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POSTER ABSTRACT BOOKLET

Poster abstracts listed in blue
Poster abstracts with presentations listed in green

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.

P02 - **Hepatocellular organelle and gene expression abnormalities following elimination of hepatitis C virus and carcinogenesis**

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Background
Direct-acting antivirals (DAAs) have been successful in achieving a sustained virologic response (SVR) in almost all chronic hepatitis C (CHC) patients. However, the development of hepatocellular carcinoma (HCC) in SVR patients is a significant global health concern. This study aimed to investigate organelle and gene expression abnormalities in the liver of SVR patients, that may be associated to pathogenesis and carcinogenesis after achieving SVR.

Methods
Liver biopsy specimens of SVR patients were analyzed using RT-PCR to detect viral genomes. Ultrastructure findings of hepatocytes in liver biopsy specimens of CHC and SVR patients were semi-quantitatively assessed using transmission electron microscopy and compared with those of Huh-7 cell and mouse models. Next-generation sequencing technology was used to analyze alterations of gene expression in SVR patients, Huh-7 cells, and mouse models.

Results
Hepatitis C virus (HCV) RNA was detected in 5 out of 88 liver samples of SVR patients. However, only 3 of the 49 post-SVR HCC samples showed the presence of HCV RNA, indicating that HCV is not necessary for the development of post-SVR HCC. Various organellar alterations including nuclei (Nu), mitochondria (Mt), endoplasmic reticulum (ER), lipid droplets (LD), and pericellular fibrosis, were observed in the hepatocytes of CHC patients. These findings were similar to those observed in HCV-infected cells and hepatocytes of human liver chimeric mice. DAA treatment eliminated these morphological abnormalities in HCV-infected cells. In contrast, SVR patients significantly improved Nu, Mt, and LD abnormalities, but not ER abnormalities and pericellular fibrosis. Moreover, Mt malformation and pericellular fibrosis were exacerbated in cured chimeric mice. Additionally, when comparing two groups of SVR patients divided into hypofibrosis and hyperfibrosis group, no significant difference in organellar abnormalities was observed. However, a significant difference was noted in ER and Mt between the group within 1 year and the group over 1 year after SVR. Interestingly, swollen/degranulated ER was associated with hepatocarcinogenesis in patients more than 1 year after SVR. Furthermore, RNA-seq analysis of liver tissue of SVR patients revealed elevated expression of genes associated with HCV protein-mediated carcinogenesis, anti-apoptosis, inflammation, innate immune response, and cell migration in HCC patients compared to non-HCC patients after SVR. Similar changes were observed in SVR chimeric mice, but these changes were eliminated in cured cells.

Conclusions
This study demonstrated that the persistence of abnormalities in organelle morphology and gene expression caused by HCV infection in the liver tissue of SVR patients, which could be referred to as post-SVR syndrome. It was particularly suggested that virulence factors such as persistent oxidative/ER stress may contribute to carcinogenesis. Long-term follow-up of patients is recommended after achieving SVR.

P03 - **Establishment of infectious genotype 5 HCVcc**

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Background
The development of the HCV replicon and infectious HCVcc system has greatly contributed the development of anti-HCV drugs. In particular, the establishment of JFH-1 strain, genotype 2a HCV, enabled detailed analysis of entire HCV life cycle in cultured cells, including HCV entry, replication, particle formation, and discovery of targeting sites of anti-HCV drugs. We have further established infectious HCV strains of another genotype 2a (JFH-2 strain), genotype 3a (S310 strain), and genotype 4a (ED43 strain), which enables further analysis such as comparative pathogenesis between different genotypes, susceptibility analysis of antiviral drugs, evaluation of neutralizing antibody efficacy, and comparison of drug resistance mutations. However, it should be noted that not all genotypes HCV culture systems have been established, and further progress in comparative analysis among genotypes is expected as more genotypes are established. Therefore, in this study, we attempted to establish an infectious genotype 5 HCVcc.

Methods
We generated SRSI (S1370R/S2205I) and EGSRSI (E1203G/S1370R/S2205I), based on full-length SA13 strain. Huh7 cells were transfected with synthesized RNA and cultured for a long period. After gene transfer, the amount of secreted virus core proteins in the culture supernatant was measured continuously. Common amino acid mutations were identified in each mutant after the viral load reached its peak. We generated additional SA13 mutants by combining four amino acid mutations. The effects of these amino acid mutations for virus life cycle were analyzed by measuring subsequent viral production after RNA transfection into Huh7 cells.

Results
We measured the viral load after transfection of two mutant RNAs. In the SRSI mutant, viral production gradually increased, while in the EGSRSI mutant, it initially decreased below the detection sensitivity and then increased after 80 days. Both mutants reached their peak viral load after 100 days. Numerous nucleotide mutations were detected from the gene sequences of each mutant at the peak. Next, we tried to increase the proportion of highly infectious clones by repeating the infection of each mutant to naive cells three consecutive times. We analyzed the virus gene sequences after infection and identified amino acid mutations. By focusing on four mutations and using the SRSI and EGSRSI mutations as a base, we generated six SA13 mutants. When we measured the viral load after RNA transfection, all six mutants reached their peak after 50 days. Additionally, the mutant carrying the E1203G mutation showed lower initial viral production after RNA transfection.

Conclusions
SA13 with three mutations (Y901C, S1370R, E1727G) showed high viral production among the six mutants. This result suggests these mutations could enhance SA13 production. We will further analyze the other mutations and aim to establish SA13 strain with even higher viral production.

P04 – **Evaluation of Hepatitis C virus Transmitted/Founder variants obtained from observed HCV infection through lung transplantation from HCV-infected donors to uninfected recipients**

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Background
In contrast to the large diversity seen in chronic hepatitis C virus (HCV) infection, samples taken from acute HCV infection have shown only a small number of viral lineages, suggesting the presence of a limited number of Transmitted/Founder (T/F) variants that are able to efficiently expand and establish infection in a new host. Characterization of T/F variants in HCV infection has been limited due to the difficulty of obtaining samples from early HCV infection and in identification of the donor virus. Using samples from lung transplant recipients who received organs from HCV-infected donors, we evaluated the presence of T/F variants in observed HCV infection.

Methods
The HCV genomes of five donor and recipient pairs were amplified. Using Illumina MiSeq, 250bp reads we obtained the sequences of donors and their respective recipients from day 7 post-transplant. ShoRAH, a bioinformatics tool used for haplotype reconstruction, was then used to identify haplotypes in the Core-E2 region and estimate their frequencies. Focusing on HVR1 in E2,we used maximum likelihood phylogenetic trees (PhyML) to identify founders and non-founders and cross-referenced identified non-founder sequence with previously reported functional analyses of HVR1.

Results
Three out of five donor/recipient pairs showed donor variants in HVR1 that were not transmitted - non founders - to recipient (DA/RA, DC/RC and DF/RF) while in two other pairs (DB/RB and DE/RE) all variants identified in the donor were detected in the recipient. In the non-founder variants in 2 donors (DA and DC) a non-polar residue (Q) was found in positions 3 or 11 compared to transmitted founders which possessed a basic residue R/H. Consistently in DB/RB and DE/RE all HVR1 variants exclusively had basic residues in at least one of these positions 3,11 and 25. Previous reports have indicated that HVR1 position 3,11 and 25 were conserved for being basic residues which contribute to more efficient entry via interaction with negatively charged host cell receptors such as LDL and SR-BI, while switching these residues with either a Q or a Y showed lower entry efficiency in liver cells.

Conclusion
Our preliminary data show that at the time of HCV transmission in organ transplant patients, we identified non-transmitted variants which possessed non-polar residues in HVR1 in positions 3 or 11 compared to transmitted founder variants which possessed basic residues in at least 1 of these positions. These sites in HVR1, in addition to position 25, are known to be occupied by basic residues to ensure efficient interaction with cell receptors and viral entry. This suggests selection of founder variants for their enhanced infectivity and absence of non-founder variants that may be less fit at establishing infection. More analysis is in progress to track founder evolution in longitudinal recipient samples and assess functional characteristics in vitro.

P05 – **A rapid point-of-care assay for HCV RNA detection using CRISPR-Cas12b**

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Background
The diagnosis of HCV infection involves serological assays that detect the antibodies to HCV and molecular assays that detect the presence of HCV RNA. In both high and low/middle income countries, the requirement for two tests for HCV diagnosis leads to major drop-offs in the cascade of care. Existing near-care HCV RNA tests are expensive and inaccessible in most regions. We aim to develop a cost-effective point-of-care test (POCT) for detection of HCV RNA to enhance screening, particularly in marginalized populations and for people living in remote locations.

Methods
We developed a novel assay for HCV RNA detection from plasma or whole blood. After an initial lysis step, the viral RNA is reverse transcribed and amplified using loop mediated isothermal amplification (RT-LAMP) with HCV specific primers. Finally, a CRISPR-Cas12b based detection of the amplified target HCV DNA with a fluorescence readout is carried out.

Results
We have successfully detected HCV RNA from patient samples for HCV genotypes 1a, 1b, 2, 3a and 4. HCV RNA above 1,000 IU/mL can be reliably detected within 40 minutes total processing time and the sensitivity can be improved to a lower limit of detection of 100 IU/mL with extension to 50 minutes.

Conclusions
Rapid diagnosis of HCV is critical to achieving HCV elimination goals. Our POCT shows high specificity and sensitivity to detect HCV RNA directly from patient blood samples within 50 mins, and hence could be utilized for efficient diagnosis of HCV infection globally.

P06 – **Structural studies of broadly neutralizing antibody-HCV E2 complexes**

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Background
Broadly neutralizing antibodies (bnAbs), isolated from patients infected with the hepatitis C virus, target the E1E2 envelope glycoprotein complex, making them a major focus of vaccine design studies. Over 20 crystal and cryoEM structures of bnAbs in complex with E2 or E1E2 constructs have revealed that the bnAbs target the E2 front layer region of the CD81 receptor binding site and block virus-receptor binding. All of the bnAbs structurally characterized thus far have heavy chains derived from the human IGHV1-69 or closely related macaque KIMDB IGHV1-138*01 genes, and typically bind to E2 with hydrophobic residues from the complementarity determining region (CDR) H2 or CDR H3 bound in or near a pocket located within the E2 front layer. Our recent crystal structure of the human bnAb HC84-1 in complex with an E2 core construct reveals a novel mode of antibody-E2 recognition, with an unusual Fab approach angle and a more open front layer conformation coupled with the extension of helix α1. This structure represents the second bnAb-E2 with an outlier front layer conformation, indicating that the E2 envelope protein may undergo conformational changes in this region during the viral life-cycle.

Methods
Recombinant Fab HC84-1 and HK6a E2mC3 core were both produced in mammalian expi293 cells. Their complex was crystallized, x-ray diffraction data were collected to 2.45Å and the structure was determined by the molecular replacement method. The structure was aligned with other previously determined bnAb-E2 and CD81-E2 structures available in the Protein Data Bank for comparison and analysis.

Results
Fab HC84-1 recognizes a novel conformation for the E2 front layer region, where helix α1 (usually 4 helical residues, 439-442) is extended to contain 11 helical residues (432-442) that shift with respect to strand 424-430 to increase the angle between these two secondary structural features and enlarge the pocket found near their apex. Two Fab residues (Thr53, Phe54) at the tip of CDR H2 bind into the enlarged pocket, with somatically mutated Thr53 forming a hydrogen bond to back layer residue Tyr613 that is accessible due to the front layer conformational change. CDR H3 then binds between the C-terminal end of helix α1 and the back layer due to the unusual overall approach angle of Fab HC84-1 to E2.

Conclusions
This crystal structure enhances our understanding of the various ways that IgHV1-69 derived bnAbs can recognize the HCV E2 envelope protein. Despite their shared germline gene usage, the antibodies can bind E2 with different angles of approach and different CDR placement. The unusual front layer conformations seen here for E2 bound to HC84-1 and also for the previously characterized E2-Fab 212.1.1 structure suggest that the E2 front layer may undergo conformational changes during infection.

P07 – **Investigate interaction between DENV NS5 and the 5' SLA and the 3' SL RNA in regulating the replication of the virus**

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In positive-strand RNA viruses, replication and translation are two mutually exclusive processes because the viral genome serves as the template for the synthesis of both viral genome and viral proteins. A common strategy employed by these viruses to regulate replication and translation is through a conformational switch of the viral genome. Dengue virus (DENV), a positive-strand RNA virus belonging to flaviviruses, has been suggested to coordinate its genome conformational switch by interaction with the viral RNA polymerases, NS5. The linear conformation of the DENV viral genome is suggested to support translation, while its circular conformation of the genome supports replication. The interaction of NS5 with the RNA element stem-loop A (SLA) at the 5' end of the viral genome blocks ribosome scanning at the 5' end, thereby disrupting translation. This activity of NS5, in conjunction with genome circularization, enhances replication. The 5' SLA is also known as replication promoter of the virus that is recognized by NS5. However, to replicate the viral genome, NS5 needs to be transferred to the 3' end of the genome. NS5 interacts with the 3' end stem-loop (SL) of the viral genome. The 3' SL element can adopt two conformations: a linear stem-loop present in the linear conformation of the viral genome or two shorter stem-loop conformations present in the circular conformation of the viral genome. To understand which conformation of the 3' SL can support replication and how NS5 interacts with the 5' SLA promoter and 3' SL, we investigated the interaction between DENV NS5 and the 5' SLA and 3' SL RNA using biochemical and biophysical techniques. We show that NS5 binds more strongly to the 5' end SLA compared to its binding to the 3' SL RNA, which contrasts with previous reports that NS5 has higher affinity for the 3' end SL RNA. *In-vitro* transcription experiments revealed that the linear conformation of the 3' SL cannot initiate transcription, whereas its circular form of the 3' SL can. This finding aligns with previous studies indicating that the linear form of the 3' SL, found in the linear conformation of the genome, supports translation, while the circular form of the 3' SL, present in the circular genome, supports replication. Moreover, while the linear form of the 3' end SL RNA is unable to compete with the 5' SLA RNA for binding with NS5, the circular form of this RNA can. Further structural studies will be conducted to investigate the three-dimensional structure of these RNA elements and NS5 to elucidate the underlying mechanisms of these interactions.

P08 – **Small RNA annealing on a defined 3'UTR functional region influences the phenotypic (and structural) outcomes of Bovine Viral Diarrhea virus**

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Background
Bovine viral diarrhea virus (BVDV) is an economically significant virus amongst worldwide livestock populations. Calves persistently infected through transmission from infected mothers shed virus, causing outbreaks. BVDV is an enveloped, single-stranded, positive-sense RNA Pestivirus belonging to the *Flaviviridae* family. The genome consists of a polyprotein-encoding region flanked by structured untranslated regions (UTRs) that regulate viral processes. Two microRNA (miRNA) families, miR-17 and let-7, interact with the BVDV 3'UTR, promoting viral RNA accumulation. miR-17 binds within a single-stranded region near stem-loop 1 (SLI) and is required for efficient virus replication, whereas let-7 binds a canonical site in SLII and a non-canonical site overlapping the miR-17 binding. These miRNAs, with several synthetic small RNAs that anneal to the 3'UTR, were assessed for their abilities to promote BVDV, defining regions within the 3'UTR to which small RNA annealing influences the virus lifecycle. Correlations between viral replication, translation, genome stability, and RNA structures were analyzed to investigate the mechanism of lifecycle promotion.

Methods
Huh7.5 cells with a knockout in the Drosha- and Ago2-encoding genes were used, creating a near miRNA-null environment (that also lacks small interfering RNA (siRNA)-directed cleavage activity). *In vitro* transcribed BVDV RNA was transfected into these cells, along with the miRNAs and/or siRNAs being assayed. A monocistronic wildtype subgenomic Rluc-expressing replicon was used to measure replication, whereas a replication-defective variant was used to measure translation, both via luciferase activity. RNA stability was assayed via Northern blots. Selective 2' Hydroxyl Acylation analysed by Primer Extension (SHAPE) was used to determine 3'UTR RNA structural changes.

Results
BVDV replication was promoted efficiently by miR-17 but not let-7 when provided individually. siRNAs designed to anneal to multiple regions within the 3'UTR mapped regions to which small RNA binding promotes the viral lifecycle: one region was identified adjacent the miR-17 binding site, and a secondary site overlapping the let-7 canonical binding site within SLII, confirming that annealing to both sites promote virus replication. While many of the siRNAs binding within these regions promoted replication, only those concentrated around the miR-17 binding site enhanced translation. Further, small RNA annealing showed no significant genome stabilization. Finally, predicted RNA structures generated from SHAPE data support a hypothesis that miRNA annealing promotes correct folding of the SLI.

Conclusions
Binding of miR-17 within the BVDV 3'UTR appears indispensable for viral fitness, showing promotion of both viral replication and translation. Conversely, let-7 binding showed no such promotion on its own. While the genome is not stabilized, the presence of pro-viral RNA structures induced by these small RNA binding events may explain their mechanisms. Those small RNAs which promoted viral fitness demonstrate correlations with key RNA structures, such as SLI, suggesting that stable formation of SLI may be required for efficient replication/translation.

P09 – **Qualitative analysis of Adenovirus and recombinant HCV E2 based immunization strategies**

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Background
Although direct acting antiviral drugs are available, an effective prophylactic vaccine to prevent HCV spread is still lacking. A successful vaccine targeting the HCV envelope should induce memory B cells, germinal center cells and T cells in addition to neutralizing antibodies. The main objective of the current study is to compare qualitative and quantitative cellular and antibody responses in mice using different vaccine platforms (viral vector and recombinant protein formulated with different adjuvants).

Methods
For prime-boost vaccination, BALB/c mice were immunized intramuscularly with Adenovirus expressing HCV envelope glycoprotein E2 (Ad-E2) or HCV E2 protein (rE2) adjuvanted with CPG ODN1826 and Alum or rE2 adjuvanted with AddaS03 at days 0 and 21. Spleens and serum samples were collected at 7, 14, and 49 days post-boost to evaluate the magnitude of cellular and antibody responses using flow cytometry, ELISA, and neutralization assays.

Results
Spleen cells from immunized mice were stimulated in vitro with peptide pools from HCV E2 and IFN-gamma, TNF-alpha and IL-2 expression were examined by intracellular staining. Ad5-E2 induced production of IFN-gamma, TNF-alpha and IL-2 by CD8+ and CD4+ T cells at day 7, 14 and 49 post-boost, with a reduced response at day 49. HCV rE2 combined with CpG/alum or
AddaS03 adjuvants induced only a weak or undetectable cytokine response in T cells at all time points. In addition, Ad5-E2 induced higher germinal center (GC) B cell and T follicular helper (Tfh) cell responses compared with HCV E2-CpG/Alum or AddaS03 adjuvated groups. HCV E2 specific antibody responses were detected in the serum of all three immunized groups. ELISA antibody titers were higher in the E2/CpG/Alum and E2/AddaS03 groups compared to the Ad5-E2 treated group at day 14 and 49 days post boost, although neutralizing antibody titers were similar at these time points.

Conclusion
In our preliminary studies, the intracellular expression of E2 using the Ad5-E2 prime-boost immunization induces a more complete immune response, with respect to CD4+, CD8+, GC, Tfh cells and antibody responses, compared to the recombinant- adjuvant strategy. In addition, Ad5-E2 seems to induce a better quality antibody response with a higher proportion of E2-specific antibodies displaying neutralizing capacity. Long-term follow-up is ongoing to assess the quality of memory responses in all groups.

P10 – **Understanding fundamental vaccinology via a hamster model of viral hemorrhagic fever**

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Background
Yellow fever virus (YFV) is transmitted by the bite of infected mosquitoes, which are endemic to parts of South America and sub-Saharan Africa. Humans infected with YFV develop viral hemorrhagic fever (VHF), although the mechanisms by which coagulation abnormalities develop in these patients remain poorly understood. A highly efficacious live-attenuated YFV vaccine (YFV-17D) was developed in the 1930s through sequential passage of a virulent YFV strain (YFV-Asibi) in chicken and mouse embryos, ultimately attenuating the virus. YFV-17D differs from YFV-Asibi by only 21 amino acids; however, the specific amino acid changes that resulted in 17D attenuation have never been mapped.

Methods
We developed a circular polymerase extension reaction (CPER) reverse-genetics system for YFV-Asibi and YFV-17D, which can be interchangeably combined to create a panel of Asibi/17D chimeric viruses. Using this system also enables us to introduce hamster-adapting (HA) mutations into these chimeric viruses, facilitating infection of the Syrian Golden hamster (*Mesocricetus auratus*). Developed by Alan Barrett's group, HA-YFV-Asibi is unique among small animal models of YFV infection, as mice and other rodents are highly resistant to developing VHF-like disease when inoculated with YFV.

Results
Hamsters infected with HA-YFV-Asibi rapidly developed VHF as measured by weight loss, the development of abnormal coagulation parameters, and the appearance of petechiae. Histopathologic analyses showed significant liver damage and virological assays showed high viral loads in the blood and liver. Introduction of the single-most-important hamster-adapting mutation into recombinant (r)HA-YFV-Asibi resulted in moderate disease as determined by the above parameters. We are currently in the process of introducing the remaining HA mutations to enable full pathogenesis of rHA-YFV-Asibi, which will then allow us to begin generating rHA-YFV-Asibi/17D chimeric viruses. Ultimately, the combination of these systems will allow us to map the determinants of YFV attenuation and, conversely, determinants of YFV attenuation.

Conclusions
Identifying the molecular determinants of YFV attenuation is a long-sought milestone in vaccinology. By combining a unique animal/disease model (the hamster) with our novel CPER reverse genetics system for YFV, our approach should enable mapping of these determinants while simultaneously shedding new light on the mechanistic drivers of VHF in YFV infection.

P11 – **A mouse model of pegivirus (PgV) infection**

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Background
Pegiviruses (PgVs) are +ssRNA viruses in the *Flaviviridae* family that infect a wide variety of mammals including humans; human (H)PgV persistently infects ~20% of the global human population. Although HPgV does not cause overt disease, our understanding of HPgV infection and its long-term impact on the host is extremely limited. Currently, no *in vitro* culture systems or small animal models exist for any PgV. Despite the abundance of PgVs in mammals, a mouse PgV has yet to be described. Thus, we sought to create a mouse model of PgV infection by adapting HPgV or pegivirus from a wild rat (RPgV) to infect laboratory mice.

Methods
We inoculated wild-type, IFNAGR-knockout mice, human STAT1 knock-in mice, and STAT1-knockout mice with HPgV+ serum (obtained from Jack Stapleton) or RPgV+ serum (obtained from Amit Kapoor), then assessed viremia via virus-specific RT-qPCR for 100 days post-inoculation (dpi).

Results
No HPgV replication was detected in mice. Low-level RPgV replication was sporadically detected in some wild-type and IFNAGR mice; however, all STAT1 knockout mice developed high-titer viremia by 60 dpi. When serum from these mice containing “mouse-adapted” (ma)PgV was used to inoculate wild-type mice, a highly-reproducible pattern of infection was observed, with peak viremia occurring between 12-15 dpi and persisting for >300 dpi. We are currently in the process of identifying the mutations that allowed RPgV to adapt to infect mice, and also examining the persistence and pathogensis of maPgV in various transgenic and immunocompromised mouse strains.

Conclusions
We develop the first small-animal model of PgV infection, opening up the toolkit of the mouse host for interrogating PgV biology. Future studies will focus on adaptive mutation that allow for adaptation to new hosts, selective pressures imparted by different immune-compromising host backgrounds, and mechanisms of PgV persistence.

