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SPEAKER ABSTRACT BOOKLET

Session 1: From discovery, clinical challenges to elimination
October 1, 2023

Keynote lecture: HCV Elimination: Where there's a will there's a way

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In 2016, the World Health Organization (WHO) provided the first roadmap for viral hepatitis elimination by 2030 – specifically targeting a 90% reduction in HCV incidence and a 65% reduction in mortality from HCV infection by 2030. This is largely made possible due to the approval of safe and highly effective direct acting antiviral (DAA) medications nearly a decade ago. Yet, the United States (US) is not on track for elimination by 2030 due to increasing incidence of new HCV infections, unrecognized diagnosis in approximately 1/3 of patients, and a drop in treatment initiations since 2015 resulting in only 1 of 3 people with chronic HCV infection cured. The White House has released a National Plan to Eliminate HCV Infection that will focus on accelerating commercialization of point-of-care (POC) diagnostics, expanding access to curative direct acting antiviral (DAA) medications, and increasing access to screening by expanding access to non-traditional sites of care. Historically, barriers to access to HCV cure included screening strategies that missed the majority of people with HCV infection, complex testing algorithms that do not allow for POC testing, cost DAA therapies resulting in barriers created by payers, and lack of effective prevention strategies. There have been developments that address some of these barriers, including the recommendation by USPSTF and CDC to conduct universal screening, access to generic DAA, and simplified treatment algorithms. Yet, challenges remain including lack of uptake of universal screening by providers, lack of POC tests for active HCV infection, slowed uptake of DAA therapy, and continued complexity of treatment algorithms at most clinical care sites. For the US to achieve elimination there will need to be (1) greater uptake of screening and treatment by general practitioners, primary care, and other non-traditional care sites, which will require simplified treatment algorithms including test and treat with rapid starts; (2) expanded access to treatment for the highest risk key populations for new infections and onward transmission including people who inject drugs (PWID); (3) treatment of HCV infection in the justice system; and (4) novel approaches to lower cost for access to cure. Current simulation models suggest an opportunity to save lives and cost by diagnosing and treating people with HCV infection.

HCV elimination is possible in the US and yet as a country we are not on track to achieve this goal by 2030. While we have safe and highly effective oral therapies that cure HCV infection in 8-12 weeks, existing state policies are perpetuating health disparities and creating artificial barriers to HCV cure. Will the White House Plan for HCV Elimination change the course for the US? In addition to securing the budget funding to invest in HCV elimination, there will need to be out of the box thinking to overcome the existing barriers. Does the US have the will to achieve HCV elimination? Where there's a will there's a way.

Session 2: Innate immunity and virus-host interaction
October 2, 2023

Keynote lecture: New insights into flavivirus – host cell interactions: a tale of proteins and lipids

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Flaviviruses comprise a large group of enveloped viruses, including dengue virus (DENV), zika virus (ZIKV), West Nile virus and yellow fever virus. They have in common a single-strand RNA genome of positive polarity encoding one polyprotein that is cleaved by cellular and viral proteins into multiple products. These provide building blocks for progeny virus particles, are required for viral RNA replication or counteract antiviral defenses of the host. For successful replication and spread, flaviviruses heavily depend on host cell proteins and lipids, which are usurped at virtually any step of the viral replication cycle. Identifying these factors and gaining insight into the mechanism how they promote or restrict the viral replication cycle is critical to understand virus-induced cellular toxicity and it might provide critical information for possible new antiviral strategies. In my presentation I will report some recent data from my group illustrating how flaviviruses exploit host cell proteins and lipids in the course of their replication cycle.

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Plenary talk: Calling the shots: micro(RNA)-management of HCV infection

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Background
Efficient hepatitis C virus (HCV) RNA accumulation is dependent upon interactions with the human liver-specific microRNA, miR-122. To date, miR-122 has been demonstrated to have at least three roles in the HCV life cycle: 1) it acts as an RNA chaperone, or “riboswitch”, allowing formation of the viral internal ribosomal entry site (IRES); 2) it provides genome stability; and 3) it promotes translation. However, the relative contribution of each role in HCV RNA accumulation remains unclear.

Methods
We used point mutations, mutant miRNAs, and HCV luciferase reporter RNAs to isolate each of the roles and evaluate their contribution to the overall impact of miR-122 on the HCV life cycle. Additionally, we used infectious virus and RT-qPCR and focus-forming unit (FFU) assays to assess effects viral RNA accumulation and virion production.

Results
Our results suggest that the riboswitch has a minimal contribution in isolation, while genome stability and translational promotion have similar contributions during the establishment phase of infection. However, in the maintenance phase, translational promotion becomes the dominant role. Additionally, we found that an alternative conformation of the 5’ untranslated region, termed SLIIalt, is important for efficient virion production. Moreover, our preliminary findings suggest that miR-122 has an additional role(s) in the HCV life cycle.

Conclusions
Taken together, we have clarified the overall importance of each of the established roles of miR-122 in the HCV life cycle an provided insight into the balance between viral RNAs in the translating/replicating pool and those engaged in virion assembly.

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P99 – **A TLR3-based directed evolution approach identifies an HCV NS3 helicase point mutation specifically affecting (-) strand synthesis**

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Background
HCV NS3 is a bifunctional enzyme, harbouring an N-terminal protease and a C-terminal helicase domain. The helicase has a 3'-5' helicase activity and has been shown to be required for viral replication. However, the exact function of HCV NS3 during HCV replication remains unknown.

Methods
We applied a directed evolution approach initially aiming at identifying viral determinants contributing to TLR3 delivery. TLR3 was expressed in HCV JFH stable replicon cell lines. TLR3 senses HCV dsRNA and induces ISG expression, reducing viral replication. HCV cell clones exhibiting a strong reduction or elimination of viral replication are losing neomycin resistance and die, while TLR3 escape mutations enable continuous replication and survival. The TLR3 escape variant was identified by clonal sequence analysis and analysed in regard to replication by luciferase assay, TLR3 activation by qPCR for IFIT1 as well as (+) and (-) strand synthesis by Northern Blot. NS3 expression as well as NS5A hyperphosphorylation was analysed by Western Blot.

Results
We identified the TLR3 escape variant D1467G in domain 2 of NS3 helicase that was present in all analysed clones in two independent experiments, indicating a strong selection advantage in presence of TLR3. D1467 is invariant among all HCV genotypes and part of the Phenylalanine loop, a conserved structure in domain 2 of NS3 helicase required for replication with yet undefined function. A JFH D1467G mutant showed slightly attenuated replication compared to wt, but completely abrogated IFIT1 induction, arguing for a specific contribution of D1467G to TLR3 activation. In addition, mutation of other selected positions in the Phenylalanine loop were either severely impairing replication or not affecting TLR3 activation, demonstrating the unique ability of D1467G to ablate TLR3 activation while still enabling replication. Mutation of the equivalent D1463G in the cell culture adapted gt1b isolate GLT1 also ablated TLR3 activation, indicating that the function of D1467/D1463 is conserved amongst different HCV genotypes. Interestingly, D1467G affected the ratio of (+)/(-) strand synthesis, comparable to replacement of the 3'X-tail of JFH with the respective Con1 sequence (JFH/XCon), suggesting a specific function of the helicase in the initiation of (-) strand synthesis. NS5A hyperphosphorylation was reduced during transient expression of JFH NS3-5B D1467G, indicating that D1467G might impair HCV replicase complex assembly and NS5A phosphorylation. In addition, NS3 D1467G displayed a lower molecular size in SDS-PAGE than NS3 wt, hinting towards a post translational modification of NS3 altered by D1467G, potentially affecting replicase assembly and (-) strand synthesis.

Conclusions
Using a directed evolution approach, we identified the TLR3 escape variant NS3 D1467G, which reduces TLR3 activation by affecting (-) strand synthesis. Identification of the mechanism will reveal the specific functions of the NS3 helicase in (-) strand synthesis.

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HCV p7-mediated inhibition of signal peptide peptidase (SPP) promotes HCV assembly by facilitating Core localization to the endoplasmic reticulum (ER) and Core-E1 interaction.

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Background
HCV Core associates with the lipid droplets (LD) following SPP-mediated cleavage of its transmembrane domain. Previous studies showed that HCV p7 induces Core localization to the ER leading to efficient HCV assembly. However, the mechanism by which p7 regulates Core localization to the ER remains incompletely defined.

Methods
The effect of p7 on SPP-mediated Core processing was determined by western blot analysis of FT3-7 cells co-expressing Core and p7-HA. Interaction between p7-HA and SPP was determined by co-immunoprecipitation analysis in the presence or absence of SPP inhibitors. The SPP interacting domain of p7 was determined by deletion analysis or introducing L(51-55)A mutations to p7. The effect of p7/L(51-55)A mutations on Core subcellular localization and HCV assembly was determined by introducing these mutations to the infectious HCV genome HJ3-5/HA-p7-EMCV. The role of SPP-processing-defective full-length Core (fCore) on HCV assembly was determined by performing co-immunoprecipitation of Core and E1, as well as employing a trans-packaging system consisting of HCV replicon cell line transfected with Core and E1-NS2 expressing plasmids.

Results
Co-expression of HCV Core and p7 inhibited Core processing by SPP. P7 also inhibited SPP-mediated cleavage of host protein HO-1 indicating that p7 inhibits SPP activity. P7 interacted with the SPP and this interaction was inhibited by the SPP inhibitors suggesting that p7 inhibits SPP by interacting with these inhibitors-binding domains in SPP. P7 mutants lacking a C-terminal domain or having L(51-55)A mutations showed reduced SPP interaction and Core processing inhibition. E2-p7 processing, but not its ion channel activity, was required for p7 and SPP interaction. Importantly, p7/L(51-55)A mutations caused reduced Core localization to the ER and virus production without affecting viral RNA replication. We also determined that fCore interacts with E1 more efficiently than the SPP-processed form of Core (sCore), and the combination of fCore and sCore supported HCV assembly more efficiently than sCore alone.

Conclusions
These results suggest that HCV p7 promotes HCV assembly by inhibiting SPP-mediated internal cleavage of Core leading to increased levels of full-length Core. The increased fCore associates with the ER via intact transmembrane domain and interacts with E1 more efficiently, likely facilitating HCV particle envelopment at the ER. As previously reported, inefficient processing of E2-p7 will ensure late-stage virus assembly by delaying p7-mediated SPP inhibition, preventing the premature accumulation of full-length Core. In aggregate, this study provides insight into how HCV fine-tunes the virus assembly process by regulating host SPP activity.

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P35 – **Identification of Phosphorylation Sites on Viral and Host Proteins Regulating West Nile Virus Replication**

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Background and Aims
During viral infection, the cellular proteome is extensively remodeled, including alterations in protein abundance, localization and post-translational modifications (PTMs). We used an enrichment and mass spectrometry approach to identify changes in PTMs during West Nile virus infection. Our studies revealed that phosphorylation of both viral and host proteins can regulate viral replication.

Methods and Results
We used quantitative mass spectrometry to identify sites of host protein phosphorylation that are significantly different between WNV-infected and uninfected cells. We further analyzed these data to focus on those phosphorylation sites that are predicted to alter protein function during infection. We found a total of 10 host proteins that are predicted to have increased activity during WNV infection. We used siRNA-mediated depletion and found that knock-down of two kinases, AMPK and PAK2, leads to an increase in infection. Thus, both AMPK and PAK2 have antiviral activity in WNV infection. AMPK is a trimeric complex that has been previously described to have antiviral activity. In our current work, we present new findings that this antiviral activity is modulated by phosphorylation of a non-catalytic subunit of this complex, AMPKβ. Our strategy also revealed an increase in phosphorylation of a serine residue in the autoinhibitory domain of PAK2, a member of the Group I family of p21-activated kinases. Phosphorylation at this site relieves autoinhibition to promote PAK2 kinase activity during WNV infection. We demonstrate that antiviral activity of PAK2 likely restricts WNV during early viral RNA replication. As our mass spectrometry analysis was performed on WNV-infected cells, we also found phosphorylation sites on viral proteins. We used site-directed mutagenesis in a WNV subgenomic replicon to generate phospho-null and phosphomimetic mutations in the viral structural proteins, NS3 and NS5. The results from these experiments suggest that phosphorylation of viral proteins can both positively and negatively regulate viral RNA replication.

Conclusions
Together, our data suggest that phosphorylation of viral and host proteins add an important layer of regulation during WNV infection. In our future studies, we will determine the mechanisms by which protein phosphorylation affect WNV replication. Further, we will identify the host kinases that are required for viral protein phosphorylation.

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P98 – **Mutations in the Interferon Sensitivity Determining Region dramatically increase Hepatitis C Virus replication leading to direct viral pathogenesis**

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Background
Chronic Hepatitis C Virus (HCV) infection can eventually create the need for a liver transplantation (LTX). Fibrosing cholestatic hepatitis (FCH) is a very severe disease recurrence after LTX marked by high viral titers and rapid organ destruction. We could previously show that the HCV genotype (gt) 1b isolate GLT1, taken from an FCH patient after LTX, has high genome replication fitness in cell culture, compared to prototype gt1b isolates like Con1. The aim of this project was to study the role of replication fitness in viral evolution and pathogenesis and to identify the underlying genetic determinants.

Methods
We studied the viral quasispecies in the GLT1 patient at various time points before and after LTX via deep sequencing and analysis of individual viral clones. We performed phenotypic characterization of replication in cultured hepatoma cells based on consensus sequences and patient isolates using subgenomic replicons (SGRs).

Results
In the GLT1 patient, a dramatic shift in the viral population was observed after LTX, resulting in increased genome replication fitness but not cell entry or virus production efficiency. Exchange of Low Complexity Sequence 1 and Domain 2 (LCS1D2) of non-structural protein 5A (NS5A) transferred the high replication efficiency of GLT1 to the prototype isolate Con1. Introducing the individual LCS1D2s from a cohort of 14 FCH patients and 9 non-FCH patients into a Con1 SGR revealed a highly elevated replication fitness of almost all FCH isolates compared to the non-FCH controls. Since FCH is a severe and highly pathogenic course of disease, these results indicate that evolution of an unleashed highly replication fit HCV variant under conditions of immune suppression can directly cause liver pathogenesis. For the Interferon Sensitivity Determining Region (ISDR), contained within LCS1D2, a significantly higher amino acid divergence from the gt1b consensus was observed for FCH patients. The number and nature of these ISDR alterations was associated with high replication fitness, thereby defining genetic markers for highly replicating HCV variants. Analysis of over 2000 sequences of the HCV Research UK cohort revealed that overall only 10% of viral genomes showed genetic signatures of high replicators, suggesting that high replication efficiency might generally not be advantageous for HCV isolates, but only evolve under certain conditions. Interestingly, an increased abundance of potential high replicator HCV variants was found not only in LTX patients, but also in HCV gt1b and gt3a patients developing HCC.

Conclusions
In conclusion, we identified LCS1D2 of NS5A to be the main regulator of genome replication fitness and showed that ISDR alterations result in increased viral replication and liver pathogenesis after LTX as well as HCC development.

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P18 - **How a virus builds a house: host factors required for flavivirus replication organelle formation**

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Background
Flaviviruses are a globally distributed group of viruses which infect more than 400 million people each year. Infection causes a febrile illness which can be fatal if encephalitic or hemorrhagic symptoms develop. Although these viruses are genetically diverse and employ numerous strategies to ensure successful infection, they all replicate their genome inside a membranous structure known as the viral replication organelle, or vRO. Several recent studies have demonstrated that disruption of vRO formation has potent antiviral activity. Thus, detailed knowledge of the underlying mechanisms which lead to the formation of these replication structures could help identify broad-spectrum therapeutic targets.
Although morphologically well-characterized, it is unknown how these vROs are formed and how they function during infection. We have previously identified a group of cellular proteins associated with the ER resident protein atlastin-2 (ATL2) which influence vRO formation; however, a mechanistic understanding of this process represents a major knowledge gap. The goal of our research is to identify how ATL2 and its interacting partners are involved in the formation of vROs during flavivirus infection.

Methods
We used cell culture models to investigate vRO formation by dengue virus (DENV) and Zika virus (ZIKV). STED microscopy was used to interrogate the association of ATL2 with viral replication centers. We employed siRNA and shRNA screens to knock down individual ATL2-associated genes, followed by high-throughput fluorescence microscopy to determine the impact of these knockdowns upon viral replication. We also utilized a novel plasmid-inducible replication organelle (pIRO) system that allows direct evaluation of flavivirus vROs biogenesis to determine the impact of gene depletions on virus-induced membrane reorganization.

Results
STED microscopy showed a significant redistribution of ATL2 during infection and an enrichment at sites of viral genome replication. Targeted knockdown of ATL2-associated genes by siRNA and shRNA resulted in the identification of three genes which have proviral roles during replication. A fourth gene was curiously identified as a proviral factor only in DENV infection. Using virus infection and the pIRO expression systems, we observed that ATL2 depletion alters vRO size, shape, and distribution, suggesting a direct role for ATL2 in vRO biogenesis.

Conclusions
Our results indicate that a network of host cell proteins centered on ATL2 has an important role in flavivirus replication, potentially by interfering with the formation of vROs. Importantly, this function of ATL2 is conserved for different flavivirus infections. By identifying common pathways in viral replication, this work has the potential to illuminate host targets for therapeutic intervention.

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P40 – Identification of hepatitis E virus restriction factors by utilizing arrayed human and porcine ISG screens

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Introduction

Hepatitis E virus (HEV) is an emerging zoonotic pathogen with pigs and deer being the main reservoir of HEV genotype 3 (HEV-3). Whereas HEV is apathogenic in pigs, it can cause fulminant hepatitis and lead to liver cirrhosis and liver failure in humans. Given the divergent progression of HEV infection in humans and pigs, we aim to examine the contribution of the innate immune response during HEV infection in human and porcine hepatocytes. Further, we aim to identify human and porcine ISGs with anti-HEV activity to ultimately define species-specific restriction factors of HEV.

