 (RBM39). Thus far, these candidates have been shown to be differentially involved in cancer progression. In the following steps, we will overexpress / knock-out these genes and study HCV replication in their context to unveil their unique features that can assist towards the identification of hepatocyte-specific factors contributing to viral persistence.

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## Immune control of hepatitis delta virus infection in vitro and in vivo

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Keywords: HDV, Innate Immunology, Virus sensing

The hepatitis delta virus (HDV) represents an incomplete virus infecting the liver and causes persistent infection. HDV is unique as it is critically dependent on the co-infection with hepatitis B virus (HBV) to provide its small (S) and large (L) surface proteins for HDV envelopment. Candidate receptors for HDV RNA recognition were the two retinoic acid-inducible gene-I-like receptors (RLRs), retinoic acid inducible gene I (RIG-I) on the one and melanoma differentiation antigen 5 (MDA5) on the other hand. During the course of this work it has been shown, that within human hepatoma cell lines, HDV is recognized by MDA5, followed by a signaling via MAVS consequently leading to an upregulation of several interferon stimulated genes (ISGs). However these observations need to be verified in murine *in vivo* experiments.
As readouts of innate immune activation in the context of HDV infection and/or replication, markers of pattern recognition receptors (PRR) activation leading to type I and III Interferon (IFN) expression, as well as secondary markers of IFN activity were studied by RT-PCR and Western-Blot. The role of RLRs in HDV recognition were investigated through knock out cell lines for RIG-I, MDA5 and MAVS employing CRISPR/Cas9, which were subsequently challenged with HDV. In those cell lines we could show, that MDA5, but not RIG-I, is responsible for HDV RNA recognition. These findings were transferred into *in vivo* experiments, which took place in transgenic mice expressing humanized NTCP (Sodium taurocholate cotransporting polypeptide) in a liver-specific manner. Furthermore, NTCP was delivered into wildtype and MDA5 -/- mice by applying high-capacity adenoviral vectors. The above mentioned animals were challenged with HDV and AAV-HDV, respectively and the innate immune response was characterized in the liver of HDV-infected mice. The results revealed, that HDV has no obvious advantage in MDA5 -/- mice compared to wildtype animals, indicating that there are further receptors involved in the recognition of HDV.

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**Photoacoustic detection of fluorescent protein via temporal analysis of light absorption variation**

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Keywords:

Multispectral photoacoustic imaging allows for detection of intrinsic chromophores and externally administered agents that can reveal *in-vivo* biological functions. Nevertheless, imaging a near-infrared agent such iRFP720 (iRFP) in mouse liver presents a unique set of challenges.

One of the major challenges regards the spectral similarity between the agent (iRFP) and the intrinsic chromophore deoxyhemoglobin (Hb). Therefore, lower levels of blood oxygenation (high Hb concentration) at the region of interest make iRFP detection harder due to overlapping Hb and iRFP spectra. We have introduced a fluorescent protein detection method that relies on temporal analysis of light absorption change as a solution to the aforementioned problem of spectrum similarity.

We have validated our approach in phantoms and *in-vivo*, and finally in mouse liver. Moreover, we assessed the sensitivity of the proposed detection method and demonstrate a significant improvement in detection sensitivity of iRFP compared to the current used multispectral unmixing method.

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

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Keywords: Innate immune response, HCV, persistence, cell culture model

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.

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. 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.

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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## Effect of antiviral signaling on cell death, proliferation and tumorigenesis

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Keywords: Innate Immunology, Crispr

Most viral infections cause a rapid and effective cell-intrinsic immune response, which plays a central role in eliminating the infection. An important part of this response is mediated by the RIG-I (retinoic acid-inducible gene-I)/interferon system. At the same time, recent results suggest that this system is also tightly linked to the induction of cell death. The elucidation of the underlying mechanism is part of current research in the lab. Furthermore, a functional antiviral immune response was surprisingly shown to be essential for the successful treatment of malignancies with certain cytostatic agents. In literature an involvement of RIG-I-like receptor signaling is described.

We focus on the question whether and how the triggering of the RIG-I/interferon signal axis affects cell susceptibility to proliferative growth factor signals and induction of cell death. In this context we are interested in the influence of the RIG-I/interferon system on carcinogenesis, pathology and the treatability of malignancies, and particularly in the underlying mechanisms.