P12 – **Hepatitis B immune globulins target epitopes on the hepatitis B virus surface antigen where clinical escape mutations emerge**

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Background
Hepatitis B Immune Globulin (HBIG) is approved for the indication of preventing hepatitis B recurrence in patients following orthotopic liver transplantation. HBIG contains high titers of neutralizing antibodies (anti-HBs) against the hepatitis B virus (HBV) surface antigen (HBsAg) that are derived from human plasma from naturally immunized or vaccinated donors. HBIG is effective in preventing HBV reinfection post-surgery, however, there have been a few breakthrough cases in transplant recipients who had undergone long-term high-dose HBIG therapy where the virus prevailed by mutating certain amino acids in its HBsAg. The mechanism by which these mutated viruses escape antibody-mediated neutralization is not yet fully understood.

Methods
We conducted a comprehensive analysis of the antibody binding sites on HBsAg and correlated them with emerging escape variants. We initially purified an enriched fraction of anti-HBs antibodies from HBIG lots and screened random peptide phage display libraries to identify antibody binders. Subsequently, we sequenced the DNA of the selected clones, deduced the amino acid sequences, and aligned them with the HBsAg protein sequence to identify anti-HBs epitopes. To confirm the critical binding amino acids at these epitopes, we synthesized biotinylated HBsAg peptides with single alanine substitutions or clinically relevant mutations. We then assessed the binding of anti-HBs antibodies to these peptides using ELISA.

Results
Our investigation identified five groups of peptides that corresponded to five distinct anti-HBs epitopes on HBsAg. Three binding sites, namely Site II (C121-C124), Site III (M133-P135), and Site IV (T140-G145), were located within the common “a” determinant found in all HBV genotypes. Two additional binding sites, Site I (Q101-M103) and Site V (I152-S154), were situated outside the “a” determinant. ELISA-based alanine mutation scanning analysis verified the key binding amino acids of these five sites. Furthermore, we conducted ELISA tests on HBsAg peptides containing clinically associated mutations reported at these sites, such as Y134S, P142S, and G145R. We observed a significant reduction in anti-HBs antibody binding activity for these HBsAg mutants.

Conclusions
Our results suggest that although the anti-HBs antibodies in HBIGs can effectively bind and neutralize HBV to prevent infections, the mutability of the HBsAg gene under the immune selective pressure of HBIG treatment may serve as a crucial resistance mechanism for the virus to evade anti-HBs-mediated neutralization. By allowing mutations to emerge strategically at the anti-HBs epitopes in HBsAg, HBV can reinfect a newly transplanted liver despite the prolonged presence and high doses of HBIG. We propose that the current HBIG treatment protocol for liver transplant patients be further improved by supplementing it with site-specific neutralizing monoclonal antibodies that could target these mutation-prone hotspots and help suppress emerging HBV variants.

Informal Communications Disclaimer: My comments are an informal communication and represent my own best judgement. These comments do not bind or obligate FDA.

P13 - **Enhancing antitumor activity by signaling pathway inhibitors and targeted CAR T cells against HCV associated hepatic tumor initiating cancer cells**

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Background
We evaluated the mechanism by which signaling inhibitors play a role in impeding hepatic tumor cell growth. Overexpression of Lin28 is detected in various cancers with involvement in the self-renewal process and cancer stem cell generation and has been associated with HCV protein expression. Glypican-3 (GPC3) is a membrane associated proteoglycan that is specifically up-regulated in hepatocellular carcinoma, but hardly detectable in adult liver, and could act as a target for HCC therapeutic intervention.

Methods
We examined Lin28 axis inhibitors and cancer antigen targeted glypican-3 (GPC3) chimeric antigen receptor (CAR T) cells to generate a strong inhibitory role for tumor-initiating cancer cells derived from human hepatocytes immortalized by HCV core using *in vitro* and *in vivo* experiments.

Results
HCC patient samples exhibited a positive correlation between indoleamine 2,3-dioxygenase-1 (IDO1); a kynurenine-producing enzyme with effects on tumor immune escape, and Lin28B. Using *in silico* prediction, we identified a Sox2/Oct4 transcriptional motif acting as an enhancer for IDO1. Knockdown of Lin28B reduced Sox2/Oct4 and downregulated IDO1 in HCV core associated tumor-initiating hepatic cancer cells. We further observed that inhibition of Lin28 by a small-molecule inhibitor (C1632) suppressed IDO1 expression. Suppression of IDO1 resulted in a decline in kynurenine production from tumor-initiating cancer cells. Consequently, modulating Lin28B enhanced *in vitro* cytotoxicity of GPC3-CAR T and NK cells. We further show that cotreatment of GPC3-CAR T cell and C1632 in an HCC xenograft mouse model enhanced anti-tumor activity.

Conclusions
Our results suggest a novel combination that harnesses the power of a Lin28 inhibitor, C1632, and GPC3 as a tumor antigen targeted by CAR T cell therapy for HCC.

P14 – **Hepatitis C virus E1 and modified E2 envelope glycoproteins as candidate vaccine using mRNA-LNP platform**

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Background
Hepatitis C virus (HCV) clearance is associated with broad, potent T-cell activation and rapid induction of cross-reactive neutralizing antibody responses. Here, we examined whether impairing the E2-CD81 interaction induces stronger HCV E2 antigen-specific immune responses by modifying soluble E2 (sE2) antigen and utilizing mRNA encapsulated in lipid nanoparticles (LNP) in a preclinical-mouse-model.

Methods
We selected nucleotide substitutions inhibiting CD81-HCVE2 interactions and introducing an N-linked glycosylation site ($E2_{F442NYT}$) in the front layer of sE2. The immune modulatory effect of E2 was evaluated *in vitro* from inflammatory cytokine induction from treated primary human monocyte-derived macrophages from healthy donors. Parameters of activation and cytokine release were measured by FACS and ELISA, respectively. Polarization of $CD4^+$T cells was examined using CXCR3(Th1) and CCR4 (Th2). BALB/c mice immunized with mRNA-LNP intramuscularly were analyzed for HCV lentiviral pseudotype neutralization activity, antibody isotype switching, and cytokine induction. Immunized mice were challenged intraperitoneally with live recombinant vaccinia virus (vv) expressing HCV E1-$E2NS2_{134-966}$ or HCV E2-NS2-$NS3_{364-1619}$ to examine for protection from challenge infection.

Results
$sE2_{F442NYT}$ exhibited a decrease in CD81 binding compared to sE2 and increased CD80 and CD86 expression from stimulated macrophages. A significant Th1 polarization with a higher proliferation rate was observed on $CD4^+$T cells cocultured with $sE2_{F442NYT}$-treated macrophages. Stimulated $CD4^+$T cells expressed a significant increase in CD69, generating higher IFN-γ and lower IL-4 as compared to sE2. The use of modified $E2_{F442NYT}$ nucleoside mRNA-LNP vaccine resulted in an improved cellular immune response, enhanced isotype switching and total IgG, increased neutralizing antibody efficacy against HCV pseudotype virus, and protection from surrogate HCV vaccinia challenge infection when compared to sE1 alone or an unmodified sE2 mRNA-LNP vaccine. Vaccination with an mRNA-LNP expressing soluble E1 (sE1) alone significantly reduced recombinant vv/HCV titer in mouse ovary.

Conclusion
The results suggest a reversal of M2 to M1 macrophage phenotype and enhanced $CD4^+$T cell response using modified $sE2_{F442NYT}$, unlike parental sE2. Interestingly, the addition of sE2 mRNA-LNP in coimmunization with sE1impairs the efficacy of the sE1 construct and fails to resist challenge infection. Selection of sE1 with modified $sE2_{F442NYT}$ as an mRNA-LNP vaccine candidate appeared beneficial for protection and will advance the development of a highly effective and robust HCV vaccine.

P15 – **Delineating mechanisms of Usutu virus pathogenesis in vivo**

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Background
Usutu virus (USUV) is an emerging flavivirus within the Japanese encephalitis virus serocomplex and is closely related to West Nile virus (WNV). Currently, USUV is mainly recognized for causing astounding lethality in wild bird species in European countries. In addition to mosquito-vectored transmission between avian amplifying hosts, USUV has been confirmed to infect a wide range of mammalian incidental hosts, including humans. While the majority of human infections are asymptomatic, instances of neuroinvasive disease have been observed in immunocompromised individuals. Determination of key cell subsets activated during early infection would suggest an immunological mechanism by which healthy hosts restrict USUV spread and immunocompromised hosts succumb to lethal infection.

Methods
To dissect the complex interplay between USUV and its mammalian hosts, we constructed genetically defined infectious clones of USUV. To gain insights into the relative contributions of innate and adaptive immunity in controlling USUV infection in vivo, we virologically and histologically characterized USUV infections in several mutant mouse strains lacking functional immune cell subsets and harboring cell type-specific disruptions in type I and III interferon (IFN) signaling. We have further constructed luminescent and fluorescent viral reporter genomes to facilitate precise spatiotemporal tracking of USUV in vivo.

Results
While high doses of USUV do not cause disease in wild-type mice, low doses result in fatal infection in type I interferon (IFN) alpha/beta receptor knockout (Ifnar1$^{-/-}$) mice within six days post-infection. Full-body STAT1$^{-/-}$ mice phenocopy Ifnar1-/- mice, but tissue-specific STAT1$^{-/-}$ mice present variable outcomes. Whereas STAT1 and accompanying IFN-mediated antiviral defenses are dispensable in hepatocytes and neuronal cells, they are essential in the hematopoietic compartment, as observed in Vav-Cre/STAT1$^{fl/fl}$ mice, for organism survival. Within the hematopoietic compartment, functional type I IFN signaling further appears to be dispensable in monocytes, macrophages, and dendritic cells — myeloid lineages whose signaling competencies were previously shown to be critical for controlling other flavivirus infection. However, the influx of M2-type macrophages in multiple organs accompanies fatal disease. Exogenous administration of type I IFNs partially protected Vav-Cre/STAT1$^{fl/fl}$ mice. Notably, the role of lymphoid lineages is minimal as mice deficient in functional B, T, and natural killer (NK) cells (NRG) do not develop any clinical signs of disease.

Conclusions
Building on our preliminary data of this hereto poorly-characterized virus, we aim to define the mechanisms by which USUV establishes infection and to track the infection dynamics in vivo using genetic reporters. While symptomatic cases in humans are currently limited to immunocompromised individuals, there is the potential for new strains to emerge with increased virulence through zoonotic transmission. Ultimately, results from our work will shed light on how USUV hijacks host immune signaling to maneuver through susceptible landscapes and promote systemic infection.

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.

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.

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.

P19 – **Mixed-strain HCV infection as indicator of transmission rate and network centrality.**

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Background
People who inject drugs (PWID) are at a significant risk of HCV infection and have high HCV incidence. Disruption of PWID transmission networks is essential for HCV elimination. However, investigation of such networks is exceptionally difficult and, as a result, the structure of PWID networks is usually unknown, which prevents their application in the field. Here, we present a new framework for gauging network parameters important for public health interventions (PHI) by using the mixed-strain HCV infections (MSI) that can be detected among PWID using next-generation sequencing.

Methods
Agent-based simulations (n=10,000) were conducted using 5 distinct HCV strains (subtypes) spreading across a network of susceptible individuals (n=1,000). Network and population parameters were estimated from published data. Each node was assessed for: (i) network importance as measured by degree (number of contacts) and betweenness centrality, and (ii) MSI status (presence of ≥2 strains at the time of sampling). Transmission rate, prevalence, incidence and MSI fraction were measured regularly during the simulations. Three PHI types were modeled: (i) Therapy (node conversion to HCV-negative status), (ii) Harm reduction (node isolation) and (iii) a combination of both therapy and harm reduction. In addition to simulated networks, a real PWID transmission network (n=266, 18.0% MSI) identified in Scott County, IN during the HIV outbreak in 2015 was used in the simulation experiments.

Results
MSI nodes were 3.6-times ($p < .0001$) more connected and 8.6-times ($p < .0001$) more central in the networks than non-MSI nodes. Incidence does not correlate with transmission rate in simulations with PHI, indicating that transmission rate cannot be accurately estimated using acute infections among PWID. However, MSI fraction was found to be strongly associated with transmission rate (Pearson correlation = 0.9768, $p < .0001$) at all stages of infection spread through the network and after PHI. Central topology of MSI nodes was confirmed using the IN network. The average degree and betweenness centrality were shown to be 2.5-times ($p = .0001$) and 9.1-times ($p = .0018$) greater for MSI than for non-MSI nodes.

Conclusions
MSI are strongly associated with the importance of a node in the network, both at the local (degree centrality) and global (betweenness centrality) level as measured on simulated and real transmission networks. These results suggest that MSI could be prioritized for contact tracing and devising efficient targeted PHI to disrupt HCV transmission on PWID networks. The high correlation of MSI with transmission rate warrants further investigation for its application to monitor efficacy of PHI among PWID.

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.

P21 – E1E2-specific broadly neutralizing antibodies isolated from single immune B cells with sE1E2.SZ

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Background and Aims

The study of HCV neutralizing antibodies is crucial for understanding human immunology and for the rational design of vaccine candidates to address viral antigenic diversity. Recently, an engineered soluble form of the E1E2 complex, known as sE1E2.SZ, has been found to mimic the full-length transmembrane E1E2 both antigenically and structurally. This study assesses the utility of this novel reagent through an immunological approach for recognizing virus neutralizing antibodies present on immune B cells. Specifically, we aim to determine whether cross-neutralizing antibodies specific to epitopes present only on natively folded E1E2 complex can be labeled by this recombinant protein complex, and evaluate the proportion of antibodies to E1E2 relative to E2 in different patients hence the immunogenicity of E2 versus E1E2 during HCV infection.

Methods

E1E2-specific single B cells of HCV-infected individuals with high or low neutralizing activities were labeled and isolated using flow cytometry, followed by cloning of the antibody variable genes of heavy (HC) and light (LC) chains by RT-PCR. The HC and LC sequences were analyzed to identify unique and shared antibodies in the subjects' antibody repertoires. Cloned antibodies were expressed and characterized for their binding to viral envelope glycoproteins as well as their virus neutralizing activities.

Results

In studying six HCV-infected individuals, sE1E2.SZ-binding B cells were isolated and over 150 paired HC and LC were cloned. Notably, antibodies derived from the VH1-69 genes were frequently identified from individuals with high virus neutralizing activities but less frequently in those with low neutralizing activities. Other frequently isolated antibodies were derived from VH1-18, VH1-46, VH3-30 and VH4-39 genes. Approximately 10% of the antibody panel bound E1E2 but not E2, indicating that the target epitopes are present only on the folded E1E2 complex. Several of these antibodies competed with the prototypic anti-E1E2 antibody AR4A in binding to E1E2 and cross-neutralized HCV strains with varying sensitivity to neutralizing antibodies. In contrast to the well characterized VH1-69 class of broadly neutralizing antibodies against the E2 neutralizing face, different VH genes were employed to generate neutralizing antibodies against the conserved E1E2 antigenic region overlapping with AR4.

Conclusions

The findings demonstrate the utility of sE1E2.SZ in flow cytometry. The recombinant soluble protein assumes a native fold, enabling the labeling of HCV immune B cells and the isolation of E1E2-specific neutralizing antibodies. This novel reagent, in addition to soluble E2, is highly valuabe for studying HCV B cell responses.

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.

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.

P24 – **Analyzing functional interactions between dengue virus non-structural protein 1 and Tolllike receptor 4 in a macrophage infection model**

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Background
Dengue virus (DENV) non-structural protein 1 (NS1) induces expression of proinflammatory cytokines, which contributes to dengue pathogenesis. The underlying mechanisms are controversial, with different studies implicating Toll-like receptor 4 (TLR4) or TLR2/6 in NS1 mediated cytokine activation.1,2 The NS1 glycoprotein is secreted from cells as a hexamer in a glycosylation-dependent manner. However, the NS1 determinants that contribute to TLR recognition, such as glycosylation sites, are unknown, and potential roles for other components of TLR signalling complexes, such as CD14, are still unclear. In this study, we used macrophage infection model to probe the role of TLR4 in the response to NS1 and to characterize how NS induces pro-inflammatory cytokine expression.

Methods
THP-1 monocytic cells were differentiated into macrophage-like cells by PMA treatment and infected with DENV-2 (strain 16681). Alternatively, NS1 was ectopically expressed in THP-1 or 293T cells, respectively, to explore the specific role of NS1. The role of NS1 secretion was tested using an NS1 expression construct with the KDEL ER retention signal. Through reverse genetics we generated a DENV-DNS1 deletion virus and DENV NS1 glycosylation mutants (N130Q or N207Q). RT-qPCR, ELISA and reporter assays were used to evaluate activation of IRF3 and NF-κB signaling pathways and induction of pro-inflammatory mediators in response to DENV infection or NS1 expression. A specific role for TLR4 was assessed using TLR4 knockout (KO) THP-1 cells, while a role for the TLR co-receptor CD14 was evaluated through stable overexpression of CD14.

Results
DENV infection of THP-1-derived macrophages induced secretion of pro-inflammatory cytokines, such as IL-6 and CXCL10. IRF3 activation and cytokine induction was slightly modulated, but not abrogated, in TLR4 KO cells, suggesting that TLR4 is not a key mediator of pro-inflammatory cytokine induction during DENV infection. Consistently, ectopic expression of NS1 induced IRF3 reporter activity in THP-1 TLR4 KO cells, but did not activate TLR4 in a 293T cell reporter assay. Interestingly, stable overexpression of CD14 in THP-1 wild-type or TLR4 KO cells enhanced the activation of IRF3 following DENV infection, despite similar viral titers, highlighting a role for the TLR co-receptor CD14 (but not TLR4) in mediating DENV-induced pro-inflammatory responses. Experiments with DENV- DNS1 and NS1 glycosylation mutants in trans-complemented cells expressing ER-retained NS1 are ongoing to evaluate the role of secreted DENV NS1 and its glycosylation sites in activating TLR signaling during viral infection.

Conclusions
TLR4 is not required for induction of pro-inflammatory cytokines by NS1 during DENV infection, while CD14 may play a role. These findings enhance our understanding of the role of NS1 in inducing pro-inflammatory responses during DENV infection of macrophages and may provide new therapeutic perspectives.
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P25 – **Induction of interferon regulatory factor-1 by cyclosporine A and its effect on dengue virus replication**

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Background
Dengue virus (DENV) infects around 400 million people every year in more than 100 endemic countries, putting half of the world's population at risk. As the planet warms up, this mosquito-transmitted disease that was once restricted to tropical areas is now spreading worldwide, including Europe and North America. Unfortunately, there are currently no approved antivirals for dengue infection, with treatment limited to supportive care. Drug repurposing might be a useful strategy to identify antiviral candidates for dengue. Recently, we showed that cyclophilin inhibitors restored antiviral responses dependent on interferon regulatory factor-1 (IRF1) to restrict hepatitis C virus (HCV) replication [1]. Similarly, cyclophilin inhibitors such as cyclosporine A (CsA) also decreased MERS-CoV replication *in vitro* and *in vivo* by upregulating type III IFN (IFN- λ) in an IRF1 dependent manner [2]. These findings suggest a link between IRF1 and the antiviral activity of CsA. However, it is still unclear how CsA induces IRF1-dependent antiviral responses and whether this activity contributes to its antiviral effect against other RNA viruses, such DENV. Since IRF1 restricts DENV infection [3], we hypothesized that CsA inhibits DENV infection via IRF1.

Method
We are employing virological, molecular, and biochemical approaches in cell culture models to determine the antiviral mechanisms of CsA against DENV infection

Results
We infected Huh7 hepatoma cells with DENV serotype-2 (strain 16681) and then treated the cells with increasing concentrations of CsA for different time intervals (0, 24, and 48 hours). DENV infection was measured by GFP reporter activity and RT-qPCR, while production of infectious virus was assessed by focus-forming assay. Use of a DENV subgenomic replicon showed that CsA specifically inhibits DENV RNA application. CsA dose-dependently inhibited DENV replication in Huh7 cells, without affecting the cell viability. However, other types of cyclophilin inhibitors lacked antiviral activity against DENV infection. Consistently, genetic depletion of the classical CsA target proteins, cyclophilin A and B did not affect DENV replication, indicating the involvement of other factors. Notably, CsA treatment induced the expression of *IRF1* and IRF1-dependent antiviral genes such as RSAD2, which may contribute to restricting DENV replication. Further characterization of the antiviral mechanisms of CsA against DENV infection and its ability to induce IRF1-dependent responses is ongoing.

Conclusions
CsA, a clinically approved immunosuppressive drug that inhibits T cell proliferation, restricts DENV replication in a manner that is independent of cyclophilin A or B but may dependent on IRF1. Understanding how CsA induces IRF1-dependent antiviral responses will aid in the design and synthesis of non-immunosuppressive CsA-like molecules for further development as antiviral drugs.

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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.

P27 – **Characterizing the landscape of DENV antigenic evolution**

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Background
Dengue virus (DENV), a positive-sense RNA virus from the *flavivirus* genus, infects more than 390 million people annually. DENV consists primarily of four serotypes, which are genetically related but antigenically distinct. Quadrivalent vaccine development assumes that including a representative virus from each serotype can broadly neutralize immunity to all variants within each serotype. However, recent studies indicate variable neutralization of variants within a serotype after vaccination. Therefore, this study aims to determine how genetic variation within a serotype leads to distinct antigenicity.

Methods
We have obtained patient serum and genomic sequences from recently circulating DENV from two Colombian cities with different disease burdens to test how genetic variability can affect antigenic variability. Samples were collected from Cali, which had a high 2021 disease burden (>5000 cases), and nearby La Virginia, which had a low 2021 disease burden (<1000 cases). Nucleic acid extracted from sera of RT-PCR-positive DENV cases underwent full genome sequencing using metagenomic and amplicon-based library preparation and reference-based assembly. To test the capacity of our identified variant mutants to evade existing immunity, we have established a pliable, single-cycle, Renilla luciferase reporter virus particle (RVP) system expressing variant E protein sequences.

Results
From these samples, we have identified: serotype 1, genotype V (n=21); serotype 2, genotype III (n=3); and serotype 3, genotype III (n=6). The serotype 1, genotype V sequences from Cali and La Virginia form different clades on a phylogenetic tree, indicating multiple contemporaneous DENV variants are circulating in this region. Based on the identified genetic variation, we hypothesize that there is reduced immunity within Cali's host population to DENV variants found in La Virginia's. To test this, we have generated an RVP panel comprised of the Cali DENV1 variant, the La Virginia DENV1 variant, and reference variants for DENV1 and DENV2. We validated the performance of our RVPs by demonstrating that a potent neutralizing monoclonal antibody (mAb) against DENV (EDE-1 C10) exhibits high activity against all DENV RVPs, whereas a ZIKV-specific mAb (G9E) exhibits minimal neutralization activity. Neutralizing antibody titers from Cali and La Virginia serum will be measured against the RVP panel to assess antigenic variability between these two populations.

Conclusions
Sequence analysis has suggested a separate introduction of DENV between both cities. Additionally, the expression of each variant was tested in neutralization assays using mAb, which indicated that our RVPs consistently produced a stable epitope with low cross-reactivity. This study aims to improve vaccine development by determining the role of genetic diversity in shaping within serotype antigenic variability, therefore informing the assessment of variant inclusivity in the immune responses elicited by candidate tetravalent DENV vaccines. Additionally, this study will compare the specificity of humoral immunity to DENV between two populations in close geographic proximity.

P28 – **Evolution of resistance-associated variants in HEV-patients with sofosbuvir mono- or sofosbuvir-ribavirin combinatory therapy**

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Background

Hepatitis E virus is a single-stranded RNA virus that causes over 20 million infections worldwide. The lack of an HEV-specific antiviral, leaves drug repurposing as a fast-tracked option to help patients with symptomatic infection. Besides off-label use of ribavirin and interferon alpha, sofosbuvir is used to treat chronically infected patients. Sofosbuvir treatment often results in a strong decrease in viral RNA during the initial phase of treatment, but viral relapse often occurs after 4-12 weeks. However, the reason for viral relapse remains unknown.

