HCV-Flavi 2022

28TH INTERNATIONAL SYMPOSIUM ON HEPATITIS C VIRUS, FLAVIVIRUSES, AND RELATED VIRUSES

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JULY 6-9, 2022

Abstract Book

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Sessions | Adaptive immunity
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Sessions | Vaccine development
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Sessions | Vaccine development continued
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Sessions | Pathogenesis
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Sessions | Innate immunity
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PLENARY LECTURE 1 | July 6, 2022 (Wed) 13:00 – 13:30

Deciphering virus-host bonds at the single virus level using atomic force microscopy

David Alsteens, *Université Catholique de Louvain, Belgium*

Dr. Alsteens heads the nanobiophysics laboratory at the Université catholique de Louvain (UCLouvain) in Belgium. He is a bioengineer, specialized in nanobiotechnology by training, and graduated in 2007. He received his PhD in nanobiophysics in 2011, developing atomic force microscopy (AFM) to probe, at the single-molecule level, specific interactions on living cells. With a long-term EMBO fellowship, he then joined ETH Zürich, where he further developed AFM to study mammalian cells under physiologically relevant conditions. Since 2015, he has been a research associate at FNRS and leads his own group at UCLouvain that focuses on the study of biological processes taking place at biological interfaces.

He has recently combined AFM and confocal microscopy to probe virus binding on the surface of mammalian cells focusing on a variety of viruses including herpesviruses, reoviruses, rotaviruses, ebolaviruses and SARS-CoV-2. He has co-authored over 100 papers and his contributions have been recognized with several awards, such as the 2019 Heinrich Emanuel Merck Award for Analytical Science, the 2019 BAEF Alumni Award, and the 2021 Princess Josephine Charlotte Award in the field of viral infection

PLENARY LECTURE 2 | July 6, 2022 (Wed) 13:30 – 13:50

Dynamics of HCV envelope glycoproteins and the link with immune evasion

Jannick Prentoe, *University of Copenhagen, Denmark*

Jannick Prentoe is a civil engineer, specializing in biotechnology, by education and graduated from the Technical University of Denmark in 2007. He has since worked on several human pathogenic RNA viruses, with a focus on hepatitis C virus (HCV). This work has revolved around understanding HCV neutralizing antibody evasion and the complex interplay between envelope protein dynamics and viral entry that governs it.

In 2017, Dr Prentoe was hired as an Associate Professor at the University of Copenhagen and in 2020 he received a 5 year Lundbeck Foundation Fellowship to support establishing his own group. His research activities now range from molecular virology, antibody discovery, fluorescent and live cell imaging, vaccine antigen engineering and in vivo studies of antibodies using various mice strains. Please grab him for a chat about science, writing and performing music, the deliciousness of fresh mozzarella di bufala and rock climbing.

PLENARY LECTURE 3 | July 6, 2022 (Wed) 13:50 – 14:10

Structural biology of flavivirus envelope proteins and antibody neutralization

Shee-Mei Lok, *Duke – NUS medical School, Singapore*

Dr Shee-Mei Lok is a Provost's Chair Professor in the Emerging Infectious program in Duke-NUS, Singapore. She was also a Singapore National Research Foundation (NRF) fellow (2009-2014) and a NRF Investigator (2016-2021). She is a structural virologist specializing in x-ray crystallography and cryo-electron microscopy. Her research interest focus on the structural changes of flavivirus particles during its infection cycle and the effect of anti-viral therapeutics on them.

She obtained her Msc and PhD in NUS and did her post-doctoral training in Purdue University under the supervision of the late Prof Michael Rossmann. Her laboratory made significant discoveries in the understanding of the structures of the Zika and dengue viruses, the morphological variants of dengue viruses, how potent human antibodies neutralize flaviviruses, how capsid orchestrate the flavivirus assembly process and the structural changes of flavivirus during maturation.