To address these questions, we will investigate if triggering of RIG-I signaling in cells impacts on their proliferative capacity. We will also extend this analysis to an authentic mix of antiviral cytokines and interferons and quantify the impact on cell proliferation. In parallel, we will investigate whether RIG-I-triggering leads to direct induction of cell death or the sensitization of cells towards apoptosis or other types of cell death. To verify that cells are less prone to dying if RIG-I-signaling is blocked we will use CRISPR/Cas9-mediated knockout of RIG-I and subject cells to different cytotoxic treatments, such as with the chemotherapeutic doxorubicin or gamma-irradiation. Once the relevance of functional RIG-I signaling in cell death induction is confirmed, we are going to investigate the molecular basis of RIG-I stimulation, which is likely mediated by endogenous RNA ligands generated upon cytotoxic insult.

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**Combinatorial knock-down / knock-out strategies to reconstitute anti-hepatitis B virus immune response and to eliminate persisting hepatitis B virus cccDNA**

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Keywords: HBV, Virology, CRISPR

Persistent infection with hepatitis B virus (HBV) is caused by deposition of HBV cccDNA and excess production of HBs and HBe antigens that likely skews with the host immune responses.

In my doctoral thesis, I will target HBV gene expression using a combinatorial knock-down and knock-out approach to reconstitute anti-hepatitis B virus immune response and to eliminate persisting HBV cccDNA. Specifically, I will apply RNAi to block HBsAg and HBeAg expression in order to revert their suppressive effect on the host immune response and utilize the CRISPR/Cas system to target multiple sites in HBV cccDNA. Therefore, gene transfer vectors based on recombinant Adeno-associated viruses (AAV) were developed, which harbor multiplexed cassettes for short-hairpin and guide RNAs (shRNA and gRNAs) expression. Our results show a strong additive effect of multiplexed gRNAs and shRNAs to tackle HBV infection in HepG2 cells.

We further laid the foundation for experiments in mice by evaluating the expression of Cas9 from *Staphylococcus aureus* (*Sa*Cas9) by different promoters in the liver. In this experiment Cas9 was directed against a simultaneously delivered luciferase reporter, which acts as an episomal surrogate for HBV. We determined that the liver- specific LP1 promoter drives the strongest Cas9 expression. There is evidence that the expression of the Cas9 endonuclease is a limiting factor in the efficiency of CRISPR mediated knock-out. Therefore, we developed a split *Sa*Cas9 system that allows packaging in AAVs as superior double-stranded genomes (dsAAVs). It has been shown that dsAAVs mediate stronger and faster gene expression than conventional single-stranded DNA counterparts (ssAAVs). However dsAAV only allows the packaging of transgenes of up to 2.2 kb. Our split *Sa*Cas9 system shows higher Cas9 expression and vector copy numbers in the liver as well as an increased luciferase target knock-out efficiency. Prospectively, the split *Sa*Cas9 approach will be tested in context of HBV cccDNA elimination.

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**Skipping transcription:**
**a mRNA based CRISPR/Cas9 approach to target viral infection**

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Keywords: HBV, Virology, CRISPR

Current gene therapy approaches mainly consist of lenti-, adeno- and adeno-associated viral vectors to introduce genes of interest into cells to reconstitute functionality of missing or defective factors. This approach has clear advantages, such as long-term gene expression after single administration or tissue specific delivery enabled by the tropism of the viral capsid, however there are safety concerns which complicate clinical translation. These include the risk of carcinogenesis arising from mutational integration, immune responses resulting from viral components or negative effects by long-term gene expression. An approach to overcome these caveats can be the transfection of *in vitro* transcribed (ivt) messenger RNA (mRNA) that is transiently expressed in targeted cells or tissue. In contrast to viral vectors, this approach lacks viral components and the risk of unspecific integration into the genome.

One potential application is an mRNA based CRISPR/Cas9 system to address viral infections like chronic Hepatitis B by targeting the episomal viral genome (covalently closed circular DNA) or host factors involved in viral lifecycle. In differentiated HepG2-NTCP, HepaRG and primary human hepatocytes mRNA transfection efficiencies were up to > 80 % with high cell viability (>95 %). Introduction of modified nucleotides (e.g. Pseudo-UTP, 5mCTP) significantly increased cell viability, protein expression and functionality. Preliminary data in HepG2.2.15 and HepG2-pEpi-H1.3 showed significant reduction in HBe antigen expression 5 days after transfection of SpCas9 mRNA/sgRNA targeting HBV genome. Significant reduction of viral DNA expression after treatment with SpCas9 mRNA was detected using qPCR. In conclusion, our data indicate that mRNA-based gene therapy is a versatile tool for many *in vitro* approaches allowing fast and transient expression of SpCas9 or other proteins in dividing and non-dividing cells.