Methods

We here analyzed virus population dynamics during sofosbuvir monotherapy in chronically infected patients with HEV relapse using high throughput sequencing. Dominant variants were identified and subsequently characterized for in vitro replication capacity and drug sensitivity using an HEV replicon system. In addition, we analyzed viral diversity in patients treated concurrently with ribavirin and sofosbuvir to understand whether the combination can suppress the emergence of variants or overcome resistance.

Results

Our results suggest that viral heterogeneity plays an important role in treatment resistance. In particular, a mutation, A1343V, in the polymerase finger domain that occurred in the majority of patients (8/9) that have been treated with sofosbuvir monotherapy resulted in a 5-fold decrease in susceptibility while maintaining replication capacity in vitro. This variant was not located in the polymerase active site, as predicted by an alpha-fold model, and it was enriched in patients treated with sofosbuvir, as indicated by its low frequency (<3%) in public databases. Furthermore, sensitivity to ribavirin treatment remained unchanged. We identified similar variants, including the one in the finger domain, in patients treated with a sofosbuvir-ribavirin combination. Despite occurring, this variant was highly sensitive to sofosbuvir-ribavirin combination therapy in vitro.

Conclusions

In summary, we identified a sofosbuvir-specific variant in the viral polymerase that was associated with treatment resistance in HEV infected patients. The variant A1343V was less susceptible to sofosbuvir in vitro without any change in replication. Importantly, the resistant phenotype could be overcome in tissue culture by co-treatment. Therefore, future antivirals should take into account the dynamics of viral populations and variants associated with drug resistance.

P29 – **Biochemical studies of the interactions between Dengue virus NS5 and NS3 proteins and the 3' terminus of the negative strand of the replication intermediate**

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Background
Dengue virus belongs to Flaviviridae and is an important human pathogen. Its positive strand RNA genome of 11 kbp is replicated by a virally encoded NS5 protein. Crucial for replication is an interaction between NS5 polymerase and the stem-loop A structure at the 5' terminus of the genome (5' SLA). The negative strand synthesis is initiated on the 3' terminus of the genome and results in the formation of the double stranded replication intermediate. Significantly less is known about the subsequent step, where the dsRNA intermediate is used as a template to produce positive-strand RNA. The replication intermediate is likely separated by NS3 helicase for subsequent positive strand synthesis by NS5. It has been shown that NS3 can only effectively separate substrates with a 3' overhang, while the replication intermediate is a blunt ended dsRNA. The 3' end of the negative strand contains a sequence complimentary to the 5' SLA (3' SLA). Here, we tested if 3'SLA folds into a stable structure and if NS3 and NS5 proteins specifically interact with 5' and 3' SLAs.

Methods
We first determined whether 3'SLA folds into a stable structure using circular dichroism. To investigate the interaction of NS3 and NS5 with 5'SLA/3'SLA, we performed *in vitro* polymerization assays, fluorescent anisotropy and EMSA binding assays. We also tested if helicase could unwind blunt-ended dsRNAs using *in vitro* helicase assays.

Results
We show that 3' SLA folds into an RNA structure with a thermal stability similar to that of the 5' SLA, suggesting that the 3' SLA sequence could fold into a structural element during Dengue genome replication. Both fluorescent anisotropy and EMSA assays showed that NS5 can bind 3' SLA albeit with a 2-fold lower affinity than 5' SLA. Interestingly, our data show that NS3 binds both 5' and 3' SLAs similarly. NS5 polymerization showed that when a 3' SLA-containing RNA is used as a template both *de novo* and elongation products are produced which resembles a 5' SLA containing template. Moreover, we performed helicase assays using recombinant NS3 and short RNA templates either with a 3' overhang or blunt-ended showing that a blunt-ended dsRNA can be separated by Dengue NS3, however the rate of this reaction is approx. 3 times lower than a reaction using a dsRNA with a 3'-overhang as a substrate.

Conclusion
Our data indicate that Dengue NS3 alone might be able to separate the blunt-ended replication intermediate, which could lead to the formation of 5' SLA and 3' SLA in the positive- and negative-strand, respectively. We show that *in vitro* both 5' SLA and 3' SLA containing templates can be replicated by the NS5 polymerase despite the protein's differential binding affinities for 5' SLA and 3' SLA.

P31 – **Domain stabilization and comparison of secreted versus membrane-bound forms of HCV E1E2 vaccine candidates**

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Background
Development of an effective vaccine for hepatitis C virus (HCV) requires a vaccine that elicits immune responses to key conserved epitopes. Based on the structures of broadly neutralizing antibodies (bnAbs) targeting key epitopes of HCV, we designed E2 and E1E2-based immunogens to modulate the structure and dynamics of E2 and E1E2 to favor induction of bnAbs. Previously, we showed that mice immunized with HCV sE2 harboring the mutation H445P exhibited an increase in breadth of neutralization. Here, we describe the antigenicity and immunogenicity of these designs, including a secreted form of E1E2 (sE1E2), in comparison to membrane-bound E1E2 (mbE1E2), in murine and macaque models.

Methods
We recently designed a secreted form of E1E2 that uses a leucine zipper scaffold to replace the E1 and E2 transmembrane domains which mediate assembly of native E1E2 (sE1E2.LZ). In anticipation of vaccine studies in humans, we adapted a synthetic structural homologue with no human sequence homology (SynZip) as an alternate scaffold (sE1E2.SZ). We analyzed immunogenicity of formulated sE1E2 immunogens as wild-type molecules or harboring the structure-guided H445P mutation in mice and macaques, in comparison to mbE1E2 and sE2. The capacity of macaque sera to neutralize antigenically diverse HCV was tested *in vitro* and efficacy against infectious virus was assessed in human liver chimeric mice. The effect of the modification on the specificity of the antibody responses is currently being probed using alanine scanning mutagenesis of the conserved neutralization site.

Results
Mice immunized with mbE1E2, sE1E2.LZ, and sE2 exhibited similar anti-E2 endpoint titers. However, sera from mice immunized with sE1E2.LZ elicited the broadest neutralization activity to HCV genotypes 1-6, in comparison to mbE1E2 and sE2, using HCVpp and HCVcc. We found that both sE1E2.LZ and sE1E2.SZ enabled proper folding of the antigenic domains as measured by binding to a panel of HMAbs that target conformational epitopes in E2 and E1E2. In macaques immunized with mbE1E2, sE1E2.SZ, and sE1E2.SZ harboring the H445P stabilizing mutation, bnAbs were detected in all cases, albeit at lower levels than in mice. Macaques immunized with mbE1E2 had higher end-point titers to itself and neutralization against selected HCVpp isolates. However, the H445P stabilizing mutation exhibited higher endpoint titers and an improved neutralization profile in comparison to sE1E2.SZ. Passive protection studies are ongoing using human liver chimeric mice infused with Ig-enriched fractions from the immunized macaques to assess the level of protection when challenged with infectious virus.

Conclusions
These results show that antigen optimization through structure-based design is a promising route for enhanced epitope presentation, and secreted E1E2 serves as a promising HCV vaccine candidate for further development.

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.

P33 – **Automated high-throughput screen discovers members of the Akt serine/threonine kinase family as targets for treatment of HEV virus infection**

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Background
Nearly four-and-a half decades after the discovery of Hepatitis E virus (HEV) as the etiological agent of viral hepatitis in human, treatment options remain limited to the off-label use of the nucleoside-analog ribavirin (RBV) and pegylated interferon-α. Although these drugs have made HEV infections manageable for the majority of patients, a considerable number of patients are either not eligible for or do not respond to currently available treatment options. To find substitute antiviral medications against HEV infections, we developed a simple and effective image-based high-throughput screening assay.

Methods
We screened up to 9,935 compounds derived from FDA-approved drug-libraries and carried out dose-response assays of up to 170 of the most promising compounds by utilizing subgenomic HEV reporter replicons of genotype 3 expressing a GFP gene as a marker for viral replication in hepatoma cells. Furthermore, we tested the top hits in infection experiments with the human-derived HEV-3 p6 and the wild-boar HEV-3 83-2 virus. We characterized pan-Akt inhibitors by time-of-drug-addition experiments and confirmed their efficacy in primary human hepatocytes.

Results
We discovered at least 5 compounds that markedly inhibit viral infection at low micromolar concentrations in hepatoma cells. Infection experiments with HEV-3 p6-FL and HEV-3 83-2 virus confirmed that ML-9, an Akt family inhibitor, exhibits antiviral properties against HEV infection. Moreover, two clinical phase III pan-inhibitors of the serine/threonine kinase Akt (Capivasertib and Ipatasertib) were found to inhibit virus infection in hepatoma cells. Ipatasertib, in particular, appears to primarily target early replication and acts additive when combined with ribavirin treatment. Moreover, when tested in primary human hepatocytes, Ipatasertib demonstrated a reduction in virus infection, achieving a minimum decrease of 50%.

Conclusions
In conclusion, screening drug-repurposing libraries proved to be a versatile tool for identifying novel drugs against HEV infections, but most importantly, our findings highlight the effectiveness of pan-Akt inhibitors in inhibiting viral infection in liver cells and provide valuable insights into the antiviral mechanisms of Ipatasertib in early-stage viral replication processes.

P34 – **Dengue virus serotype 1 inhibition of T cell receptor signaling.**

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Background
Recent work in our laboratory found that dengue virus serotype 1 (DENV 1) reduces IL-2 release following stimulation of the human T cell receptor (TCR) while DENV 3 did not. In this study we assessed DENV 1 inhibition of activation of proximal TCR signaling molecules to facilitate understanding of the mechanism employed in DENV 1.

Methods
DENV 1 or DENV 3 was added to either Jurkat cells (human CD4+ T cell line) or primary peripheral blood mononuclear cells (PBMCs) prior to stimulation with anti-CD3 +/- anti-CD28. Early TCR signaling events were monitored by assessing activation of signaling molecules Lck (lymphocyte-specific protein tyrosine kinase), ZAP-70 (zeta chain-associated protein kinase-70), and the linker for activated T cells (LAT). TCR- mediated activation was measured via immunoblot to analyze phosphorylation and TIRF microscopy to measure protein cluster formation. Overall TCR-mediated signaling was confirmed by measuring IL-2 release. Since DENV 1 env protein was sufficient to inhibit TCR-mediated IL-2 release and DENV 3 was not, a series of chimeric envelopes and deletion mutants were created to determine viral requirements for TCR inhibition.

Results
DENV 1 but not DENV 3 inhibited LAT clustering following stimulation of the TCR. ZAP-70 and LAT activation were reduced in cells expressing DENV 1 env compared to controls while Lck activation did not appear different suggesting that DENV 1 interferes with a step(s) following Lck activation. Expression of DENV 1-3 chimeric envelopes determined that the N-terminal 65 amino acids of DENV 1 were required for TCR inhibition. Deletion mutations of DENV 1 found that a 14 amino acid region between amino acids 49 and 62 of DENV 1 was sufficient to inhibit IL-2 release, and selected point mutations in this region identified critical amino acids involved in TCR inhibition.

Conclusions
Epidemiological data suggest that DENV 2 and 3 are more likely associated with severe immunologically mediated dengue infection 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. Understanding the mechanisms involved in DENV 1 TCR signaling inhibition may provide novel targets for mitigating DENV diseases. Studies are underway to determine intracellular binding partners with DENV 1 env involved in TCR inhibition, and to determine if the TCR inhibitory effects of DENV 1 may be introduced into DENV 3.

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.

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.

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.

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.

P39 – **Understanding the mechanisms of viral-induced p38 activation for viral replication**

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Background
Recent studies have shown that atypical activation of p38 is critical for HCV replication and is broadly applicable to the regulation of other viruses. Despite the broad roles of p38 in regulating cellular proliferation, differentiation, and inflammation the molecular mechanisms that underlay atypical p38 activation by viruses remain a critical gap in our understanding of viral physiology. The advancement of current direct-acting antivirals (DAA's) has resulted in the potential to cure roughly 95% of HCV infections, however, this excludes ~2.4 million people. The extreme genetic variation of HCV also represents an enhanced risk of resistance-associated substitutions (RAS) in individuals who failed current treatment regimens. Treatment failure, therefore, increases the risk of transmission for DAA-resistant viral strains. Because of this, and the potential conservation of atypical p38 signaling in viral infections. The identification of molecular targets as an additional broad-range antiviral could significantly increase the impact of patient treatment outcomes.

Methods
To address this unmet need we are exploring the potential of novel biologics that can suppress virus-induced activation of atypical p38 for replication and inflammation. Our working hypothesis is that atypical p38 signaling is a potential therapeutic target for hindering viral replication and virus-induced inflammation during infection. Using a phage display library of humanized nanobodies we screened for a candidate panel that can suppress atypical p38 activation. Candidates were confirmed using in vitro transfections, binding assays, immunoprecipitation, and microscopy. To explore the role of atypical p38 in viral replication we have used cell culture permissive HVC and HUH7.5, siRNA, chemical inhibitors, and intrabody expression.

Results
Using two specific avenues we have addressed our central hypothesis. Initially, we generated a panel of inhibitory nanobodies that suppress atypical p38 activation. Confirming suppression of atypical p38 activation and binding to the critical adaptor protein. We are currently screening their capacity to suppress HCV replication as a model virus. Secondly, we have identified a molecular adaptor that co-associates with the HCV core during replication that we predict regulates the replication of multiple viruses. We are examining the conservation of this adaptor's functionality for other viruses.

Conclusions
We have discovered lead intrabodies that can block atypical p38 activity and that atypical p38 signaling can drive viral replication. By defining these molecular drivers and examining intrabody regulators we will outline the conserved mechanisms of virus-induced atypical p38 activation and explore the potential to support current anti-viral therapies.

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

Volker Kinast, Eleftherios Michailidis, Sarah Schlienkamp, Yannick Brüggemann, Daniel Todt, Florian WR Vondran, Axel Hamprecht, Finn Grey, Charles M Rice, Eike Steinmann

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.

P41 – **Hepatitis E virus signaling landscape identifies Yes1 as a druggable host factor**

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Background
Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus of the *Hepeviridae* family. Although HEV accounts for more than 3 million symptomatic cases of viral hepatitis per year, anti-HEV therapy is currently restricted to the off-label use of ribavirin and peg-IFN-α both offering only limited effectiveness. Since the HEV infectious cycle is still poorly understood, the identification of virus-host interactions bears the potential to help to understand the HEV infectious cycle and guide the development of novel antiviral strategies against HEV.

Methods
Human hepatoma (HepG2) cells and an antibody-based microarray with 613 phosphosite- and 265 pan-specific antibodies were used to detect differences in protein expression and phosphorylation in cell signaling pathways, caused by HEV replication. The influence of individual factors on HEV infection were analyzed by utilizing RNAi. The antiviral potential of an inhibitor of the top hit of the RNAi mini screen was characterized in different HEV infection assays including primary human hepatocytes.

Results
54 alterations of protein expression and phosphorylation were detected upon HEV replication. A subsequent RNAi mini screen of 28 factors revealed a two-fold reduction of HEV susceptibility upon knockdown of the SRC-family kinase Yes1. Additionally, selective inhibition of the Yes1 kinase activity resulted in a ~20-fold reduction of HEV infectivity in hepatoma cells. Time-of-addition and HEV replicon assays indicate that Yes1 contributes to the HEV entry process. Enhanced phosphorylation of the catalytically activating Tyr416 of Yes1 upon HEV infection indicate that HEV triggers Yes1 kinase activity.

Conclusion
HEV infection triggers a variety of host cell signaling molecules, including Yes1, which acts as a potential host factor during HEV's early life cycle steps. Given that a specific Yes1 inhibitor efficiently reduced HEV infection, the drug might help to elucidate the HEV entry process and shows potential to contribute to the development of novel antivirals against HEV.

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.

P43 – **Combating Yellow Fever Virus With A Novel 7-Deaza-7-Fluoro-Adenine Nucleoside Analog**

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Background
Yellow fever virus (YFV) is a deadly zoonotic flavivirus endemic in tropical/sub-tropical Africa and South America. The WHO classifies YFV as a "high impact, high threat disease" with resurgent epidemic potential, however, there are no approved antiviral agents for YFV. This work investigated the anti-YFV activity of a 7-deaza-7-fluoro-adenine nucleoside analog (DFA) which also has activity against other deadly flaviviruses such as Dengue and Japanese encephalitis viruses.

Methods
DFA was evaluated for anti-YFV activity in target human hepatoma cells against vaccine strain 17DD and the neuroinvasive clinical strain DakH1279 by dose-response assay (0 – 10 **μM)**. It was further evaluated for anti-YFV17DD activity in primary human macrophages and in a 3D spheroid human hepatoma model as well as in the YFV murine A129 model. For mouse studies, equal numbers of male and female mice were infected with 10^6 PFU/mL of 17DD, left uninfected, or mock infected by intravenous injection of the tail vein. At 1 hpi, and every 24 hr for 2 days, 10 mg/kg of compound was administered intraperitoneally. After 3 days, mice were assayed for liver injury via indocyanine green injection and imaging (IG) then sacrificed and blood and liver obtained to test for serum alanine aminotransferase (ALT) levels via BioClin kinetic kit and viral burden via qRT-PCR. Histological analysis was performed on liver sections. To examine proposed mechanism of action of binding to the YFV RdRp, molecular modeling studies were performed and Prime MM-GBSA dG (NS) values were calculated from Schrödinger.

Results
DFA exhibited sub-micromolar anti-YFV activity against both vaccine and clinical strains. This compound had low cytotoxicity (CC_{50} >100 μM) in Vero and BHK cell lines as well as in human hepatoma spheroids and primary human macrophages. DFA had potent anti-YFV 17DD activity in hepatomas (EC_{50} 0.4 *μM*; EC_{90} 2.8 μM), primary macrophages (EC_{50} 0.3 *μM*; EC_{90} 1.3 μM), and 3D hepatoma spheroids (EC_{50} 5.3 *μM*; EC_{90} 10.5 μM) as well as against clinical strain DakH1279 in human hepatomas (EC_{50} 3.6 *μM*; EC_{90} 8.1 μM). In A129 mice, DFA significantly reduced YFV-induced liver damage with a 79% reduction in IG ($p < 0.0001$) and 68% reduction in ALT levels ($p < 0.0001$) and provided protection to the liver with a significant reduction in virus burden (0.7 log reduction; $p = 0.013$). Finally, molecular modeling revealed that DFA should readily bind in the active site of the YFV RdRp with a slightly higher affinity than ATP natural substrate (binding free energy -103.30 kcal/mol vs -101.51 kcal/mol, respectively).

Conclusion
We demonstrated that DFA is an efficacious and effective anti-YFV agent in culture and in an animal model for YFV. Collectively, our data support its further pre-clinical development as an attractive new anti-YFV candidate and possible pan-anti-flavivirus inhibitor.

P44 – **Construction of a human pathogenic flavivirus library for pandemic preparedness**

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Objectives
The experience of the COVID-19 pandemic suggests that potential pandemic pathogens will continue to pose substantial global threats, and we must prepare for future pandemics. It is well known that viruses among the same genus and species exhibit various pathogenicity. Therefore, a comprehensive understanding of viruses is crucial for pandemic preparedness. In this study, we established a human pathogenic flavivirus library that is currently prevailing worldwide by using reverse genetics and in vivo experiments to determine the pathogenicity of the viruses. We believe the flavivirus library we constructed will contribute to developing antivirals against yet-unknown X viruses.

Methods
Circular polymerase extension reaction (CPER) is an efficient technology for constructing infectious clones and has emerged as a primary technique for generating SARS-CoV-2 variants. Viral genome information was obtained from the NCBI database, and multiple fragments covering the full length were synthesized. Using CPER, circular nucleic acids containing the full-length flavivirus genome flanked by the CMV promoter and HDV ribozyme were generated. HEK293 cells transfected with the CPER solution were co-cultured with Huh7, Vero, or C6/36 cells until the appearance of cytopathic effects. Viral infectious titers in the culture supernatants were determined by the focus-forming assay. For in vivo experiments, sequentially diluted viruses were intraperitoneally injected into four mice in each group and monitored daily for survival and body weight changes for two weeks.

Results
Twenty-eight strains of 14 human pathogenic flaviviruses from various families currently prevailing were generated by CPER. After transfection of the CPER solution into HEK293 cells and co-culturing with susceptible cells, infectious viruses ($1x10^{5-8}$ FFU/ml) in the culture supernatants determined by focus forming assay using specific antibodies were recovered. Among flaviviruses previously shown to be lethal to wild-type mice, the viruses exhibited weight loss at various time points up to 7 days to 14 days post-inoculation.

Future directions
Here, we generated a human pathogenic flavivirus library by CPER and examined the pathogenicity in mice. Further investigation of characteristics of the viruses, and pathogenicity and organ specificity in mice is now underway.

P45 – **Interaction of West Nile Virus Polymerase NS5 with Flaviviruses Stem-loop A (SLA)**

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Background
West Nile virus (WNV) is a single stranded, positive-sense RNA virus in *Flaviviridae*. Flavivirus encompasses dengue (DENV), Zika (ZIKV), and Japanese encephalitis virus (JEV), all of which have been implicated in human disease and are considered etiologies of emerging infectious diseases. WNV causes West Nile fever in humans, the most common mosquito-borne disease in the United States. Flaviviral replication depends upon the presence of the stem-loop A (SLA) structure in the 5' untranslated region of the genome. The viral polymerase, NS5, interacts with the 5'SLA and initiates synthesis of the negative strand. The secondary structures of SLA and protein sequences of NS5 polymerase are both highly conserved across flaviviruses, suggesting that they utilize a similar replication mechanism mediated by SLA. For example, DENV is shown to recognize the WNV SLA and replicate the WNV genome and vice versa.

Methods
To test if WNV Lineage 2 NS5 recognizes virus-specific 5'SLA, we determined NS5 interactions with various flavivirus 5'SLAs using an electrophoretic mobility shift assay (EMSA). Next, we determined the NS5-binding site on SLA utilizing WNV SLA mutants using EMSAs. To determine the effects of SLA mutations and replacement with other Flavivirus SLAs on WNV replication, we used a luciferase reporter system.

Results
Binding studies of WNV NS5 and WNV SLA mutants by EMSA show that NS5 binds to the top loop and side loop (the upper and lower side loop) of the SLA. When these SLA mutations were introduced in WNV replicon, the side-loop and top loop mutants reduced viral replication, while the bottom stem did not, consistent with the binding results. Further, WNV NS5 binds the SLA of DENV, JEV, and ZIKV, consistent with the interchangeability of SLA between DENV and WNV. However, the shifts in the EMSA show that WNV NS5 has a reduction in binding to JEV and ZIKV SLAs compared to WNV and DENV SLAs. Replacement of WNV SLA with that of DENV mildly reduces viral replication, while replacement with JEV SLA drastically reduced viral replication.

Conclusions
SLA sequence and structure plays a critical role in WNV NS5 recognition of SLA leading to initiation of genomic replication.

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P46 - **Insights into early acute HCV Infection in uPA-SCID Mice with Humanized Livers versus Chimpanzees using Mathematical Modelling Approach**

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Background
Understanding the early evolution of HCV is essential in pinpointing crucial aspects of initial HCV infection. This knowledge is vital for developing an urgently needed prophylactic HCV vaccine. In this study, we investigated HCV kinetics from inoculation to steady state in uPA-SCID chimeric mice with humanized livers and developed a mathematical model to provide insights into early HCV-host dynamics. We then compared the results to estimates from a previous mathematical modeling study of acute HCV in 10 chimpanzees reaching steady state and before seroconversion (Gastroenterology.2005;128(4):1056-66).