PLENARY LECTURE 4 | July 6, 2022 (Wed) 15:30 – 15:50

A molecular scar in exhausted virus-specific CD8+ T cells hinders functional memory formation after HCV cure

Maike Hofmann, *University of Freiburg, Germany*

In 2012, Dr. Maike Hofmann obtained her PhD in Molecular Medicine from the Faculty of Biology and the Faculty of Medicine of the University of Freiburg, Germany. Since 2016, she is co-heading a research group together with Prof. Dr. Robert Thimme at the Medical Center - University of Freiburg, Department of Medicine II - Gastroenterology, Hepatology, Endocrinology and Infectious Diseases. A main focus of her work in the last years relates to mechanisms underlying the T cell and NK cell dysfunction in the context of chronic viral hepatitis caused by HBV and HCV and in liver cancer.

In addition, she is interested in understanding shared and diverging principles of T cell and NK cell memory in chronic versus self-limiting viral infections with HBV, HCV, cytomegalovirus (CMV), Epstein-Barr virus (EBV), Influenza viruses and most recently also SARS-CoV-2 as well as after vaccination. Her research has been funded by the German Research Foundation, the German Ministry of Research and Education and since 2019, she is a Margarete von Wrangell-fellow funded by the Ministry of Science, Research and Arts of Baden-Wuerttemberg (Germany) and the European Union.

PLENARY LECTURE 5 | July 7, 2022 (Thu) 09:00 – 09:20

In silico, in vitro and in vivo models to study vaccine efficacy

Thomas Pietschmann, *TWINCORE, Hannover, Germany*

Thomas Pietschmann, born in 1971 in Würzburg, studied biology with emphasis on biochemistry, animal physiology, virology and immunobiology at the University of Würzburg and the Duke University (Durham, NC, USA). After completing his studies in 1996, he received his Ph.D. degree in biology at the Institute for Virology of the University of Würzburg and subsequently worked as postdoc at the Institute for Virology in Mainz and in the Department for Molecular Virology in the University Clinic of Heidelberg.

Thomas Pietschmann established there an independent research group that investigated the mechanisms of morphogenesis and cell entry of the hepatitis C virus. From the year 2006 his group was supported by an Emmy Noether fellowship from the German Research Community (Deutsche Forschungsgemeinschaft). In the spring of 2007, he was appointed with his work group to TWINCORE. He now leads the Department for Experimental Virology there.

PLENARY LECTURE 6 | July 7, 2022 (Thu) 09:20 – 09:40

An epitope-focused hepatitis C virus vaccine – a perspective

Thomas Krey, *University of Lübeck, Germany*

Thomas Krey obtained his doctoral degree in virology from the faculty of veterinary medicine in 2004 from the University of Giessen, Germany. He switched from molecular to structural virology and received his post-doctoral training at the Institut Pasteur, Paris, under the supervision of Felix Rey. In 2015, he moved to Hannover Medical School to establish his own research group and since 2019 he is head of the Institute of Biochemistry at the University of Luebeck.

He studies the structure-function relationship of viral and cellular proteins involved in virus entry and assembly, with a particular focus on how viral glycoproteins interact with the humoral immune response. In addition, he is interested in the complex capsid assembly process of large DNA viruses. His lab uses structural biology techniques and state-of-the-art protein biochemistry to understand key processes during the virus replication cycle.

PLENARY LECTURE 7 | July 7, 2022 (Thu) 09:40 – 10:00

Are T cells still relevant in the age of neutralizing antibodies?

Naglaa Shoukry, *Université de Montréal, Canada*

Dr. Shoukry obtained her Pharmacy degree from Cairo University (1991) and Ph.D. in Immunology from McGill University (2000). Her postdoctoral research has established the essential and complementary roles of CD8 and CD4 T lymphocytes in resolution and protection from hepatitis C virus (HCV) infection. Since joining the Université de Montréal and CRCHUM in 2005, she has established a translational research program focused on studying immunity to HCV, immunological mechanisms of liver fibrosis progression, and liver cancer.

She has published over 80 articles in high impact journals. She has maintained uninterrupted funding from the Canadian Institutes of Health Research (CIHR), Fonds de recherche du Québec – Santé (FRQS), and the National Institutes of Health (NIH). She has served on various review committees on the national and international level including CIHR, FRQS, Canada Research Chairs, NIH, DFG and the Wellcome Trust. She is an Academic Editor for PLoS One, and Frontiers in Immunology. In recognition, CIHR profiled her achievements on World Hepatitis Day in 2013 and 2017. In 2019, she was selected as Professor of the Year by the Department of Medicine, University of Montreal and was awarded the CLF 50th Anniversary Recognition Medal. Since 2015 she has been the Director of the Canadian Network on Hepatitis C (CanHepC), federally funded network with over 100 investigators, trainees and knowledge users working towards establishing a pipeline from research to implementation and to improve the lives of Canadians living with hepatitis C and to work towards the eradication of HCV.