Methods

We employed primary human and porcine hepatocytes (PHH and PPH, respectively) for HEV infection experiments and utilized JAK-inhibitors and IFNs to modulate the innate immune response. Moreover, we took advantage of arrayed human and swine ISG libraries each comprising ~400 ISGs. HEV infection experiments, HEV subgenomic replicon systems, microscopic analysis and IFN-beta reporter assays were used to characterize individual ISGs.

Results

HEV infection experiments in PHH and PPH revealed a 50-fold and 1000-fold increase of progeny virus production upon JAK- inhibition indicating that the canonical IFN-response is essential to control HEV propagation. By utilizing the arrayed ISG libraries we identified ISGs with anti-HEV activity including well known ISGs (i.e. TRIM25, MDA5 and the IRF proteins), but also uncharted factors such as SLC1A1. Following experiments suggest that SLC1A1 restricts the entry process of HEV.

Conclusion

The innate immune response is critical to control HEV infection in primary hepatocytes of humans and pigs. Our screening of human and porcine ISGs sheds light on the antiviral strategies of different HEV hosts and thereby serve as a first step towards a profound understanding of species-specific innate immune responses potentially determining HEV pathogenesis.

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P16 – **NS5A Oligomerization-Dependent Membrane Remodeling Activity Correlates with HCV Replicative Fitness and is a Direct and Common Target of Different NS5A Inhibitors.**

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Background
The NS5A inhibitors (NS5A-I) are an essential component of direct-acting antiviral therapy for HCV infection and are the most potent antiviral drugs currently available. NS5A-I prevents the formation of double-membrane vesicles (DMV) representing the HCV replication compartment. However, the mechanism of action of NS5A-I is incompletely defined. Our previous study described the prototype NS5A-I (daclatasvir)-mediated disruption of oligomerization and giant unilamellar vesicles (GUVs) membrane remodeling by full-length NS5A or NS5A-NTD (N-terminal domain). The goal of this study is to determine the mechanism of action of different NS5A inhibitors and the relationship between HCV replicative fitness and NS5A-mediated GUV remodeling efficiency using NS5A mutants.

Methods
NS5A-NTD and its mutants were expressed and purified from BL-21 Rosetta cells and labeled with NTA-Atto647. GUVs were generated by electroformation. The interaction between purified NS5A-NTD and GUVs was determined in the presence or absence of NS5A-I using a confocal microscope. Analytical ultracentrifugation was performed to determine the NS5A oligomeric states.

Results
We determined that different NS5A-I, including Daclatasvir, Ledipasvir, Ombitasvir, and Velpatasvir could inhibit NS5A-NTD-mediated GUV membrane remodeling at a 1 nM concentration. Introducing NS5A-I resistant mutation Y93H to NS5A-NTD permitted its GUV remodeling activity in the presence of above NS5A-I even at a 100 nM concentration. These results suggest that NS5A-I-mediated membrane remodeling is a direct and common target of all of the NS5A-I we tested. Proline-rich linker (PRL) region between NS5A amphipathic helix and domain 1 within NS5A-NTD is a frequent target where NS5A-I-resistant mutations develop and different mutations at this region were shown to confer differential replicative fitness. We determined that P29A or P32A mutations in PRL, which prevented HCV replication, also prevented the NS5A-NTD to form the oligomers and remodel GUV membranes. L31M or L31V mutations in PRL, which significantly reduced HCV replicative fitness, also reduced the NS5A-NTD binding to membrane and GUV remodeling. On the other hand, HCV replication-competent L31I mutation showed no impact on NS5A-NTD-mediated membrane remodeling. These results suggest that in vitro GUV remodeling activity of NS5A-NTD could accurately represent the HCV RNA replication fitness in cells.

Conclusion
This study revealed that different NS5A-I directly inhibit NS5A-mediated membrane remodeling. It also revealed the critical importance of the PRL region in NS5A oligomerization and its oligomerization-dependent membrane remodeling activity, which correlates with the HCV replicative fitness, while also providing an explanation of why some NS5A-I-resistant mutations in this region impaired HCV replicative fitness although others did not. In aggregate, our data provide insights into the mechanistic function of NS5A in HCV replication, and the common fundamental mechanism of action of different NS5A-I used in HCV treatment.

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P01 - **PLA2G4C induced by HCV infection is involved in the accumulation of lipid droplets via the inhibition of lipolysis**

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Background
Hepatic steatosis is one of the histological features observed in the livers of individuals infected with HCV. The mechanisms responsible for HCV-induced steatosis have been shown to be driven by HCV proteins, which affect the cholesterol/lipid metabolism within host cells. Previous reports showed that the phosphatidylcholine (PC)-hydrolyzing enzyme phospholipase A2 G4C (PLA2G4C) is involved in lipogenesis as well as HCV replication and assembly. However, it is still unclear how the phospholipid metabolism pathways may be involved in the pathogenesis of HCV-related liver diseases.

Methods
RNA expression of genes involved in the de novo pathway and recycling pathway for PC synthesis was examined using RT-qPCR in HCV-infected Huh7.5.1 cells. Imaging mass spectrometry was conducted to analyze the expression of lipid species in the livers of human hepatocyte chimeric mice, both with and without HCV infection. For the promoter reporter assay, Huh7.5.1 cells were transfected with a luciferase reporter vector containing the PLA2G4C promoter (-1182 /+332) along with expression plasmids for HCV proteins. The level of triglyceride was measured using ELISA, and lipid droplets (LD) were stained with BODIPY 493/503. Analysis of LD was performed using an in-cell analyzer and confocal microscopy.

Results
Among 32 kinds of genes involved in PC biosynthesis, the most significant change in mRNA expression due to HCV infection was PLA2G4C, which was up-regulated about 100-fold by the viral infection. The levels of PC were significantly lower in the livers of HCV-infected human liver chimeric mice by imaging mass spectrometry. PLA2G4C promoter-reporter experiments revealed that 114 nucleotides upstream from the transcription start site, which contains possible recognition sites for NFkB and c-Myc, was a minimum sequence responsible for increase in PLA2G4C expression mediated by HCV infection or Core-p7 expression. Mutational analyses and experiments with inhibitor and activator treatment demonstrated involvement of both NFkB and c-Myc in HCV-mediated up-regulation of PLA2G4C transcription. Total lipid content and LD size were decreased in PLA2G4C-knockout cells compared to control cells. The degradation rate of LD in PLA2G4C-knockout cells was higher than in parental cells, indicating the involvement of PLA2G4C in LD stability. We further found that the increased expression of PLA2G4C by HCV infection contributes to the decreased localization of lipolysis-related factors such as ATGL, PLIN1 and ABHD5 in LDs.

Conclusions
We found that PLA2G4C expression is upregulated through NFkB and c-Myc activation upon HCV infection, and this upregulation is associated with a decrease in PC species. The increased expression of PLA2G4C resulted in changes in the phospholipid composition of LDs, led to the dissociation of lipolysis-related factors from the LD surface and the accumulation of lipid contents within LDs. These findings suggest that the disturbance of phospholipid metabolism pathway caused by HCV infection is potentially involved in the development of HCV-associated fatty livers.

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P36 – **Elucidating the switch from translation to replication in the HCV life cycle**

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Background
As a positive-sense RNA virus, the Hepatitis C virus (HCV) genome acts a template for both translation and replication. Interestingly, translation occurs in the in the 5' to 3' direction, while viral replication occurs 3' to 5', and as such, these events are mutually exclusive on a single molecule of genomic positive-sense viral RNA. Thus, there must be a mechanism to "switch" off translation, clear the RNA of ribosomes, and to initiate viral RNA replication. HCV has been documented to recruit two argonaute (Ago)-microRNA-122 (miR-122) complexes to the 5ÅL terminus of its genome which results in: 1) an RNA chaperone-like switch in conformation (or riboswitch); 2) viral RNA stability; and 3) translational promotion. Subsequently, the Ago:miR-122 complexes recruit trinucleotide-repeat containing gene 6 (TNRC6), the RNA silencing effector protein. Recruitment of TNRC6 suppresses translation, allowing replication organelle (RO) biogenesis, primarily mediated by the NS5A protein. We propose these processes are mediated by phase separation at the 5' and 3' end of the viral RNA, respectively. Herein, we are exploring the roles of Ago:miR-122, TNRC6, and NS5A in the switch from translation to replication and RO biogenesis in the HCV life cycle.

Methods
We are using a G28A-BoxB system to explore the switch from translation to replication and RO biogenesis in the HCV life cycle. We previously demonstrated that a viral mutant (G28A), can accumulate to low levels in miR-122 knockout (KO) cells. In the absence of miR-122, G28A is defective in the "switch" from translation to replication; however, due to its translational advantage, it can establish ROs, albeit inefficiently. In combination with a BoxB loop in lieu of SLI, we can tether λN fusion proteins to the 5ÅL terminus of the G28A-BoxB genome, to test if we can restore the switch from translation to replication. Moreover, to explore whether overexpression of NS5A can augment G28A-BoxB replication, we are using an NS3-5A cis-overexpression system.

Results
Our preliminary data suggests that tethering Ago2 to the G28A-BoxB RNA restores the "switch" from translation to replication, while direct tethering of TNRC6B does not have a significant impact on G28A-BoxB RNA accumulation. Direct tethering of other host and viral phase separation proteins is underway. Using the NS3-5A cis-overexpression system we plan to test whether overexpression of NS5A in cis improves the efficiency of G28A-BoxB RO biogenesis.

Conclusions
We have shown that tethering Ago2 is sufficient to restore the switch from translation to replication and rescue G28A-BoxB RNA accumulation in the absence of miR-122. Future research will focus on exploring the roles of TNRC6 and NS5A in the switch and RO biogenesis. We anticipate that this will reveal the mechanism(s) behind the switch from translation to replication and RO biogenesis in the HCV life cycle.

Session 2: Innate immunity and virus-host interaction
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P60 – **Genetic and molecular characterization of species-specific receptor transport protein 4 (RTP4)-mediated HCV repression**

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Background & Aims
The flying fox ortholog of receptor transport protein 4 (RTP4) was previously shown to be a potent antiviral effector against several ER-replicating RNA viruses, inhibiting viral genome replication. The murine ortholog is a specific inhibitor of hepatitis C virus (HCV), despite the human ortholog bearing no such effect. This suggests a role for RTP4 as a restriction factor for HCV replication in mice. To this end, we sought to understand its role in HCV infection in mice versus humans. Specifically, we aimed to determine the functional domains driving this ortholog-specific effect, along with the molecular mechanism thereof.

Methods
To map precise regions within mouse RTP4, we created mouse-human domain swap chimeras whose functions have been tested in HCV-infected Huh7 cells. We further generated FLAG- and c-myc-tagged versions of both human and murine RTP4 in order to interrogate the capabilities of murine and human RTP4 to oligomerize upon replication-intermediate HCV dsRNA. These were overexpressed in Huh7 cells that were subjected to co-immunoprecipitation (co-IP) and immunofluorescence microscopy (IF) in mock and infected conditions. Complementing our *in vitro* data, we sought to assess RTP4 knockdown in HCV-susceptible mice. We previously generated mice in which the second extracellular loops of the HCV entry factors CD81 and occludin (OCLN) were replaced with the equivalent human sequence (mCD81$^{EL2[h/h]}$ mOCLN$^{EL2[h/h]}$), which support HCV glycoprotein-mediated uptake. mCD81$^{EL2[h/h]}$ mOCLN$^{EL2[h/h]}$ mice crossed to an RTP4$^{-/-}$ background were infected with cell culture-derived HCV, and HCV RNA in serum and liver tissue were quantified longitudinally. To monitor HCV infection in the absence of adaptive immune responses which might suppress viral infection, we transplanted hepatocytes from mCD81$^{EL2[h/h]}$ mOCLN$^{EL2[h/h]}$ RTP4$^{-/-}$ donors into immunodeficient liver-injury FAH$^{-/-}$ NOD Rag1$^{-/-}$ IL2Rg^{NULL} (FNRG) recipients devoid of functional B, T and NK cells.

Results
In vitro, we established that murine RTP4 bears a dominant effect regardless of human RTP4 expression. Our domain-swapping experiments demonstrated the necessity of specific domains of murine RTP4 to inhibit HCV replication. Our co-IP and immunofluorescence experiments further delineated a mechanism underpinning RTP4's species-specific effect upon HCV replication. *In vivo*, serum and liver HCV RNA copies were indistinguishable between mCD81$^{EL2[h/h]}$ mOCLN$^{EL2[h/h]}$ RTP4$^{-/-}$ and mCD81$^{EL2[h/h]}$ mOCLN$^{EL2[h/h]}$ mice at all timepoints tested. Identical results were obtained in immunodeficient mice transplanted with these hepatocytes.

Conclusions
Our results indicate that specific regions within murine RTP4 are responsible for murine RTP4's dominant anti-HCV activity. Our data further delineates a model for the species-specific effect of RTP4-mediated inhibition of HCV replication. Disruption of RTP4 in mCD81$^{EL2[h/h]}$ mOCLN$^{EL2[h/h]}$ mice is not sufficient to render animals permissive to HCV infection. These data solidify RTP4 as another crucial factor in the complex set of barriers to HCV infection in mice.

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P86 – **Regulation of protein kinase R by hepatitis C virus non-structural protein 5A**

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Background
Chronic hepatitis C virus (HCV) infection affects 71 million people worldwide and increases the risk of developing hepatocellular carcinoma (HCC). HCV is genetically diverse, with 6 genotypes reported to date that differ in their severity and pathogenicity. Although direct-acting antivirals against HCV can cure the infection, they do not eliminate the risk of developing HCC. Protein kinase R (PKR), a multifaceted kinase, is implicated in liver disease and cancer, but its regulation in the context of chronic HCV infection and HCC is poorly understood. During infection, PKR is normally activated by binding to viral double-stranded RNA (dsRNA), leading to protein translation shutdown and stress granule (SG) formation. Interestingly, PKR has been reported to interact with HCV non-structural protein 5A (NS5A)[1], and mutations in the PKR-binding domain of NS5A are associated with HCC.[2] However, the functional consequences of the NS5A-PKR interaction are not fully understood. Here, we show that NS5A activates PKR, leading to translation shutdown and SG formation.

Methods
We infected Huh7.5 cells with HCVcc (JFH1) or transfected 293T/17 cells with mammalian expression constructs for HCV-NS5A from two different genotypes: HCV-NS5A genotype 1b (J4; HCV-gt1b-NS5A) or HCV-NS5A genotype 2a (JFH1; HCV-gt2a-NS5A). We evaluated PKR activation by western blot for phosphorylated PKR and assessed downstream effects by evaluating translation shutdown and SG formation. To assess protein translation, we used luciferase reporter assays, while SG formation was visualized by immunofluorescence to evaluate the localization of G3BP1, a well-reported SG marker.

Results
Consistent with previous literature, our results show that PKR is activated in Huh7.5 cells infected with HCV JFH1 at a multiplicity of infection of 0.5 or 0.25. To investigate the mechanism of PKR activation, we ectopically expressed NS5A (genotype 1b or 2a) in 293T/17 cells and similarly observed PKR activation and SG formation. Furthermore, ectopic expression of NS5A decreased translation of luciferase in a constitutive luciferase reporter assay, indicating translation shutdown. Interestingly, HCV-gt1b-NS5A more potently induced PKR activation, inhibition of protein synthesis and SG formation than HCV-gt2a-NS5A, suggesting that genotype differences may affect the ability of NS5A to modulate PKR activation. Furthermore, PKR activation by NS5A does not require cyclophilin A, a known interaction partner, as the cyclophilin inhibitor cyclosporin A had minimal effect. Experiments to understand the mechanisms are ongoing, using a polyprotein expression construct encoding NS3-5A in Huh7 cells.

Conclusions
Our findings show that HCV-gt1b-NS5A and HCV-gt2a-NS5A differentially activate PKR, restrict protein translation, and induce SG formation. These differences may contribute to different pathogenicity of HCV gt1b relative to 2a. Overall, these findings contribute to understanding the regulation of PKR during HCV infection and may provide insight into new chemopreventive strategies for HCC.

[1]Gale et al. (1998) *Mol Cell Biol. 18:*5208
[2]Paolucci et al. (2020) *Viruses 12:*255

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P38 – **What do HCV Core and NS5A partners in crime tell us about hepatocyte deregulations? Interacting host factors and enriched pathways identified in an infection system.**

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Background
HCV-induced pathology can be driven both by direct and indirect mechanisms. The direct mechanisms are thought to be mainly mediated by two viral proteins: the capsid protein or Core and the nonstructural protein 5A (NS5A), which have been reported to be involved in the deregulation of several host pathways. However, this has mainly been studied in systems comprising transiently over-expressed isolated HCV protein(s), and/or nonhepatic cells. We aimed at identifying HCV Core and NS5A cellular interacting partners that would be essential to the virus life cycle and/or linked to HCV-induced pathobiology during HCV infection.

Methods
A panel of recombinant viruses encoding tagged HCV Core or NS5A was generated within the backbone of a JFH1-derived, highly cell culture-adapted strain (Jad). For protein purification purposes, a twin strep tag (ST) was fused in frame within the aminoterminal region of Core or at either of two positions within the carboxy-terminal segment of NS5A, without significantly impacting viral replication and morphogenesis. Protein complexes were affinity-purified from infected Huh-7.5 cells using streptactin beads. Interacting partners were identified by liquid nano-chromatography coupled to tandem mass spectrometry (nano- LC-MS/MS). To discriminate strong interactions, we developed a novel scoring algorithm, incorporating statistical analyses of ST-derived hits with respect to non-binding V5-tagged controls, the Mass Spectrometry interaction STatistics (MiST) and the Significance Analysis of INTeractome (SAINT) scores of the Nano-LC-MS/MS data.

Results
We identified 134 interacting partners of Core and 527 of NS5A, including some common hits. Using the CytoScape and STRING tools, the highest scored pathways enriched among the interacting partners of Core were involved in the regulation of host gene expression. NS5A partner enrichment highlighted mainly metabolism-related pathways, notably the TCA cycle and oxidative phosphorylation, but also pathways implicated in transport, immune system, and hepatocellular carcinoma processes. In addition, we were able to identify domains of interaction of previously described NS5A cellular partners based on differential retrieval with NS5A fused to ST at the two different insertion positions.