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## High-throughput compatible, minimally trans-complementation dependent hepatitis B virus reporter vectors

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Keywords: HBV, Virology, cccDNA

Formation of covalently closed circular (ccc)DNA is crucial for HBV persistence. Reporter-encoding HBV (rHBV) variants should allow for sensitive and quantitative detection of cccDNA-dependent reporter expression, providing a high-throughput compatible readout to screen for cccDNA-relevant host factors. However, incorporation of foreign sequence into the tiny, intricately organized HBV genome, generally requires deletion of equally sized viral information, often resulting in multiple deficiencies that necessitate trans-complementation with full length helper plasmids. Here we present a series of HBV reporter designs in which parts of the core (HBc) gene sequence are replaced by a full-length *Gaussia* Dura-luciferase (DLuc) gene. Such vectors should depend exclusively on trans-complementation by HBc. Production of DLuc and polymerase (Pol) proteins is controlled by various combinations of translation control elements, i.e. 2A peptides and small internal ribosome entry sites (sIRES). All tested vectors expressed active DLuc and replicated upon provision of HBc *in trans,* however with markedly varying efficiencies. Based on DLuc activity and intracellular staining of hepatitis B surface antigen at least some rHBV variants are infectious for HepG2-NTCP cells. Importantly, viral entry inhibitor myrcludex B (Myr B) as well as capsid protein assembly modulators (CpAMs) known to counteract cccDNA formation strongly reduced DLuc expression upon infection, providing first evidence that such rHBVs can qualify as surrogates for the wildtype virus.
A second generation rHBV design aims for DLuc production only from precore (preC) mRNA to rule out reporter expression from different templates, e.g. linear genomes, integrates or transfected plasmid. To expedite reporter HBV production, HBc expression cassettes were site-specifically integrated into the AAVS1 safe harbor loci of HepG2 and Huh7 cells via CRISPR/Cas9 technology. Selected cell clones of these new helper cell lines efficiently produce HBc capable of trans-complementing HBc-deficient genomes and will be used generate larger stocks of HBV vector virions.

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**Harnessing an effective antiviral response targeting hepatitis B virus (HBV)**

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Keywords: HBV, Innate Immunology, Systems Biology, Signaling, viral proteins

HBV infections are a major health burden with around 240 million chronically infected people worldwide. Affected patients are at high risk of developing severe liver disease including hepatocellular carcinoma. There is an effective vaccine, but currently available drugs are unable to cure chronically infected patients as the virus persists in the cell and can therefore be reactivated. As the outcome of an infection is determined by highly dynamic interrelations of the viral infection cycle and host-cell responses, a systems biology approach is necessary to identify possible targets for intervention.

In order to develop a mathematical model of the HBV infection cycle, the abundance of core-protein was measured over time by quantitative immunoblotting and an assay to measure the envelope protein was established. Additionally, a targeted mass spectrometric approach is developed to allow sensitive detection and quantification of the viral proteins.

To link the dynamics of the HBV infection to the interferon-α (IFNα) mediated host-cell-response, a previously established mathematical model of the IFNα-signal-transduction-pathway was adapted to HepG2-hNTCP cells, which serve as a suitable cellular system to study HBV infection. In a first step, absolute numbers of molecules per cell of the key components of the IFNα-signaling-pathway were determined and used as starting conditions for the mathematical model. Second, IFNα-induced changes in phosphorylation and total amounts of the key proteins were detected by immunoblotting. With the generated data it was possible to successfully adapt the mathematical model of the IFNα-signal-transduction-pathway to HepG2-hNTCP cells. The results showed that the overall dynamic behavior of the signaling pathway was very comparable.

The aim of this project is to combine both processes in an integrative mathematical model to identify mechanisms that set the threshold for acute versus persistent infection with the long-term goal to decipher novel strategies towards curing chronic HBV infection.

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**Hepatitis B virus X (HBx) SUMOylation by the host cell regulates cccDNA establishment**

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Keywords: HBV,cccDNA, HBx, SUMOylation

PML-NBs are multiprotein nuclear complexes associated with biological functions such as DNA repair, tumorigenesis and antiviral response. SUMOylation represents a PTM (*posttranslational modification*), which is mainly occurring within the molecular environment of PML-NBs. SUMOylation severely affects transcriptional functions, localization and binding capacity of proteins that undergo this PTM due to cellular signaling. Recently, it became apparent that SUMOylation also used by human viruses to extensively exploit host-cell networks and homeostasis.

During HBV infections, cccDNA is essential for chronic persistence. Various cellular repair proteins, which are required for cccDNA formation, are associated with PML-NBs due to SUMOylation. HBx is the key player to promote transcription of the cccDNA. However, this virus mediated process is not understood in detail.

During this study immunoprecipitation assays were performed to investigate the interaction of transfected proteins with Sepharose beads. Ni-NTA pulldown was performed to investigate the SUMOylation of transfected HBx. Therefore, Ni-NTA beads were distributed to each sample to bind the $His_6$-tagged SUMO2. Proteins were detected by Westernblot analysis. Additionally immunofluorescence stainings were performed to confirm our IP and NINTA-assay results. Furthermore, the quantitative qPCR was used to monitor the relative quantification of intracellular HBV DNA as well as cccDNA was performed by real time PCR.