PLENARY LECTURE 8 | July 7, 2022 (Thu) 10:00 – 10:20

Pathways for vaccine development for hepatitis C

Heidi Drummer, *Burnet Institute, Australia*

Professor Heidi Drummer is Scientific Director of the Burnet Diagnostics Initiative and Program Director for Disease Elimination at the Burnet Institute in Melbourne, Australia. She was awarded her PhD degree in 1993 and has worked across diverse animal and human viruses.

For the last 20 years, her work has focused on developing candidate antigens for Hepatitis C vaccines and was the first to describe a minimal core domain of glycoprotein E2 and variable region 3. Her work has investigated the use of protein, virus-like particle, mRNA and viral vectored vaccines for HCV with an aim of developing a universal prophylactic vaccine to prevent HCV.

PLENARY LECTURE 9 | July 7, 2022 (Thu) 10:50 – 11:10

Preparing a vaccine for the next pandemic – lessons learnt from COVID-19

Teresa Lambe, *Univesity of Oxford, Great Britain*

Teresa Lambe is a Professor of Vaccinology & Immunology and the Calleva Head of Vaccine Immunology at the University of Oxford. Prof Lambe is leading a research group which improves human health by controlling disease through vaccination – stopping epidemics before they become pandemics.

Her team work on emerging and outbreak pathogens including Ebola, CCHF and SARS CoV-2. Prof Lambe is one of the Principal Investigators overseeing the Oxford/AstraZeneca vaccine programme. In January 2020, Prof. Lambe co-designed the vaccine, led the preclinical studies and then oversaw the delivery of the immune results needed to support regulatory approval in late 2020. The vaccine has played a pivotal role in the fight against COVID-19. Prof. Lambe was appointed as an honorary OBE for her services to Sciences and Public Health in the 2021 Queen's Birthday Honours

PLENARY LECTURE 10 | July 7, 2022 (Thu) 14:55 – 15:15

Leveraging the design of mRNA vaccines to increase their succes in a prophylactic setting

Leonie Wyffels, *Ziphius Vaccines, Belgium*

Leonie Wyffels obtained her degree of Pharmacist and PhD in Radiopharmaceutical Sciences at the University of Ghent after which she joined the University of Arizona (Tucson, UZA) for a post-doctoral study on the development of radiopharmaceuticals for imaging of cardiac apoptosis and inflammation. During her career in academia (University of Antwerp) and biotech industry (Ablynx, a Sanofi company), Leonie has gained extensive experience with working in an international, multi-disciplinary and GMP environment, 'bench to bedside' drug development, quality control and project management.

She is still affiliated with the University of Antwerp Faculty of Medicine and Health Sciences - Department of Nuclear Medicine as Visiting Professor and recently joined Ziphius Vaccines as R&D director.

PLENARY LECTURE 11 | July 7, 2022 (Thu) 15:15 – 15:35

The case for and against adjuvanted HCV protein vaccines

Michael Houghton, *University of Alberta, Canada*

Dr. Michael Houghton was the Canada Excellence Research Chair in Virology from 2010-2018 and is the Li Ka Shing Professor of Virology at the University of Alberta where he is also the Director of the Li Ka Shing Applied Virology Institute. He was jointly named the 2020 Nobel Prize winner in Physiology or Medicine along with Harvey J. Alter and Charles M. Rice in recognition of the discovery of the hepatitis C (HCV) virus. His research in the field of viral hepatitis has led to improved blood safety, and hepatitis C treatment to the point where the viral infection can now be cured in virtually all patients. He has also been working on a HCV vaccine for the last 30 years in the US & Canada

Born in the United Kingdom, Houghton graduated from the University of East Anglia with a BSc in biological sciences in 1972, and subsequently completed his PhD in biochemistry at King's College, University of London in 1977.