Conclusion
In a relevant infection system, we found that Core and NS5A may contribute to the progression of HCV-induced liver damage by deregulating the host transcriptome, or by directly interacting with proteins involved in multiple host pathways, respectively. The functional significance of selected, previously unreported interactors is now being examined. In parallel, we have generated tagged NS5A intergenotypic recombinants viruses within the Jad backbone, by replacing the NS5A sequence by that found in clinical strains of diverse HCV genotypes. We are currently performing Nano-LC-MS/MS experiments in order to identify common and distinct NS5A interacting partners across genotypes, data which we plan to also present during this meeting.

Session 3: Developing next generation vaccines
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Keynote lecture: Defining humoral correlates of protection and risk in Dengue viral infection

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While antibodies represent the primary correlate of immunity for most licensed vaccines, antibodies can both contribute to protection against disease or cause pathology, depending on how they modulate the host:pathogen interaction. Specifically, beyond their ability to neutralize pathogens, pathogen-specific antibodies are able to drive a number of antiviral and inflammatory responses that can tip the balance in favor of the host or the pathogen, via interactions with Fc-receptors found on all immune cells. To begin to define whether antibody properties, beyond their ability to neutralize, contribute to either protection against symptomatic disease or contribute to antibody dependent enhancement, here we deeply profiled the functional humoral immune response in cohorts of dengue/zika pre-immune young children prior to re-infection with a heterologous Dengue virus, that either developed asymptomatic disease or pathogenic enhanced disease. Enhanced Envelope-specific complement-fixing antibodies were significantly associated with the development of asymptomatic infection, pointing to a critical role for E-specific antibody mediated virolysis as a key mechanism in attenuating viral infection. Conversely, and surprisingly, E and NS1-specific high affinity IgA responses were associated with the evolution of hemorrhagic disease, via the induction of neutrophil activation. These data point to specific immunologic humoral immune pathways that lead to differential outcomes following Dengue viral re-infection, providing critical insights for vaccine or therapeutic development.

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P110 – Design and non-viral delivery of live attenuated virus vaccine to prevent chronic hepacivirus (HCV-like virus) infection.

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Background
The development of an effective prophylactic vaccine for hepatitis C virus (HCV) is an unmet medical need.

Methods
Since HCV lacks an immunocompetent animal model, we used rodent hepacivirus of rats (RHV-rn1/RHV) as a surrogate to determine if a live attenuated virus (LAV) can be designed using targeted mutagenesis and can be used as a vaccine to prevent chronic HCV-like virus infection. We used in silico RNA secondary structure prediction methods to select less structured regions of the virus genome and then introduced hundreds of synonymous mutations in these regions to increase the frequencies of dinucleotide UpA or CpG. The hypermutated RHV genome clones were transcribed *in vitro* to either rescue infectious LAV or to produce lipid nanoparticle-encapsulated viral RNA (LNP-vRNA) for vaccinating the laboratory rats.

Results
Both groups of rats, injected with the infectious in vivo-rescued virions or the LNP-vRNA developed RHV viremia that was spontaneously cleared within 2-3 weeks in most infected rats. A second infection of the LAV produced either shorter and low titer or no viremia. The vaccinated animals developed functional memory T cells against multiple epitopes in both structural and nonstructural proteins. The phenotype and function of vaccine-induced T cells were determined using rat-MHC tetramers and *ex vivo* stimulation with RHV peptides using IFN-γ ELISPOT and intracellular cytokines staining assays. The vaccinated rats were challenged with a wild-type RHV-RN1 stock that invariably led to HCV-like lifelong chronicity in non-immunized naïve inbred Lewis rats. Importantly, 78% rats (18/23) vaccinated using LAV virions and 76% rats (16 of 21 rats) vaccinated using LNP-vRNA showed clearance of RHV-RN1 viremia within 2-5 weeks of the challenge and remained cleared of infection throughout follow-up. The control and clearance of wild-type RHV infection correlated with a 10-100-fold increase in the frequencies of vaccine-specific memory T cells.

Conclusions
Overall, we demonstrated that attenuated RHV administered as an mRNA induced protective immunity against wild-type virus, a strategy with a clear translational path towards HCV immunization in humans.

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P120 – Preclinical evaluation of T and B cell targeting DNA/MVA-based HCV vaccine candidates in mice and rhesus macaques

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Background
The hepatitis C virus (HCV) is a major cause of liver cirrhosis and cancer in humans. Currently, there is no approved HCV vaccine. A deeper understanding of the correlates of HCV protection and infection resolution indicates both T cell and antibody responses are important for protection. Here, we tested the immunogenicity of DNA prime and modified vaccinia Ankara (MVA) boost (DNA/MVA) based vaccine candidates that are designed to induce a potent and broad CD4 and CD8 T cell response, and neutralizing antibody response in mice and rhesus macaques.

Methods
The DNA and MVA vaccines expressed either HCV (genotype 1a) nonstructural (NS) proteins NS3-NS4-NS5 or structural proteins Core-E1-E2-p7 to produce virus-like particles (VLPs). In some constructs, we introduced point mutations to inactivate polymerase (Pol) and protease (Pro) activities of NS5 and NS3, respectively to enhance safety. In mice, we tested the immunogenicity of NS and VLP constructs individually and also compared the influence of Pro mutation on the induction of T cell response. In macaques, we compared the immunogenicity of NS (with Pol and Pro) and VLP constructs individually (NS-only, VLP-only) or together (NS and VLP) to test the influence on T cell and antibody responses. Animals received 2 doses of DNA 4-8 weeks apart, and a single dose of MVA 8 weeks later.

Results
The DNA/MVA vaccination induced robust frequencies of HCV-specific CD4 and CD8 T cell responses both in mice and macaques with poly-functional profiles based on co-expression of IFN, TNF and IL-2. NS immunogens induced CD4 and CD8 T cell responses against multiple NS proteins. CD4 T cells targeted towards NS3, NS4b, NS5a and NS5b but not NS4a, with the highest response against NS5a. However, the CD8 T cells were mostly targeted against NS3. NS immunization with and without protease activity in NS3 showed comparable T cell response. Impressively, the hierarchy of T cell targeting was comparable between mice and macaques. The VLP construct induced neutralizing antibody response against H77 HCV pseudotyped particles. In addition, it also induced a strong CD4 and CD8 T cell response that was directed against E1 and E2. Co-delivery of NS and VLP vaccines induced a strong T cell response against both NS and structural proteins, and neutralizing antibody response that was comparable to vaccination with individual vaccines. However, co-delivery resulted in a trend towards lower T cell response to NS proteins.

Conclusion
Our results show that DNA/MVA-based expressing NS and VLP antigens induce a potent and broad CD4 and CD8 T cell response targeting practically the entire proteome of HCV and neutralizing antibody response against HCV genotype 1a in mice and rhesus macaques. These findings are key towards the development of a successful HCV prophylactic vaccine.

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P32 – **Supramolecular assembly of Toll-like receptor 7/8 agonist into multimeric virus-mimicking polymer assemblies of E1E2 and core and evaluation of immune responses**

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Background
Clinical trials of HCV vaccines focused on B-cell responses to E1E2 or T-cell responses to non-structural proteins (NS3-NS5b) showed promise in animal models but were unsuccessful in humans. We have recently optimized a secreted form of HCV E1E2 (sE1mE2) that elicits broadly neutralizing antibodies (bnAb) in mice and macaques. We further developed a novel PEGylated polyphosphazene adjuvant (PPZ-PEG2) bolstered with TLR7/8 agonist R848, (PPZ-PEG2+R848), that allowed co-presentation of sE1mE2 and core antigens as virus-mimicking polymer assemblies (VMPAs). We evaluated murine and macaque B and T-cell responses to sE1mE2 and core VMPAs using this PPZ-PEG2+R848 adjuvant system.

Methods
(1) Antigens**:** sE1mE2 was developed, by replacing the transmembrane domains of native E1E2 with a synthetic scaffold (SynZip) to allow secretion and insertion of a stabilizing mutation, H445P. (2) VMPA formulation: PPZ-PEG, containing 2% (mol) of 5 kDa PEG chains, was mixed with R848 to associate a cationic small-molecule agonist with negatively charged carboxylic acid groups of the PPZ polymer. The resulting macromolecule (PPZ-PEG2+R848) was combined with core and sE1mE2 to derive the final VMPA vaccine. In parallel, vaccines containing sE1mE2 and core proteins were formulated with PPZ-PEG2 (minus R848) or alum alone. Dynamic Light Scattering (DLS) and Asymmetric Flow Field Flow Fractionation (AF4) were used to ascertain integrity of VMPA vaccines. (3) Immunological analysis: Balb/c mice, 5 per group, or macaques, 3 per group, were immunized with a prime and 3 boosts (mice), or a prime and 4 boosts (macaques). Humoral and cellular responses were evaluated.

Results
DLS and AF4 profiling showed unimodal size distribution with the diameter of VMPAs ranged between 68-78 nm based on core protein content. In contrast, similar formulations using PPZ homopolymer (no PEG grafts) showed aggregation which demonstrates the importance of light PEGylation of PPZ for fabrication of ternary sE1mE2-core-R848 VMPAs. Mice immunized with VMPAs showed the highest endpoint titers, bnAb responses, and balanced IgG1/IgG2a ratios in comparison to PPZ-PEG2 (no R848) or alum alone. Frequency of $CD44^{hi}$ T cells producing IFNg or TNFa to sE1mE2 and core proteins were 2-3-fold higher when using the VPMA adjuvant compared to alum formulation. Increased E1E2-specific effector T cells were evident in VMPA-immunized macaques by Day 37 with a sustained increase in IFNg producing CD4 and CD8 T cells past 9 months.

Conclusions
The PPZ-PEG2+R848 adjuvant system was capable of co-formulating sE1mE2 and core proteins into VMPAs that elicited higher magnitude of antibody titers, balanced IgG1/IgG2a response, and a superior breadth of neutralization compared to PPZ-PEG2 alone or alum adjuvant. These results suggest that the VMPA vaccine may be promising approach in development of a B and T-cell based HCV vaccine.

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P37 – **Structural and biochemical studies of flaviviruses in complex with antibodies and attachment factors**

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Background
Flaviviruses comprise an important genus in the family Flaviviridae with more than 70 viruses causing disease in humans. Numerous studies have revealed the structure and dynamics of flavivirus virions, their morphogenesis, and the role of antibodies in neutralizing virus infection. Here we will show structures of a unique neutralizing antibody bound to a flavivirus as well as attachment factor bound to a virion that promotes virus entry.

Methods
Preparations of virus, antibodies and cell surface glycosaminoglycans (GAGs) were appropriately complexed at fixed molar ratios. The samples were flash frozen and single particle cryo-electron microscopy reconstructions were performed to obtain high resolution structures of the complexes. In addition, virus and biochemical assays were performed to evaluate the role of GAGs in promoting the entry of specific flaviviruses.

Results
The structure of a potently neutralizing Zika virus IgM was determined using Fab bound to virus and computational modeling to infer the structure of a bound IgM to a virus particle. The ability to bind to both 2-fold and quasi-2-fold axes presents a unique binding mode that may reveal a new role of IgM in controlling virus infection. Furthermore, it will be shown the role of the dengue virus glycan at residue 67 to bind to heparin. Biochemical studies demonstrates that all four dengue serotypes can utilize heparin in cell-specific attachment.

Conclusions
The studies presented here will provide new insights into the integration of structure and function to probe the important interactions between viruses and their hosts. These data will be presented in terms of novel IgM function and the role of cell-surface GAGs for virus entry.

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Lipid/mRNA HCV E1E2 vaccines elicit broadly neutralizing antibodies and the requirement of transmembrane domains

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Background
The HCV gpE1E2 envelope glycoprotein heterodimer is the optimal vaccine antigen to elicit broadly neutralizing antibodies (bNAbs) against the heterogeneous clades of HCV around the world. This antigen contains bNAb epitopes in E2 and in the E1E2 heterodimer with the greatest breadth of protection. When tested in the chimpanzee challenge model, animals vaccinated with adjuvanted recombinant E1E2 containing E1 and E2 transmembrane domains (TMDs) strongly reduced the incidence of viral persistence following experimental challenge by 75%. We explored the delivery of E1E2 including native TMDs via a mRNA vaccine to meet global vaccine requirements.

Results
Using a large panel of bNAbs, we investigated both adjuvanted recombinant E1E2 proteins and mRNAs of different variants of E1E2 (that included point mutations in E1E2) to assess enhancements of critical epitopes of bNAbs. We produced lipid/mRNA nanoparticles expressing the E1E2 heterodimer that showed strong immunogenicity in vaccinated guinea pigs. All animals vaccinated with proteins or mRNA elicited very high titers of binding and neutralizing antibodies against the homologous isolate employed to produce the vaccines. The vaccine-induced antisera also neutralized heterologous viruses. This cross-neutralization activity is consistent with the data, by competition ELISA, that antisera contained antibodies against epitopes related to well-established bNAbs (HC-1, HC-11, HC84.26, HC33.4, CBH-7 and AR4A) to E2 and E1E2. Mutation at the E2 445 residue position in our vaccines, previously shown to enhance access of important bNAbs, did not enhance induction of antibodies targeting this region. Taken together, these findings validate the utility of RNA technology to investigate immunogenicity of E1E2 based vaccines against HCV.
Furthermore, we compared purified E1E2 proteins with and without their natural TMDs and noted that E1E2 proteins without native TMDs showed a 5-fold reduction in CD81 binding and a similar 4.5-fold reduction in binding against an E2 bNAb epitope that shares contact residues involved in virus binding to CD81.

Conclusions
Our data shows that wild-type and mutant derivatives of HCV E1E2 vaccine antigen can be delivered either by use of adjuvanted recombinant protein or via lipid/mRNA formulations that will further facilitate and accelerate global vaccine delivery. In addition, we believe that E1 and E2 TMDs are required for native gpE1E2 structures.
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Extensive C->U transition biases in the genomes of HCV and a wide range of mammalian RNA viruses; host-mediated editing of viral RNA?

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Background
The rapid evolution of hepatitis C virus (HCV) has been long considered to result from a combination of high copying error frequencies during RNA replication, short generation times and the consequent extensive fixation of neutral or adaptive changes over short periods. While both the identities and sites of mutations are typically modelled as being random, recent investigations of sequence diversity of SARS coronavirus 2 (SARS-CoV-2) have identified a preponderance of C->U transitions, proposed to be driven by an APOBEC-like RNA editing process. We investigated whether this phenomenon extended to HCV and other mammalian RNA viruses.

Methods
We constructed datasets of aligned whole genome sequences of genotypes 1a, 1b, 2a and 3a, SARS-CoV-2, other coronaviruses and a range of mammalian RNA viruses using sequences from GenBank and the VIPR database. Calculation of pairwise distances and nucleotide composition was performed using the SSE package version 1.4. Sequence changes were compiled using the program Sequence Changes with a variability threshold typically set at 3% heterogeneity, where heterogeneity was calculated as the cumulative frequency of all non-consensus bases. RNA secondary structure prediction though computation of MFE and MFED values and association index calculations were carried out in in the SSE package.

Results
HCV sequences from all genotypes showed a pronounced 2.1-3.0 fold base-normalised excess of C->U transitions over U->C, comparable to those observed in SARS-CoV-2. Overall, 18 from 36 datasets of aligned coding region sequences from a diverse range of mammalian RNA viruses showed C->U transition asymmetries (range 2.1x–7.5x), with a consistently observed favoured 5' U upstream context. The presence of genome scale RNA secondary structure (GORS) was the only genomic or structural parameter significantly associated with C->U/U->C transition asymmetries by multivariable analysis (ANOVA), potentially reflecting RNA structure dependence of sites targeted for C->U mutations. Using the association index metric, C->U changes were specifically over-represented at phylogenetically uninformative sites, potentially paralleling extensive homoplasy of this transition reported in SARS-CoV-2. Although mechanisms remain to be functionally characterised, excess C->U substitutions accounted for 14% of standing sequence variability of HCV and may therefore represent a potent driver of its sequence diversification and longer-term evolution.

Conclusions
Although the mechanism for C->U editing in HCV is undetermined biochemically, the data obtained for a potent mutational driver may substantially damage HCV's ability to replicate. The phenomenon is potentially akin to genome editing of HIV-1 and other retrovirus genome sequences by APOBEC, one of the principal components of vertebrate antiretroviral defence mechanisms. Its occurrence indicates the powerful role of C->U hypermutation in the generation of HCV and other RNA virus diversity and represents a potent driver of its sequence diversification, generation of quasispecies and its longer-term evolutionary trajectory.

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P97 – **Rationally designed attenuated HCV variants for vaccine Development**

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Background
Hepatitis C virus (HCV) is a leading cause of liver disease, and no vaccine is currently available for HCV. It is widely accepted that an efficient HCV vaccine should stimulate both B-cell and T-cell responses. Therefore, a Live attenuated vaccine containing weakened viruses, that replicate the natural infection without causing serious diseases and produce natural B and T cell anti-viral immunity, may be a good candidate for HCV vaccine. Our aim was to rationally design attenuated HCV variants by introducing synonymous mutations to disrupt HCV mRNA structure thus weakening the viral fitness and pathogenicity, while maintaining its original antigentic structure and immunogenicity.

Methods
We used novel bioinformatics tools to analyze HCV genomes from databases for identifng ‘silent’ patterns of HCV mRNA folding. We utilized this information to design HCV variants containing synonymous mutations that affect the mRNA structure, but maintain the original amino acid sequence. The reversion of the inserted mutaions was evaluated by NGS the viral genome following long period of infection. To evaluate the effect of the synonymous mutations on viral fitness, we measured the ability of the HCV mutants to replicate and spread. Moreover, we evaluated the mutants’ pathogenesis by measuring their effect on epigenetics and expression of host genes related to oncogenic pathways, and and cancer- related phenotypes. The immunogenicity of the attenuated viruses was determined using the modular immune in vitro construct (MIMIC) system, a useful in vitro clinical trial system that recapitulates multiple elements of the human immune response.