Methods
Five PXB uPA-SCID mice with hepatocyte donor: JFC (1 year, male Caucasian) and human albumin > 9mg/mL, inoculated intravenously with HCV (genotype 1a)-infected serum of $1x10^6$ copies/animal. Viral levels were frequently measured from blood samples up to 35 days post infection (p.i.). We modified our previous mathematical model (ibid) to account for the lack of adaptive immune response and hepatocytes proliferation in the uPA-SCID mouse model. Model fits with experimental data was performed using the scipy and lmfit Python packages.

Results
Modeling indicates an HCV half-life in mice serum of 45-49 minutes, reminiscent of the HCV half-life estimated in patients treated with direct-acting agents (PNAS.2013;110(10):3991-6.) and liver transplantation (Elife. 2021;10:e65297). As seen in chimpanzees before seroconversion, the virus had a biphasic increase with a transient decline in between, reaching a steady state. Compared to the chimpanzees, the mice had a transient decline 3-12 days earlier and reached steady state 26-34 days earlier. This increased speed in kinetics in mice compared to chimpanzees could be due to the ~1000-fold larger quantity of inoculated virus in the mice. In both mice and chimpanzees, the model suggests that the biphasic increase is due to blocking of HCV production, with a similar efficacy of 90-95% and 85-95% in mice and in chimpanzees, respectively. While an endogenous type I interferon response was suggested to explain the transient HCV decline (i.e., blocking of HCV production) seen in chimpanzees (ibid), further research is needed to provide insights into the nature of this observation and modeling prediction in mice. Lastly, we found that the HCV steady state level was about at an average of 8.5 log copies/mL in mice reminiscent of HCV peak levels seen in patients after lung transplantation from HCV-positive donors (Lancet Respir Med. 2020;192-201). The 100-fold higher viral level in mice, compared to 6.1 log copies/mL in chimpanzees, may suggest a role of the adaptive immune response in reducing the number of infected hepatocytes at steady state (before seroconversion) in chimpanzees.

Conclusions
The uPA-SCID mouse model with humanized liver exhibits several features reminiscent of the chimpanzee model, suggesting that this model is suitable to investigate HCV-host dynamics from infection to steady state.

P47 – **Hepatocyte Gab1 promotes cell proliferation and liver fibrosis via potential involvement of HGF/c-Met signaling**

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Background and Aims
Chronic liver diseases are caused by HCV, chemicals, and metabolic stress. Grb2-associated binder 1 (Gab1) is an adapter protein binding to various growth factor receptors such as tyrosine protein kinase Met (c-Met) and triggers cell differentiation/survival signaling pathways. However, it is not known how the expression of Gab1 in hepatocytes contributes to liver fibrosis.

Methods
To identify signaling molecules involved in the progression of liver diseases, we performed proteomic analysis of hepatocytes isolated from liver tissues of humanized liver mice after 7 days of HCV infection.

Results
Acute HCV infection in humanized liver mice significantly decreased the level of hepatocyte p-Gab1. The *in vitro* kinetic studies for Gab1 expression showed that p-Gab l expression is decreased in Huh7.5.1 cells at early time points of infection (D1 to D3) but is increased at later time points of infection (D4, D5). Notably, p-Gab1 levels was inversely related to the production of TGF-β, a pleotropic cytokine associated with regulation of cell growth. To further determine the role of hepatocyte Gab1 expression in liver fibrosis progression *in vivo*, we assessed the expression of Gab1 in the liver of CCL4-induced fibrotic mice. Interestingly, the level of p-Gab1 was increased in CCL4-induced fibrotic liver. Hepatoma cells showed elevation of p-Gab1, along with an increase in STAT3 and ERK activation, upon treatment with HGF (ligand of HGF receptor/c-Met) and CCL4. In Gab1 knockdown hepatoma cells, cell proliferative signaling activity (STAT3, ERK) was reduced but the level of activated caspase-3 was increased.

Conclusions
These findings suggest that hepatocyte Gab1 expression may play a role in promoting liver fibrosis progression by triggering ERK activation and inhibiting apoptosis. It implies that the Gab1-mediated signaling pathway would be potential therapeutic target to treat chronic liver diseases.

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.

P49 - **Production of single-round infectious particles for human pathogenic flaviviruses with DNA-based replicon**

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Background
Many flaviviruses are transmitted to vertebrates by mosquitoes, ticks or unknown vectors, and cause disease in human beings and animals, resulting a serious impact on public health in the world. We have established a method for production of single-round infectious particles (SRIPs) of representative flaviviruses, that are useful for viral mutagenesis studies, diagnostic applications and the production of safe vaccines with no risk of productive infection. In this study, we generated SRIPs for more than 30 species of various human pathogenic flaviviruses.

Methods
SRIPs were generated by transfection of 293T cells with prME-expression plasmids, capsid-expression plasmid and replicon plasmid including the nano-luciferase reporter gene. The capsid-expression and replicon plasmids derived from dengue virus type 1 (DENV1), Japanese encephalitis virus (JEV), yellow fever virus (YFV) and Zika virus (ZIKV) were used. 36 different prME-expression plasmids for DENV1-4, JEV, YFV, ZIKV, Alkhurma, Apoi, Bagaza, Banzi, Bussuquara, Edge Hill, Ilheus, Karshi, Kokobera, Koutango, Kyasanur Forest disease, Langat, Louping ill, Murray Valley encephalitis, Ntaya, Modoc, Omsk hemorrhagic fever, Powassan, Rio Bravo, Rocio, Sepik, Royal Farm, Spondweni, St Louis encephalitis, Tick-borne encephalitis, Tyuleniy, Usutu, Wesselsbron and West Nile virus were used for production for SRIPs. The infection of SRIPs was evaluated by inoculating the SRIPs into Vero cells, followed by measurement of luciferase activity.

Results
SRIPs generated with the YFV-based replicon showed a high infectious titer compared to that observed with the ZIKV, DENV-1 and JEV-based replicon. All examined flavivirus SRIPs except Tyuleniy and Kokobera virus were successfully generated using the YFV-based replicon. In addition, high luciferase activity was demonstrated earlier after infection with YFV-based SRIPs compared with ZIKV-, DENV1- and JEV-based SRIPs.

Conclusions
With the prM and E nucleotide sequence information available, various flaviviral SRIPs can be easily produced by transfection. Production of the YFV-based SRIPs is superior in terms of infectious titer and rapid assay / analysis for multiple human pathogenic flaviviruses. This system has important value as a basic research and diagnostic tool, and could be used to enhance the safety and speed of neutralization assays, as well as novel vaccine production.

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.

P51 – **Characterizing of hepatitis E virus infection in a rat model with and without ribavirin interventions**

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Background and Aims
Hepatitis E virus (HEV) is the most common cause for acute viral hepatitis and is also responsible for chronic hepatitis in immunosuppressed patients worldwide. We previously reported that athymic nude rat model can support the productive replication of rat HEV and therefore resemble the situation in immunocompromised individuals with chronic HEV (PMID: 27483350). In this study, we aim to characterize chronic rat HEV kinetics from the initiation of infection to the steady state.

Method
Rat HEV LA-B350 (~ 2 x10^7 viral RNA copies) was injected in the tail vein of athymic nude rats in both the 12-day and 26-day experiments (n=25). In the 12-day experiment, rats were treated once daily by oral gavage with either vehicle (n=5) or ribavirin at a dose of 30 mg/kg (n=5) or 60 mg/kg (n=5) for 12 days. In the 26-day experiment, rats were left untreated (n=10) for 26 days. Feces were collected at various time points after infection and analyzed for the presence of viral RNA by quantitative RT-qPCR. The HEV RNA kinetics were categorized into four main groups by empirical analysis. Each identified slope was calculated by linear regression as previously done to characterize HEV kinetics in organ transplant patients under ribavirin treatment.

Results
In the 12-day experiment, three main viral phases were identified in the control group and the group treated with ribavirin (30 mg/kg, once daily oral administration): a LLOQ phase (i.e., P1) that lasts ~4 days, a plateau phase (i.e., P2) that lasts ~4 days, and the beginning of a rapid ascension phase (i.e., P3). No statistically significant difference was identified in HEV replication kinetics between the control group and the group treated with ribavirin 30mg/kg. Only the group treated with ribavirin 60 mg/kg resulted in the viral load remained in the LLOQ without showing any severe side effects. In the 26-day experiment, similar P1 and P2 phases were seen as in the 12-day experiment. The 26-day experiment helped to fully characterize phase 3 (P3) that lasted until ~day 18 post infection (with viral doubling time of ~5 days) that was followed by a high viral plateau (7.9±0.5 $\log_{10}$ copies g^{-1} feces) (P4).

Conclusion
This study presented here provides important insights into rat hepatitis E virus kinetics with and without ribavirin interventions. The robust and convenient rat model could be used to elucidate the dynamics of the HEV life cycle and possibly to predict the efficacy of novel vaccine candidates (e.g., antiviral therapeutics). Future studies need to reveal whether the viral kinetics in feces can reflect the viral kinetics in the blood.

P52 – **Generation and Characterization of HCV E1-Specific Nanobodies for Structural and Functional Study of HCV Envelope Proteins**

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While effective treatments for HCV are available, there is no vaccine to prevent infection. The HCV envelope glycoproteins, E1 and E2, are prime targets for vaccine development as they facilitate viral entry into the host cell. The structure and function of E1 is still poorly understood. Since E1 requires E2 to fold correctly, its structure needs to be studied as part of the E1E2 heterodimer. Using various well-characterized antibodies against HCV E1E2 and cryoEM, the 3D structure of recombinant E1E2 heterodimer has recently been elucidated, though the resolved E1 structure lacks many regions including the fusion domain of the full-length E1 protein (Torrents de la Pena et al, 2022, Metcalf et al, Nat Comm 2023). Since E1 plays an integral role in the structure and function of HCV envelope proteins, it is important to fully characterize this protein in the context of E1E2 heterodimer and HCV virion. In the current study, we generated HCV E1-specific antibodies. Using phage display technology, we identified over 17 clones of specialized single-chain antibodies, or “nanobodies”, specific to E1. These nanobodies consist of the variable portion of a camelid heavy-chain-only antibody fused to a humanized Fc region. We then characterized 10 of these nanobodies with recombinant E1E2 via ELISA, immunoprecipitation and transmission electron microscopy. ELISA data showed that the selected nanobodies have varying affinities for recombinant E1E2 and E2. Three of the ten examined nanobodies showed high affinity for E1E2 with negligible binding to E2, supporting that they are E1-specific. In addition, all the nanobodies successfully pulled down E1E2 but not E2 alone, through immunoprecipitation. Several of the nanobodies were observed under TEM in negative staining with direct binding to HCV E1E2. Using a combination of anti-E2 antibodies AR1B and AR2A to define domains of E1E2 on TEM (Kanai et al, JV 2023), we showed that the binding sites of the nanobodies are distinct from those of anti-E2 antibodies. These results confirm that these nanobodies are E1-specific and can be valuable tools for the study of HCV E1E2 structure and function.

P53 – **Enhancement of alphavirus replication in mammalian cells at sub-physiological temperatures**

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Background
Chikungunya virus (CHIKV) is a re-emerging *Alphavirus* transmitted by *Aedes* mosquitoes and causing fever, rash and arthralgia. Currently there are no vaccines or antiviral agents against CHIKV, therefore it is important to understand the molecular details of CHIKV replication. CHIKV is a positive sense, single-stranded RNA virus, containing a genome of ~11.8 kb and consists of two open reading frames (ORF1). While ORF1 is translated from full length genomic RNA, OFR 2 is translated from a sub-genomic RNA.

Methods
In this research, we utilized transfection of CHIKV replicon and ONNV infection. Viral replication was detected and indicated by Firefly luciferase and protein translation were indicated by Renilla luciferase. Viral protein express levels were also detected by western blot. Viral dsDNA, host protein G3BP1 or mCherry were detected by immunofluorescence. Cell viability were detected by MTT assay. Quantification of protein levels or dsRNA colocalization with viral protein or host proteins were conducted via imageJ.

Results
we generated a panel of mutants in a conserved, surface exposed cluster in the nsP3 alphavirus unique domain (AUD), and tested their replication phenotype using a subgenomic replicon (SGR) in a variety of mammalian and mosquito cells. We identified three mutants that replicated poorly in mammalian cells but showed no defects in mosquito cells. Further investigation showed that these mutants were temperature-sensitive, rather than species-specific as they showed no replication phenotype in mammalian cells at sub-physiological temperatures (28°C). We also observed similar effects in CHIKV as well as a closely related virus: O'Nyong Nyong virus (ONNV). Intriguingly, this analysis also revealed that the wildtype SGR replicated much more efficiently at sub-physiological temperatures as compared to 37°C. This was not due to impaired interferon responses as this enhancement was also observed in *Vero* cells. Neither was this due to a defect in the unfolded protein response as treatment with ISRIB, an inhibitor of global translation attenuation, did not compensate replication defects at 37°C for mutants. However, we noticed different levels of colocalization among viral dsRNA, nsP3 and host proteins at various temperatures.

Conclusions
Those AUD mutants generated in CHIKV nsP3, as well as the WT, showed temperature-sensitive phenotype. These observations provide new insights into alphavirus replication favoured by sub-physiological temperatures, and might indicate how the virus adapt to different temperatures, either jumping from mosquitoes to humans, or from human skin to internal body, all involving a temperature increase.

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.

P55 – **Roles of epidermal growth factor receptor, claudin-1 and occludin in multi-step entry of hepatitis C virus into polarized hepatoma spheroids**

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Abstract
The multi-step process of hepatitis C virus (HCV) entry is facilitated by various host factors, including epidermal growth factor receptor (EGFR) and the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN), which are thought to function at later stages of the HCV entry process. Using single particle imaging of HCV infection of polarized hepatoma spheroids, we observed that EGFR performs multiple functions in HCV entry, both phosphorylation-dependent and -independent. We previously observed, and in this study confirmed, that EGFR is not required for HCV migration to the tight junction. EGFR is required for the recruitment of clathrin to HCV in a phosphorylation-independent manner. EGFR phosphorylation is required for virion internalization at a stage following the recruitment of clathrin. HCV entry activates the RAF-MEK-ERK signaling pathway downstream of EGFR phosphorylation. This signaling pathway regulates the sorting and maturation of internalized HCV into APPL1- and EEA1-associated early endosomes, which form the site of virion uncoating. The tight junction proteins, CLDN1 and OCLN, function at two distinct stages of HCV entry. Despite its appreciated function as a “late receptor” in HCV entry, CLDN1 is required for efficient HCV virion accumulation at the tight junction. Huh-7.5 cells lacking CLDN1 accumulate HCV virions primarily at the initial basolateral surface. OCLN is required for the internalization of virions. This study produced further insight into the unusually complex HCV endocytic process.

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.

P57 – **Protein kinase D (PKD) 1 promotes HCV Replication by suppressing the TBK1/IRF-3/IFNs pathway**

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Abstract
Hepatitis C virus (HCV) has acquired multiple means to overcome host innate immune responses initiated by pattern-recognition receptors (PRRs). Protein kinase D (PKD) 1 is a serine/threonine kinase involved in cell proliferation and lipid metabolism. In this study, we investigated the role of PKD1 during HCV infection. We found that PKD1 was phosphorylated and activated by protein kinase C (PKC) in HCV-infected cells. PKD1 played a positive role in HCV replication, as its overexpression increased the HCV core protein level. On the contrary, the depletion of PKD1 either by siRNA silencing or CRISPR knockout decreased the levels of the HCV core protein and HCV RNA, which was corelated with the phosphorylation of TBK1 and IRF3 and the induction of interferons (IFNs) and IFN-stimulated genes (ISGs). Re-introduction of PKD1 into the PKD1-knockout cells reduced the expression of IRF-3 and enhanced HCV infection. PKD affected the replication of HCV RNA, which was confirmed by the analysis of HCV subgenomic RNA replicon cells. Interestingly, PKD1 knockout also impaired the ability of HCV to activate caspase 1 and induce the expression of IL-1β and IL-18. Taken together, our results indicate that PKD1 suppresses the TBK1/IRF-3/IFN pathway to enhance HCV RNA replication and in the meantime activates inflammasomes to result in the release of IL-1β and IL-18.

P58 – **The Effect of Fentanyl on Tight Junction Protein Expression and HCV Replication**

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Background
The opioid crisis constitutes a worldwide public health emergency, with over 100,000 overdose deaths recorded in 2021. People who inject drugs (PWID) make up an increasing proportion of overdoses and have heightened risk for comorbidities, especially viral diseases like human immunodeficiency virus (HIV) and hepatitis C virus (HCV). Despite both being blood-transmitted, new HCV cases far outnumber HIV cases in PWID. The rise in HCV cases is largely driven by the opioid epidemic, constituting a “syndemic” of overlapping crises. HCV cases may be elevated in PWID for many reasons ranging from environmental stability of virions to opioids influencing viral infection. Opioids have been shown to increase HCV replication but the mechanism responsible for this remains undefined other than indirect effect caused by suppression of innate antiviral signaling by the opioids. In the gut and blood brain barrier, it has been demonstrated that opioids compromise tight junction expression, localization and integrity. Because HCV requires tight junction proteins claudin-1 (CLDN-1) and occludin (OCLN) to spread in the liver, we hypothesize that the opioid fentanyl changes tight junction protein expression to promote increased HCV transmission in the liver.

Methods
To test this hypothesis, we first measured the expression of tight junction proteins CLDN-1 and OCLN in hepatocytes following fentanyl treatment. Western blotting and qRT-PCR were utilized to show expression changes. Next, we determined the effects of fentanyl on HCV replication by measuring HCV protein expression with western blotting, HCV RNA levels with qRT-PCR, and infectivity with focus-forming assays.

Results
Our results showed an increase in CLDN-1, but not OCLN protein levels 30 to 48 hours after fentanyl treatment in hepatocytes. We also detected an increase in viral protein core and nonstructural protein 3 (NS3) levels 48 hours following fentanyl treatment. Additionally, initial focus-forming assays indicate an increase in HCV infectivity after fentanyl treatment.

Conclusions
These results suggest a direct effect of fentanyl on HCV spread by affecting hepatic tight junction and viral protein levels in liver cells. Further studies will seek to elucidate the role of fentanyl-dependent changes in hepatic tight junctions on cell-free and cell-cell HCV spread.

P59 – **HCV promotes the selective lysosomal degradation pathways, Chaperone-mediated autophagy and endosomal microautophagy, for viral proliferation.**

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Background
We previously reported that hepatitis C virus (HCV) enhances Chaperone-mediated autophagy (CMA) by association of the cellular chaperone protein HSC70 with HNF-1α by HCV NS5A protein to promote the lysosomal degradation of HNF-1α, resulting in downregulation of Glucose transporter 2 expression. We also reported that HCV infection enhances endosomal microautophagy (eMI) by association of HSC70 with DGAT1 by NS5A protein to promote the selective degradation of DGAT1 protein, resulting in efficient production of infectious HCV particles. Both HNF-1α protein and DGAT1 protein contain a pentapeptide KFERQ-motif, which is recognized by HSC70, leading to the trafficking to the lysosome or the endosome, respectively. We proposed that HCV promotes these two selective lysosomal degradation pathways, CMA and eMI in hepatocytes for viral pathogenesis and proliferation. However, little is known about the biological roles of CMA and eMI in HCV infection. In this study, we aimed to elucidate the biological roles of CMA and eMI in HCV proliferation.

Methods
To determine the roles of CMA and eMI in HCV infection, we generated the shRNA-mediated knockdown Huh-7.5 cells for HSC70, LAMP-2A, VPS4A, and VPS4B, respectively. LAMP-2A is a critical factor for CMA and VPS4B is a critical factor for eMI. VPS4A is an important factor for ESCRT pathway. To examine HCV proliferation in shHSC70 cells, shLAMP-2A cells, shVPS4B cells, and shVPS4A cells, we analyzed the HCV RNA levels and HCV protein levels by real-time RT-PCR and western blotting, respectively.

Results
HCV RNA levels and HCV protein levels were markedly reduced in shHSC70 Huh-7.5 cells, suggesting that HSC70 is essential for HCV proliferation. HCV RNA levels were lower in shLAMP-2A cells than control cells. HCV RNA levels were markedly reduced in the shVPS4B cells compared to shLAMP-2A cells, shVPS4A cells and control cells. HCV protein levels were also reduced in both shLAMP-2A cells and shVPS4B cells. Taken together, these results suggest that the both CMA and eMI pathways are required for efficient HCV proliferation and eMI pathway especially plays a crucial role in HCV proliferation.

Conclusions
We provide evidence suggesting that HCV infection promotes CMA and eMI, leading to downregulation of cellular proteins. These results suggest that eMI plays a crucial role in HCV proliferation. We are currently investigating the details of the viral survival strategy via the eMI pathway.

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.

P61 – **Tripartite motif containing protein 26 restricts hepatitis C virus replication in vivo.**

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Background & Aims
The murine ortholog of tripartite motif-containing protein 26 (TRIM26) was previously shown to be insufficient to support HCV genome replication. This is in contrast to the human ortholog of TRIM26, which associates with and ubiquitinates hepatitis C virus (HCV) NS5B, enabling proper assembly of genome replication components. Previous data indicates that murine cell lines harboring human TRIM26 (along with entry factors) can sustain moderate HCV replication. To this end, we sought to generate a minimally humanized mouse model possessing human entry factors and TRIM26 that would, in theory, support HCV infection. We further examined these mice in the presence and absence of an adaptive immune response and analyzed *in vivo* HCV genome replication.

Methods
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, termed mCD81/EL2[h/h] mOCLN/EL2[h/h], which support HCV glycoprotein-mediated uptake. mCD81/EL2[h/h] mOCLN/EL2[h/h] mice were modified via 2-cell homologous recombination CRISPR (2C-HR-CRISPR) to knock-in the human ortholog of TRIM26 into the first coding exon of the mouse ortholog thereby also genetically disrupting the expression of the murine gene. The resultant mice, mCD81/EL2[h/h] mOCLN/EL2[h/h] TRIM26[h/h], 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] TRIM26[h/h] donors into immunodeficient liver injury FAH-/- NOD Rag1-/- IL2Rg^{NULL} (FNRG) recipients, which are devoid of functional B, T and NK cells.

Results
Serum and liver HCV RNA copies were indistinguishable between immunocompetent mCD81/EL2[h/h] mOCLN/EL2[h/h] TRIM26[h/h] and mCD81/EL2[h/h] mOCLN/EL2[h/h] mice at all timepoints tested. Notably, however, we detected moderate, sustained HCV serum viremia in FNRG mice transplanted with mCD81/EL2[h/h] mOCLN/EL2[h/h] TRIM26[h/h] donor hepatocytes.

Conclusions
Expression of the human ortholog of TRIM26 in mCD81/EL2[h/h] mOCLN/EL2[h/h] mice appears to be sufficient for rendering animals permissive to HCV infection in the absence of B, T, and NK cells. Our data indicate that these transplant-recipient mice are able to permit HCV replication *in vivo* and sustain viremia for several weeks. Future studies will seek to develop this model further by incorporating additional factors implicated in HCV tropism and by interrogating the specific immune subsets implicated in HCV restriction in immunocompetent mCD81/EL2[h/h] mOCLN/EL2[h/h] TRIM26[h/h] mice. Results from this work will advance the ultimate goal of an immunocompetent mouse model that sustains human-like HCV infection, while further highlighting the importance of understanding viral tropism to small-animal model development.

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.

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.

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.

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.

P66 – **Lineage-related bNAbs with distinct neutralization profiles are associated with spontaneous clearance of reinfection**

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Background
Approximately 25% of people spontaneously clear primary hepatitis C virus (HCV) infections without treatment. Of those infected again following clearance, ~80% of individuals clear the subsequent reinfections. A major challenge to clearance of HCV is the rapid replication of the virus with an error-prone polymerase, facilitating viral escape from antibody pressure. In this study, we evaluated both antibody and viral evolution in a participant who spontaneously cleared multiple infections with high plasma neutralizing breadth. We explored the dynamics of the antibody-virus relationship in the third cleared infection.