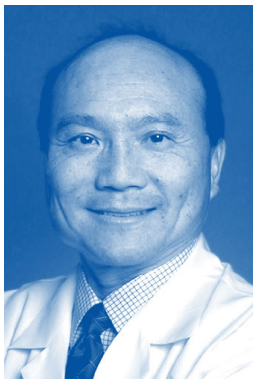
Houghton joined G. D. Searle & Company in the UK studying human intereferon genes before moving to Chiron Corporation in 1982 where, together with colleagues Qui-Lim Choo and George Kuo, and Daniel W. Bradley from the Centers for Disease Control and Prevention, they first discovered HCV in 1989. Houghton was co-author of a series of seminal studies published in 1989 and 1990 that identified hepatitis C antibodies in blood, particularly among patients at higher risk of contracting the disease, including those who had received blood transfusions. This work led to the development of a blood screening test in 1990; widespread blood screening that began in 1992 with the development of a more sensitive test has since virtually eliminated hepatitis C contamination of donated blood supplies in Canada and around the world.

Houghton was recruited to the University of Alberta in 2010 as the Canada Excellence Research Chair in Virology in the Li Ka Shing Institute of Virology. In 2013, Houghton's team at the University of Alberta showed that a vaccine derived from a single strain of Hepatitis C could prevent infection of cell cultures by most global strains of the virus.

PLENARY LECTURE 12 | July 7, 2022 (Thu) 15:35 – 15:55

Leveraging the design of mRNA vaccines to increase their succes in a prophylactic setting

Jake Liang, *NIDDK, NIH, USA*



T. Jake Liang, M.D. is a NIH Distinguished Investigator and the Chief of Liver Diseases Branch, NIDDK, NIH. He is a graduate of Harvard College and Harvard Medical School. He completed his postgraduate training at Harvard Medical School, where he was an Assistant Professor of Medicine from 1990-1996. Dr. Liang is an internationally renowned thought leader and investigator in the field of viral hepatitis and liver diseases. His research program focuses on the virology, pathogenesis, vaccine and therapeutic development of viral hepatitis and liver cancer. He has published over 300 papers and has a h-index of 107. Dr. Liang has been an Associate Editor for top medical journals such as Gastroenterology, Hepatology and Gut. He has been in various leadership roles at NIH. He served on the Governing Board of the AASLD and was its President in 2011. His outstanding contributions has been recognized with numerous national awards including the AASLD Distinguished Mentor/Clinician Educator Award and the AGA Distinguished Achievement Award in Basic Science. He was elected to the

American Society for Clinical Investigation, the Association of American Physicians, the AAAS Fellow, the American Academy of Microbiology, and the National Academy of Medicine.

PLENARY LECTURE 13 | July 8, 2022 (Fri) 09:00 – 09:20

Drug development for flaviviruses

Johan Neyts, *KU Leuven, Belgium*

Johan Neyts is full professor of Virology at the University of Leuven, Belgium. He teaches virology at the medical school and at the school of dentistry. The laboratory has a long-standing expertise in the development of antivirals strategies and drugs against emerging and neglected viral infections (such as dengue and other flaviviruses), Chikungunya and other alphaviruses, enteroviruses, noroviruses, HEV and rabies. The lab has and is intensively involved in developing antiviral strategies against SARS-CoV2. A second focus of the lab is the development of a novel vaccines.

His team developed/develops a number of very potent single dose vaccines including against COVID-19. The approach is based on the yellow fever vaccine as a vector. The laboratory also developed a platform technology, the PLLAV (Plasmid Launched Live Attenuated Virus) vaccine approach. This technology allows to rapidly engineer highly thermostable vaccines against multiple viral pathogens. Johan Neyts is past president of the International Society for Antiviral Research (www.isar-icar.com). Five classes of antivirals discovered in his laboratory have been licensed to major pharmaceutical companies (two on HCV, one on dengue, one on rhino/enteroviruses and one on RSV). He is co-founder of KU Leuven spin-off Okapi Sciences (which was acquired within 5 years of incorporation). He published ~585 papers in peer reviewed journals, is inventor of a large patent portfolio, has given ~280 invited lectures and a large number of interviews to lay-press