Results
We generated eight HCV mutants that varies in number and positions of inserted mutations, each contains up to more than 100 variations. The mutants demonstrated an overall reduction in their fitness compared to WT, while maitaing their original antigenic structure. The spread and replication levels of the mutants varied with correlation to the level and positions of mutations inserted. No reversion to WT was observed in log term infection in culture due to the high numer of mutations. The pathogenesis of the mutants varied also with correlation to the level of viral replication, with minimal effect on oncogenic epigenetic and gene expression changes, and cancer related phenotypes, for the most attenuated viruses. Both HCV-specific cytotoxic T and neutralzing antibody responses were induced by the attenuated virus.

Conclusions
The findings of this study highlight the potential of viral attenuation generated by synonymous mutations affecting viral mRNA folding to reduce viral fitness, as a potential tool for developing rationally designed live attenuated HCV-vaccine.

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P105 – **Bispecific antibodies against hepatitis C virus E1E2 show enhanced breadth and potency in HCV pseudoparticle and authentic virus assays**

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Background
To reach the elimination of hepatitis C virus (HCV), currently causing 1.5 million new yearly infections worldwide it is critical to develop a prophylactic vaccine and next-generation therapeutics. Broadly neutralizing antibodies (bNAbs) target the E1E2 glycoprotein on the outside of the virus and are able to neutralize a majority of the highly diverse circulating HCV strains. HCV bNAbs not only provide sterilizing protection in animal models, but are even capable of mediating clearance of an established infection. This makes bNAbs potentially useful as (additional) therapeutics. Several bNAbs have been isolated from HCV infected individuals which target several distinct E1E2 epitopes. However, bNAbs targeting a single epitope might be sensitive to viral escape, while previous research has shown combinations of bNAbs can show synergy.

Methods
We have generated 60 IgG-like bispecific antibodies (bsAbs) that simultaneously target two distinct epitopes on E1E2. The heavy and light chain plasmids of the parental antibodies were engineered to enable accurate pairing during transfection, or to enable controlled Fab-arm exchange. This resulted in the generation of three types of bsAbs, each containing a different hinge type. After production and quality control, we used different binding assays to test the bispecificity and affinity of our constructs to E1E2. We screened the bsAbs against a panel of HCV pseudoparticles (HCVpp), which display the E1E2 of different genotypes on their surface, to select the candidates with the highest neutralizing potential. Finally, we tested the seven most promising candidates in neutralization assays against authentic HCV viruses of genotypes 1a, 2a, 3a, 4a and 5a produced using the HCV cell culture system (HCVcc).

Results
We have successfully produced a panel of novel bsAb constructs, combinations of previously existing bNAbs, and confirmed their (thermos)stability and retained binding properties. Most of the bsAbs retain their neutralization activity to all tested viruses. Some bsAbs showed increased breadth and potency compared to the parental antibodies or a corresponding cocktail of monospecific antibodies. We have shown neutralizing activity of the bsAbs in both HCVpp and HCVcc systems.

Conclusions
Previously only shown for cocktails of bNAbs, we now demonstrate that a similar cooperative effect can be achieved by making bsAbs with 2 arms targeting different epitopes. By strategically pairing bNAb candidates with different E1E2 specificities, we can learn about the modes of action of both individual parental bNAbs as well as bispecific combinations. The knowledge gained by studying these bsAbs and their interaction with a diverse panel of E1E2 can be used to inform vaccine design and opens up novel therapeutic and prophylactic strategies.

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P72 – **Optimization of HCV vaccine antigens to enhance binding of broadly neutralizing antibody unmutated ancestors**

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Background and Aims
Eliciting broadly neutralizing antibodies (bNAbs) should be a major consideration in hepatitis C virus (HCV) vaccine design, but the antigenic features of HCV envelope glycoproteins (E1E2) necessary for the induction of bNAbs are poorly understood. In particular, very little is known about the E1E2 features necessary for binding of bNAb unmutated ancestors. In a recent study (Frumento et al., doi: 10.1172), we showed that HCV E1E2 variants could be grouped into antigenic clades based on their sensitivity to binding by a panel of mature and unmutated ancestor bNAbs. We found that repeated infection with bNAb-sensitive 'antigenic clade 1' viruses was associated with potent bNAb induction in humans, while infection with bNAb-resistant 'antigenic clade 4' viruses was associated with poor antibody induction. In this study, we sought to identify the polymorphisms that are responsible for the phenotypic differences between antigenic clade 1 and clade 4 E1E2.

Methods
We used the Subject-adjusted Neutralizing Antibody Prediction of Resistance (SNAPR) algorithm to identify significantly enriched polymorphisms in antigenic clades 1 or 4. Polymorphisms at six positions were predicted to mediate the antigenic difference between clades. We introduced clade 4 polymorphisms at each of these positions into a representative clade 1 E2 or E1E2 sequence using site-directed mutagenesis (SDM) and used ELISAs to measure the effect on the binding of a panel of mature and unmutated ancestor bNAbs. Since H77 E1E2, a commonly used vaccine antigen, has clade 4 (potentially deleterious) polymorphisms at two of these positions, we also used SDM to convert these positions in H77 E1E2 to the clade 1 (potentially favorable) amino acids.

Results
Each substitution in the clade 1 E1E2 protein significantly decreased binding of at least one bNAb, and binding of each bNAb was significantly inhibited by at least one substitution. Three substitutions significantly impacted binding across the entire bNAb panel– D431E, Q482D, and K500L. For H77 E1E2, binding of most mature front-layer-targeting bNAbs was increased by substitution E431D. Binding of most non-front layer bNAbs was increased by E482Q, and binding of all bNAbs was significantly improved by the substitutions in combination. Overall, the substitutions had a greater effect on the binding of unmutated ancestor bNAbs than mature bNAbs.

Conclusions
We identified polymorphisms that contribute to phenotypic differences between antigenic clades 1 and 4 E1E2s. Notably, the commonly used vaccine strain H77 has deleterious polymorphisms at two of these positions, and mutation of these positions to the favorable clade 1 polymorphism improved binding of many bNAbs and their unmutated ancestors. These data could allow optimization of vaccine antigens to favor binding of mature and germline ancestor bNAbs, which could enhance vaccine induction of these desirable antibodies.

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P104 – **Structure-based design of a stable soluble hepatitis C virus E1E2 heterodimer**

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Background and Aims
An effective preventive vaccine for hepatitis C virus (HCV) is a major unmet need. Protein-based vaccine attempts have failed to elicit the desired immune response and a crucial obstacle has been the limited knowledge on the E1 and E2 glycoprotein complex, main target for broadly neutralizing antibodies (bNAbs). The recent resolution of the structure has offered the field with a blueprint for structure-based vaccine design. This work, presents the efforts and successes to stabilize the E1E2 glycoprotein utilizing heterodimerization domains Jun-Fos and structure-based design, leading to our immunogen candidate: a soluble, stable, well-folded E1E2 heterodimer.

Methods
In this work we combine the available computational tools and the knowledge on structure-guided protein design to stabilize the E1E2 heterodimer. We used the recently published cryo-EM structure and previous related studies to perform strategically designed mutations across E1 and E2 to improve its folding and stability as determined by the complex's antigenic profile against reference antibodies including the bNAb AR4A, used as a proxy for native-like folding. We show all preliminary data including design, ELISA and biolayer interferometry (BLI) results, as well as more in-depth characterization like site-specific glycosylation and electro-microscopy.

Results
We first designed an AMS0232 E1E2 heterodimer using JunFos heterodimerization domains based on the design published by Guest and colleagues (PNAS, 2021). Guided by the structure and with the aid of the AI tool Alphafold2, we designed our first mutated version of E1E2 (E1E2.v2) which consistently showed significantly improved overall folding by indirect testing in ELISA and BLI. This mutation not only improved our initial design, but showed consistently improvement regardless of the genotype or the dimerization domains used. We then screened over 40 mutants to identify the combination that would result in best antigenic profile and folding. This candidate which includes critical proline mutations is our E1E2.v3, a candidate that drastically improved the thermostability of the protein retaining binding of AR4A to over 80 °C. Finally, we designed a new strategically position disulfide bond in our construct (E1E2.v4) that further stabilized the protein as established by all aforementioned techniques including proof on protein gels of this newly formed bond. E1E2.v3 and v4 outperformed all our previous candidates in every assessment performed including better more native-like.

Conclusions
We show that the use of structure-guided design combined with Alphafold2 predictions can lead to better folded immunogens. Strategically designed mutations, including a newly formed disulfide bond, revealed improved mutants that consistently outperformed the current designs across genotypes and platforms. Altogether, we have designed new HCV immunogens that are well folded, native-like and stable. The upcoming immunization studies will unveil how these immunogens perform *in vivo* and give insights on how these stabilized proteins can help us meet the global goal of developing an effective and preventive HCV vaccine.

Session 4: Flavivirus structure, function and viral life cycle
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Plenary talk: Receptor binding and entry of hepatitis C virus

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Hepatitis C virus (HCV) is a causal agent of chronic liver disease, cirrhosis, and hepatocellular carcinoma in humans, afflicting more than 70 million people worldwide. HCV envelope glycoproteins E1 and E2 are responsible for host cell binding. HCV uses a hybrid entry mechanism that requires both low pH and the receptor cluster of differentiation 81 (CD81), but the exact process remains undetermined. HCV E2 interacts with CD81, and most broadly neutralizing antibodies preclude interaction between HCV E2 and the large extracellular loop of CD81 (CD81-LEL). To obtain insights into the mechanism of HCV entry, several structures of E2 fragments in the presence and absence of CD81-LEL were determined. In the absence of CD81, E2 has an internal loop (the CD81-binding loop) tucked against Ile422 of the amino terminal region. Upon binding to CD81 at low pH, E2 undergoes two conformational changes: the amino terminal region of E2 folds around CD81, and the CD81-binding loop stretches away from the core of E2. Docking of the E2/CD81-LEL complex onto the structure of the membrane-embedded, full-length CD81 places Tyr529 and Trp531 of the CD81-binding loop proximal to the cellular membrane. Isothermal calorimetry and liposome flotation assays demonstrate that low pH increases E2 affinity for CD81-LEL and membranes. A new structure of an amino-terminally truncated E2 shows the membrane binding loop in a bent conformation and the aromatic side chains sequestered. A comparison of four E2 structures determined with the same Fab indicates that this internal loop is flexible, and that local context influences the exposure of hydrophobic residues. These data support the model that acidification and receptor binding result in a conformational change in E2 in preparation for membrane fusion.

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P120 – Structure of the hepatitis C virus E1/E2 heterodimer in a homodimeric complex

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Background
The formation of higher-order complexes of hepatitis C virus (HCV) E1/E2 heterodimers at the virion surface has been a subject of long-standing speculation. The recently solved structure of HCV E1/E2 from an isolate of genotype 1b revealed exposed hydrophobic and conserved regions of the E2 ectodomain, notably in the back layer (BL) and the base, that are likely involved in oligomerization. However, the topology of such oligomers remains unknown and is commonly speculated to be E1/E2 trimers mediated partly by the transmembrane domains (TMs) of E1.

Methods
We have characterized the capacity of detergent-extracted HCV E1/E2 derived from various HCV genotype isolates to assemble into higher-order complexes using blue-native PAGE. We stabilized and affinity purified these complexes by introducing strep tags as well as substitutions in the TMs of E1 and E2. Finally, we determined the structure of these purified E1/E2 complexes using cryo-electron microscopy.

Results
The structural analysis of purified detergent-extracted HCV E1/E2 from isolates of genotype 1a and 3a revealed the presence of anti-parallel E1/E2 homodimers (2 x E1/E2), at resolutions up to 3.5 angstrom for the genotype 3a isolate. This homodimer represents the most complete E1/E2 structure to date and includes detailed information on the interface of the homodimer, as well as the N-terminal segments of the E2 hypervariable region 1 (HVR1) and the TMs of both E1 and E2. Furthermore, a distinctive arrangement of the E2 front layer (FL) was observed. Notably, the homodimer interface displays a substantial buried surface area, involving the central ß-sandwich, the BL, and the base domains of E2. Surprisingly, the N-terminus of HVR1 extends into a cavity situated in the stem region of E2. Lastly, the detergent reconstituted part of the complex suggests membrane association of an internal region within E1. This region encompasses a part of E1 that has been previously documented to play a critical role in viral fusion.

Conclusions
We have successfully elucidated the structure of HCV envelope proteins in an anti-parallel homodimeric arrangement. Intriguingly, we observed that epitopes targeted by non-neutralizing antibodies are predominantly concealed within this dimeric configuration. Consequently, future vaccine antigens based on such an E1/E2 homodimeric complex have the potential to elicit antibody responses that surpass that of previously constructed recombinant E1/E2 vaccines for HCV.

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Using machine learning to map membrane fusion mechanisms across the Flaviviridae

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Background
Enveloped viruses encode specialised glycoproteins that mediate fusion of viral and host membranes. Discovery and understanding of the molecular mechanisms of fusion has been achieved through experimental determination of glycoprotein structures from many different viruses. Nonetheless, there is an incomplete understanding of membrane fusion mechanisms across the Flaviviridae. The E glycoprotein of the Orthoflaviviruses, e.g. Dengue virus, is a prototypical class II fusion protein. However, the glycoproteins from the Jingmen Viruses, Large Genome Flaviviruses, Pesti-, Pegi- and Hepaciviruses remain poorly characterised and unclassified. This knowledge gap is a barrier to mechanistic understanding and vaccinology for important animal and human pathogens including hepatitis C virus.

Methods
We have combined phylogenetics and state-of-the-art machine learning approaches to predict structures for all viral glycoproteins across the Flaviviridae. We used detailed structural analysis and cross comparison to provide insights on both mechanism and evolution of membrane fusion in each viral genus.

Results
We initially modelled the E1E2 glycoproteins from sixty viral species in the Hepaci-, Pegiand Pestivirus genera. Whilst the predicted structure of E2 varied widely, E1 exhibited a very consistent fold across these genera, despite little or no similarity at the sequence level. Importantly, our predictions were verified by recent experimental structures of HCV E1E2. The structure of E1 and E2 are unlike any other known viral glycoprotein, this suggests that the Hepaci-, Pegi- and Pestiviruses may possess a novel membrane fusion mechanism, distinct from that of the Orthoflaviviruses. Next, we widened our approach to encompass the entire Flaviviridae, allowing us to unambiguously identify fusion mechanisms for >400 species, including those from the Jingmen Viruses and Large Genome Flaviviruses. This revealed a complex pattern of genus defining evolutionary events, with fusion mechanism often correlating with commitment to specific replication strategies and/or ecological niches.

Conclusions
This work provides a unique perspective on the emergence and evolution the viral genera that comprise the Flaviviridae. Based on our findings we propose that membrane fusion glycoproteins are a critical determinant of viral tropism and lifestyle. We expect this work to guide future experimental virology to uncover molecular mechanisms and to inform structural vaccinology for a diverse range of existing and emerging human pathogens.

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Flavivirus STAT2 antagonism and host coevolution

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Background
For efficient infection and pathogenesis, all Flaviviruses need to suppress the host interferon response. Many of these viruses do so by using their nonstructural 5 (NS5) proteins to antagonize the Signal Transducer and Activator of Transcription 2 (STAT2) in infected cells. Analyses done by us, and others, indicate that STAT2 has evolved under positive selection within multiple mammalian lineages. This correlates with the observation that STAT2 antagonism by Flaviviruses is species-specific, which is best illustrated in the resistance of mouse STAT2 to Zika virus (ZIKV), dengue virus (DENV), and yellow fever virus (YFV) NS5 antagonism.

Methods
Here we evaluate the capacity of the NS5 proteins from various Flaviviruses to antagonize the STAT2 from a range of mammalian species. Using a luciferase-based reporter assay, we determined the susceptibility of the STAT2 from 38 mammalian species to antagonism by the NS5 proteins of ZIKV, DENV, YFV, and Spondweni virus (SPOV).

Results
We have identified that the STAT2 susceptibility determinants vary between the different Flavivirus NS5 proteins despite their sequence and structural conservation. In mapping the resistance determinants of rodent STAT2 proteins, we found that resistance was acquired at least twice in rodent evolution as evident by those determinants differing between rodent lineages. We also observed that STAT2 resistance to DENV NS5 antagonism was acquired in multiple mammalian lineages. Most strikingly we identified that resistance to DENV NS5 was acquired early in the evolution of lemurs, but was then lost by some species. While the amino acid changes which confer resistance to DENV NS5 are conserved across lemurs, there are additional changes in some species which overcome that resistance and allow for antagonism in those species.

Conclusions
These results suggest the possibility that antagonism by flaviviruses has impacted STAT2 evolution. The observed resistance to STAT2 antagonism in some mammalian lineages likely limits the host tropism of Flavivirus infection and pathogenesis, and illustrates hurdles and possible routes of adaptation for the creation of small animal models.

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Identifying the Molecular Determinants of Epitope Specificity in hepatitis C virus

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Introduction
The current strategy for HCV vaccines is to preferentially enrich for responses that target the envelope 2 (E2) epitopes associated with broad neutralising antibodies (BnAb) e.g. Domain B/AR3, Domain D and domain E. However, the properties of the Abs that target these epitopes have only been investigated in a small number of mAbs and have not been examined more broadly. Therefore, it is not known if the features identified in the mAbs studied to date are unique or generalisable to that epitope. Immense diversity exists in the possible repertoire of Abs, due to broad and differential gene usage and the highly variable process of somatic hypermutation (SHM) during pre- and post- antigen selection during B cell development and maturation. By understanding the molecular determinates of BnAb epitopes it may help direct vaccine design towards preferential induction of these responses.