Methods
We isolated two closely-related broadly neutralizing antibodies (bNAbs), hcab55 and hcab64, from the same B cell lineage by flow-cytometrically sorting antigen-specific B cells using a mixture of soluble E2 proteins. We also single-genome amplified and sequenced longitudinal E1E2 variants from the participant's third cleared infection. We used these E1E2 sequences to generate pseudoparticles (HCVpp) and measured their neutralization by hcab55, hcab64, or variants of those mAbs generated by site-directed mutagenesis of individual somatic mutations. We also solved the crystal structures of hcab64 and hcab55 Fabs in complex with E2 ectodomains.

Results
While Hcab64 and hcab55 bNAbs are from the same B cell lineage, share V_H, D, and J_H gene segments, and have the same HCDR3 length, they differ by 10 amino acids in HCDR1-3. Fab- E2 ectodomain structures demonstrated that both mAbs bind to the same site in the front layer of E2 and share most of their E2-contact residues. When we tested neutralization of autologous, longitudinal HCVpp from the third infection, we found that naturally-selected substitutions in E1E2 conferred increased neutralization resistance to hcab64 (4-fold increase in IC_{50}), but increased sensitivity to hcab55 (7-fold decrease in IC_{50}). Variants of hcab55 and hcab64 with individual somatic mutations reverted to germline had similar neutralization profiles to the mature bNAbs, indicating that the somatic substitutions tested thus far do not explain the functional difference between hcab55 and hcab64.

Conclusions
Substitutions that naturally arose in E1E2 over the course of a third cleared infection conferred resistance to the autologous bNAb hcab64 while increasing sensitivity to the closely related autologous bNAb hcab55. Studies to understand the molecular mechanism of this observation are ongoing. The evolutionary 'dead end' for the virus caused by cooperativity between this bNAb pair may have contributed to spontaneous viral clearance.

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.

P68 – **Tomography studies of the dengue fusion process**

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Background
Dengue virus (DENV), a flavivirus, utilizes the endocytic pathway for cell entry. Previous crystal structures of envelope (E) protein ectodomain pre-fusion dimers and the post-fusion trimers reveal only the start- and end-stages of the fusion process, with intermediate remaining uncharacterized.

Methods
Here, we used a combination of cryoEM tomography and biochemical assays to study the fusion intermediate structures of DENV with liposomes at pH conditions mimicking those of the early (pH6.5) to late endosomal (pH6.0) environment.

Results
At pH6.5, virus is attracted to liposomes but no E protein insertion is observed. At pH6.0, segmentation of cryoET tomograms collected with a Volta-phase plate consistently showed that the E protein monomers located at the center of the interaction site have flipped up to interact with the adjacent liposomal membrane, whereas flipped-up monomers located at the periphery of the interaction site have their previously membrane-associated stem regions likely extended. The E protein trimers in the extended form were also observed consistently at the periphery of the interaction site, suggesting trimerization between the extended E monomers. We also collected cryoET data without using a Volta phase plate and manually picked E protein densities at these interaction sites. Subtomogram averaging and classification of these densities yielded three structural classes: (i) short E monomers normal to the liposomal membrane, (ii) longer E protein monomers tilted relative to the liposomal membrane, and (iii) a C3-symmetric density consistent with three E protomers in a looser association than seen in the post-fusion trimer. We then applied a machine learning approach to automatically segment the viral and liposomal membranes in order to measure viral-to-liposomal membrane separation at each of these aligned subtomogram positions, for each class. These results showed that the tilted monomer class likely represents a mixture of tilted E proteins of different lengths, based on whether the stem region is extended. These results are consistent with the conclusions from our segmentation experiments.

Conclusion
Our results document important intermediate stages of the virus-liposome fusion process.

P69 – **Specific labeling, isolation, phenotyping, and BCR-sequencing of E1-dependent and independent anti-HCV B cells using soluble E1E2 and E2 proteins**

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Background
The humoral immune response against hepatitis C virus (HCV) is directed against the E1E2 glycoprotein heterodimer, which mediates viral entry into hepatocytes. Compared to individuals with chronic infection, individuals who spontaneously clear HCV develop broadly neutralizing antibodies (bNAbs) earlier in infection. Due in part to technical limitations, most previously described anti-HCV bNAbs target E2, while plasma studies suggest that a diverse antibody response, including antibodies recognizing intact E1E2 heterodimer, is important for clearance. Improved methods are needed to assess the full repertoire of E2 and E1E2-reactive B cells.

Methods
Recombinant purified soluble E1E2 syn-zip (sE1E2.SZ) (H77) and soluble E2 (sE2) (H77, 1a157, 1b09, and 1b21) were biotinylated, conjugated with streptavidin-fluorophores and used to label cryopreserved PBMCs from the Baltimore Before and After Acute Study of Hepatitis (BBAASH). We performed single-cell sorting on class-switched B cells (CD3-CD14-CD19+CD20+IgM-IgD-) reactive to sE1E2.SZ or sE2 into 96-well plates with 3T3-msCD40L feeder cells as well as cytokines and growth factors. After incubating for 13-15 days, we harvested cell culture supernatants and measured IgG production as well as binding to HCV E2 or E1E2 proteins in ELISAs. RNA from each cell was used for cDNA synthesis and the amplification, barcoding, pooling, and next-generation sequencing of IgH, IgK and IgL loci.

Results
We specifically labeled distinct populations of B cells from HCV+ donors which were reactive with sE2 only, sE1E2 only, or both sE2 and sE1E2. Interestingly, the proportions of these populations varied amongst donors. We sorted 208 sE1E2-reactive and 36 sE2-reactive B cells from an individual who spontaneously cleared their HCV infection and developed high plasma neutralization breadth. Of these B cells, 68% of the sE1E2-sorted and 61% of the sE2-sorted B cells secreted measurable IgG in culture. Of the IgG+ supernatants, 85% were reactive in HCV ELISAs. Interestingly, 20% of supernatants from sE1E2-reactive B cells exhibited E1-dependent binding. Compared to HCV-non-reactive B cells, E2/E1E2-reactive cells significantly utilized V_H1-69 and V_H3-33 germline genes ($p<0.05$). There was a trend towards greater V_H1-69 usage by E1-independent cells and greater V_H3-33 usage by E1-dependent cells.

Conclusions
We have developed a novel method to flow cytometrically sort the diverse repertoire of E2 and E1E2-reactive B cells from HCV-infected individuals, which allowed us to quickly phenotype the antibody produced by each B cell while also obtaining authentically-paired heavy and light chain B cell receptor (BCR) sequences from the same cells. These BCR sequences can be used for repertoire analysis and monoclonal antibody production. In validating this method, we found interesting trends in the B cell phenotypes and sequences, which we will follow up as additional individuals are studied and monoclonal antibodies are generated.

P70 – **Incorporation of native E1E2 into a nanoparticle vaccine platform**

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Background
The worldwide HCV disease burden makes development of an effective vaccine a significant unmet medical need. Since HCV rapidly accumulates mutations, vaccines must elicit the production of broadly neutralizing antibodies (bnAbs). Decades of research have generated a number of HCV vaccine candidates. Based on the available data and research through clinical development, a vaccine antigen based on the E1E2 glycoprotein complex appears to be the best choice, but robust induction of humoral and cellular responses leading to virus neutralization has not yet been achieved. One issue that has arisen in developing an HCV vaccine (and many other vaccines as well) is the best platform to use for antigen delivery. The majority of viral vaccine trials have employed subunit vaccines. However, subunit vaccines often have limited immunogenicity, as is seen for HCV, and thus multiple formats must be examined in order to elicit a robust anti-HCV immune response. Nanoparticle vaccines are gaining prominence in the field due to their ability to facilitate a controlled multivalent presentation and trafficking to lymph nodes, where they can interact with both arms of the immune system. Here we used a soluble, secreted form of E1E2 (sE1E2) to assemble native E1E2 into a nanoparticle platform and examined its biochemical properties and immunogenicity.

Methods
Nanoparticles were assembled by purifying sE1E2 containing a C-terminal SpyTag and an mi3-SpyCatcher fusion separately and coupling sE1E2-SpyTag to mi3-SpyCatcher via overnight incubation. Free sE1E2-SpyTag was removed from nanoparticle preparations via gel filtration. Nanoparticles were assessed for solution behavior and antibody binding and incorporation of sE1E2 was confirmed by electron microscopy. Immunogenicity of sE1E2-mi3 nanoparticles was examined relative to sE1E2 alone and membrane-bound E1E2 (mbE1E2) following inoculation of groups of CD1 mice.

Results
Optimization of coupling conditions yielded a high degree of sE1E2-SpyTag incorporation into the mi3-SpyCatcher nanoparticles. Relative to the sE1E2-SpyTag alone, we observed enhanced binding to select bnAbs for the sE1E2-mi3 nanoparticles. Electron microscopy analysis showed a clear incorporation of sE1E2 on the surface of the nanoparticle when compared to mi3-SpyCatcher alone samples. Assessment of the immunogenicity of the sE1E2-mi3 nanoparticles as compared to sE1E2 alone and mbE1E2 is ongoing.

Conclusions
These studies show that E1E2 can be incorporated into a nanoparticle format in a native-like state that preserves binding to important bnAbs, most notably AR4A which is strongly correlated with viral clearance. This proof-of-principle study sets the stage for further exploration of nanoparticles and other multivalent platforms for the development of E1E2-based vaccines.

P71 – **A glycoengineered CHO (geCHO) biomanufacturing platform for HCV vaccine production**

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Background
An effective HCV vaccine must elicit the production of broadly neutralizing antibodies in a reproducible fashion. The HCV envelope E1E2 glycoprotein complex is a natural target of neutralizing antibodies, but there are challenges associated with its production. First, it is critical to produce E1E2 with reproducible glycan content, as glycans regulate the exposure of epitopes that give rise to a potent and broadly neutralizing antibody response. Ideally, the glycan content would maximize epitope exposure without compromising antigen stability or exposing epitopes that are hidden during a natural infection. However, variation across host cells or clones can lead to substantial variation in batch-to-batch or inter-batch glycosylation, potentially affecting epitope exposure, the antibody response, and neutralization potency. Second, it can be difficult to maintain an expression host that consistently produces functional E1E2 glycoforms. We addressed these challenges using glycoengineered CHO (geCHO) cell lines that impart nearly homogeneous glycosylation as a means to examine the extent to which controlling glycan content influences downstream readouts of antigenicity and immunogenicity for the secreted E2 ectodomain (sE2).

Methods
sE2 was expressed in ten geCHO cell lines and in wild-type CHO (wtCHO) and HEK293 cells as controls. Binding affinity measurements (Kd) were performed using a panel of neutralizing antibodies representing the major antigenic domains of E2. The top two sE2 glycoforms exhibiting the best Kd profiles were selected for further analysis. Each antigen was purified to homogeneity using the same protocol. Following biochemical, biophysical, and structural characterization, the immunogenicity of the two glycoforms of sE2 and wtCHO and HEK293 controls were examined following inoculation of groups of CD1 mice.

Results
The antigens with nearly homogenous glycans exhibited a modest but reproducible increase in affinity for some mAbs relative to wtCHO- and HEK293-derived sE2, particularly for antigenic domains B and D. Structural analysis by cryo-EM using the domain D antibody HC84.26 confirmed engagement with the target epitope on the geCHO-derived antigen. The total antibody response was the same for all four groups of mice for both total IgGs and IgG1 and IgG2a subtypes. However, the geCHO sE2-inoculated mice exhibited a tighter clustering of IC50 values against the homologous HCV pseudoparticle relative to the wtCHO and HEK293 and sE2 inoculated groups. Moreover, for one of the geCHO groups we observed a statistically-significant improvement in neutralization against the homologous HCV pseudoparticle. Cross-neutralization activity was low, but the best performing geCHO group had the most responders (i.e. observed neutralization activity) against genotypes 1b, 2a, 2b, and 3.

Conclusions
These studies show that glycan content impacts biochemical properties of antigens in subtle ways that can influence the quality and uniformity of immune responses. Moreover, they demonstrate the potential utility of the geCHO system as a biomanufacturing platform for HCV vaccine production.

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.

P73 – **Determining antigen-specific T-cell phenotypes in cases of Hepatitis C virus clearance versus chronic infection**

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Background
To understand protective immunity to HCV infection in high-risk populations, we prospectively followed HCV antibody negative high-risk people who inject drugs (PWIDs) in a protocol designed for monthly follow-up through the Baltimore Before and After Acute Study of Hepatitis (BBAASH). We demonstrated previously that HCV reinfection in PWIDs is associated with a reduction in the magnitude and duration of viremia, enhanced frequency of HCV clearance, and broadened cellular immune responses versus the initial infection, consistent with protective immunity against chronic disease. The goal of this project is to determine molecular determinants of CD8+ T-cell responses that differentiate cleared HCV infection versus chronic infection.

Methods
Upon infection and reinfection, interferon gamma (IFNg) ELISpot assays were performed on peripheral blood mononuclear cells (PBMCs) to identify specific Major Histocompatibility Complex class I (MHC I) epitopes recognized by HCV specific CD8+ T-cells. Using a spectral flow cytometry-based platform that combines single cell analysis of pentamer labeled HCV-specific CD8+ T-cells with traditional cell surface and intracellular staining for proteins involved in metabolic programming, we assessed 27 molecules on HCV-specific CD8+ T-cells. Time points from the acute infection phase in participants who cleared one or multiple HCV infections and those that progressed to chronic infection were used for this analysis.

Results
As in previous studies, T-cell breadth and magnitude as measured by ELISpot did not significantly differ based on outcome, suggesting that the phenotype of CD8+ T-cells and functions other than IFNg secretion may impact infection outcome. Our high parameter flow cytometry demonstrated that HCV-specific CD8+ T-cells from those who progress to chronic infection have a unique acute phase phenotype that clusters separately from those who later successfully control HCV. Acute phase CD8+ HCV-specific T-cells from those who subsequently clear an initial HCV infection show increased expression of CD127 and CXCR3 and decreased expression of glucose transporter 1, programmed death-1 and CD39 compared to acute phase T-cells in those who later progress to chronic infection.

Conclusion
We have characterized CD8+ HCV-specific T-cells from the acute phase of HCV using high dimensional spectral flow cytometry and identified features of CD8+ T-cell responses associated with control of HCV infection. Understanding HCV-specific T-cell features in spontaneous control of HCV infection and reinfection will inform goals for vaccine induced immunity to prevent chronic HCV infection.

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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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.

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.

P77 – **Cryo-EM structure of a secreted hepatitis C virus E1E2 ectodomain in complex with neutralizing antibodies**

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Background
Hepatitis C virus (HCV) is a major cause of liver disease and hepatocellular carcinoma. Despite the development of curative direct-acting antiviral treatments, an effective prophylactic vaccine is desired for controlling HCV infection rates, as the virus remains a persistent burden on health care systems across the world. The membrane-associated E1E2 surface glycoprotein is the primary target for neutralizing antibodies, but due to its membrane association, biochemical preparation of E1E2 heterodimers for structural, biochemical, and vaccine studies possesses an elevated level of complexity over solubilized ectodomain forms. Utilizing a design strategy in which the transmembrane domains of the E1 and E2 were substituted with leucine zipper scaffolds, we previously engineered soluble E1E2 ectodomains that were recognized by a wide-array of neutralizing antibodies, including those specific to the E1E2 heterodimer. Here, we describe the structural analysis of one such scaffolded, soluble E1E2 heterodimer ectodomain, sE1E2.SZ, bound by neutralizing antibodies.

Methods
Single particle cryo-EM was used to investigate the structure of a soluble E1E2 ectodomain sE1E2.SZ of genotype 1b in complex with Fabs of neutralizing antibodies AR4A, HEPC74, and IGH520.

Results
The structure of sE1E2.SZ in complex with antibodies was determined to ~3.65 Å resolution. The structure reveals the three-dimensional heterodimeric organization of the E1 and E2 subunits liberated from membrane association, including resolved regions within E1 and the C-terminus of E2 that comprise the E1-E2 interface. sE1E2.SZ maintains essential structural features observed in the structure of non-engineered full-length membrane-extracted E1E2. Comprehensive analysis of antibody epitopes within E1E2 provides the structural basis for antibody breadth of recognition and neutralization, and delineates antibody navigation of a glycan shield that falls predominantly on one face of the heterodimer.

Conclusions
The findings presented in this study provide a structural model for an engineered secreted E1E2 heterodimer ectodomain, and confirm that it maintains native structural features observed in full-length forms of the glycoprotein. The study validates the design strategy used to engineer sE1E2.SZ, and opens the door for further rational vaccine development utilizing soluble E1E2 antigens.

P78 – **Structure of HCV E2 envelope glycoprotein bound to neutralizing and non-neutralizing human antibodies**

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Background
Hepatitis C Virus (HCV) is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma and is considered as a major public health problem worldwide (**1**). No vaccine is available against HCV and the high cost of current therapeutics agents restricts access in developing nations where the disease burden is greatest (**2**). The HCV E2 envelope glycoprotein is a major target for broadly neutralizing antibodies and considered as a potential candidate for the immune prophylaxis of HCV infection and vaccine development.

Methods
We determined structures of E2 bound to monoclonal antibodies (mAbs) from HCV-infected individuals using single-particle cryoEM. The relatively small size of E2 (~70 kDa) presented a challenge for cryoEM and prompted us to develop fiducial markers to facilitate cryoEM analysis. We engineered bivalent versions of Fab fragments of anti-E2 mAbs that doubled the effective size of Fab–E2 complexes and rendered them highly identifiable in shape, thereby facilitating particle selection for image processing (**3**).

Results
Using bivalent Fab HC84.26, we determined cryoEM structures of Fab HC84.26–E2 and Fab HC84.26–E2–Fab CBH7 complexes. HC84.26 and CBH7 recognize non-overlapping E2 domains D and C, respectively. Furthermore, we determined the cryoEM structure of a bivalent Fab CBH4B–E2 complex. CBH4B recognizes domain A on the non-neutralizing face of E2. Analysis of the Fab HC84.26–E2 complex revealed that the footprint of HC84.26 on E2 resembles the footprint made by vaccine-induced neutralizing mAbs (**4**). The angle of approach for CBH7 is unique among all mAb–E2 structures as its epitope spans both neutralizing and non-neutralizing faces of E2. CBH7 is the only known mAb whose light chain overlaps the CD81 binding site but whose heavy chain does not. The Fab CBH4B–E2 structure revealed an angle of approach for CBH4B distinct from the angle of approach of other non-neutralizing mAbs.

Conclusion
Chronic infection with HCV is a major risk factor for the development of hepatocellular carcinoma worldwide. An effective HCV vaccine must overcome the ability of this virus to escape host immune responses. The goal of structural vaccinology is to control immunogenicity at the atomic level. The mAb–E2 complexes reported here delineate the structure of neutralizing and non-neutralizing epitopes of E2. We will use this information for structure-guided design of E2 and E1E2 variants with optimal immunogenicity for incorporation into a prophylactic HCV vaccine.

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P79 – **Optimizing secreted HCV E1E2 antigens using sequence- and structure-based design**

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Background
A major goal of hepatitis C virus (HCV) vaccine development is an E1E2 glycoprotein antigen that effectively induces potent broadly neutralizing antibodies (bnAbs). While many previous and current efforts have utilized E2 and E1E2 ectodomains from well-defined HCV strains, such as H77, these may not necessarily be optimal as vaccine antigens. Here we describe the design and experimental validation of new E1E2-based antigens that were optimized by distinct design approaches and validated for antigenicity with a panel of HCV bnAbs.

Methods
Consensus ectodomains were generated using multiple sequence alignments of E1E2 sequences collected from various databases and filtered to optimize diversity. Alternative sE1E2 scaffolds were identified from known heterodimeric structures. Structure-based designs of E1E2 ectodomain substitutions were generated using Rosetta protein modeling software. Designs were cloned in secreted E1E2 (sE1E2) format, expressed in mammalian cells, and assessed for antigenicity with E1E2 bnAbs using ELISA.

Results
We found that multiple new sE1E2 designs displayed comparable antigenicity to the previously designed sE1E2 form based on H77 ectodomains and leucine zipper scaffold. These included sE1E2 with the Spytag/Spycatcher heterodimer used as scaffold, as well as a sE1E2 with genotype 1 consensus ectodomains. One design with consensus ectodomains representing genotypes 1-7, named cons80, showed superior antigenicity to the current sE1E2 construct and will be tested in vivo in planned comparative immunogenicity studies.

Conclusions
We have shown that multiple rational design approaches can result in alternative sE1E2 designs with maintained or improved antigenicity, which may result in a more effective HCV vaccine. Upcoming studies will further test these new designs in comparison with sE1E2 containing HCV ectodomains associated with spontaneous viral clearance to validate their antigenicity and immunogenicity in vitro and in vivo.

P80 – **Characterization and developmental pathway of a highly neutralization-resistant HCV strain**

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Background
Hepatitis C virus (HCV) infection remains a global health threat and consequently there is an urgent need for the development of effective prophylaxis. Defining correlates of protection is crucial for the development of a vaccine. Several studies have demonstrated that broadly neutralizing antibodies (bNAbs) play a key role in protection against (chronic) infection. However, the HCV glycoprotein E1E2, targeted by these antibodies, has been shown to escape from autologous antibodies during chronic infection. To obtain a better understanding of how evolutionary processes during chronic infection may shape E1E2 neutralization sensitivity, we performed a detailed analysis of an HCV E1E2 variant (AMS230) and its ancestor sequences, obtained from a patient who had remained low viremic for over a year following a failed treatment episode with an interferon-based regimen, until he fully rebounded to pre-treatment viral load levels.

Methods
Neutralization sensitivity of AMS230 was determined by constructing an HCV pseudoparticle (HCVpp), which was tested against a comprehensive panel of 21 monoclonal antibodies (18 bNAbs and 3 non-neutralizing), targeting the most important epitopes on both E1 and E2. In addition, to analyze evolutionary changes in E1E2 over time from acute infection until the patient ultimately cleared the infection, E1E2 genes were sequenced from 8 timepoints over 6 years, starting during acute infection, prior to interferon treatment. Finally, to determine how the polyclonal antibody response evolved over time, serum samples from the same timepoints were tested against a panel of 11 different HCVpps from multiple genotypes with a wide range of neutralizing sensitivities.

Results
AMS230 is an extremely neutralization-resistant virus; only AR4A, which targets a metastable epitope on E1E2, neutralized AMS230 with an IC_{50} of 5 µg/mL. In contrast, none of the bNAbs against epitopes in and surrounding the CD81 receptor binding site (CD81bs), including HEPC3, AR3C, 1416_01_E03, AT1209, AP33 and HC84.26, neutralized AMS230.
AMS0230 acquired a number of amino acids in and in the proximity of the CD81bs as well as the hypervariable region 1 (HVR1), which were not present at the beginning of the infection, and only became dominant following viral load rebound. However, since some of the CD81bs bNAbs did bind the sequence-matched AMS230 E1E2 protein, the observed neutralization resistance likely involves additional factors besides CD81bs and HVR1 amino acid changes.
Emergence of these changes appeared to coincide with the emergence of a polyclonal serum neutralizing response, which broadened over time to a geometric mean titer of 1:1000. Notably, the response against 'autologous' AMS0230 remained relatively low (< 1:1000) compared to the response against heterologous HCVpps.

Conclusion
We have isolated a remarkably neutralization-resistant HCV variant, which should be included in panels used for characterizing vaccination responses. Additional analysis to explain the phenotype of this variant is pending. The results of this study highlight the importance of investigating autologous and heterologous immune responses and virus-host co-evolution in individuals with a unique disease course.