PLENARY LECTURE 14 | July 8, 2022 (Fri) 09:20 – 09:40

HCV Elimination

Heiner Wedemeyer, *Hannover Medical School, Germany*

Heiner Wedemeyer is Professor and Chairman of the Department of Gastroenterology, Hepatology and Endocrinology at Hannover Medical School since April 1, 2020. He was Professor and Chairman of the Department of Gastroenterology and Hepatology at the University Clinic Essen from February 2018 to April 2020. He received his medical degree from the University of Göttingen in 1996 and subsequently started his training in Internal Medicine at Hannover Medical School in Germany. From 1998 to 2000, he was a research fellow in immunology at the Liver Diseases Branch, National Institutes of Health, Bethesda, USA. Since 2001, he completed his training in Internal Medicine and Gastroenterology at Hannover Medical School, where he became Professor of Medicine in 2011. Professor Wedemeyer has been involved in the scientific coordination of the German Network of Competence on Viral Hepatitis (Hep-Net) and the German Liver Foundation for more than 15 years. Currently, he serves as the Managing Director of the German Hepatitis C-Registry.

Heiner Wedemeyer is member of several scientific organizations and was Secretary General of the European Association for the Study of the Liver from 2009 to 2011. Professor Wedemeyer has a long-term research interest in liver diseases with a main focus on viral hepatitis, liver transplantation and hepatocellular carcinoma. He has been principal investigator in numerous clinical trials, focusing on antiviral therapy and immunotherapy of viral hepatitis B, C, D and E. He has authored over 500 original articles; his current Hirsch-Index is 101 (google scholar; October 2021) and his work has been quoted

more than 45,000 times. Heiner Wedemeyer has received numerous awards including the Hans Popper Award of the International Association of the Study of the Liver in 2002, the Innovation Award of the German Medical Faculties (2011) and the Rudolph-Schoen-Award (2011). His research has been funded by the Deutsche Forschungsgemeinschaft, the German Ministry of Research and Education, the European Union, the European Association for the Study of the Liver and the Bill and Melinda Gates Foundation.

PLENARY LECTURE 15 | July 8, 2022 (Fri) 11:40 – 12:00

Host-factors involved in flavivirus infection

Charles M Rice, *Rockefellar University; USA*

Dr. Rice is the Head of the Laboratory for Virology and Infectious Disease at The Rockefeller University. He is a prominent figure in research on members of the Flaviviridae including hepatitis C virus. Dr. Rice received his bachelor's degree from University of California Davis in 1974 and earned his Ph.D. from the California Institute of Technology in 1981. From 1986-2000, Dr. Rice was a faculty member at Washington University in St. Louis before moving to The Rockefeller University. Dr. Rice has co-authored over 500 articles in the field of virology and his laboratory's contributions have been widely recognized by numerous prizes and awards, such as the 2007 M.W. Beijerinck Virology Prize, the 2015 Robert Koch Award, the 2016 InBev-Baillet Latour Health Prize, the 2016 Lasker-DeBakey Clinical Medical Research Award and the 2020 Nobel Prize in Physiology or Medicine. Dr. Rice is a member of the National Academy of Sciences.

PLENARY LECTURE 16 | July 8, 2022 (Fri) 14:30 – 14:50

Lipid droplets grease viral replication: role in assembly and beyond

Gabrielle Vieyres, *Leibniz Institute for Experimental Virology (HPI), Germany*

Dr. Gabrielle Vieyres graduated with a Master degree in Molecular and Cellular Biology at the „Ecole Normale Supérieure de Lyon" with emphasis on virology. In 2010 she obtained a joint PhD from the Universities of Glasgow and Lille for her work on the hepatitis C virus envelope glycoproteins and neutralizing antibodies. During her subsequent postdoc at Twincore, in Hannover, she investigated the process of hepatitis C virus assembly and dissected the involvement of both the host and viral machinery. In particular, she reported the role of lipid droplet degradation in the morphogenesis of the HCV lipo-viro-particle. In 2020, she was recruited as an independent junior group leader at the Leibniz Institute for Experimental Virology in Hamburg.

Her group belongs to the Leibniz ScienceCampus InterACt and combines quantitative imaging approaches to traditional cell biology and virology techniques. At the frontier between these fields, Dr. Vieyres investigates the interaction between the pathogen and its host and unravels the subcellular compartimentalization of viral infections. Her primary focus is the interplay between host lipid droplets and positive-stranded RNA viruses, including hepatitis C and related viruses.