Methods
rE2 positive IgD- B cells were sorted from 21 HCV infected individuals and 305 mAbs were expressed (with native Vh and Vl pairing) and analysed for their E2 binding and epitope specificity by competition mapping against a panel of well-known mAbs: AR3A, HC84.26, HCV1, AR2A, AR1A, CBH7, CBH4G. The 305 patient derived mAbs were then clustered based on their epitope specificity and the BCR properties were analysed. VDJ useage, SHM and physiochemical properties of CDR2 and CDR3 were examined to determine if there were unique properties associated with specific E2 antibody epitopes.

Results
This study found that the antibodies grouped into 7 clusters, with each cluster generally representing a unique epitope. Analysis of the different epitope clusters revealed that there were Ab characteristics associated with the epitope that they targeted. For gene usage, it was found that Abs clustered with HCV1 all used VH1-69, as with 58% of cluster 6AR3A/HC84.26 Abs. Interestingly, it was found that the Abs of cluster 2AR2A were consistently dominated by a single VDJ gene for both the heavy and light chains. SHM across all heavy chain genes were similar between clusters, however the VH genes of cluster 6AR3A/HC84.26 Abs were significantly more varied from germline than those of any other cluster. Epitope specific characteristics of hydrophobicity and polarity were also reported for the CDR2 and CDR3 regions.

Discussion
In conclusion, this study has identified that there are unique molecular determinants associated with epitope recognition and may help provide a more refined understanding of BCR-development driven epitope specificity and may inform future HCV Ab research for a more rational approach to vaccine design.

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P108 – **Differential T cell signaling by memory-like and severe exhausted HCV-specific CD8 T cells in chronic HCV infection revealed by highly multiplexed mass-based phosphoflow analysis**

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Background
T cell exhaustion is a major contributor to CD8+ T cell dysfunction in chronic viral hepatitis and linked to persistent antigen stimulation and altered cellular metabolism. Recently, progenitor-progeny relationships of exhausted HCV-specific CD8+ T cells have been identified that have implications for the maintenance of the exhausted T cell pool and respond differently to direct acting antiviral (DAA) therapy. However, the signaling and metabolic determinants of these distinct exhausted T cell subsets in chronic HCV infection remain unclear.

Methods
We performed comprehensive flow-based immunometabolic characterization of HCV-specific CD8+ T cells during DAA therapy *ex vivo* and evaluated the signaling dynamics of HCV-specific CD8+ T cell subsets using a 42-plex mass cytometry phosphor-signaling panel after TCR stimulation *in vitro*.

Results
$PD1^{+}CD127^{+}$ HCV-specific T_{PEX} cells enriched during DAA therapy. This finding was connected to the higher mitochondrial fitness of the $PD\text{-}1^{+}CD127^{+}$ population compared to $PD1^{+}CD127^{-}$ HCV-specific T_{EX} cells. However, the mitochondrial programs were not significantly modified in T_{PEX} cells, whereas there was an improvement of mitochondrial metabolism in severely exhausted HCV-specific T cells after DAA therapy, suggesting a role for antigen recognition in driving the metabolic programs. We therefore investigated the connection between signaling and metabolism at the subset level using highly multiplexed time-lapsed phosphoflow analysis by mass cytometry. Our analysis of $PD1^{+}CD127^{+}$ T_{PEX} versus $PD1^{+}CD127^{-}$ T_{EX} cells from cHCV patients revealed major differences in the proximal TCR signaling. We observed an increased activity of PLCγ and the NFAT signaling downstream of calcium signaling in T_{EX} cells compared to T_{PEX}. Interestingly, in chronic HCV, Tpex cells also showed a different activation pattern of the NFkB pathway compared to Tex cells. Finally, we found an increase in the phosphorylation of protein-tyrosine phosphatase (SHP-2) in $PD1^{+}CD127^{-}$ T_{EX} cells and a reduced activity of ZAP-70.

Conclusion
Our data suggests a different signaling wiring of HCV-specific T_{PEX} and severe T_{EX}. Severely exhausted T cells exhibit high levels of NF-κB and calcium signaling, which is associated with their metabolic state. Notably, removal of antigen stimulation allows for a partial recovery of the mitochondrial phenotype. Our data suggest that signaling interventions may modify exhaustion subset programming.

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P67 – **Neutralizing antibodies exploit vulnerable E2 amino acids to mediate repeated, spontaneous HCV clearance**

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Background
Through poorly defined mechanisms, individuals who clear primary hepatitis C virus (HCV) infections spontaneously clear subsequent reinfections more than 80% of the time. These reinfections are associated with a rapid rise in neutralizing antibody (NAb) titers, shorter duration of infection, and lower peak viremia, demonstrating protective adaptive immunity that can serve as a model for a desired vaccine response. In this study, we investigated the mechanism of repeated immune-mediated clearance of HCV reinfections.

Methods
We sequenced the viral quasispecies at longitudinal timepoints in individuals with repeated HCV clearance (n=8) or persistent infection (n=8) to characterize the evolution of the envelope glycoprotein E2. We assessed the prevalence of each substitution arising in E2 using an HCV sequence database (HCV-GLUE), and we measured the effect of these E2 substitutions on virus sensitivity to neutralization by longitudinal autologous plasma samples, monoclonal broadly neutralizing antibodies (bNAbs), and bNAb unmutated ancestors. We also measured the effect of these substitutions on E2 binding to CD81, the primary HCV receptor.

Results
We found that substitutions in cleared infections are enriched in E2 relative to the rest of the HCV genome ($p=0.0001$). We also found that cleared infections acquired a greater number of lower prevalence amino acid substitutions in E2 relative to persistent infections, and we identified amino acid substitutions in E2 that were shared among multiple cleared infections. We determined that these substitutions were selected by early autologous plasma NAbs ($p=0.01$), and they reduced E2 binding to CD81 ($p=0.007$). Later, peri-clearance plasma samples regained neutralizing capacity against these variants. We then identified a set of bNAbs for which these same loss-of-fitness E2 substitutions conferred resistance to unmutated bNAb ancestors, but increased sensitivity to mature bNAbs. Interestingly, these substitutions were distributed across E2 and did not fall at known bNAb-E2 or CD81-E2 binding residues.

Conclusions
These data suggest a mechanism by which neutralizing antibodies contribute to repeated immune-mediated clearance of HCV infection. We identified public, plasma NAb-selected amino acid substitutions in E2 that led to loss of viral fitness. We also identified specific bNAbs that exploit these fundamental vulnerabilities in E2. Induction of these bNAb-types should be a goal of HCV vaccine development.

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P65 – **A new assay for the quantification of HCV E2- or E1-dependent neutralizing antibody responses in polyclonal plasma**

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Background
Previous studies have shown a wide range of plasma neutralizing breadth both in individuals with spontaneous HCV clearance and in those with persistent infections, and a key difference in responses may lie in the specific epitopes targeted in envelope glycoproteins E1 and E2. Little is known about the relative contribution to plasma neutralizing activity of NAbs targeting epitopes across E1E2. An assay that can sensitively and specifically quantitate the contribution of each NAb-type to plasma neutralizing activity would be useful for natural history studies and vaccine development.

Methods
We developed a modified HCV pseudoparticle (HCVpp) neutralization assay that depends on the prior development of natively-folded soluble E2 (sE2) with the key amino acid residues in the front layer (FRLY) domain knocked out via alanine mutagenesis (FRLYko sE2). Mutations were selected to abrogate the binding of known FRLY-targeting NAbs without affecting the binding of antibodies against other conformational epitopes. HCVpp neutralization was measured by plasma alone or plasma preincubated with native, unmutated sE2 or FRLYko sE2, which can compete with the HCVpp for binding to NAbs. Comparison of results from each condition allowed for the calculation of the proportion of neutralization resulting from NAbs targeting the E2 FRLY, E2 non-FRLY, or E1-dependent epitopes.

*E2 FRLY response = (%neut with FRLYko sE2/%neut plasma only) * (1 - %neut with native sE2/%neut plasma only) * 100%*
*E2 non-FRLY response = ((1 - %neut with native E2/% neut plasma only) * 100%) - FRLY response*
*E1-dependent response = 100% - (1 - %neut with native sE2/%neut plasma only) * 100%*

The monoclonal antibodies HEPC74 (front-layer E2), HCV-1 (non-front layer E2), and AR4A (E1-dependent) were used as controls. We selected polyclonal plasma from individuals in two cohorts (the Baltimore Before and After Acute Study of Hepatitis (BBAASH) and the Multicenter AIDS Cohort Study/Women's Interagency HIV Study Combined Cohort Study (MWCCS)) who either spontaneously cleared HCV infection or remained persistently infected to identify differences in targeted epitopes between individuals in these two outcome groups.

Results
Eight of 9 (88%) individuals who cleared their infections demonstrated a dominant E1-dependent NAb response (49%-100% of the neutralizing response in each individual). In contrast, this E1-dependent profile was found in only 6 of 14 (43%) individuals with persistent infection ($p = 0.04$, Fisher's exact test).

Conclusions
This assay provides a new way to quantify epitope specificities of NAbs in polyclonal plasma. Individuals who cleared HCV infections had significantly more dominant E1-dependent antibody responses than individuals who remained infected. This challenges conclusions drawn from more limited data that HCV NAbs primarily target the front layer of E2. The use of this assay on a larger number of samples may further elucidate key differences between effective and ineffective plasma antibody responses.

Session 4: Flavivirus structure, function and viral life cycle
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P26 – **Dengue virus serotype-specific inhibition of T cell responses is due to a single amino acid polymorphism in the envelope protein.**

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Background
Clinical outcomes differ among dengue virus (DENV) serotypes; however, few serotype-specific biological differences have been identified. Using cell lines that express flavivirus envelope (env) proteins from HCV, YFV, HPgV, ZIKV and DENV-2 env found that only ZIKV and DENV-2 did not inhibit T cell receptor (TCR) signaling. Here, we investigated the effect of virus and env expression of all DENV serotypes on T cell functions.

Methods
DENV 1, 2, 3, 4 (1-4) were added to primary peripheral blood mononuclear cells (PBMCs) or Jurkat T cells prior to stimulation with anti-CD3. TCR signaling was monitored by measuring IL-2 release. DENV replication was assessed by measuring serotype-specific binding at 4^{o}C, entry, viral RNA production and release of infectious virus. The effects of DENV env (1-4) expression in Jurkat cells was measured by TCR-mediated IL-2 release. Alternatively, DENV (1-4) env effects on primary human TCR responses were measured by seeding transwell™ plates with 293T cells expressing various DENV envs, adding primary PBMCs to the top well and measuring TCR-stimulated IL-2 release. DENV env regions required for TCR inhibition were mapped by creation of env chimera's, deletions, or specific amino acid mutations. Results were confirmed by reverse genetics with key env mutations inserted into infectious cDNA parental viruses and replication competent DENV generated by CPER.

Results
DENV 1 and 4 viruses inhibited TCR signaling in primary and transformed human T cells while DENV 2 and 3 did not. DENV incubation with T cells led to abortive infection without release of infectious virus. No differences in DENV 1-4 binding, entry or RNA production were observed. DENV envelope expression recapitulated the findings in both 293 transwell methods and in Jurkat cells expressing DENV envs, with serotypes 1 and 4 envs inhibiting TCR and 2 and 3 envs not inhibiting TCR. The functional region of DENV 1 and 4 was mapped and found to require the N-terminal 65 amino acids. Alignments identified potential amino acids involved. CPER generated DV1 and 4 parental viruses inhibited TCR while 2 and 3 did not. Replacing a key env amino acid in DENV 1 and 4 with the amino acid present in 2 and 3 reversed TCR inhibition, confirming that this single amino acid is sufficient to reverse the TCR inhibitory phenotype.

Conclusions
Epidemiological data suggest that DENV 2 and 3 are more likely associated with severe, immunologically mediated dengue diseases including hemorrhagic fever and shock syndromes. Since DENV 1 and 4 interfere with TCR, it is possible that this TCR effect blunts host immunologic responses during infection, mitigating immune-mediated pathogenic effects of DENV. Studies are underway to introduce TCR inhibition into DENV 2 and 3, which could provide a method to potentially enhance safety of live-attenuated, quadrivalent DENV vaccines.

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P87 – **Understanding the virus-host protein-protein interaction network of the hepatitis E virus**

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Background
The hepatitis E virus (HEV) is an underestimated RNA virus and currently the most common cause of acute viral hepatitis. The HEV viral life cycle and pathogenesis remain poorly understood and no specific therapies are currently available. Throughout their life cycle, viruses interact with cellular host factors, thereby determining host range, cell tropism and pathogenesis; and ensuring their propagation. The HEV virus-host interaction network to date is limited, however it is crucial to unravel as it will lead to novel fundamental findings and may identify highly-needed antiviral targets.

Methods
Two related high-throughput mammalian two-hybrid approaches based on cytokine-receptor complementation (MAPPIT & KISS) were used to screen for HEV interacting host proteins. As bait we used ORF2 and ORF3 from both genotype (gt) 1 and gt 3 as well as ORF4 (gt 1). Promising hits were examined on protein function, involved pathway(s), cellular expression and their relation to other viruses, mostly by using the DAVID functional annotation tool.

Results
We identified 37 ORF2 hits, 187 ORF3 hits and 91 ORF4 hits. Based on clusters and literature search, SHARPIN (ORF3 & ORF4 hit) and RNF5 (ORF3 hit) were selected for further study. Both hits target proteins belonging to the RLR-MAVS pathway leading to IFN induction and have shown to be of importance during different virus infections. Interestingly, our PPI analysis showed that ORF3 also interacts with the attachment factor TIM1, for which we previously demonstrated that it is involved in the entry of enveloped HEV.
We used CRISPR-Cas9 to create SHARPIN and RNF5 knock-out (KO) cells permissive to HEV transfection and/or infection. A qPCR approach was set-up to investigate effects on IFN type I and type III as well as ISG induction. In accordance to previous literature, we found that HEV transfected cells could induce a type I and type III IFN response, which was substantially augmented upon poly(I:C) stimulation. Using the HEV replicon system, our preliminary data indicated no effect on viral replication in one of the investigated KO cell lines for both proteins. We confirmed the interaction of ORF3 with TIM1 via co-localization and PLA assays. Moreover, our preliminary replication data in TIM1-KO cells shows that this protein also influences replication.

Conclusions
MAPPIT and KISS are valuable tools to study virus-host interactions, as we found over 200 host proteins interacting with HEV ORF2-4. Two proteins, SHARPIN and RNF5 were selected and the role of the protein in the life cycle will be investigated using qPCR approaches, followed by further characterizing (downstream) pathway. The effect of KO on viral replication and infection will also be investigated further. We confirmed the interaction of ORF3 and TIM1, future work will need to elaborate more on this interaction in the potential trafficking of the virus during its life cycle.

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P42 – IL-15-induced activation of liver damaging bystander CD8$^+$ T cells is counteracted by Ca^{2+} signals in viral hepatitis

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Background
IL-15-induced bystander activation of memory CD8$^+$ T cells exert NKG2D-dependent innate-like cytotoxicity which is associated with liver injury in patients with acute hepatitis A virus (HAV) infection (Kim et al. *Immunity* 2018, 48(1):161-173) or chronic hepatitis C virus (HCV) infection (Huang et al. *Hepatology* 2022, 76(3):803-818), indicating an immunopathological role of bystander-activated memory CD8$^+$ T cells in viral hepatitis. In a consecutive study, we found that upregulated CCR5 mediates migration of bystander-activated CD8$^+$ T cells into inflammatory liver tissues which also correlates with liver injury among patients with acute HAV infection (Seo et al. *Cell Reports* 2021, 36(4):109438). Importantly, we found that the expression of NKG2D and CCR5, a hallmark of IL-15-induced bystander activation, is not induced by TCR stimulation, rather, it is diminished by concurrent TCR stimulation. However, regulatory mechanisms underlying IL-15-induced bystander activation counteracted by the concurrent TCR stimulation have not been examined before. Therefore, we aimed to identify mechanisms involved in TCR-mediated inhibition of IL-15-induced bystander activation of memory CD8$^+$ T cells.

Methods
We obtained memory CD8$^+$ T cells from healthy donors and performed bulk RNA sequencing following ex vivo stimulation with IL-15, anti-CD3, or IL-15 with anti-CD3. In a following bulk RNA sequencing analysis, we used ionomycin, a Ca^{2+} ionophore, instead of anti-CD3. We also obtained CD8$^+$ T cells from patients with acute HAV infection and performed single-cell RNA sequencing following labeling them with CITE-seq antibodies and dCODE dextramers specific to HAV, CMV, EBV, and influenza. Finally, we confirmed our findings by flow cytometry analysis.

Results
We identified a bystander activation-specific set of genes upregulated by IL-15 but down-regulated by the concurrent TCR stimulation which is significantly enriched with genes associated with NK cell-mediated cytotoxicity and IFN responses. We validated that this unique gene signature represents IL-15-induced bystander activation by demonstrating its significant enrichment in non-HAV-specific bystander CD8$^+$ T cells during acute HAV infection. We found that such IL-15-induced bystander activation features are counteracted by Ca^{2+}-mediated signals. Furthermore, we demonstrated that upregulation of IL-15-induced bystander activation features was not suppressed, rather enhanced in the presence of the concurrent anti-CD3 stimulation by calcineurin inhibitors, including tacrolimus and cyclosporin A.

Conclusions
Our data show that Ca^{2+}-mediated signals counteract IL-15-induced bystander activation of human memory CD8$^+$ T cells. Gaining a deeper understanding of the regulatory mechanisms underlying bystander activation of memory CD8$^+$ T cells could have significant clinical implications for effectively managing T cell-mediated immunopathological liver injury.

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P54 – **A structural perspective into hepatitis c virus e1e2 glycoproteins using a lentivirus-pseudoparticle approach**

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Background
Hepatitis C virus (HCV) infections remain a major global health burden, with more than 58 million cases recorded worldwide. As preventative vaccines and therapeutics remain elusive because of a lack of structural information, genetic variabilities among strains, and frequent selective mutations, HCV remains at the forefront of severe liver diseases like cirrhosis, hepatocellular carcinoma, and liver failure. The E1E2 glycoproteins of HCV are involved in receptor binding, viral entry, and fusion with target cells. However, there is limited information available on the oligomeric organization and molecular assembly of these proteins on the viral membrane.