P81 – **A longitudinal analysis of Hepatitis C virus envelope and antibody response in a patient infected with an antibody resistant variant**

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Background
Hepatitis C virus (HCV) infection remains a global health threat and consequently there is an urgent need for the development of effective prophylaxis. Understanding correlates of protection is crucial for the development of a vaccine. Several studies have shown that neutralizing antibodies play an important role in protection against (chronic) infection. However, the glycoprotein E1E2, targeted by these antibodies, has been shown to continuously escape from autologous antibodies during chronic infection. Understanding the evolutionary pathways resulting in viral escape and a neutralization resistance is important for vaccine design.

Methods
Here we studied the evolutionary pathway of AMS230 a virus derived from a patient after 4 years of chronic infection, who experienced a relapse following treatment with interferon during the acute phase of infection. Sensitivity of this virus to broadly neutralizing antibodies was tested against a comprehensive panel of monoclonal antibodies targeting E1 and antigenic regions 3 and 4 on E2, including the potent broadly neutralizing antibody AR4A. E1E2 sequence was determined for 8 different timepoints over 6 years, starting directly after infection. In addition, serum samples from the same time points were tested against a panel of 12 different HCVpps with a wide range of neutralizing sensitivity.

Results
AMS230 is an extremely resistant virus, and was only neutralized by AR4A at a concentration of 5 ug/ml, with a geometric mean titer of 29 ug/ml against the full panel. During the first 2 years of infection, during which a treatment initiated sharp drop in viral load was sustained during 17 months, polyclonal response was almost absent. Antibody response broadened after that to a geometric mean titer of 1:1000, although ‘autologous’ response against AMS230 remained relatively low (< 1:1000). Once polyclonal response became more potent, consistent changes in the HVR1 were observed at position 401, 404 and 405. In addition, 3 mutations around the CD81 binding region were present in AMS230, although 2 of these were no longer detected at a later time point.

Conclusion
we have isolated an extremely resistant HCV variant, which should be included in panels used for characterizing vaccination responses. Additional analysis to explain the phenotype of this variant is pending. The results of this study highlight the importance of investigating autologous and heterologous immune responses and virus-host co-evolution in individuals with a unique disease course.

P82 – **Host sequence snatching during hepatitis E infections enhances viral replication**

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Background
Hepatitis E virus (HEV) infections are usually asymptomatic and self-limiting, while immunocompromised or other risk group patients may develop chronic courses. The hypervariable region (HVR) within HEV's first open reading frame is known to integrate sequence snippets of human origin. We collected longitudinal samples of a chronic infected patient experiencing ribavirin (RBV) treatment failure. In order to gain insight on HVR insertions during the course of disease, we analyzed the patient's viral population using Illumina sequencing. Our aim was to identify insertions connected to the patient's treatment failure.

Methods
A specific sequencing protocol was developed exclusively targeting HEV's HVR. In order to process Illumina data, we developed the bioinformatic pipeline Hyper-EINS. Identification of insertions was achieved by running a sequence search on the provided data using the human genome as reference. All reads classified as the same insertion formed the necessary basis for building assemblies that represent complete insertion sequences. Those insertions, as well as eight insertions of human origin identified in public databases were cloned into a sub genomic luciferase encoding replicon system and in our state-of-the-art robust authentic virus cell culture system.

Results
In silico analysis performed by Hyper-EINS identified seven previously unknown insertions. Insertions derived from human SERPINA1 and TRIM22 are the most frequent ones. Both insertions are already present pretreatment. During RBV administration, the frequencies of viral genomes harboring insertions rises. Furthermore, frequencies remain at a considerably higher level beyond treatment than before. All insertions were shown to increase the replication capacity of the virus, while not affecting the sensitivity towards RBV in the sub genomic replicon system. Furthermore, insertion containing constructs produced higher viral titers than the parental HEV strain without insertion. Distinct sequence patterns both preceding and following the insertions were found to be essential for the observed effects. The activity and role of the predicted NLS sequences are still under investigation.

Conclusion
In conclusion, this study linked insertions and peripheral sequence patterns in the HVR to an increased replication capacity and infective particle production. The mechanism for the replication advantage and the reason for the selection of those clones in vivo could reveal new therapeutic targets for the development of antiviral drugs.

P83 – **Maintenance of hepatitis C virus within-host population structure after DAA treatment failure**

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Background
Using viral genetic data, it has been shown that untreated chronic hepatitis C virus (HCV) infection is often characterised by a distinctive pattern of within-host population structure in which multiple subpopulations are present, but not all subpopulations are observed at each sampling time point. Patterns consistent with within-host population structure have also been observed after liver transplantation, but it is not known whether similar patterns are also present after Direct Acting Antiviral (DAA) treatment failure. Structured within-host populations are highly suggestive of compartmentalisation of the virus within individuals. Characterising the within-host HCV populations after treatment failure may help to inform why some individuals fail treatment, and the consequences of treatment failure for the spread and evolution of the virus.

Methods
To determine whether within-host viral population structure is evident among individuals who experience viral rebound after DAA treatment, we analysed high throughput sequencing data generated from a randomised trial (the BOSON study, N=570) of sofosbuvir, ribavirin and peginterferon-alfa in genotype 2 and 3 infected patients. This included single timepoints for patients who achieved sustained virological response (SVR) (N=485) and multiple timepoints for patients who did not achieve SVR (N=85). Briefly, viral nucleic acid was extracted from plasma samples, prepared into sequencing libraries, amplified, enriched for viral content, and sequenced on Illumina MiSeq v2 platform. The raw sequencing output were cleaned by QUSAR, Cutadapt, Bowtie and mapped by shiver. We used phyloscanner to produce sliding window alignments with continuous 210bp reads and to decontaminate the sequencing reads. We used CliqueSNV to reconstruction haplotypes for each sample. All phylogenetic trees were built using iqtree2. Finally, we used fastbaps to cluster haplotype and within-host sequences separately to detect signals of population structure.

Results
We observed patterns of viral evolution consistent with the presence of distinct viral subpopulations, with the viral population circulating soon after treatment failure often subsequently replaced by viral populations evolved from variants that circulated earlier in the infection. We also summarised the genetic diversity and mutation signatures of known drug resistant sites and candidate sites determined by previous GWAS studies to assess their relationship to treatment outcome.

Conclusions
As has been previously observed in longitudinally sampled HCV chronic infections, here we report within-host viral population structure maintained in patients who have undergone DAA treatment but failed to achieve SVR. As structured populations suggest potential compartmentalisation of viruses and/or viral replication, these results could have implications for future clinical treatment guidelines of chronic HCV infections.

P84 - **Investigation of the HCV - lnc-ITM2C-1 - GPR55 - ISG regulation axis**

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Background
Long noncoding RNAs (lncRNAs) play critical roles in the regulation of different biological processes. We demonstrated previously that Hepatitis C Virus (HCV) replication and infection affect the levels of intergenic lncITM2C-1. This lncRNA stimulates expression of its neighbored G protein-coupled receptor 55 (GPR55) gene which in turn negatively regulates ISGs like *ISG15*, *Mx1,* and *IFITM1* at early time points after HCV infection. According to our findings, *lnc-ITM2C-1* was renamed GCSIR for GPR55 *cis*-regulatory suppressor of immune response RNA by the HUGO gene nomenclature committee. To gain a more detailed understanding of the HCV – GCSIR - GPR55 regulation axis, we first analyzed which splice variants of GCSIR and GPR55 RNAs are expressed in Huh-7 cells.

Methods
Since the extremely low expression of GCSIR and GPR55 in Huh-7 cells required complete removal of genomic DNA from RNA used for RT-qPCR analysis of GCSIR expression, we developed an improved TRIzol-based method for RNA isolation. Then, GCSIR and GPR55 splice variants expressed in Huh-7 cells were detected by specific RT-(q)PCR primers.

Results
Bioinformatic analysis of GCSIR and GPR55 genes revealed the need for slight corrections of the gene annotations in Ensemble and NCBI databases. Two out of 13 GCSIR transcription candidates were specifically identified to be individually expressed in Huh-7 cells, whereas a third expressed transcript cannot be unambiguously assigned to a specific splice variant. Among the nine GPR55 splice variants, two could be shown to be expressed. We analyzed expression of these GCSIR and GPR55 splice variants in HCV transfected and infected Huh-7 cells.

Conclusions
Only a few splice variants of the GCSIR and GPR55 RNAs are expressed in Huh-7 cells. We found that the annotation of GCSIR and GPR55 genes need to be revised in Ensemble and NCBI databases.

P85 – **Epitope tagging of the hepatitis E virus ORF1 PCP domain as a tool for functional studies**

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Background
Hepatitis E virus (HEV) is a leading global cause of acute viral hepatitis. The virus is primarily transmitted between humans via the faecal-oral route, however zoonotic transmission is also possible particularly from porcine species. A lack of molecular reagents has hampered both fundamental research into HEV biology and the development of antiviral therapeutics. In this study we focused on the controversial **p**utative **c**ysteine **p**rotease (PCP) domain within the HEV ORF1 polyprotein with a view to developing unique tools to permit functional studies.

Methods
To begin with we used transposon mutagenesis to randomly insert 15 nucleotide insertions into the ORF1 region of a sub-genomic replicon (SGR) derived from genotype 1 HEV. Using Illumina and MinION sequencing platforms we identified insertion sites across ORF1 that were compatible with genome replication.
In this study we focus on replication-permissive insertion sites within the PCP. To permit functional studies we introduced epitope tags (e.g. HA) at these sites in the context of an SGR. Using immunofluorescence and confocal microscopy we analysed the spatial and temporal localisation of PCP during HEV replication. Finally we generated Huh7 cell lines stably harbouring an SGR with HA-tagged PCP for downstream proteomic and functional studies.

Results and conclusions
We demonstrate that the PCP domain of HEV ORF1 can tolerate the insertion of epitope tags at several closely clustered positions with a minimal effect on the rate and level of genome replication compared to the untagged precursor. These data support the suggestion that the PCP domain is not a protease.
Using stable Huh7 cell lines harbouring either HA or splitGFP-tagged PCP SGRs we conducted a detailed analysis of the sub-cellular localisation of the PCP domain by immunofluorescence. In Huh7 cells actively replicating the HEV SGR the PCP domain appeared as both ring-like structures and distinct puncta in the cytoplasm. There was no colocalisation between PCP and a range of cellular organelles (ER, Golgi or endosomes). Although dsRNA abundance was increased as expected in HEV SGR-harbouring cells, we did not observe colocalisation of PCP with this marker of genome replication.. Interestingly, cells harbouring the SGR showed changes to morphology and abundance of lipid droplets, again without any direct colocalisation with the PCP domain.
We are now using the HA-tagged PCP SGR harbouring cells in the context of western blot and immunoprecipitation approaches to investigate post-translational modifications of ORF1. This work is being extended to include other tolerated insertion sites and proteomic approaches to investigate the interactions between PCP and host proteins as well as other putative ORF1 domains.

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.

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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.

P88 – **Computational models for HCV genomic surveillance: phenotype classification using SMILES data**

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Background
Chronic hepatitis C virus (HCV) infection is a serious global health problem. Approximately 30% – 50% of acutely infected persons spontaneously clear HCV within 6 months of infection. Among chronically infected, 15% - 30% develop liver cirrhosis within 20 years. Viral hepatitis surveillance is critical for devising national hepatitis prevention and elimination strategies. Application of machine-learning computational methods to next-generation-sequencing (NGS) data accelerated the development of viral genomic surveillance, which is fundamentally based on efficient approaches to extraction of public health relevant information from genomic data. Traditional feature-engineering approaches require massive amounts of NGS data to avoid inadequate representation of useful HCV properties, which, being time-consuming and costly, prevents their effective application to genomic surveillance. Novel deep learning (DL) methods deliver a data-driven method to automatically learn deep expressive representations from raw data. The application DL models to simplified molecular input line entry specification (SMILES) strings, which contain all the composition and structure information of molecules, have outperformed the state-of-the art technologies used for classification tasks and drug discovery. However, these methods have been applied exclusively to small chemical molecules. In this study, we describe a novel computational application for generating SMILES from NGS data of the HCV hyper-variable region 1 (HVR1) and, in addition to traditional approaches, we investigate suitability of DL methods to automatically mine complex HCV properties from the SMILES data generated from each sequenced intra-host HCV variant.

Methods
We developed a computational tool using the cheminformatics RDKit toolkit for the fast and accurate extraction of variant-specific SMILES from NGS data. We used the T-distributed Stochastic Neighbourhood Embedding (tSNE) unsupervised machine learning algorithm to measure the class-specific clustering of HVR1 variants based on SMILES, structure and biomolecular property information. The DL method, Bidirectional Encoder Representations from Transformers (BERT), was used to mine relevant properties from the SMILES data.

Results
The SMILES strings were efficiently generated from a multitude of the intra-host HVR1 variants sampled by NGS from acute and chronic stages of HCV infection, demonstrating feasibility for the extraction of complex molecular information from RNA or DNA biomolecules of up to 264nt long. Clustering tests indicate that the deep expressive SMILES representations outperform the physicochemical-based representations for differentiating between the chronic- and acute-associated HVR1 variants. Classification performance of the BERT model strongly supports applicability of language models for identification of acute-associated HVR1 variants from NGS data.

Conclusions
Findings presented in this work provide a new framework for the development of cyber-molecular assays to enhance HCV genomic surveillance. This novel engineering of molecule-specific properties is not only applicable to the identification of acute HCV infections but to modeling of other clinically and epidemiologically important HCV phenotypic traits such as disease severity and rate of disease progression.

P89 – **NS5A of genotype 3 HCV exhibits distinct molecular characteristics that may contribute to both the unique disease phenotype and DAA resistance.**

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Background
HCV genotype 3 (GT3) accounts for ~25% of global HCV infections and ~50% of infections in the UK, and causes more severe and rapid liver disease compared to other GTs. In addition, GT3 infected patients are the most likely to fail DAA treatment. We sought to investigate whether the distinct characteristics of GT3 could be explained by differences in the functions of NS5A. To do so we directly compared NS5A of the GT2 isolate JFH-1 with the GT3 infectious clone, DBN3a.

Methods
Working with both infectious clones and sub-genomic replicons (SGR) of JFH-1 and DBN3a we used a combination of mutagenesis, western blotting, immunofluorescence and replication/infectivity analyses to study the functions and characteristics of NS5A. We also constructed intragenotypic chimeras in which the NS5A coding sequences were exchanged between JFH-1 and DBN3a.

Results and Conclusions
The kinetics of DBN3a infection and replication were distinct from JFH-1, specifically we observed a lag in both the infectious clone and SGR, before DBN3a then attained similar levels of genome replication and higher virus production than JFH-1. Western blot analysis confirmed this, and revealed that DBN3a NS5A exhibited only one species, compared to the hyper- and basally phosphorylated species observed for JFH-1. Further analysis with phospho-specific antibodies (gift from Ming-Jiun Yu, Taiwan) revealed that, despite the fact that the serine-rich low complexity sequence 1 was conserved between the two GTs, DBN3a NS5A was not phosphorylated.
To further investigate the lack of phosphorylation of DBN3a NS5A, we produced a panel of mutants in which phosphorylated serines previously identified in JFH-1 were mutated to alanine (phospho-ablatant) or aspartic acid (phospho-mimetic). Intriguingly, this analysis revealed replication phenotypes of these mutants, suggesting that very low levels of NS5A phosphorylation (below the level of detection by western blot) might be required for GT3 replication.
Generation of intragenotypic chimeras in which the NS5A coding sequences were exchanged revealed a non-reciprocal tolerance for the chimeric NS5A. Although JFH-1 could replicate when its cognate NS5A was replaced with that of DBN3a, the reciprocal chimera (DBN3a containing JFH-1 NS5A) was replication-defective. In addition, we observed a key role for NS5A in the replication kinetics, such that the JFH-1 chimera containing DBN3a NS5A exhibited a lag in replication, similar to that observed for the parental DBN3a.
DAA testing of the chimera also revealed that the sensitivity to NS5A-targeting DAAs was an intrinsic property of NS5A and not influenced by the viral genetic context. This was also true for the phosphorylation status of NS5A – the chimera exhibited a lack of serine phosphorylation in LCS1.
This study points to fundamental differences in the function of NS5A between GT2 and GT3 and implicate NS5A in the unique disease presentation and DAA resistance of GT3.

P90 – **Modulation of host lipid metabolism during HCV infection**

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Background
All (+)RNA viruses modify cytoplasmic membranes, such as the endoplasmic reticulum (ER), to establish replication compartments (RCs). These RCs are thought to form a platform for membrane-associated replicases, in addition to protecting the viral RNAs from cytosolic innate immune signaling and RNA-degradation machinery. Our lab and others have shown that a key component in the viral mechanism of RC formation is the modulation of RC membrane lipid composition. Previously we demonstrated that at least three (+) RNA virus families (*Bromoviridae, Picornaviridae*, and *Flaviviridae*) share the property of stimulating phosphatidylcholine (PC) accumulation at RCs. This suggests that understanding viral modulation of PC synthesis may have broad implication as a conserved mechanism in RC formation.

Methods
We checked *de novo* PC synthesis in HCV-infected Huh 7.5 cells by metabolically pulse labeling newly synthesized PC with propargylcholine, a choline analog consisting of terminal alkyne moiety. Next, the incorporated propargylcholine was covalently linked with Alexa 488-labeled azide via the Click chemistry technique.
To confirm the membrane association of PC synthesizing enzymes during HCV infection, we performed membrane floatation assays in naïve or Huh7.5 cells containing HCV sub-genomic replicon. We determined the effects of reducing PC synthesizing enzymes on HCV life cycle by harvesting supernatants and cellular RNA at 96 hpi, followed by the quantification of viral RNA and titer by RT-PCR and by limiting dilution analysis, respectively.

Results
We find that HCV infection induces de novo PC synthesis at the viral replicating site. We also find that HCV infection and expression of NS3/4A results in the re-localization of the rate-limiting enzyme in PC synthesis, CCTa, from the nucleus, where it is inactive, to viral RCs, suggesting the role of Kennedy pathway in HCV-induced PC synthesis. Surprisingly, this requires the NS3/4A protease activity. We also provide evidence that NS3 cleaves the enzyme downstream of CCTa in PC synthesis, CHPT-1, that lead to the re-localization of the active domain of CHPT1 from the Golgi to RCs likely via an interaction with NS5A. Also, we demonstrate that the cleavage of CHPT1 is important for PC synthesis as well as HCV replication.

Conclusions
Our study will help us understand viral modulation of PC synthesis that may have broad implications as a conserved mechanism in RC formation. The most conceptually innovative aspect of this study is the requirement of the viral protease activity of NS3/4A, in the function of RCs.

P92 – **Adenosine sulfamate analogs inhibit HBV RNA synthesis and accelerate the decay of viral transcripts**

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Background
The neddylation pathway is required for HBV replication. Targeting the NEDD8-activating E1 enzyme with MLN4924 leads to inhibitory effects on both HBV gene transcription and surface antigen expression in preclinical models. However, how this new class of inhibitor modulates neddylation signaling and HBV gene transcription remains unclear.

Methods
Adenosine sulfamate analogs (ASAs) MLN4924 and TAS4464 were evaluated for their ability to target host components of the neddylation pathway and inhibit HBV gene transcription in HepG2-NTCP, HepaRG-NTCP and HepAD38 cells, in addition to explanted primary human hepatocytes (PHHs) from multiple donors. Host protein expression was determined by Western blot. Viral transcription rates were determined by RT-qPCR and nascent transcript production determined by click-chemistry. RNA-sequencing of HBV infected PHHs (ASA-treated and untreated) is underway to determine the global effects of ASAs on both viral and host transcriptomes.

Results
In HepG2-NTCP cells, MLN4924 and TAS4464 reduced neddylated cullin protein expression by 31% and 65% respectively, although no decrease in NAE1, Ubc12 and Cul4A levels was observed. In contrast, in PHHs, both ASAs reduced expression of NAE1, Ubc12, Cul4A and neddylated cullins. Upon SMC5/6 gene knockdown or when HBx protein was absent (using HBx-knockout virus), the inhibitory effects of both analogs on viral transcription were alleviated, suggesting a partial dependence on the HBx-SMC5/6 complex. TAS4464 was consistently more potent than MLN4924, with lower IC50 values. To further investigate the mode of action, 5-ethynyl-uridine was incorporated into infected HepG2-NTCP cells, allowing the detection of nascent transcripts using click chemistry. MLN4924 and TAS4464 reduced synthesis of HBV transcripts by 60% and 74%, respectively. Using HepAD38 cells, in which cccDNA and pgRNA formation were blocked by the addition of doxycycline (tet-on), ASAs in combination with triptolide (a transcription inhibitor) accelerated the decay of subgenomic transcripts (*t1/2* = 10.8 and 14.2 hours) when compared to DMSO/triptolide treatment (>24 hours).

Conclusions
We demonstrate that ASAs reduce protein expression of host components of the neddylation pathway, inhibit HBV RNA synthesis and accelerate viral transcript decay. ASAs have potential for future development and repurposing as a novel class of anti-HBV therapeutic.

P93 – **Discovery of a Novel 2'-Bromo-2'-Fluoro-Uridine Nucleoside Prodrug Inhibiting Hepatitis C Virus Replication**

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Globally, an estimated 58 million people worldwide are chronically infected with hepatitis C virus (HCV), with about 1.5 million new infections occurring per year. About four hundred thousand people die each year as a result of HCV infection, mostly from cirrhosis and hepatocellular carcinoma (primary liver cancer). Today, the most widely used drug regimens, Epclusa and Vosevi, contain the nucleoside analog Sofosbuvir. In general, pan-genotypic nucleoside HCV inhibitors display a high genetic barrier to drug resistance and are the preferred direct acting agents (DAA) in the pursuit to achieve 100% sustained virologic response. Although Sofosbuvir-based regimens have improved the HCV treatment prospects significantly, the treatment course remains long at anywhere from eight to twelve weeks. Furthermore, treatment has been dampened due to high cost of currently available DAA. Therefore, there remains a need for additional safe, pan-genotypic nucleoside analogs with a high barrier to resistance that could be cost effective and when combined with potent pangenotypic DAAs could lead to a shorter duration of treatment while maintaining very high cure rates. In our search for such compound, we discovered a -D-2'-deoxy-2'--bromo-2'--fluoro-uridine phosphoramidate prodrug (cmpd 1), that is a potent, pan-genotypic HCV polymerase inhibitor. The active triphosphate form of cmpd 1 is highly selective and does not inhibit human , β, and γ DNA polymerase and showed low incorporation by human mitochondrial RNA polymerase. No mitochondrial and bone marrow toxicities were observed up to 10 μM and only mild toxicities were observed for both cmpd 1 and Sofosbuvir at 50 μM. No toxicities were observed in a large number of cell lines and a Mini Ames was negative versus five strains. Cmpd 1 was highly stable in human blood for up to 2 h, was rapidly metabolized in human hepatocytes to the nucleoside-5'-monophosphate and showed low metabolism in human intestinal microsomes. Cmpd 1 has an excellent preclinical profile, suggesting further development to establish its potential as a clinical anti-HCV nucleotide analog.

P94 – **Mouse-adaptation expands the species tropism of HCV in vitro by diverse mechanisms including facilitated cell entry**

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Background and Aim
HCV species tropism is limited to humans and chimpanzees, a fact that has hampered HCV vaccine research. Making use of the error-prone replication of HCV, which allows it to adapt to changing environments, we adapted the virus in vitro towards infection of murine cells.

Methods
We used a cell-culture adapted HCV and stepwise adapted it to murine cells, starting with a co-culture of highly permissive Huh-7.5 cells and mouse liver tumor cells with human HCV entry factors with a MAVS knockout (MLT-5H), MLT-5H cells alone and finally primary mouse hepatocytes from receptor-transgenic mice with a type I interferon receptor knockout (PMH hOChep IFNAR-/-). We sequenced the viral population, generated a molecular clone, and compared the replication kinetics in human and murine cells. We used antibodies directed against the viral glycoproteins and against HCV receptors to assess the changes in virus neutralization and entry. Usage of mCD81 for virus entry was tested using cells expressing undetectable levels of human CD81 and high levels of mCD81.