PLENARY LECTURE 17 | July 8, 2022 (Fri) 14:50 – 15:10

Cellular regulation of HCV release

Jin Zhong, *Chinese Academy of Sciences, Institut Pasteur of Shanghai, China*

Dr. Jin Zhong is a professor at Institut Pasteur of Shanghai, Chinese Academy of Sciences. He received his Ph.D degree from the Department of Microbiology at the University of Texas at Austin, USA, where he studied microbial mobile elements under the guidance of Dr. Alan Lambowitz. He was further trained in molecular virology as a postdoctoral fellow in Dr. Francis Chisari's group at The Scripps Research Institute, USA. During his postdoctoral training period, he and colleagues developed a robust cell culture model for HCV infection. Since 2007 he has been the head of Unit of Viral Hepatitis at Institut Pasteur of Shanghai, Chinese Academy of Sciences. He has also been Adjunct Professor at ShanghaiTech University since 2013. Dr. Zhong's major research interest is to study biology of HCV and other human RNA viruses. He has published over 100 research articles. Dr. Zhong is the Chair of the Virology Division in Shanghai Society for Microbiology, and serves the national committee of the Virology Division in Chinese Society for Microbiology. Dr. Zhong has won several awards, including 100-Talents Excellence Award by Chinese Academy of Sciences.

PLENARY LECTURE 18 | July 9, 2022 (Sat) 09:00 – 09:20

Immune modulation of Abs via FcR signaling

Taia Wang, *Stanford University, USA*

Taia Wang MD, PhD is an Assistant Professor at Stanford University where her lab studies human immunity and susceptibility to viral pathogens. She completed MD, PhD training at Mount Sinai School of Medicine where her graduate thesis studies were in influenza virology with Dr. Peter Palese. She then performed postdoctoral training in Fc receptor biology with Dr. Jeffrey Ravetch at the Rockefeller University. There, she conducted studies demonstrating how distinct polyclonal IgG Fc domain repertoires impact vaccine responses and susceptibility to viral infections, including the first studies demonstrating that afucosylated IgGs can contribute to the pathogenesis of severe dengue virus infections.

Ongoing studies in the Wang laboratory are driven by the hypothesis that the heterogeneity in IgG Fc domain repertoires among individuals,defined by IgG subclass and Fc glycoform distributions, is a central driver of diversity in human immune functioning and susceptibility to infectious diseases.

PLENARY LECTURE 19 | July 9, 2022 (Sat) 10:50 – 11:10

Antibody fucosylation and dengue disease

Tineke Cantaert, *Institut Pasteur de Cambodge, Cambodia*

Dr. Tineke Cantaert obtained a PhD in Clinical Immunology in 2008, from the University of Amsterdam, the Netherlands. In 2009, she received the Young Investigator Award from the European League against Rheumatism. In 2010, she moved to Yale University undertaking a second postdoctoral study. Here, she studied the mechanisms that establish both central and peripheral B cell tolerance in humans. As her interest was to understand the response of the human immune system to infectious diseases, she moved to Cambodia in 2015, where she is currently head of the Immunology Unit at Institut Pasteur Cambodia.

Her research is focused to understand the immunopathology of arboviral infections, where her studies have identified protective B and T cell responses in individuals that can control dengue virus infection without excessive immune activation leading to vascular damage. Moreover, she has shown that the absence of specific sugar moieties on anti-dengue antibodies can lead to severe dengue disease. In 2017, Dr Cantaert was awarded the Early Career Research Price in Vaccinology R&D from the International Union of Immunological Societies. Since 2017, she has been a HHMI/Wellcome Trust International Research Scholar.