Methods
One of the bottlenecks in the structural study of HCV/HCV glycoproteins has been the limited ability to produce samples with high purity owing to HCV's pleomorphism and their secretion as lipoviral particles. One alternative to address these problems is a selection of a system that closely resembles the native system but bypasses some of the bottlenecks. The current study addresses some of the problems by utilizing lentivirus-based HCV pseudoparticles (HCVpp). The HCVpp system is generated by the incorporation of unmodified HCV E1E2 protein in the lentiviral particles by co-expressing full-length HCV glycoproteins, lentiviral core protein, and packaging genome in Expi293F cells.

Results
A multistep purification scheme has been developed to produce high-quality samples for structural studies. A preliminary study of obtained samples using cryo-electron microscopy revealed the presence of HCVpps of varying sizes and shapes, resulting in an unsuccessful initial attempt to classify them two-dimensionally. Additionally, HCVpps frozen with the receptor CD81, or AR4A Fab, yielded limited success. More recently, samples have been frozen to optimize the concentration of BSA-gold tracer beads, a fiducial marker, in preparation for cryo-electron tomography data collection.

Conclusions
With the newly developed HCVpp purification scheme in combination with cryo-electron microscopy and tomographic studies, we will provide insights into the HCV glycoproteins oligomeric organization and molecular assembly, thus forming a platform for future vaccine design.

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P56 – **Investigating the Role of NS2A in the Zika Virus Life Cycle**

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Background
One of the least understood Zika virus (ZIKV) proteins is NS2A; a small ~22-kDa (226 amino acids), positively-charged transmembrane protein, that has been suggested to form oligomers. NS2A is critical for both genome replication and virion assembly; however, there are different requirements for the protein at each of these stages. During replication, NS2A is required in *cis*, but can be complemented in *trans* for virion assembly. Importantly, in the related Yellow Fever Virus, NS2A has also been shown to be present in a truncated form, called NS2Aα, with a predicted role in virion assembly. However, NS2A has largely remained unexplored in most flaviviruses due to its small, hydrophobic nature and a lack of NS2A-specific reagents/antibodies. As such, the importance of NS2A oligomerization in replication and/or assembly, and whether an α-form is present in other flaviviruses remains unclear.

Methods
We have established a ZIKV infectious cDNA with an N-terminally-tagged NS2A protein. Additionally, we created expression constructs for full-length and truncated N-terminally (HA- or V5)-tagged NS2A. We also have a construct that contains N-terminally-tagged NS2A in the context of NS2A-2B-3. To study NS2A during infection, we used the N-terminally-tagged NS2A containing infectious cDNA to generate infectious viral particles. To map the putative NS2A oligomerization interface we transfected full-length and truncated NS2A expression constructs into 293T cells and used co-immunoprecipitation assays to map the interface.

Results
Our preliminary data indicates that the predominant form of NS2A during ZIKV infection is a truncated form, NS2Aα. Subsequently, we show that NS2Aα biogenesis is mediated by an alternative NS3 cleavage site. We have mapped the alpha cleavage sequence to the final cytoplasmic linker before the C-terminal amphipathic helix, specifically SVKK|NLPF (| indicates the cleavage site). Interestingly, we also found that NS2Aα biogenesis is not dependent on prior cleavage at the NS2A-2B boundary. Additionally, using co-immunoprecipitation assays, we also have preliminary data which suggests that the putative NS2A oligomerization interface lies within the N-terminal 1-103 amino acids of NS2A. Moreover, full-length NS2A can pull down NS2Aα, indicating the different forms of the protein can interact. Moving forward, we intend to develop *cis*- and *trans*-complementation assays to elucidate the requirements of full-length NS2A and NS2Aα during ZIKV replication and assembly.

Conclusions
Our research suggests that a truncated form of NS2A, NS2Aα, is the predominant form of the protein during infection. Furthermore, we have mapped the oligomerization interface to the N-terminus of NS2A, identified the NS2Aα cleavage site, and verified that NS2Aα can contribute to the formation of NS2A oligomers. We anticipate that these studies will allow us to better define the requirements of NS2A and NS2Aα during viral replication and assembly. Moreover, due to the conservation of NS2A across related viruses, these findings may be applicable to other mosquito/tick-borne flaviviruses.

Session 5: Replication systems and animal models
October 3, 2023

Keynote lecture: Breaking the species barrier of hepatitis C virus

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The highly restricted species tropism of HCV, which is limited to chimpanzees and humans, has made in vivo study of this virus notoriously difficult. This has become even more challenging with the NIH moratorium on research in chimpanzees, underscoring the need for a small animal model suitable for studying HCV pathogenesis and for testing preclinically vaccine candidates. Several alternative, yet partially complementary approaches are being pursued to address this challenge: adapting the host cellular environment of non-permissive species genetically (i.) or through xenotransplantation (ii.); adapting HCV to infect and replicate in non-permissive species (iii.); using surrogate viruses which are genetically similar to HCV (iv). Here, I will highlight our lab's efforts to render mice susceptible to HCV and other hepatotropic pathogens and compare and contrast the different approaches.

Session 5: Replication systems and animal models
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P22 – **Impact of HBV pre-core mutation and IFNα on hepatocyte proteome in chronically-infected primary human hepatocytes**

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Background
HBV pre-core mutations arise during chronic HBV infection and impact HBV pathogenesis and treatment responses. Launching infectious HBV from DNA remains a bottleneck. Studies focusing on HBV pre-core mutations have largely relied on clinical isolates without isogenic controls or DNA transfection of hepatoma cells over primary human hepatocytes (PHH). Studying pre-core mutations is further limited by the need for very high inocula to establish *in vitro* infections, limited viral spread, and challenges maintaining PHH functions in culture. Therefore, these systems only partially recapitulate chronic HBV, including cccDNA copy numbers.

Methods
To model chronic HBV *in vitro* we developed a system based on culturing mouse-passaged (mp)PHH isolated from HBV-infected humanized mice. These HBV-mpPHH can be maintained in culture for several weeks. A major advantage is that nearly all hepatocytes are infected and contain high levels of cccDNA. In addition, we established an innovative method to generate HBV stocks of different sequences including HBV with pre-core mutations. This method relies on transplanting transfected/infected mpPHH in already humanized mice and passaging the virus-containing sera into new mice. We used the HBV-mpPHH as a tool to compare wild-type and isogenic pre-core mutant HBV in terms of the impact on hepatocyte proteome and response to IFNα treatment.

Results
To our surprise, we identified very distinct protein expression patterns with 2,860 differentially expressed proteins between wild-type and pre-core HBV-mpPHH. While these studies are ongoing, most of the differences were found to be relevant to lipid and mRNA metabolic processes. In addition, treatment with IFNα revealed both induced and suppressed proteins. Among these differentially expressed proteins, we highlight here 42 proteins that are downregulated in the presence of HBV.

Conclusions
Together, these data are expected to identify host factors that have a crucial role in the context of HBV pre-core mutations and response to IFNα treatment. Moreover, the in vitro systems we developed together with CRISPR-based applications and systems biology analyses can be extended in other hepatotropic viruses.

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P50 – **Function and therapeutic potential of cathepsin peptidases during Hepatitis E Virus infection**

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Background
HEV is estimated to be responsible for 44,000 deaths annually, yet its therapy is still limited to off label use of Ribavirin and pegIFNα, despite unsatisfactory cure rates and severe side effects, emphasizing the need for new and improved therapy options. In the pursuit of effective antiviral therapies, targeting viral entry holds promise, as it disrupts the initial stage of viral replication and has been proven effective for other hepatropic viruses. However, the precise mechanisms and host factors required for during HEV entry remain unclear. Hence, our objective was to employ a drug repurposing strategy to identify potential antiviral targets specifically related to viral entry.

Methods
In this study, we tested several anti HCV protease inhibitors in our recently established HEV cell culture model for their ability to interfere with infections of non-enveloped HEV in human hepatoma cells by immunofluorescence staining for the HEV capsid protein and microscopic analysis. The cytotoxicity of the compounds was evaluated using MTT assays, while the potential impact on viral replication was assessed by employing HEV subgenomic replicon systems. To identify the specific stage of the HEV life cycle targeted by the compounds, we performed time-of-addition kinetic experiments. Additionally, we employed Western Blot analysis to monitor the processing of the HEV capsid protein during viral entry.

Results
We found that the antiHCV HCV NS3/4A protease inhibitor Telaprevir inhibited HEV late entry, but not replication. The antiviral activity of Telaprevir stems from its ability to hinder the cysteine protease family, specifically by targeting lysosomal proteases belonging to the cathepsin family. Remarkably, the cathepsin inhibitor K11777 exhibited potent suppression of HEV infections with an IC_{50} of approximately 0.01 nM and no significant toxicity until micromolar concentrations in hepatoma cells. Notably, the inhibitory effects of K11777 were consistently observed in HepaRGs and primary human hepatocytes. Furthermore, western blot analysis of HEV-inoculated cells revealed that the HEV capsid was processed during entry, a process that is potently inhibited by K11777.

Conclusions
Our findings suggest that inhibition of cathepsins is an attractive antiviral strategy to target HEV entry. Especially the cathepsin Inhibitor K11777 represents a promising antiviral candidate with nanomolar efficacy and minimal toxicity. Our data indicate that K11777 prevents the proteolytic processing of the viral capsid during entry, resulting in restriction of HEV infection. Considering that RBV targets viral replication, advantages of possible synergistic antiviral effects need to be characterized to fully evaluate the clinical potential of cathepsin inhibitors. In addition to their attractiveness as a therapeutic target to combat HEV, characterization of the role of cathepsins can broaden our fundamental understanding of the HEV entry process.

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P64 – **Comprehensive epitope mapping and structural studies of antibodies from an HCV Elite Neutralizer reveal bNAbs that bind multiple antigenic regions on the E2 glycoprotein**

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Background
Structural studies of E1E2 glycoprotein-specific broadly neutralizing antibodies (bNAbs) isolated from HCV-infected individuals informs HCV vaccine design efforts. We learned so far that the front layer of E2 glycoprotein (antigenic region 3, AR3) is the main target of *VH1-69*-encoded bNAbs. Two regions on E2 are thought to contain either non-neutralizing epitopes (AR1 in the E2 -sandwich) or epitopes targeted by antibodies with a narrow neutralizing breadth (AR2 in E2 back layer). While bNAbs that bind E1-stabilized metastable epitopes in the back and stem region of E2 (AR4A-like bNAbs) are elicited in individuals who clear HCV, E1E2 heterodimer expression and purification for HCV vaccine studies remain a big challenge due to protein oligomerization and aggregation. The information about the existence of potent bNAbs targeting non-AR3 neutralizing epitopes on E2 can inform the design of easier-to-produce E2 immunogens that could elicit synergistic antibody responses.

Methods
We used longitudinally-collected blood samples from an HCV Elite Neutralizer who cleared multiple infections to isolate bNAbs recognizing non-overlapping E2 antigenic regions. We characterized the gene usage of the isolated bNAbs and determined their neutralization potency and breadth. To determine the epitope specificity of the bNAbs, we performed epitope binning experiments using Octet BLI biosensors and binding experiments with E2 knockout probes. Finally, we determined crystal structures of E2 glycoproteins complexed with selected bNAb Fabs (hcab40, hcab55, hcab64, and hcab17) representing key E2 antigenic regions.

Results
Epitope mapping of E2-specific bNAbs revealed three major antigenic regions, with potent bNAbs being represented in each region. The structural analyses of E2 complexed with bNAb Fabs from each antigenic region revealed that isolated bNAbs use diverse V_H-genes to target AR3 (*VH1-46* encoded hcab55 and hcab64), AR2 (*VH4-34* encoded hcab40) and AR1 (*VH4-34* encoded hcab17). The structures of the front layer-specific *V_H1-46* bNAbs, hcab55, and hcab64, were remarkably similar to the previously described crystal structure of AR3-specific *V_H1-69* bNAb HEPC74, which was also isolated from the same subject. AR1-specific hcab17 and AR2-specific hcab40 competed for binding with several bNAbs, including the ultrapotent bNAb hcab5, indicating that both AR1 and AR2 contain neutralizing epitopes. Finally, the structural analysis of the Hcab40-E2 complex indicated that the hcab40 binding footprint spanned both AR2 and recently characterized AR4A epitope in the stem region of E2. However, unlike E1E2-specific AR4A, hcab40 did not require E1 stabilization of E2 for binding, suggesting that AR2/AR4 interface might represent a novel target for E2-based immunogen design.

Conclusions
The existence of antibodies that target non-overlapping neutralizing E2 epitopes in humans suggests the possibility of a synergistic antibody response between bNAbs that utilize multiple V_H-genes. The structural characterization of novel AR1 and AR2 bNAbs will facilitate lineage-targeted E2-based immunogen design to induce the development of bNAbs to multiple conserved epitopes.

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P75 – **The role of B cells and their antibodies in the clearance of an HCV-related rodent hepacivirus**

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Background
Evidence assessing the role of B cells and their antibodies, or lack thereof, in the spontaneous resolution of acute hepatitis C virus (HCV) infection is conflicting. Utilization of a strictly hepatotropic, HCV-related rodent hepacivirus (RHV) model circumvents many of the challenges facing the field in characterizing the immunological correlates of dichotomous infection outcomes. While adult C57BL/6J mice clear this infection within ~4 weeks, select immunological perturbations can induce infection persisting for the lifetime of the mice, like chronic HCV infection in humans. As such, this study seeks to elucidate the importance of B cells in the timely clearance of acute RHV infection.

Methods
C57BL/6J, μMT, MD4-HEL heterozygotes, and $AID^{cre/cre}$ mice were obtained from Jackson Laboratories and infected with 10^4 GE RHV via intravenous tail vein injection. Virus-specific $CD8^+$ and $CD4^+$ T cells were characterized using newly developed MHC class I and II tetramers and ex vivo intracellular cytokine analysis using peptides incorporated in the tetramers. IgG was purified from the serum of RHV- or LCMV Armstrong-infected mice at the post-clearance time points of 6- and 3-weeks post-infection, respectively.

Results
μMT mice, congenitally lacking mature B cells, developed chronic RHV infection for over a year. After identifying their defective antiviral Th1 responses, the direct B cell-dependent nature of RHV clearance was confirmed by the persistent infection of C57BL/6J mice depleted of B cells despite mounting fully functional T cell responses. The persistent infection developed by MD4-HEL and $AID^{cre/cre}$ mice revealed that antigen-specific, class-switched B cells or their antibodies were crucial for viral resolution. Passive transfer of IgG purified from RHV- or LCMV-cleared donors into $AID^{cre/cre}$ recipients led to timely clearance of infection only in αRHV IgG recipients. Further, transfer of RHV-specific IgG into B cell depleted recipients, which normally develop persistent infection, led to timely clearance.

Conclusions
After determining that antigen-specific, class-switched B cells are essential for timely resolution of RHV infection, we directly attributed this effect to their virus-specific IgG production. Clearance of B cell-depleted recipients following passive transfer of RHV-specific IgG corroborated our findings that B cell-depleted, MD4-HEL, and $AID^{cre/cre}$ mice generate fully functional T cell responses, suggesting that antigen presentation by B cells is dispensable and that IgG is the sole effector component of the B cell compartment required for clearance. Due to the concerted synergy employed by these immunoglobulins and the T cell compartment in driving RHV resolution, HCV vaccine regimens should aim to simultaneously elicit robust HCV-specific antibody and T cell responses for optimal protective efficacy.

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P63 – **Phenotype and fate of liver resident CD8 T cells during acute and chronic hepacivirus infection**

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Background
Approximately 60-80% of humans fail to clear the hepatitis C virus infection, and the mechanism of HCV persistence and immune evasion remains elusive. T cells play an essential role in the control and clearance of HCV infection in humans and chimpanzees. We developed lab mouse and rat models for rodent HCV-like virus (RHV). Further development of these new rodent models is highly significant due to the lack of relevant animal models to study HCV immunity and pathogenesis.

Methods
We developed mouse MHC class I and class II tetramers to characterize the serial changes in RHV-specific CD8+ and CD4+ T cells during acute and chronic infection in C57BL/6J mice. To determine the nature and function of RHV-specific T cells, leukocytes isolated from the liver and spleen of RHV infected mice were stimulated *ex vivo* with peptides representing different RHV proteins and those incorporated in the T cell tetramers.

Results
RHV infection induced rapid expansion of T cells targeting viral structural and nonstructural proteins. After virus clearance, the virus-specific T cells transitioned from effectors to long-lived liver-resident memory T cells (TRM). The effector and memory CD8+ and CD4+ T cells primarily produced Th1 cytokines, IFN-γ, TNF-α, and IL-2, upon *ex vivo* antigen stimulation, and their phenotype and transcriptome differed significantly between the liver and spleen. Rapid clearance of RHV reinfection coincided with the proliferation of virus-specific CD8+ TRM cells in the liver. Chronic RHV infection was associated with the exhaustion of CD8+ T cells (Tex), as determined by their reduced ability to produce antiviral cytokines and higher expression of PD-1 and TOX. Interestingly, the virus-specific CD8+ Tex cells continued proliferation in the liver despite the persistent viremia and retained partial antiviral functions, as evident from their ability to degranulate and produce IFN-γ upon *ex vivo* antigen stimulation.

Conclusions
We developed mouse MHC class I and class II tetramers to characterize the nature and fate of liver-resident hepacivirus-specific effector and memory T cells during acute and chronic hepacivirus infection. We determined that the control and clearance of primary and secondary hepacivirus infections were associated with the expansion of liver-resident virus-specific T cells. Altogether, we defined the fundamental characteristics and unique transcriptome signature of liver-resident RHV-specific CD8+ T cells during acute and chronic hepacivirus infection.