Results
The mouse-adapted HCV population as well as its molecular clone infect and replicate in MLT-5H cells and PMH hOChep IFNAR-/- while maintaining high replication fitness in Huh-7.5 cells. Non-synonymous mutations were necessary and sufficient for the mouse-adapted phenotype. Introduction of the adaptive mutations into a subgenomic replicon did not affect replication fitness. The mouse-adapted virus exhibited enhanced susceptibility to neutralizing antibodies which could be restored to the levels of the non-adapted lab strain Jc1 by reversion of the glycoprotein mutations. The glycoprotein mutations also facilitated the susceptibility to antibodies directed against the HCV entry factors SR-BI and CD81 and they allowed for increased use of murine CD81 for virus entry compared to Jc1. Reversion of the glycoprotein mutations partially but not completely attenuated the viral fitness in both human and murine cells.

Conclusions
We generated a mouse-adapted viral population which replicates in primary mouse hepatocytes from receptor transgenic, interferon receptor knockout mice, and a molecular clone which reflects its replication phenotype. While the glycoprotein mutations were not solely responsible for this phenotype, we found that they enhanced neutralization susceptibility while rendering the virus less dependent on SR-B1 and enhancing usage of both human and murine CD81 for entry. This mouse-adapted virus lays the basis for a better understanding of HCV species tropism.

P95 – **Evolutionarily Conserved Disruption of STING-mediated Antiviral Defenses by Flavivirus NS4B protein**

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Background

Flaviviruses such as Zika virus and Dengue virus cause major health and economic problems worldwide. However, the crosstalk between flavivirus and their host remain elusive, especially the way especially in how the virus evades host defenses to establish infection. Flavivirus nonstructural proteins have been shown to play a major function in antagonizing the interferon pathway. Based on previous work, we hypothesized that all flaviviruses share the same strategy of hijacking STING through the interaction of NS4B and STING, and blocking downstream immune responses to evade host innate immunity.

Methods

We used co-immunoprecipitation, RT-qPCR, confocal microscopy, interferon promoter-reporter system and immunoblotting to investigate the molecular mechanism by which NS4B targets STING to inhibit interferon signaling. Coimmunoprecipitation and the NanoBiT system were used to probe the critical amino acid residues for the interaction of NS4B with STING. Reverse genetic tools, plaque assay, infection kinetics and animal model were used to determine the infectivity and pathogenesis of recombinant ZIKV both in vitro and in vivo.

Results

In this study, we demonstrated that flavivirus non-structural protein 4B (NS4B) selectively targeted STING orthologs from numerous species, such as Homo sapiens, Mus musculus and Drosophila melanogaster to escape antiviral response. In addition, mechanistic studies indicated that NS4B could not impair the dimerization of STING or the recruitment of TBK1, but rather inhibited the phosphorylation and translocation of IRF3 which is critical for STING-mediated antiviral immunity. NS4B is a multiple-transmembrane protein and we identified the critical residues within the NS4B which are responsible for the interaction with STING. Notably, we generated the recombinant ZIKV in which the NS4B is unable to evade STING mediate antiviral response, and the recombinant ZIKV exhibited comparable infection kinetics and cytopathic effect with that of WT virus in vitro. Furthermore, the morbidity and mortality of both viruses were comparable in type I IFN receptor knockout mice.

Conclusions

Collectively, our data suggest an evolutionarily conserved mechanism that enables flavivirus NS4B to evade host interferon responses induced by STING pathway. In the future, we sought to characterize the infectivity, pathogenicity, and host immune response of the recombinant ZIKV virus with NS4B mutations in an immunocompetent model, which could reveal the biological significance of evasion of STING antiviral responses by flaviviruses and give us some implications for flavivirus treatment potentially.

P96 – **Zika Virus Infection Critically Depends on Neutral Lipid Biosynthesis**

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Background
Flaviviruses have (re)emerged around the globe, with increasing numbers of infections and substantial morbidity, but no potent antiviral treatment has been developed yet. All viruses rely on host metabolic pathways for replication. Host cell lipid droplets (LDs) are the storage organelles for neutral lipids and thus play a central role in lipid metabolism. During flavivirus infection LDs are degraded to fuel replication and Zika virus (ZIKV) infection depends on diacylglycerol O-acyltransferase (DGAT)-mediated triglyceride biosynthesis. In contrast, the related hepatitis C virus (HCV) requires DGAT1 activity to target LDs and cause lipid accumulation.

Methods
Here, we systematically investigated the function of neural lipid synthesizing enzymes including DGAT1 and DGAT2 and the sterol O-acyltransferase (SOAT) 1 in ZIKV infection using different cell culture systems.

Results
Downregulation of DGAT1 and SOAT1 using RNAi significantly reduced ZIKV titers. In line, ZIKV plaques were smaller in SOAT1- and DGAT1-knockdown cells and viral RNA and protein levels decreased. Immunofluorescence analysis revealed fewer and smaller ZIKV double-stranded RNA (dsRNA) foci in SOAT1-depleted cells, pointing to impaired replication organelle formation. In contrast, depletion of DGAT1 did not change dsRNA foci, highlighting potential mechanistic differences. As ZIKV is neurotropic, we additionally depleted SOAT1 and DGAT1 in neuronal cell lines. Preliminary data suggest that downregulation of SOAT1 but not DGAT1 impairs ZIKV infection of microglia and neural-like cells. Importantly, treatment with two different SOAT1 inhibitors reduced ZIKV plaques and titers indicating that enzymatic function of SOAT1 is required for efficient ZIKV infection.

Conclusion
Taken together, these data suggest an important role of cholesterol ester synthesis in ZIKV infection and may point to novel targets for antiviral intervention.

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.

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.

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.

P100 – **Elevated levels of genome replication of Hepatitis C Virus are associated with response to interferon treatment but do not correlate with viral serum titers or DAA therapy failure**

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Background
We recently identified a small region in the non-structural protein (NS) 5A of the hepatitis C virus (HCV) to regulate viral replication fitness, encompassing the Interferon Sensitivity Determining Region (ISDR). The ISDR was initially identified as a determinant for efficient response to interferon (IFN) treatment by accumulation of 3 or more amino acid deviations from the genotype (gt) 1b consensus (Enomoto et al., 1995). In a cohort of HCV gt 1b infected liver transplant (LTX) patients, we could previously show that ≥3 ISDR substitutions were associated with increased replication fitness, resulting in very high titers and severe liver damage. This study aims to understand if highly altered ISDR sequences and therefore high replication fitness are generally associated with clinical parameters such as Interferon sensitivity, DAA-resistance and establishment of high titers.

Methods
To examine the correlation of ISDR mutations and viral titers, we sequenced the ISDR of 30 immune-competent gt 1b patients with titers >10^7 IU/ml. We further aimed to correlate the previously identified genotypic determinants of IFN sensitivity with replication fitness. We therefore introduced the previously published ISDR sequences of 15 IFN treated patients (Enomoto et al., 1995) into the Con1 subgenomic replicon (SGR) and studied the effect on replication fitness in cell culture. We investigated ISDR sequences from 30 patients with typical resistance associated substitutions (RAS) in NS5A and 10 patients failing DAA-treatment without RAS.

Results
We found no accumulation of mutations in the ISDR in high titer patients. This indicates that in immune-competent hosts the serum titers do not correlate with the replication fitness of the viral population, but might be determined by more complex parameters. However, we indeed observed a higher replication fitness for almost all IFN sensitive patients with an altered ISDR, underlining the crucial role of the ISDR in regulating HCV replication efficiency. Finally, we hypothesized that increased replication fitness, mediated by ISDR mutations, might contribute to failure of current therapies. Indeed, we identified one patient with a potential high replicator ISDR in presence of Y93H, suggesting that ISDR mutations might be capable to compensate for fitness costs of RAS.

Conclusions
Overall, we could show that response to interferon therapy depended on viral replication fitness, whereas viral serum titers and replication fitness do only correlate in the setting of immunosuppressed transplant patients. Selection of high replicating ISDR variants appears to happen in individual patients upon DAA-therapy failure to compensate for the fitness cost of RAS. These findings highlight the complex interplay of viral replication fitness and the immune system and suggest that highly replicating HCV variants are only selected under specific conditions, but are not generally favorable for viral persistence.

P101 – **Secreted NS1 proteins of tick-borne encephalitis virus and West Nile virus block dendritic cell activation and effector functions**

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Background
The effective initiation of protective host immune responses controls the outcome of infection, and dysfunctional T cell responses have previously been associated with symptomatic human flavivirus infections. The flavivirus non-structural protein 1 (NS1) is secreted from infected cells into the circulation of infected individuals and its serum levels correlate with disease severity. However, the effect of exogenous secreted NS1 (sNS1) on mammalian immune cells in the absence of other viral components is largely unknown. Here, we expressed recombinant sNS1 proteins of tick-borne encephalitis virus (TBEV) and West Nile virus (WNV) and investigated their effects on dendritic cell (DC) effector functions.

Methods
We treated primary murine bone marrow-derived DCs (BMDC) as well as human monocyte-derived DCs (moDC) with recombinant sNS1 proteins of TBEV and WNV that were produced in a mammalian expression system. After poly(I:C) stimulation of the DCs, we assessed their activation status based on pro-inflammatory cytokine secretion measured by ELISA or bead array, and surface expression of co-stimulatory molecules detected by flow cytometry. Transcriptional profiles of BMDC upon poly(I:C) stimulation with or without prior sNS1 treatment were determined by RNA sequencing.

Results
BMDCs showed reduced surface expression of co-stimulatory molecules and decreased release of pro-inflammatory cytokines when treated with sNS1 of TBEV or WNV prior to poly(I:C) stimulation. Transcriptional profiles of BMDCs that were sNS1-exposed prior to poly(I:C) stimulation showed two gene clusters that were downregulated by TBEV or WNV sNS1 and that were associated with innate and adaptive immune responses. Functionally, both sNS1 proteins modulated the capacity for BMDCs to induce specific T cell responses indicated by reduced IFN-γ levels in both $CD4^+$ and $CD8^+$ T cells after BMDC co-culture. In human monocyte-derived DCs, poly(I:C) induced upregulation of co-stimulatory molecules and cytokine responses were even more strongly impaired by TBEV sNS1 or WNV sNS1 pre-treatment than in the murine system.

Conclusions
We demonstrate that secreted flavivirus NS1 proteins modulate innate immune responses of uninfected bystander cells. Our findings indicate that exogenous flaviviral sNS1 proteins interfere with DC-mediated activation of T cells. Hence, by modulating cellular host responses that are required for effective antigen presentation and initiation of adaptive immunity, sNS1 proteins may contribute to severe outcomes of flavivirus disease. Collectively, our data determine flaviviral sNS1 as a virulence factor that may be responsible for a dampened immune response to flavivirus infections.

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.

P103 – **Determinants of HCV genotype 1a resistance to clinically relevant NS5A inhibitors**

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Background
In the absence of a prophylactic vaccine, efficient treatments are essential for control of hepatitis C virus (HCV). Current treatment regimens involve combinations of direct-acting antivirals (DAA) that target key HCV proteins, including the NS3 protease, NS5A and the NS5B polymerase; NS5A inhibitors are included in all regimens and are essential for high sustained virological response rates. However, despite this high DAA efficacy a sub-population of treated individuals fails to clear the virus. Such individuals harbor HCV variants with resistance associated substitutions (RAS) in the DAA targets. Although NS5A inhibitor resistance is most widespread, NS5A RAS have not been comprehensively explored. Among the 8 major HCV genotypes, genotype 1 is most prevalent worldwide. We aimed to characterize HCV genotype 1a resistance to clinically relevant NS5A inhibitors, focusing on inhibitor- and isolate-specific differences.

Methods
Cell-culture infectious full-length HCV genotype 1a recombinants of isolates TN, H77 and HCV1 were subjected to escape experiments in Huh7.5 cells using clinically approved NS5A inhibitors. For 1a(TN), resistance selection was carried out with ledipasvir, elbasvir, velpatasvir, and pibrentasvir. For 1a(H77) and 1a(HCV1), escape experiments focused on the pangenotypic inhibitors velpatasvir and pibrentasvir. Putative RAS were identified by Sanger and deep sequencing and engineered singly and in combination into the original recombinants. Fitness of the resulting variants was monitored following transfection of in vitro transcripts and viral passage in Huh7.5 cells by evaluation of viral spread and NS5A genetic stability. Viral passage virus stocks were used for concentration-response assays to evaluate the impact of RAS on NS5A inhibitor resistance and cross-resistance.

Results
In escape experiments pibrentasvir showed the highest barrier to resistance, while the other inhibitors showed comparable barriers to resistance. Further, 1a(HCV1) showed the lowest barrier to resistance, because it escaped higher inhibitor concentrations faster than 1a(H77) and 1a(TN). Conversely, 1a(TN) showed the lowest genetic barrier to resistance, because it acquired fewer RAS to escape high inhibitor concentrations than 1a(H77) and 1a(HCV1). The three recombinants showed similar RAS-hotspots at NS5A positions 26, 28, 30, 31 and 93 but isolate-specific combinations of RAS. Overall, the identified RAS had low fitness cost, however, 1a(TN) variants had higher genetic stability than 1a(H77) and 1a(HCV1) variants. Across isolates, position 93 was the major determinant of resistance. Resistance of up to 440,000-fold was conferred by RAS combinations that included position 93. Ledipasvir, elbasvir, and velpatasvir showed similar potencies, while pibrentasvir showed higher potency against all resistant variants.

Conclusion
We showed inhibitor- and isolate-specific differences in barriers to NS5A inhibitor resistance. Furthermore, we characterized a large panel of NS5A inhibitor escape variants and demonstrated RAS- and inhibitor-specific resistance levels. These findings may contribute to DAA resistance monitoring and improved individualized patient treatment, and thus help prevent the development of viral resistance.

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

Joan Capella-Pujol[1,2], Fabian Mulder[1,2], Stan Peters[1,2], Gabriel Ozorowski[3], Maddy Newby[4], Ian Zon[1,2], Laura Radić[1,2], Max Crispin[4], Andrew Ward[3], Janke Schinkel[1,2], Rogier W. Sanders[1,2], Kwinten Sliepen[1,2]
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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.

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.

P106 – **Design of a native-like germline-targeting E1E2 immunogen for hepatitis C virus**

Fabian Mulder[1,2*], Joan Capella-Pujol[1,2*], Stan Peters[1,2], Gabriel Ozorowski[3], Maddy Newby[4], Ian Zon[1,2], Laura Radić[1,2], Max Crispin[4], Andrew Ward[3], Janke Schinkel[1,2], Rogier W. Sanders[1,2], Kwinten Sliepen[1,2]
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Background and Aims
An effective preventive hepatitis C virus (HCV) vaccine is a major unmet need and such a vaccine should probably induce broadly neutralizing antibodies that neutralize most HCV strains. The E1E2 envelope glycoprotein on the outside of the virion is the only target of bNAbs. To induce bNAbs, it is necessary to first engage the desired cognate germline precursors of bNAb-producing B cells. However, most HCV immunogens do not bind germline bNAbs (gl-bNAbs) and are thus unable to activate the desired bNAb-producing B cell lineages. Here, we present the design and characterization of a soluble native-like germline-targeting E1E2 (gtE1E2) capable of engaging a broad panel of (inferred) gl-bNAbs.

Methods
We introduced a set of stabilizing mutations (version 3 and 4 (Capella-Pujol et al. HCV-Flavi 2023 abstract)) in a designed E1E2 sequence. We characterized these constructs with a broad panel of HCV bNAbs and their (inferred) germline precursors using ELISA binding assays and Biolayer-interferometry (BLI). This panel included bNAbs targeting antigenic region (AR) 3, which overlaps the CD81 binding site, and AR4, which is a metastable epitope that is only available on native-like well-folded E1E2. We are currently investigating whether gtE1E2 engages naïve B cells from isolated peripheral blood mononuclear cells (PBMCs) and immunogenicity in different animal models.

Results
The stabilized soluble gtE1E2 efficiently engaged all tested mature bNAbs, including those that target AR4, indicating it represents a native-like mimic of virion-associated E1E2. We compared binding of 11 AR3-targeting (inferred) gl-bNAbs by gtE1E2 to our previous best germline targeting E1E2 candidate (Capella-Pujol et al, Nat Commun. *accepted*). We observed that gtE1E2 displayed significantly broader and stronger binding to AR3 gl-bNAbs, including germline precursors of AR3A, AR3B, AR3C, HEPC3 and HEPC74 with up to 100-fold higher affinity. Remarkably, gtE1E2 also showed high affinity to inferred germline versions of conformational antibodies against AR4 (gl-AR4A and gl-AT1618), making it a suitable candidate for multi-epitope germline targeting vaccine strategies. Site-specific analyses revealed that gtE1E2 is fully glycosylated and that its enhanced gl-bNAb binding is not due to lack of glycans surrounding AR3 or AR4. Further high-resolution structural analyses are pending. The ongoing binding studies against donor naive B cells will allow us to further characterize our gtE1E2 construct and its feasibility to succeed in immunization studies.

Conclusions
We present a gtE1E2 immunogen capable of remarkably strong binding to a broad panel of (gl-)bNAbs against AR3 and AR4. This immunogen is well folded, well glycosylated and hyperstable, making it a promising candidate for future immunogenicity studies and germline-targeting vaccines in particular.

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.

108 – **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.

P109 – **Genome-Wide CRISPR Screening Identifies Cullin-RING Ubiquitin Ligase Required for STAT2 degradation by ZIKV**

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Background
Zika virus (ZIKV) has evolved multiple strategies to evade the immune response for establishment of infection. ZIKV nonstructural protein 5 (NS5) has been shown to inhibit the type I interferon (IFN) signaling by degrading the STAT2, a critical component in JAK/STAT pathway. However, the mechanisms of NS5 mediated STAT2 degradation is not well characterized.

Methods
In this study, we generated a stable cell line that expressing STAT2-mCherry and ZIKV NS5, in which NS5 expression was under the control of a doxycycline (Dox)-dependent transactivator (rtTA). By adding or withdrawing the Dox, we could control NS5 expression and monitor STAT2 levels using fluorescence signals. This reporter system allowed us to transduce a genome-wide CRISPR KO library to identify the host factors required for NS5 mediated STAT2 degradation by cell sorting of the STAT2-mCherry positive cells after Dox treatment. The sgRNA pools from sorted and input cells were amplified by PCR and quantified by next-generation sequencing. The MAGeCK algorithm was employed to identify significant hits, and biochemical experiments *in vitro* were performed to validate the function of the hits.

Results
We identified several enriched genes and performed Gene Ontology (GO) analysis to consider whether the screening hits were functional classified with STAT2 degradation. The analysis indicated that the cullin-RING ubiquitin ligase (CRL) complex as well as its core CRL components, such as elongin B (ELOB), are required for NS5-mediated STAT2 degradation. The interaction between NS5 and CRL complex was confirmed by immunoprecipitation and the results indicated that the NS5 promotes the interaction between STAT2 and CRL complex. Specifically inhibiting neddylation, which is required for Cullin mediated ubiquitination, by MLN4924 increased STAT2 accumulation. Likewise, Cas9-mediated knockout of CRL component resulted in increased endogenous STAT2 level after ZIKV infection, and as expected, expression of the CRL component in the knockout background restored the process of STAT2 decrease. Notably, viral growth analyses revealed that restoring STAT2 after the depletion of CRL components in turn inhibited ZIKV infection, and thereby protect cells from the cytopathic effects (CPE) induced by ZIKV infection.

Conclusions
Here we use whole-genome CRISPR-Cas9 screening and determine that ZIKV NS5 could hijack the cellular cullin-RING ligase complex to target the STAT2 for degradation. Blocking this event promotes the IFN signaling and inhibits the viral replication. This study provides insight into how ZIKV NS5 antagonizes the IFN signaling by hijacking the host CRL complex and could support potential therapeutic intervention and vaccine development.

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.

P111 – **Protection without pathology: altered cell tropism and modulation of innate immune activation contribute to Yellow fever vaccine attenuation**

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Background
Yellow fever virus (YFV) is spread by mosquito vectors and represents a significant cause of hospitalization in endemic tropical regions, causing a viral hemorrhagic fever with high mortality rates. YFV represents a neglected tropical disease and is chronically understudied, with many aspects of YFV biology incompletely defined including host range, host-virus interactions and correlates of host immunity and pathogenicity. Despite the availability of a highly effective vaccine against YFV, no antiviral treatment exists. On the virus side, serial passage of a patient derived isolate in mice and chicken embryos resulted the 17D vaccine strain, with over 30 attenuating mutations distributed throughout the viral proteins. For over 80 years 17D has been safely injected into over 800 million people, providing life-long protection. While the immunogenicity of the vaccine strain is retained, these mutations result in a complete loss of pathogenicity. From the host side, understanding how host responses to the 17D vaccine elicits life-long protective immunity in the absence of pathology could guide future vaccine development, but currently remains poorly understood.

Methods
Live virus infections of cell lines and primary human cells were performed with the YFV-17D vaccine strain and two genetically distinct pathogenic strains (Asibi and Uganda). Plaque assay was used to determine virus production rates and RT-qPCR was used to determine YFV genome copies in infected cells. Flow cytometry was used to determine virus induced cell death. Host transcriptional responses to infection were determined using bulk and single cell RNA sequencing (RNA-seq):

Results
While all YFV strains entered cells by clathrin mediated endocytosis, 17D exhibited impaired entry in human peripheral blood mononuclear cells (PBMCs) and primate VeroE6 cells, and reduced attachment to hepatoma cell lines. Compared to pathogenic strains, increased rates of apoptosis/necrosis were observed in 17D infected cells, which was more pronounced in hepatoma cell-lines. Despite reduced attachment compared to pathogenic strains, 17D infection induced significantly enhanced antiviral and inflammatory responses in HepG2 cells, indicating a loss of immunosuppressive properties. In contrast, infection of PBMCs with YFV resulted in viral entry but infections were non-productive. However, 17D infection of PBMCs was associated with dampened host antiviral responses and impaired induction of chemotactic cytokines associated with monocyte recruitment, when compared to pathogenic strains.

Conclusions
In cell-lines of hepatic origin, 17D infection resulted in enhanced antiviral responses and increased cell death rates, indicating a loss of immunosuppressive properties during vaccine attenuation. In contrast, reduced 17D tropism for PBMCs likely reflects a loss of ability to infect certain PBMC subsets, which is currently being investigated by scRNA-seq and flow cytometry. In summary YFV vaccine attenuation is associated with an altered cell tropism in PBMCs and enhanced innate immune activation and cytolytic responses in the liver.

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.

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.

P114 – **Immunogenicity of lipid/mRNA vaccines eliciting HCV neutralizing antibodies**

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Background
We consider the HCV gpE1E2 envelope glycoprotein heterodimer as the optimal vaccine antigen to elicit broadly neutralizing antibodies (bNAbs) against the heterogeneous clades of HCV around the world. This antigen contains bNAbs in E2 and E1E2 with the greatest breadth of protection. When tested in the reliable chimpanzee challenge model, animals vaccinated with adjuvanted recombinant gpE1E2 strongly reduced the incidence of viral persistence following experimental challenge with either homologous or heterologous genotype 1a viruses which predominate in many countries of the world. Although we have now increased the commercial production of recombinant gpE1E2 to meet global vaccine requirements, we wanted to explore the delivery of gpE1/gpE2 via a mRNA vaccine.