PLENARY LECTURE 20 | July 9, 2022 (Sat) 11:55 – 12:15

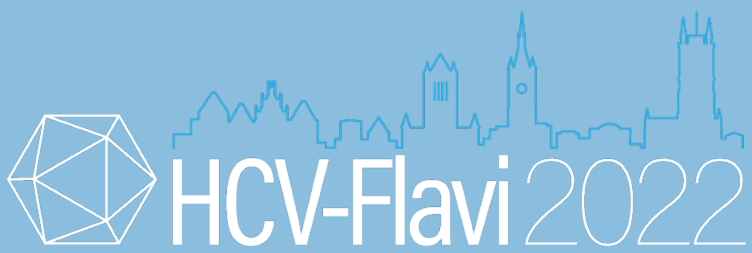
Innate immune responses to flaviviruses

Sonja Best, *NIH, NIAID, USA*

Dr. Best received her Ph.D. from the Australian National University (ANU) and conducted her postdoctoral research at Rocky Mountain Laboratories (RML) campus of NIAID, NIH in the USA. She is currently Deputy Chief of the Laboratory of Virology, and Chief of the Innate Immunity and Pathogenesis Section, also at RML. Her laboratory focuses on mechanisms by which highly pathogenic RNA viruses induce and evade innate immunity, with a focus on flaviviruses and filoviruses.

Work from her lab uses animal models and viral reverse genetics systems to understand how these critical virus-host interactions influence host response and disease. She has been awarded the Presidential Early Career Award for Scientists and Engineers (PECASE), and currently serves on multiple Editorial Review boards including Science Translational Medicine, PLoS Pathogens, and Journal of Virology.

Session 1
VIRAL ENTRY



O1 **The role of the phosphatidylserine receptor TIM1 in the entry of the hepatitis E virus.**
Laura Corneillie, Ghent University, Belgium

O2 **ACE2 is a mutual entry factor for HCV and SARS-CoV-2**
Meital Gal Tanamy, Bar-Ilan University, Israel

O3 **Post-translational modifications of flaviviral proteins modulate entry and RNA replication.**
Zachary Walter, Thomas Jefferson University, USA

The role of the phosphatidylserine receptor TIM1 in the entry of the hepatitis E virus.

Laura Corneillie[1], Irma Lemmens[2], Claire Montpellier[3], Karin Weening[1], Laurence Cocquerel[3], Ali Amara[4], Jan Tavernier[2] and Philip Meuleman[1]

[1]Laboratory of Liver Infectious Diseases, Department of Diagnostic Sciences, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium; [2]VIB-UGent Center for Medical Biotechnology, Department of Biomolecular medicine, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium; [3]Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 8204 - CIIL- Center for Infection and Immunity of Lille, Lille, France; [5]INSERM U944-CNRS 7212, Laboratoire de Pathologie et Virologie Moléculaire, Institut Universitaire d'Hématologie, Université Paris Diderot Sorbonne Paris Cité, Paris, France

Background and Aims: 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 its pathogenicity 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, pathogenesis and ensuring their propagation. Unraveling these virus-host interactions will lead to novel fundamental insight and may identify potential antiviral targets.

Methods: Two related high-throughput mammalian two-hybrid approaches were used to screen for HEV interacting host proteins. Over 200 host proteins were identified that interacted with HEV ORF2-4 of two different genotypes (gts). Promising hits were examined on protein function, involved pathway(s), cellular expression and their relation to other viruses. Based on this analysis, the phosphatidylserine (PS) receptor T-Cell Immunoglobulin Mucin Receptor 1 (TIM1) was selected for further study. This protein showed an interaction in our assay with both gt1 and gt3 ORF3 and has previously been shown to be an attachment factor or receptor for a variety of viruses.

Results: Using TIM1 knock-out (KO) cell lines and infection with HEVcc collected from culture supernatant, comprising mainly enveloped hepatitis E virus (eHEV), we demonstrated a 90% infection inhibition in the KO cell cultures compared to the wild-type control. The eHEV virion type resembles plasma-derived HEV and is possibly associated with ORF3 and PS. In contrast, infection with intracellular HEVcc, containing mainly naked virus was similar in both control and KO cells. This surrogate for feces-derived virus is not associated with ORF3 and PS.

Rescue of TIM1 KO cells by recombinantly expressing TIM1 increased the eHEV infection rate by more than a 100-fold. Moreover, immunostaining experiments show a co-localization of TIM1-expressing cells and HEV infection. Additionally, transient TIM1 expression in HEK293T cells rendered these cells susceptible to eHEV infection, while mock transfected cells are resistant to infection.