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P125 – **HCV-Specific CD4+ T-Cells are Targeted by HIV-1 for Infection and Viral Reservoir Persistence.**

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Background
Hepatitis C virus (HCV) infection is a serious comorbidity in people living with human immunodeficiency virus (HIV) (PLWH) [1]. Antiretroviral therapy (ART) reduces HIV replication to undetectable levels, but it does not eliminate HIV reservoirs, which persist in long-lived memory CD4+ T-cells of various antigenic specificities [2]. HCV coinfection is associated with a larger HIV-DNA reservoir size in CD4+ T-cells as compared to mono-infected subjects [3] suggesting that HCV-specific CD4 T-cells maybe highly susceptible to HIV infection. Herein, we aimed to investigate whether HCV-specific CD4+ T-cells are permissive to HIV infection and whether they contribute to HIV reservoir persistence in ART-treated subjects.

Methods
Memory CD4+ T-cells from chronic HCV patients and negative controls (n=20 per group) were infected with $HIV_{NL4.3BaL}$ and HIV_{THRO} strains in vitro. HIV integration and replication were measured by real-time PCR and HIV-p24 flow cytometry/ELISA analysis, respectively. The CFSE-based T-cell proliferation assay was used to examine the phenotype and susceptibility of HCV-specific CD4+ T-cells to $HIV_{NL4.3BaL}$ infection in eight HCV resolvers and two HCV chronic subjects. *S. aureus, C. albicans*, CMV lysates, and Staphylococcal Enterotoxin B (SEB) were used as controls. HIV replication was measured by intracellular/soluble HIV-p24 expression. A monocyte-derived dendritic cell-based viral outgrowth assay (MDDC-based VOA) was used to detect the presence of replication-competent HIV reservoir in HCV-specific CD4+ T-cells from HCV+/HIV+ subjects on ART.

Results
We observed that HCV-specific CD4+ T-cells from chronic HCV-infected individuals compared to HCV-negative controls were more susceptible $HIV_{NL4.3BaL}$ infection in vitro, as demonstrated by intracellular ($p=0.332$), soluble HIV-p24 expression ($p=0.0298$), and integrated HIV-DNA levels ($p=0.0559$). Similar to C. albicans and S. aureus but not SEB, a fraction of HCV-specific T-cells supported productive HIV infection. HCV-specific CD4+ T-cells expressed relatively high levels of CXCR6+ (a minor HIV co-receptor and a liver homing marker), which correlated positively with HIV replication in vitro (Spearman $r=0.788$, $p=0.0466$). The MDDC-based VOA demonstrated that HCV-specific CD4+ T-cells harbor replication competent HIV reservoir. UMAP clustering algorithm combined with Leiden phenograph showed that HCV-specific T- cells distinguish from the other antigenic specificities by a predominant Th1 biased Tfh phenotype (CXCR3+/Tbet+/BCL6+).

Conclusion
Our results provide evidence that HCV-specific CD4+ T-cells are highly susceptible to HIV infection and may represent long-lived HIV reservoirs in ART-treated PLWH co-infected with HCV.

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P102 – **Insertions in hepatitis C virus hypervariable region 1 as a novel antibody evasion mechanism**

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Background
Despite the availability of effective antivirals, hepatitis C virus (HCV) still afflict around 58 million individuals globally, causing an estimated 290,000 deaths yearly. Developing a vaccine could be instrumental in preventing HCV transmission. However, vaccine development poses challenges, partly due to the virus's ability to evade neutralizing antibodies. Hypervariable region 1 (HVR1), located at the N-terminus of envelope glycoprotein E2, plays an important role in HCV evasion and it can protect distinct neutralization epitopes, but its exact protective mechanism remains poorly understood. HVR1 is 27 amino acids in length with high sequence variability and prior studies on genotype 1b patients have even identified 3-4 amino acid insertions in the N-terminus of HVR1. However, the prevalence and functional implications of these HVR1 insertions remain unknown.

Methods
We analyzed the prevalence of HVR1 insertions by examining E1E2 sequences from 131 HCV RNA samples obtained from Danish patients. To assess the functional significance of the insertions, we inserted these non-canonical HVR1 sequences into HCVcc and evaluated their effect on infectivity. When HVR1 insertions attenuated the recombinants, we adapted them to cell culture and performed reverse genetic analysis. Among the viable recombinants, we generated sequence-confirmed virus stocks and studied the effect of the HVR1 insertions on receptor dependency and sensitivity to neutralizing antibodies.

Results
We identified patient samples with HVR1 insertions of 1, 3 or 4 amino acids in genotype 1a, 2b or 3a. The insertions were predominantly found at the N-terminus or HVR1, except for genotype 1a where they were towards the C-terminus. When observed, HVR1 insertions were the dominant (>70%) quasispecies in the patients, emphasizing their significance. Except for genotype 3a, recombinants with HVR1 insertions were attenuated. We adapted the attenuated recombinants in cell culture and identified putative adaptive substitutions within and outside HVR1. Notably, in some adapted genotype 1b recombinants, we observed deletions of 10 or 13 amino acids in HVR1, likely rendering HVR1 non-functional. For the genotype 3a recombinants, we were able to directly test the effect of the insertions on receptor dependency and neutralization sensitivity. The
insertions did not affect receptor dependency. However, we found that the 3 amino acid insertion, GSG, in the N-terminus of HVR1 increased resistance to the broadly neutralizing antibody, AR3A, while having no effect on AR4A and AR5A sensitivity. This indicates that HVR1 insertions can serve as an epitope-specific mechanism to evade neutralization.

Conclusion
In conclusion, dominant HVR1 insertions of 1-4 amino acids occur in different genotypes of HCV at a frequency of around 3% in infected individuals. This highlights their relevance in natural infection. Interestingly, HVR1 insertions can cause epitope-specific resistance to broad neutralizing antibodies, suggesting that they may represent a novel antibody evasion mechanism.

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P76 – **The role of Foxp3+ regulatory T cells in orchestrating anti-viral T and B cell responses during an acute Hepacivirus infection**

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Background
Rat hepacivirus (RHV) can establish hepatotropic infections in rats and mice and provide a unique and indispensable model for understanding immune responses needed for the development of an effective hepatitis C virus vaccine. The suppression of antiviral T cell responses by regulatory T cells (Tregs) may be critical for establishing chronic RHV infection, however the contributions of Tregs in orchestrating anti-viral T and B cell humoral immune responses during an acute RHV infection is not well understood.

Methods
$Foxp3^{DTR}$ knock-in mice, in which $Foxp3^{+}$ Treg cells express the human diphtheria toxin receptor (DTR)-eGFP transgene, were infected with 10^4 GE RHV via intravenous tail vein injection. Tregs were depleted by diphtheria toxin treatment during an acute RHV infection. Virus-specific $CD8^{+}$ and $CD4^{+}$ T cells were characterized using novel MHC class I and II tetramers and by ex vivo intracellular cytokine analysis.

Results
Continuous depletion of Tregs resulted in a reduced frequency of intrahepatic virus-specific class-II tetramer^{+} $CD4^{+}$ T cells and class-I tetramer^{+} $CD8^{+}$ T cells and an impaired antiviral Th1 responses, resulting in increased viremia and intrahepatic viral load. Interestingly, transient depletion of Tregs initiated before the infection resulted in a delayed virus clearance, while depletion immediately after the infection cleared the virus rapidly which correlated to increased antiviral Th1 response. Persistent Treg-depletion remarkably reduced B cell frequencies, however, B cell depletion did not alter the frequencies of intrahepatic Treg populations nor the frequencies of virus-specific $CD4^{+}$ or $CD8^{+}$ T cells or Th1 response. Nonetheless, B cells depletion resulted in an elevated viremia and intrahepatic viral load, suggesting an important role for B cell humoral response in controlling acute RHV infection.

Conclusions
The timing and frequency of Treg depletion regulate the fate of effector $CD4^{+}$ T and B cell responses and virus clearance. The constant depletion of Tregs resulted in an impaired anti-viral $CD4^{+}$ T cell response, increased viremia and intrahepatic virus load leading to an aggravated liver injury during acute viral infection. These findings suggest a crucial role for Tregs in maintenance of liver homeostasis, regulation of B cell responses, antiviral Th1 responses and viral clearance during an acute RHV infection.

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P17 – **Analysis of Intrahepatic CD8+ T Cells with Different Viral Epitope-Specificity During Primary and Secondary Hepacivirus Infection**

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Background
A lack of immune-competent small animal models has impeded the study of the hepatic immunological mechanisms that contribute to viral clearance and protective immunity versus viral persistence during hepatotropic virus infections, such as hepatitis C virus (HCV) infection. Vaccination strategies are difficult to develop without this information, as evident by the difficulties in developing an effective HCV vaccine. The development of an infection mouse model based on HCV-related Norway Rat Hepacivirus (NrHV) has opened new avenues for researching hepatic viral infections. Similar to studies on HCV infection, initial work on NrHV infection has highlighted the essential role of not only CD4+ T cells but also CD8+ T cells in hepacivirus clearance. In this study, we aimed to gain a better understanding of the role of distinct hepatic virus-specific CD8+ T cell subsets in viral clearance and the impact of CD4+ T cell help on the CD8+ T cell response.

Methods
We analyzed the intrahepatic CD8+ T cell response to a primary and secondary NrHV infection in C57BL/6 mice using full spectrum flow cytometry. MHC class I tetramers for several distinct viral epitopes allowed us to view the NrHV-specific CD8+ T cell response and how it differed by antigen recognition. Stimulation with the peptides corresponding to these epitopes provided insight into the differences in cytokines and cytotoxic molecules elicited by these distinct antigens. Finally, we performed CD4 depletion experiments to assess the intrahepatic CD8+ T cell response without CD4+ T cell help.

Results
Our analysis of acute NrHV infection showed a robust virus-specific effector CD8+ T cell response that reached its peak at day 21 post-infection (when viremia is resolved), followed by a slow contraction weeks after viral clearance and the accumulation of memory subsets in the liver months after infection. Secondary infection induced a rapid induction of effector functions in hepatic memory cells. Different epitopes appeared to elicit distinct phenotypes of CD8+ T cells and unique cytokine responses. CD4 depletion reduced the total number of antigen-specific CD8+ T cells and caused phenotypical changes in CD8+ T cell subsets.

Conclusions
Our results provide detailed insights into the kinetics, phenotype, and functional properties of hepatic CD8+ T cells targeting distinct viral epitopes during primary and secondary hepacivirus infection. Our data suggest that differences in CD8+ T cell phenotypes and cytokine response are associated with differences in epitope recognition. A lack of CD4+ T cell help during primary infection leads to specific phenotypical changes in CD8+ T cells, which may contribute to the establishment of viral persistence in the absence of CD4+ T cells.

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P107 – **Hepatitis C viral evolution after childbirth bears signatures of both CD8+ T cell and antibody pressure in women with postpartum suppression of viral replication**

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Background
Some individuals infected with hepatitis C virus (HCV) control viral replication and clear the infection after onset of adaptive immune responses. In most patients, any initial control of viral replication fails with loss of CD4+ T cell help, emergence of viral variants with mutations that escape recognition by HCV-specific CD8+ T cells and neutralizing antibodies, and exhaustion of CD8+ T cells targeting remaining intact epitopes. Lifelong infection ensues with steady state viremia. However, a surprising decrease in viral load (VL) occurs in some chronically infected women postpartum. Given evidence for restored CD4+ T cell help postpartum, we hypothesized that this spontaneous drop in VL may be associated with selection of viral variants bearing signatures of enhanced CD8+ T cell and antibody pressure.

Methods
To assess postpartum HCV evolution, we collected plasma from 17 pregnant women during their third trimester (T3) through 12-24 months postpartum (12-24PP). Of these patients, 8 experienced a VL reduction ≥1 $\log_{10}$ between T3 and 3 months postpartum (3PP) ("Controllers"), and 9 experienced a <0.5 log10 VL decrease between T3 and 3PP ("Non-controllers"). Viral genomes at T3 and 12-24PP were assessed by Illumina sequencing of near-full-length PCR products from viral cDNA. Reads from T3 were aligned to reference genomes to develop consensus sequences for each subject's viral quasi-species. Synonymous and nonsynonymous mutations away from the T3 consensus sequence were identified when nucleotide substitutions increased from ≤20% of reads at T3 to ≥80% at 12-24PP.

Results
Across the viral genome, there was a significant increase in non-synonymous mutations between T3 and 12-24PP in controllers when compared to non-controllers (p=0.0014), and this correlated with increased viral control (VC) (p=0.0012). Roughly 1/3 of mutations emerged in the HVR1 region, ~1/3 in the structural genes (-HVR1), and the remaining 1/3 in non-structural genes. The number of mutations in the HVR1 region did not correlate with VC, however the number of mutations in the structural (-HVR1) region and non-structural regions did (p=0.0006; p=0.0152). Most mutations occurred within predicted class I epitopes and correlated with VC in both structural (-HVR1) and non-structural regions (p<0.0001; p=0.0055). Additionally, in the structural (-HVR1) region and E2 specifically, the number of mutations occurring outside of predicted class I epitopes also correlated with VC (p=0.0119; p=0.0359) with enrichment in the front layer of the neutralizing face of E2.

Conclusions
Selection of viral variants encoding amino acid substitutions specifically in predicted class I epitopes across structural and non-structural proteins and outside class I epitopes in E2 suggest that transient postpartum suppression of viral replication is mediated by resurgent pressure from both CD8+ T cells and neutralizing antibodies. A better understanding of these renewed effector responses may provide novel insight into mechanisms of immune restoration against chronic viral infections.

Session 6: Emerging and reemerging flaviviruses, vector transmission and biology
October 4, 2023

Keynote lecture: Zika virus vaccine development: Experience is not preparedness

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In 2016, the World Health Organization (WHO) declared Zika virus (ZIKV) a Public Health Emergency of International Concern in response to the association of ZIKV infection with an increased frequency of infants born with microcephaly and cases of Guillain-Barre syndrome in adults. In response to this declaration and informed by significant experience with related flaviviruses, vaccine development for ZIKV proceeded quickly. We developed DNA vaccines encoding two ZIKV structural proteins sufficient for producing non-infectious virus-like particles. Both DNA vaccines were evaluated in phase I clinical trials, and the most promising candidate was studied further in a phase II trial conducted at sites throughout the Americas. To identify vaccine-elicited correlates of protection, we conducted vaccine dose de-escalation studies in animals. These studies revealed neutralizing antibodies as an imperfect correlate of vaccine-elicited protection and identified qualitative characteristics of the neutralizing antibody response critical for protection. Furthermore, we demonstrated that virus-like particles inefficiently elicit the most protective antibodies when compared to natural infection. To address these limitations, we investigated features of ZIKV structural proteins that define the antigenic structure of virus-like particle vaccine antigens and strategies to manipulate these antigens to elicit the most protective responses. A more complete understanding of the interplay between flavivirus antibody repertoires, immunogenicity, and viral antigen structures may accelerate vaccine development to the degree required to impact short-lived but explosive arbovirus epidemics.

Session 6: Emerging and reemerging flaviviruses, vector transmission and biology
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Plenary talk: Flavivirus-vector interactions

Greg Ebel
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Arthropod-borne flaviviruses perpetuate in nature in transmission cycles that require alternating replication in vertebrates and arthropods. In recent history, but going back several hundred years, these agents have emerged as significant global pathogens. This seminar will review the biology of the virus-vector interactions with a particular focus on the West Nile virus (WNV) – mosquito interaction. In addition to discussing foundational concepts in the biology of this interaction, recent research into the evolutionary implications of this interaction, which differ significantly from those that operate in the vertebrate-virus interaction, also will be discussed. Finally, research into the impact of climate change on the evolutionary biology of viruses within mosquitoes will be presented.

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P23 – Treating Dengue infections in vivo using mRNA encoded Cas13

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Background
Dengue is currently one of the world's most important neglected tropical diseases, and its incidence has increased dramatically in recent decades due to the geographical expansion of the *Aedes* mosquito. Transmission of dengue viruses (DENV) occurs in many parts of the world, with new cases occurring and spreading to non-endemic areas in the United States and Europe. Even with the discovery of small molecules against specific flaviviruses, there is a clear need for drugs that have efficacy against multiple DENV serotypes, have resistance to viral adaptation and mutation, and have limited cytotoxicity.

Methods
We envisioned a drug that consists of a lipid nanoparticle (LNP) that encapsulates LbuCas13a-encoding mRNA along with a crRNA guide targeting the DENV genome. Intravenous injection of the LNP delivers the mRNA and guide to infected cells in the liver, spleen, and circulating macrophages. To evaluate our approach, we performed *in vitro* experiments, including qPCR, RNAseq, plaque assay and immunofluorescence, and *in vivo* challenge studies using DENV 2 and DENV 3 serotypes.

Results
In the *in vitro* studies, we demonstrated significantly reduced intracellular viral RNA levels due to Cas13 activity, a reduced antiviral innate response, and no significant off-target effects. Critically, we showed that an LNP-formulated Cas13 mRNA-guide RNA drug protected mice in both DENV 2 and DENV 3 lethal challenge models, providing the basis for the development of a pan-DENV mRNA-based Cas13 drug.

Conclusions
Our results demonstrate that the mRNA-expressed Cas13 approach is very potent *in vitro* and *in vivo* in lethal mouse models of DENV 2 and 3 infection using only one dose of 2 mg/kg at 24 hours after infection. This level of therapeutic efficacy against DENV infection has rarely been demonstrated in the field.

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P113 – **Genetic and Functional Characterization of the Membrane region of the Pestiviral NS2 Protein**

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Background
The pestivirus bovine viral diarrhea virus (BVDV) belongs to the family *Flaviviridae*. Members of the *Flaviviridae* family use non-structural (NS) proteins in both RNA genome replication and virus morphogenesis. The pestiviral NS2 protein also serves multiple roles within the BVDV life cycle. With its autoprotease activity the NS2 protein mediates the release of NS3 that is essential for pestiviral RNA replication, whereas uncleaved NS2-3 is indispensable for producing viral progeny. Accordingly, the extent of NS2-3 cleavage is temporally regulating the switch from RNA replication to virion morphogenesis. Because of its central role in the pestiviral life cycle a detailed knowledge of the functional properties of pestiviral NS2 and NS2-3 is mandatory. Recently, we determined the membrane topology of BVDV-1 strain NCP7 NS2 and fine-mapped its autoprotease domain. This knowledge allowed a membrane-topology-based mutational analysis for the functional characterization of the BVDV NS2 membrane region.