Results
We have produced lipid/mRNA nanoparticles expressing the gpE1E2 heterodimer and show their strong immunogenicity in vaccinated guinea pigs. All vaccinated animals elicited very high titers of neutralizing antibodies against the HCV strain used to produce the mRNA vaccine. The breadth of cross-neutralization of heterogeneous HCV isolates by the mRNA vaccine is now being determined as is the possible augmentation of this mRNA vaccine by the introduction of specific mutations within E1E2 that may enhance the presentation of certain bNAb epitopes. These data will be presented.

Conclusions
Our data indicates that the wild-type and mutant derivatives of HCV gpE1E2 vaccine antigen can be delivered either by use of adjuvanted recombinant protein or via lipid/mRNA formulations which could further facilitate and accelerate global vaccine delivery.

P115 – **Unpacking the complex relationship between flaviviruses and hypoxia**

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Background
Virus replication is highly dependent on the host cellular environment, which can be dramatically altered by hypoxia or low oxygen tension and corresponding stabilisation of the HIF (hypoxia inducible factor) family of transcription factors. It is therefore not surprising that hypoxia and HIF signalling have been shown to impact virus lifecycles, with some viruses manipulating this pathway to enhance infection. This is the case with Flaviviridae members Dengue and Hepatitis C virus in which low oxygen tension can enhance replication, although the role of HIF signalling in infection and pathogenesis is not clear. However, little is known about the role of hypoxia in Zika virus (ZIKV) infection, a virus of global concern that caused significant outbreaks in South America and was linked to alarming increases in microcephaly and other neurological complications in newborns. Several hypoxic tissue microenvironments are important clinical targets of ZIKV infection, however interactions between ZIKV and hypoxic pathways, and the impact on viral replication have not been well explored.

Methods
To investigate interactions between ZIKV, oxygen tension and HIF signalling, Huh7 (human hepatoma) and HTR8 (human first trimester placental trophoblast) cell lines were infected with ZIKV under atmospheric (21%) or low (1-3%) oxygen conditions. Virus replication was analysed by qRT-PCR and plaque assay, while HIF expression and stabilisation was quantified by western blot and HIF and target gene expression (qRT-PCR). Roxadustat was used to chemically induce HIF signalling under non-hypoxic conditions to investigate HIFdependent mechanisms at play during virus infection. Flavivirus replicons are also being used to look at viral translation and genome replication under low oxygen conditions.

Results
Both HTR8 and Huh7 cells showed enhanced viral replication under low oxygen conditions, particularly at 48 hours post infection, which corresponds with the results for other Flaviviridae family members. Results suggest HIF signalling alone can enhance ZIKV replication, although there may also be HIF-independent mechanisms involved. Both HCV and DENV infection has been shown to stabilise HIF1a under normoxic conditions, however ZIKV infection resulted in a decrease in HIF1a protein even under low oxygen conditions. Interestingly, using DENV and ZIKV replicons we noted increases in translation and/or RNA stability under low oxygen conditions that could mechanistically be responsible for the enhanced replication observed.

Conclusion
Replication of Flaviviridae viruses, including ZIKV, is enhanced by conditions of low oxygen, although the role HIF signalling plays in infection and pathogenesis is still not clear. Understanding how ZIKV interacts with HIF and other hypoxic pathways during infection to enhance replication may allow us to further understand virus-host interactions for ZIKV and other Flaviviridae viruses, and potentially reveal novel therapeutic targets.

P116 – **HCV infection leads to reprogramming of host cell response to IL-1β by enhancing NFκB-dependent signaling**

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Background
Despite the development of adequate therapeutic options, hepatitis C virus (HCV) remains a major cause of chronic liver disease and a risk factor for hepatocellular carcinoma worldwide. In up to 70% of infected individuals, the virus persists, with, despite of persistent replication, infection often leading to overt disease only after several decades of asymptomatic progression. The low antigenicity of the virus underlying this course of disease suggests that it can subvert both the inflammatory and antiviral responses of the host. In doing so, HCV interferes with host cell signaling at multiple levels, including altering the signaling of mediators produced in response to the viral infection itself. The goal of this work is to better understand the mechanisms that allow HCV to affect the host cell response to inflammatory mediators, which are often slightly elevated in chronically HCV infected individuals.

Methods
Cytokine and chemokine analysis in serum by ELISA. Analysis of gene expression and signal transduction by immunoblot, chromatin IP and qPCR in control cells or in cells harboring a sub genomic HCV replicon or infected with HCV.

Results
Serum concentrations of IL-1β and CXCL8 are increased in patients with chronic hepatitis C compared with healthy controls. Thereby, IL-1β concentration correlates directly with viral load while CXCL8 concentration correlates directly with IL-1β but not with viral load. In vitro studies suggest that HCV leads to a substantial enhancement of IL-1β-inducible CXCL8 expression in the host cell. This is accompanied by a reduction in basal protein levels of IκBα in the presence of HCV, as well as a significantly delayed recovery of IκBα protein levels after their IL-1-induced degradation. This effect of HCV on IL-1β-induced signaling at the level of IκB molecules results in enhanced NFκB activation. Chromatin immunoprecipitation analyses show that HCV significantly enhances IL-1β-induced binding of the p65 subunit of the NFκB complex to the NFκB binding region in the CXCL8 promoter. This leads to a consecutive reprogramming of the host cell response to IL-1β.

Conclusion
HCV reprograms the host cell response to inflammatory cytokines such as IL-1β by enhancing the activation of NFκB. This is likely mediated by accelerated degradation as well as delayed reconstitution of IκBα.

P117 – **Propagation of hepatitis E virus in human neuronal cells as infection model system for extrahepatic manifestations**

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Background
HEV infections are the most common cause of acute viral hepatitis, annually causing over 3 million symptomatic cases and 70,000 deaths worldwide. Historically, HEV was described as an hepatotropic virus, but has recently been linked to various extrahepatic manifestations including neurological disorders like Guillain-Barré syndrome and neuralgic amyotrophy. However, underlying pathogenesis of these neurological injuries remains largely unknown, and it is also uncertain whether or not HEV can directly infect neuronal cells. The aim of this study was to investigate whether neuronal cell lines support the full life cycle of HEV and, in a further step, to develop a neuronal model system using induced primary neurons (iPNs) to study extrahepatic HEV manifestations under authentic conditions.

Methods
Five human neuronal-derived cancer cell lines (MO3.13, SK-N-MC, DAOY, DBTRG and U-373 MG) and human iPNs, derived from urinary renal epithelial cells, were used in this study. Employing a state-of-the-art HEV genotype 3 (Kernow-C1 p6) cell culture model, quantification of viral infection of enveloped and naked HEV particles was possible. Illumina RNA sequencing was further applied to monitor dynamic responses in transcript expression levels to HEV challenge in iPNs.

Results
All neuronal-derived cell lines were susceptible to HEV infection with focus forming units (FFU) ranging from 10^2 – 10^4 FFU/mL. Of note, DAOY cells were the least, while U-373 MG cells were the most infectable. Production of infectious particles derived from neuronal cells ranged between 10^3 – 10^5 FFU/mL, demonstrating that HEV can complete its full life cycle in neuronal cancer cells *in-vitro*. Moreover, iPNs and their neuronal progenitor cells supported HEV entry and replication. Volumetric three-dimensional reconstitution revealed that mainly differentiated neuronal cells were infected, while non-differentiated neuronal cells were less susceptible. Immunofluorescence analysis of neurons further indicated that HEV affects neurite length. In addition, transcriptomic analysis revealed comparable expression levels of antiviral innate immune signalling genes or dsRNA sensing genes in infected and uninfected iPNs.

Conclusions
In summary, our results indicate that neuronal cell lines are capable of supporting the entire replication cycle of HEV including entry, replication, assembly and release. Moreover, we use primary neurons, which are susceptible to HEV and characterize the effect of HEV infections. As a result, iPNs serve as a model system as they provide an authentic cellular background for neurological HEV studies and thus the opportunity to gain deeper insights into the relationship between HEV and extrahepatic manifestations.

P118 – **Identification of HCV Resistant Variants against Direct Acting Antivirals in Plasma and Liver of Treatment Naïve Patients**

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Background
Current standard-of-care treatment of chronically infected hepatitis C virus (HCV) patients involves direct-acting antivirals (DAAs). However, concerns exist regarding the emergence of drug -resistant variants and subsequent treatment failure. In this study, we investigate potential natural drug resistance mutations in the NS5B gene of HCV genotype 1b from treatment-naïve patients.

Methods
A paired plasma samples and liver biopsies of eighteen patients chronically infected with HCV genotype 1b (HCV-1b) and naïve to any treatment were included. Population based Sanger sequencing and 454 deep sequencing of NS5B gene were performed on plasma and liver samples obtained from 18 treatment- naïve patients.

Results
The quasispecies distribution in plasma and liver samples showed a remarkable overlap in each patient. Although unique sequences in plasma or liver were observed, in the majority of cases the most dominant sequences were shown to be identical in both compartments. Neither in plasma nor in the liver codon changes were detected at position 282 that cause resistance to nucleos(t)ide analogues. However, in 10 patients the V321I change conferring resistance to nucleos(t)ide NS5B polymerase inhibitors and in 16 patients the C316N/Y/H nonnucleoside inhibitors were found mainly in liver samples.

Conclusions
In conclusion, 454-deep sequencing of liver and plasma compartments in treatment naïve patients provides insight into viral quasispecies and the pre-existence of some drug-resistant variants in the liver, which are not necessarily present in plasma.

P119 – **Development of an Activation-Induced Marker (AIM) Assay to Identify Hepatitis C Virus (HCV)-specific T cells**

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Background
Activation-induced marker (AIM) assay is a robust and effective tool that enables the identification of antigen-specific T cells *ex vivo* irrespective of HLA-type or cytokine production capacity, enabling the characterization of hard-to-detect antigen-specific T cells. Although the established use of tetramers to identify virus-specific T cells, they are limited by their availability and affinity. Herein, we developed an AIM assay to monitor the kinetics, magnitude, phenotype and transcriptomic signature of hepatitis C virus (HCV)-specific CD4+ and CD8+ T cell response in a cohort of HCV reinfected patients.

Methods
PBMCs from HCV-reinfected subjects were rested for 3 hours at 37 °C then incubated with CD40-blocking antibody and stimulated for 6, 9, 18, 21 and 24 hours by overlapping HCV peptide pools. Unstimulated and Staphylococcal enterotoxin B (SEB) stimulated cells served as negative and positive controls, respectively. HCV-specific CD8 and CD4 T cells were identified by flow cytometry based on the expression of different combinations of activation markers including CD40 Ligand (CD154), CD69, 4-1BB (CD137) and OX40 (CD134). A panel including the lineage markers (CD3, CD4, CD8, CD45RA), polarization (CXCR3, CCR4, CCR6, CXCR5), activation/exhaustion (ICOS, PD-1, TIGIT, CTLA4) and degranulation (CD107a) was used to characterize HCV-specific T cells. Moreover, CD40L+ CD69+ CD4+ and 4-1BB+ CD69+ CD8+ T cells were sorted for downstream single-cell RNA sequencing analysis and T cell receptor (TCR) clonotyping analysis using 10X genomics technology.

Results
CD40L+CD69+ HCV-specific total CD4 and circulating T follicular helper (cTfh ($CXCR5^+$, $PD1^+$)) T cells were detectable as early as 6 hours post-stimulation. However, cells co-expressing either OX40+CD69+ or 4-1BB+ CD69+ were detectable only at late time points (as of 18 hours). HCV-specific CD8+ T cells were only detectable at 18 hrs and distinguished by upregulation of 4-1BB and CD69 but not OX40 or CD40L. Sequencing data allowed the identification of 6 and 10 different clusters of CD4+ and CD8+ HCV-specific cells, respectively.

Conclusion
We optimized an 18-hours AIM assay that enabled the simultaneous detection, phenotyping, and single-cell gene expression and TCR analysis of HCV-specific CD4+ and CD8+ T cells.

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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.

P121 – Frequency of interferon-lambda 4 genotypes in Egyptian patients infected with hepatitis C virus genotype 4

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Background and Aims
Genome-wide association studies conducted by groups worldwide have reported host genetic markers and single nucleotide polymorphisms (SNPs) in the interferon-lambda family of cytokines, especially interferon-lambda-4 (IFNL-4) and 3 associated with the resolution of chronic HCV infection and better responses to antiviral therapy. The IFNL-4 rs12979860 genotypes' frequency varies in different populations, with the CC genotype associated with resolving chronic HCV infection and better responses to antiviral therapy. Egypt has a large population infected with HCV genotype 4 who have been diagnosed and treated with nationwide efforts for screening and offering affordable treatment with directly acting antivirals. Thus, we aimed to investigate frequencies of IFNL-4 genotypes in an Egyptian cohort.

Methods
Blood samples were collected from chronic HCV patients (n = 83) mean age 52.5 (18–84) years, 38.6% males infected with genotype 4a recruited from the National Hepatology and Tropical Medicine Research Institute, Cairo, and Faqous Central Hospital, Sharqia, Egypt. Patients had a mean viral load before the start of treatment of 949,683 IU/mL (35 –14,118,373 IU/mL). Genomic DNA was isolated and used for genotyping IFNL4 rs12979860 by PCR using our previously published protocol [1].

Results
Analysis of genotyping of IFNL4 rs12979860 in the Egyptian cohort showed that 53% carried the CT genotype and 35% the TT genotype, while only 12% had the CC genotype.

Conclusions
Our findings agreed with previous studies on Egyptian cohorts infected with HCV genotype 4a that the most prevalent genotype is CT 53% vs. 43–57%, while differed in the frequencies of TT 35% vs. 11–19% and CC 12% vs. 23–45% [2–4]. Observed frequencies of s12979860 SNP TT genotype in the current cohort were similar to the African ancestry population studies.

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P122 – **A cellular phosphatase regulated by Yellow Fever Virus (YFV) interferes with release of infectious virions and inhibits TCR-mediated IL-2 release**

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Background
YFV-17D vaccine strain infection regulates the phosphatase PTPRE using a short, genome-derived RNA (vsRNA) mechanism. YFV-vsRNA-mediated PTPRE knockdown results in enhanced production of YFV by Huh7D and T cells and reduces T cell receptor (TCR)-mediated signaling. Here, we further examined how PTPRE regulates virus replication.

Methods
Attempts to knockout PTPRE using CRISPR did not generate viable Huh7D or Jurkat cell lines. Thus, we overexpressed a PTPRE lacking the 3'UTR complementary sequences targeted by YFV vsRNA under the control of tetracycline-regulated promoter. YFV 17D and Zika (PR strain - control) were used to infect these cell lines using doxycycline to regulate PTPRE. YFV binding at 4^{o}C, entry at 37^{o}C, and kinetics of viral RNA and infectivity released from cells were measured. Intracellular virions were observed by TEM and TCR-mediated signaling determined by measuring IL-2. Mutations altering the YFV vsRNA genome region were introduced into YFV-17D to maintain the native a.a. sequence and virus generated using CPER.

Results
Infectious YFV released from cells that expressed PTPRE without the complementary sequences targeted by the YFV vsRNA was reduced by more than 100-fold in Jurkat and Huh7D cells. In contrast, viral RNA released was only reduce by 3 to 10-fold in these PTPRE expressing cells. The differences were not observed when the cells were grown in doxycycline. PTPRE overexpression had no effect on Zika virus replication kinetics. YFV attachment and entry was not altered in the cells overexpressing the "non-targeted" PTPRE compared to those with native PTPRE. Despite reduced infectivity of supernatant virus produced by PTPRE-nontargeted cells, YFV envelope protein content in culture supernatants was similar to that released by cells that did not express the nontargeted PTPRE. Following YFV infection, electron microscopy demonstrated large, empty-appearing particles in cells expressing nontargeted PTPRE, and these were not present in cells that did not express nontargeted PTPRE. In preliminary experiments, infection of primary PBMCs and Jurkat cells with CPER generated recombinant YFV containing genome RNA mutations to prevent targeting PTPRE but maintaining the envelope a.a. sequence partially, and significantly, restored TCR-mediated IL-2 release.

Conclusions
These data demonstrate that PTPRE is a YFV restriction factor that interferes with the generation of infectious YFV particles without reducing total cellular production of viral RNA. TEM data support a role of PTPRE in preventing YFV RNA encapsidation, though further studies are needed to confirm this. Studies are underway to determine the potential role of the genome sequence on PTPRE regulation, replication kinetics in cells with and without targetable PTPRE, and YFV envelope phosphorylation during virus assembly and morphogenesis. These studies may identify novel targets for the treatment of YFV infection.

P123 – **Virus-specific CD8$^+$ T cells mediate spontaneous clearance of multiple HCV infections in the absence of neutralizing antibodies - A case study**

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Background
The development of a successful vaccine against hepatitis C virus (HCV) requires the identification of signature pathways of protective immunity against this virus, since they could serve as indicators of efficacy for a given vaccine candidate. Around 30% of people who become acutely-infected with HCV clear the virus spontaneously. The likelihood of clearance increases upon reinfection in people who spontaneously resolved a previous infection. Expansion of HCV-specific CD8+ T cells with high functional avidity has been associated with protection upon reinfection. However, elements responsible for this process are yet to be fully understood. Here, we report the detailed immune characterization of a subject with multiple HCV infections with different outcomes.

Methods
We used samples collected from a person who inject drugs who experienced three episodes of infection with HCV genotype 1a, 1, and 3a, respectively. The second infection occurred ~1 year after primary infection, while the third episode occurred ~3 years after. He resolved the first and second infections spontaneously, while the third episode ended up in viral persistence. We performed high-resolution immunophenotyping using MHC class I tetramers B27/NS5b-2841 and a combination of cell surface and intracellular markers, and measured neutralizing antibodies of key time points from each infection: 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).

Results
HCV-specific CD8$^+$ T cells expanded during each episode, regardless of the outcome. Most of these cells maintained a memory-like (CD127$^+$PD-1$^+$) phenotype in all three episodes, while the highest frequency of effector (CD127$^-$PD-1$^+$) T cells was observed during early primary infection. In contrast, total and activated cTfh cells expanded at higher levels during the early acute stage of the third infection, showing as well an increased expression of the proliferation marker Ki67+. The cTfh1 (CXCR3+ CCR6-) phenotype was predominant during all episodes of infection. However, low or absence of expansion of HCV-specific memory B cells was observed during reinfection. Neutralizing antibodies were also low or absent during the first two resolving episodes. Although we observed an early development of antibodies against E2 protein in the third episode, they were not able to neutralize HCV *in vitro*, and the patient became chronically infected.

Conclusions
This case study shows that clearance of HCV infections of a similar genotype can be achieved by a virus-specific CD8$^+$ T cell response alone, in the absence of neutralizing antibodies. However, upon reinfection with a different HCV genotype, the early expansion of CD8$^+$ T cells and development of non-neutralizing antibodies together did not prevent chronicity.

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P124 – **Unveiling Intricate Immune Responses During Acute HCV Infection: Insights from Single-Cell RNA Sequencing**

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Background
CD4+ and CD8+ T cells are critical for HCV clearance but remain poorly characterized in the HCV-infected liver. HCV's rapid adaptability often leads to T cell exhaustion, hindering effective immune responses. Recent advances in single-cell sequencing technology enable detailed examination of immune responses within the liver, offering insights into heterogeneity and identifying key regulators of T cell exhaustion. Integrating this technology with our understanding of HCV pathogenesis holds promise for improving outcomes during this critical phase.

Methods
We conducted an investigation using cryopreserved intrahepatic mononuclear cells isolated from a chimpanzee with acute HCV infection and post-clearance. Subpopulations of liver-infiltrating immune cells were sorted based on the presence of HCV-specific CD4 T cells, identified by high PD-1 and ICOS co-expression, along with dominant HCV-specific CD8 T cells stained with MHC I tetramers. The 10x Genomics platform was utilized for single-cell RNA sequencing to capture the transcriptomic profiles of these intrahepatic immune cells. Data analysis was performed in R, encompassing quality control, clustering, and differential gene expression analysis. This approach facilitated a comprehensive exploration of the transcriptional landscape of intrahepatic immune cells, unveiling key molecular signatures associated with HCV-specific T cell responses.

Results
Single-cell RNA sequencing revealed a complex CD4+ and CD8+ T cell response in liver during acute infection. The acute CD4+ T cell response was comprised of dominant regulatory (Treg) and effector clusters that were not present in liver after resolution of infection. The Treg clusters were identified by high expression of *FOXP3* and related genes. The effector CD4+ T cell clusters had a unique Tfh1 transcriptional signature marked by high expression of *PDCD1* and *ICOS* but limited or no *CXCR5*. They expressed multiple genes associated with the Tfh and Th1 subsets including *IL21*, *CXCL13* and *IFNG* genes. Transcriptional analysis also revealed HCV-specific $CD8^+$ T cells in the acute phase of infection that persisted in liver 2 years after spontaneous resolution of infection. Further investigations aim to define transcriptional signatures of acute-phase effector and late-phase memory CD8+ T cells in the liver. These findings demonstrate that liver-infiltrating CD4+ and CD8+ T cell responses are transcriptionally complex and represent a response to infection as that contracts sharply after resolution of infection.

Conclusions
Our study utilized single-cell RNA sequencing to probe intrahepatic immune responses in HCV infection, importantly at the site of infection. Our data suggests that HCV-specific T cells comprise multiple subpopulations during acute infection. Studies to further characterize CD4+ and CD8+ T cells by scRNAseq, including trajectory analysis to define the transition towards intrahepatic memory populations, are ongoing. These studies are expected to provide insight into successful HCV-specific T cell responses that will aid development of a protective vaccine.

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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P126 – 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.

P127 – Hybrid agent-based and viral kinetics modeling of hepatitis C micro-elimination among people who inject drugs with direct-acting antivirals in metropolitan Chicago

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Background
Persons who inject drugs (PWID) are at high risk for acquiring and transmitting hepatitis C virus (HCV) infection. Direct-acting antiviral (DAA) therapy leads to high cure rates among PWID, however high rates of reinfection after cure and may require multiple DAA treatments to reach the World Health Organization's (WHO) goal of HCV elimination by 2030. In this modeling study we use an agent-based model (ABM) with a kinetic model of HCV transmission via syringe sharing to show that continued courses of DAA treatment among networks of HCV infected PWID is required to achieve elimination in a large population of PWID from Chicago, IL and surrounding suburbs.

Methods
We extended our previously developed agent-based Hepatitis C Elimination in PWID (HepCEP) model to identify and optimize direct-acting antiviral (DAA) therapy scale-up and treatment strategies. We previously used a fixed probability (1%) of HCV transmission through syringe sharing among infected PWID. The updated HepCEP uses a mathematical model to determine transmission probabilities relative to the HCV RNA titers of needle/syringe-sharing donors. Individual temporal viral load profiles are sampled from unique distributions for acute and chronic naïve and reinfected individuals, and for individuals undergoing DAA treatment. We compared the rates of new chronic infections using the fixed and variable HCV transmission models for different combination(s) of DAA enrollment and frequency of DAA treatment courses.

Results
When retreatment limitations are not imposed, annual DAA enrollment rates of >= 5% of the PWID population are able to achieve elimination using a fixed HCV transmission probability of 1%. The updated viral kinetics model shows that elimination is possible only for DAA enrollment rates >= 7.5% and that the elimination goal is shifted later by several years. When a retreatment limitation is imposed such that PWID are only eligible for four total DAA treatments (after cure and reinfection), the fixed HCV transmission model again shows that elimination is feasible for DAA enrollment rates of >= 5%, while the viral kinetics model shows that elimination is not possible for DAA enrollment rates up to 10% of the PWID population.

Conclusions
HepCEP model simulations underscore the importance of DAA scale-up without any re-treatment prohibition to achieve significant reductions in HCV incidence when the rates of reinfection among PWID networks are high. An unbiased DAA scale-up of >7.5% (or 75 per 1000 PWID) is projected to achieve the WHO target of 90% HCV incidence reduction by 2030 using the updated kinetic model of HCV transmission probability.