Here, we show that HBx colocalizes with specific PML isoforms as well as with PML-NB associated proteins. Consistent with the fact that PML-NBs represent the SUMO hotspots in the cell, we have evidence that HBx represents a novel target of the SUMO machinery covalently attaching SUMO1, 2 and 3 to the viral regulator. Intriguingly, generation of SUMO deficient HBx variants by site-directed mutagenesis and subsequent infection with HBx negative viruses revealed reduced cccDNA synthesis by qPCR. Based on our observations, HBx SUMOylation orchestrates the efficacy of cccDNA conversion most presumably happening juxtaposed to PML-NB compartments in the infected cell.

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**HBV Core protein SUMOylation promotes PML association and chronic infection.**

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Keywords: HBV, PML, SUMO, Core protein, host factors

Despite an effective vaccine, more than 350 million people worldwide are chronically infected with HBV. Downside of recent therapies is that they do not target the persistence reservoir of the virus, which is the episome-like covalently closed circular DNA (cccDNA). The cccDNA is generated from the viral polymerase-linked relaxed circular DNA (rcDNA) in a process probably involving several host cell DNA damage response (DDR) factors, a molecular mechanism far from being understood. The HBV Core protein is involved in almost every step of the HBV life cycle, including critical steps like transport of the rcDNA to the nucleus and promotion of transcriptional activity and stability of the HBV cccDNA

Here, we show that the HBV Core protein, involved in the import of the rcDNA into the nucleus, is SUMO modified and interacts with specific PML proteins. Additionally, by generation of SUMOylation deficient mutants of the HBV Core protein, we were able to show that posttranslational modification of the HBV factor by SUMO2 is a prerequisite for its association with PML-NBs and regulates the generation of cccDNA from its rcDNA precursor.

Based on these results, we hypothesize, that the SUMO modification of the HBV Core protein and the subsequent association with PML-NBs is a key factor involved in the HBV rc- to cccDNA conversion and therefore a promising target for inhibition of the formation of the HBV persistence reservoir.

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**Hepatitis B Virus X protein-mediated transcription of covalently closed circular DNA and its inhibition by a neddylation inhibitor**

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Keywords: HBV, Virology, Genome replication and regulation

Hepatitis B Virus (HBV) persistence requires establishment and maintenance of covalently closed circular (ccc)DNA, serving as an episomal template for transcription in the nucleus of infected hepatocytes. Using a quantitative PCR, the kinetics of cccDNA formation in *in vitro* infection systems and the effect of drugs on cccDNA were analysed. Copy numbers of cccDNA in infected cells were low, and entry inhibitor, Myrcludex B, efficiently blocked cccDNA formation.

Maximal cccDNA-dependent gene expression requires HBV X protein (HBx). Transiently expressed HBx by its authentic promoter showed nuclear localization. Using a lentiviral-based trans-complementation assay to restore the transcription of HBx-minus virions to the wild-type level, key fragments and residues of HBx were mapped. The C-terminus of HBx (51-154 amino acids) was sufficient and indispensable to rescue the replication of HBx-minus virus, whereas N-terminal HBx (1-50 amino acids) displayed no function. Remarkably, one HBx mutant (R96E) with weak binding activity to DNA damage-binding protein 1 (DDB1) abolished its transactivation activity.

Transcription from cccDNA requires degradation of the host restriction factors, structural maintenance of chromosome 5/6 (SMC5/6). A prerequisite for ubiquitin-dependent SMC5/6 degradation is the binding of HBx to the DDB1-Cullin 4 ring ligase complex. Since neddylation of the Cullin is required prior to ubiquitination, MLN4924, a neddylation inhibitor, was identified for its antiviral potential. MLN4924 potently reduced HBV transcription without affecting cccDNA levels. Remarkably, transcription from cccDNA of HBx-minus virions was marginally affected indicating HBx dependency. A minor reduction of HBs transcription in cell lines with stable integrates suggested the selectivity of MLN4924 on cccDNA. MLN4924 prevented SMC6 from degradation and in turn, the reappearance of SMC6 silenced transcription from cccDNA.

To sum up, nuclear HBx induces SMC5/6 degradation and promotes transcription from low-copy cccDNA. Transcription of persistent cccDNA is profoundly declined and MLN4924 treatment therefore traps cccDNA in a “transcriptional silent” status.

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The participants of our SFB TRP 179 IRTG Retreat in February 2017!

We wish a wonderful 3rd PhD retreat!