Methods
A comprehensive mutagenesis screen of NS2 block mutations in the loop-regions and single amino acid mutations in the transmembrane segments (TMs) residues was conducted. Mutations were analyzed for their impact on NS2-3 cleavage, RNA replication and infectious virus production.

Results
Revers-genetic analysis revealed that none of the NS2 mutations inhibited NS2-3 processing. Interestingly, several of the mutations affected RNA replication, indicating that the N-terminal membrane region of NS2 can affect replicase assembly without interfering with the release of NS3, pointing to a role for NS2 in modulating replicase assembly. Furthermore, fine mapping identified several critical NS2 residues that, when mutated, yielded a specific defect in infectious virus production. Passaging these mutant viruses led to rescue of infectious viruses. These rescued viruses are currently further analyzed to identify potential second-site mutations and viral NS2 interaction partners.

Conclusions
Our report reinforces the hypothesis that NS2 acts as a central player in virus assembly, most likely by promoting key interactions between viral nonstructural and structural proteins as well as by regulating critical virus-host interactions. We now have evidence that certain NS2 mutations can specifically influence particle formation and infectivity. Further mechanistic dissection of these mutant phenotypes is now needed to tease apart the wide complement of NS2 interactions that are required for infectious BVDV production.

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P112 – **A viral footprint provides clues on how dengue virus escapes the acid bubble**

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Background & Aims
Due to their highly error prone replication, the flaviviruses form genetically highly heterogeneously populations of quasispecies. This tremendous genetic heterogeneity enables flaviviruses to rapidly adapt to distantly related hosts or different cellular environment during transmission by expanding the preexisting genetic variant which is more fit in a particular host environment. In this study, we sought to adapt dengue virus (DENV) to robust infection of lowly permissive human keratinocyte (HaCaT) cells – a physiologically relevant cell type during early infection events. By studying how adaptive mutations enhance the infectivity, we aim to elucidate the interplay between virus and host factors in this specific cellular environment and help complete the understanding of DENV or flavivirus molecular virology.

Methods
We serially passaged a genetically defined DENV4 virus in HaCaT cells resulting in adapted genomes with significantly increased infectivity. Using an amplicon-based viral whole genome sequencing, we identified several mutations including a nonsynonymous mutation in DENV envelope (E) protein. To test whether these mutations were causative for the observed infection enhancement in HaCaT cells, we introduced individual mutations into the parental genome. To enable analysis of the putatively altered interactome due to the adaptive mutations we constructed replication competent epitope-tagged DENV4 genomes.

Results
We confirmed that DENV4 harboring a specific mutation in the E protein resulted in significantly increases in viral RNA, the frequencies of viral antigen-bearing cels and viral titers in HaCaT cells. The E mutation did not impact negatively the high replicative capacity of the parental genome in Huh7 hepatoma cells. Introducing the E mutation into infectious clones of three phylogenetically closely related DENV 4 strains increased their infectivity specifically in HaCaT cells but did not enhance infection of two other DENV4 strains or selected DENV1, 2 and 3 genomes. We further determined that this mutation increased viral RNA copy numbers very early during infection but did not affect viral binding, early steps of viral entry and genome replication, suggesting that this mutation enhances the viral infectivity by boosting a late entry step.

Conclusions
We have identified a mutation in DENV E protein that can increase the infectivity of a group of DENV4 strains specifically in HaCaT cells but not Huh7 cells, indicating a change of virus-host interaction might be responsible for the enhancement. Our preliminary data suggest that this mutation might overcome a bottle neck at a late entry step. Future experiments using epitope-tagged viruses focus on identifying putatively altered interactions between the mutant E proteins and cellular factors using IP/MS. Collectively, results from our work will help to increase the understanding of DENV and conceivably even more generally flavivirus molecular virology and highlight new targets for therapeutic intervention.

Session 6: Emerging and reemerging flaviviruses, vector transmission and biology
October 4, 2023

P48 – **Two is better than one: exploring the helicase and capping interfaces between NS3 and NS5 in flavivirus infection**

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Background
Interactions between the viral NS3 (helicase, protease) and NS5 (RNA-dependent RNA polymerase [RdRp], methyltransferase [MTase]) proteins are critical for synthesis of new positive (+)-strands from the dsRNA intermediate generated during viral negative (-)-strand RNA synthesis. To processively unwind the 11 kb dsRNA, the $NS3_{Hel}$ requires direct interaction with NS5. NS3 then feeds the newly single-stranded (-)-strand into the $NS5_{RdRp}$ active site. Together, NS3 and NS5 also possess all the enzymatic activities required to generate the 5´ type I cap on newly synthesized (+)-strands. Existing models of the interactions between NS3 and NS5 either do not account for the function of these proteins during (+)-strand synthesis or are not based on experimental data.

Methods
We sought to model the interactions of NS3 and NS5 during (+)-strand RNA synthesis, and explore the model(s) experimentally. Using Zika virus (ZIKV) as a case study, Alphafold-Multimer and molecular dynamics simulations were used to model putative interactions between NS3 and NS5. Models of NS3-NS5 interactions were then integrated with existing solved structures for ZIKV NS5 monomers and multimers. Using previous studies in Yellow Fever virus and Kunjin virus, we are developing an NS3 helicase *trans*-complementation system to assess the roles of the identified NS3-NS5 interaction interfaces in (+)-strand RNA synthesis.

Results
Our modelling suggests that the base unit of the (+)-strand RNA synthesis complex in ZIKV involves two NS5-NS3 heterotrimers, with a central NS5 dimer supported by four NS3 monomers each tethered to the ER membrane of the viral replication organelle by NS2B and NS4B. Excitingly, each heterotrimeric unit involves two novel NS3-NS5 interaction interfaces. The first, termed $NS5_{RdRp}$-$NS3_{Hel}$, feeds the (-) strand directly from the NS3 helicase into the ssRNA entry channel of the NS5 RdRp. Meanwhile, the second, termed $NS5_{MTase}$-$NS3_{Hel}$ aligns a putative RNA binding channel on NS3, containing residues previously implicated in NS3-NS5 interactions, with the NS5 MTase active site. Our NS3 *trans*-complementation system will allow us to test the importance of each of these interfaces in (+)-strand synthesis and capping during ZIKV replication.

Conclusions
Our data predicts a novel protein complex that would serve as the basic unit of (+)-strand synthesis in ZIKV. This putative complex contains two novel interfaces between the viral NS3 and NS5 proteins that may provide the structural basis for (+)-strand RNA synthesis and capping. Furthermore, these models suggest that capping and synthesis of new (+)-strands may be tightly coupled processes in replication organelles. These complexes and their roles in the viral life cycle may be applicable across the *Flaviviridae* and in other (+)-strand RNA viruses.

Session 7: Correlates of protective immunity
October 4, 2023

Epigenetic scars in regulatory T cells are retained after successful treatment of chronic hepatitis C with direct-acting antivirals

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Background
During chronic hepatitis C virus (HCV) infection, many compartments of the immune system undergo quantitative and qualitative alterations associated with chronic inflammation. Despite having nearly 100% cure rate, whether the altered immune system is normalized after viral clearance after direct-acting antiviral (DAA) treatment is still controversial. In the present study, we conducted longitudinal study examining the quantitative and qualitative alteration of regulatory T (TREG) cells from patients with chronic HCV-infection, before, during and after DAA treatment using multi-omics, including transcriptomic, epigenomic, and flow cytometry analysis.

Methods
Patients with chronic genotype 1b HCV infection who achieved sustained virologic response (SVR) by DAA treatment (n = 11, treated with daclatasvir/asunaprevir for 24 weeks; n = 4, treated with sofosbuvir/ledipasvir for 12 weeks) and healthy donors (n = 12) were recruited. Peripheral blood mononuclear cells (PBMCs) were longitudinally obtained before treatment, during treatment (week 8 and week 16 for the daclatasvir/asunaprevir-treated group; week 8 and week 12 for the sofosbuvir/ledipasvir-treated group), and 12 weeks after achieving sustained virologic response (SVR). Phenotypic characteristics of TREG cells were longitudinally investigated through flow cytometry analysis both ex vivo and after in vitro stimulation with anti-CD3/CD28 antibodies. In addition, transcriptomic and epigenetic landscape of TREG cells from patients before treatment and after SVR and from healthy donors were analyzed using RNA-seq and ATAC-seq analysis.

Results
We identified the elevated frequency of peripheral TREG cells, especially activated TREG cells, in patients with chronic HCV infection compared to healthy donors. Transcriptomic analysis revealed that TREG cell population from patients with chronic HCV infection showed uniquely altered features that are sustained even after viral clearance following DAA treatment, distinguishing them from healthy donors. These altered characteristics of TREG cell population were featured with immune activation and TH17-related genes. In addition, epigenetic analysis showed increased and retained chromatin accessibility of genes related to immune activation from the patient both before treatment and after SVR, compared to healthy donors. Furthermore, we identified that the expanded TREG cell population from the patients had an enhanced capability to secrete inflammatory cytokine TNF, upon anti-CD3/CD28 stimulation, and this increased capacity sustained after SVR. In addition, these TNF secreting TREG cells exhibited activated phenotype, which were maintained even after viral elimination.

Conclusions
In summary, our data showed that the expanded TREG cell population during chronic HCV infection acquires sustained inflammatory features at phenotypic, transcriptional and epigenetic levels, even after successful viral clearance. Further studies are required to investigate the clinical significance of sustained inflammatory features in the TREG cell population after recovery from chronic HCV infection.

Session 7: Correlates of protective immunity
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P62 – **Analysis of circulating and intrahepatic CD4+ T cell response in acute resolving HCV infection.**

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Background
CD4+ T cells are essential to prevent hepatitis C virus persistence but remain poorly characterized. Surrogate markers are needed to visualize and enrich HCV-specific CD4+ T cells. CD4+ T cells activated by virus infection typically express PD-1 (a co-inhibitory receptor) and ICOS (a co-stimulatory receptor). Here, PD-1 and ICOS co-expression was evaluated for identification and enrichment of circulating and intrahepatic HCV-specific CD4+ T cells from chimpanzees during acute HCV infection. Our objective was to define the transcriptional, functional, and phenotypic profile of protective CD4+ T cells relevant to vaccine development.

Methods
We studied cryopreserved mononuclear cells from the peripheral blood (PBMC) and liver (LMC) of 8 chimpanzees that spontaneously cleared acute HCV infection over 1 year of follow-up. CD4+ T cells with a PD-1^{hi}ICOS^{hi} phenotype were assessed for HCV specificity by class II tetramer staining, intracellular cytokine staining (ICS), and TcR repertoire analysis. Transcriptional activity of circulating PD-1^{hi}ICOS^{hi} CD4+ T cells that were predominately HCV-specific was evaluated by RNA sequencing. Immunostaining was undertaken to confirm expression of Th lineage defining chemokines, chemokine receptors, cytokines, and transcription factors by PD-1^{hi}ICOS^{hi} CD4+ T cells from blood and liver.

Results
CD4+ T cells with a PD-1^{hi}ICOS^{hi} phenotype were detected in blood several weeks after HCV infection. The peak response was transient but significantly associated with peak ALT, seroconversion, and initial control of HCV replication. The CD4+ T cells were predominantly HCV-specific as assessed by tetramer and intracellular cytokine staining along with TcR repertoire analysis. Bystander activation of CD4+ T cells targeting other viruses was not observed. Significant differential expression of Tfh and Th1 associated genes was detected in PD-1^{hi}ICOS^{hi} CD4+ T cells that were sorted from blood at the peak of the response. A Tfh1 subset assignment was confirmed by immunostaining for lineage defining cytokines, chemokines, and transcription factors. Significant enrichment of HCV-specific PD-1^{hi}ICOS^{hi} CD4+ T cells with a Tfh1 functional profile was observed in liver at the point of initial virus control. Most (>80%) circulating PD-1^{hi}ICOS^{hi} CD4+ T cells did not express CXCR5 despite a clear Tfh lineage relationship defined by expression of IL-21 and CXCL13. Instead, they expressed multiple chemokine receptors (CXCR3, CXCR6, CCR5, CCR2) that direct T cells to inflamed tissues like the liver.

Conclusions
PD-1^{hi}ICOS^{hi} CD4+ T cells that expand in blood and liver during acute HCV infection are predominately HCV-specific and belong to the Tfh1 lineage by transcriptional, phenotypic, and functional analysis. The majority did not express CXCR5 and instead were similar to tissue-infiltrating CXCR5-negative Tfh recently described in autoimmune disease and cancer. Identification of these protective multi-functional CD4+ T cells in blood and liver is relevant to HCV vaccine development.

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P20 – **Memory B cell responses in chronic hepatitis C patients following direct-acting antiviral treatment**

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Background and Aims
Memory B cells (MBCs) are critical for the rapid recall of protective immunity against re-infection, although their role in HCV re-infection remains uncertain. HCV infects ~1% of the world population, predisposing the infected individuals to an increased risk of liver cirrhosis and liver cancer. Highly effective direct-acting antivirals (DAAs) can cure over 95% chronic HCV patients, yet reinfections have been reported in individuals who have cleared a previous infection, either spontaneously or post-therapy. These observations underscore that immune memory to HCV, acquired through infection, is not sufficiently effective against reinfection. To guide the development of a vaccine and the public health strategy to protect high-risk populations, understanding immune memory to HCV in DAA-cured patients is critical. Several studies have identified functionally and phenotypically altered T cells in chronic HCV patients, which are not fully restored after DAA-mediated virus clearance. However, a detailed analysis of antibody and B cell responses, particularly those targeting conserved viral epitopes, is still lacking despite their apparent importance in recalled immunity. This study explores how human memory B cell response is altered by chronic infection and its recovery in chronic HCV patients undergoing DAA treatment.

Methods
We profiled MBC responses against the E1E2 glycoprotein complex in 24 chronic HCV patients undergoing DAA treatment and compared the responses to spike-specific MBC responses in 16 normal blood donors (NBDs) after SARS-CoV-2 vaccination. High-dimensional flow cytometry and single-cell RNA sequencing were utilized to characterize the phenotypic, transcriptional and B cell receptor (BCR) repertoire features of antigen-specific MBCs. Corresponding monoclonal antibodies are generated for functional and structural analysis.

Results
Antigen-specific MBCs from both pre- and post-DAA-treated chronic HCV patients display comparable phenotypic and transcriptomic profiles, distinct from those observed in NBDs. These cells harbor a notably higher proportion of unswitched and IgM^+ only MBCs, comprising heterogeneous populations of atypical MBCs (atMBCs). The isotype switched MBCs are mostly IgG1 and exhibit a convergent VH1-69 gene usage. The VH1-69 MBCs, representing the main nAb-producing cells in HCV patients, target a previously undefined epitope on the E1E2 complex, in addition to the conserved neutralizing face on E2. The VH1-69 MBCs decline while atMBCs persist as infection resolves, corresponding to compromised nAb responses in the blood.

Conclusions
E1E2-specific MBCs are phenotypically, transcriptionally, and functionally altered by chronic HCV infection, in comparison to acute infection. As reported recently, MBCs persist but DAA-mediated cure does not fully reverse the alternations in some subsets. Understanding the immune checkpoints and transcriptional control of antigen-specific MBCs will help evaluate how these cells may subsequently impact immune responses to reinfection and vaccination in the convalescent individuals.

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P74 – **Activation of CD4 T follicular helper cells correlates with B cell expansion and neutralizing antibodies during HCV reinfection and clearance**

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Background
Hepatitis C virus (HCV) infection resolves spontaneously in 25% of the cases and generates long-lived immune memory responses. People who inject drugs (PWID) remain at high risk of HCV reinfection post spontaneous clearance and/or cure and represent a natural rechallenge experiment to identify correlates of long-term protective immunity against HCV. We have previously demonstrated that during primary HCV infection, activation of circulating CD4 T follicular helper (cTfh) cells occurred at an early stage in spontaneous resolvers but not in those who developed chronic infection. This was associated with expansion of HCV-specific B cells. During reinfection, we and others have also demonstrated that clearance was associated with increased breadth and magnitude of HCV-specific T cells, and the appearance of neutralizing antibodies (nAbs). However, the role of the cTfh population during reinfection and its cognate interaction with memory B cells (MBCs) to generate nAbs remain poorly understood. Herein, we aimed to evaluate the interactions between cTfh cells, B cells and nAbs during reinfection in PWID who cleared two successive episodes of HCV infection (SR) and in those that spontaneously cleared one infection but failed to clear a subsequent one (CI).

Methods
We used longitudinal samples of PBMCs and plasma from PWID (n = 23) with documented HCV reinfection recruited from the Montreal Hepatitis C cohort. The following time points were studied: early acute (≤ 3 months after estimated date of infection (EDI)), late acute (3 to 7 months after EDI) and follow-up (> 7 months after EDI). We characterized CD4 T cells and HCV E2 glycoprotein-specific MBCs using flow cytometry. We monitored HCV antibodies by ELISA and neutralization assays using a panel of HCV pseudoparticles (HCVpp) [3].

Results
We observed an early expansion of activated cTfh ($CD3^+CD4^+CXCR5^+PD1^+ICOS^+FoxP3^-$) in SR patients (n = 14). More specifically, the activated cTfh1 subset ($CXCR3^+CCR6^-$) correlated with neutralization breadth (r = 0.8669, p = 0.0286, Spearman) and potency (r = 0.7143, p = 0.0881, Spearman) of antibodies at the early acute stage of reinfection. We also observed higher frequencies of HCV-specific MBCs ($CD19^+CD27^+IgM^-$E2 Tet^+) at early acute time points in these patients. In contrast, in CI patients (n = 9), frequencies of HCV-specific MBCs and nAbs peaked at the follow-up stage of the reinfection once persistent infection was established.

Conclusions
These results suggest that the early expansion of the activated cTfh1 subset is key to the development of an effective nAb response that may contribute to clearance of reinfection.

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