Finally, TIM1-antibody neutralization experiments showed a dose-dependent decrease in eHEV infection, ultimately reaching 95 % neutralization compared to control IgG.

Conclusions: Our findings support a role for TIM1 in eHEV-mediated cell entry, a strategy HEV may use to promote viral spread throughout the body. Ongoing binding experiments are needed to further elaborate on the role of PS and ORF3 in the entry step. Moreover, the potential role of TIM1 in other steps of the viral life cycle is investigated.

Contact: Laura.Corneillie@ugent.be

ACE2 IS A MUTUAL ENTRY FACTOR FOR HCV AND SARS-COV-2

Samer Ayoub1, Tom Domovitz1, Michal Werbner1, Joel Alter2, Lee Izhaki Tavor2, Moshe Dessau2, Meital Gal-Tanamy1

1 Molecular Virology Lab, The Azrieli Faculty of Medicine Bar-Ilan University, Israel
2 The Laboratory of Structural Biology of Infectious Diseases, The Azrieli Faculty of Medicine Bar-Ilan University, Israel

SARS-CoV-2 entry into its host cell is mediated via its interaction with the cellular receptor ACE2. Hepatocytes, the target cells of hepatitis C virus infection, express low level of ACE2. Recently, we have demonstrated that SARS-CoV-2 and HCV coinfect and coreplicate in hepatocytes. Moreover, we reported the enhanced SARS-CoV-2 entry into HCV-pre-infected hepatocytes via increase in ACE2 expression in HCV-infected cells. Here we aimed to evaluate whether ACE2 plays a role in HCV life cycle as well.

We demonstrate that Huh7.5 cells overexpressing ACE2 were more susceptible to HCV infection, while ACE2 silencing in Huh7.5 cells resulted in decreased susceptibility to HCV infection. We investigated which step in the HCV life cycle is influenced by ACE2, and identified that ACE2 overexpression in Huh7.5 cells increased HCVpp uptake and HCV cell binding as compared to control Huh7.5 cells. In contrast, RNA levels were similar in replicon cells with versus without ACE2 overexpression. These observations show that ACE2 increases viral entry into Huh7.5 cells at the cell binding step, without affecting HCV RNA replication. Furthermore, we demonstrate higher binding of HCV envelop protein E2 to ACE2-expressing Huh7.5 cells compared to control Huh7.5 cells, but not direct ACE2-E2 binding in in vitro assays, providing evidence for the involvement of ACE2 in HCV entry as a cofactor. We also demonstrate that the enhanced ACE2 expression in HCV infected cells is regulated via HCV-induced HIF1-alpha expression.

This study reveals that ACE2 is a novel entry cofactor for HCV, which is upregulated in response to HCV infection. The HCV-induced increased ACE2 expression results in enhanced HCV and SARS-CoV-2 infections and efficient coinfection of both viruses in hepatocytes.

Post-translational modifications of flaviviral proteins modulate entry and RNA replication.

Zachary Walter1, Andrew Isopi1, Chike Nwaezeapu1, Minghua Li2, Sara Cherry2, Jeffrey Johnson3, Holly Ramage1

1Thomas Jefferson University, Philadelphia, USA; 2University of Pennsylvania, Philadelphia, USA; 3Icahn School of Medicine, Mt. Sinai, New York, USA

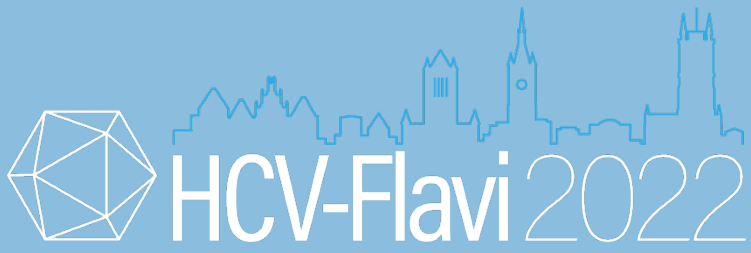
Background and Aims: The proteome is extensively remolded during viral infection, including changes in post-translational modifications (PTMs). Ubiquitination and phosphorylation are common PTMs that alter the function, stability, and localization of proteins. We used mass spectrometry to identify PTMs on West Nile virus (WNV) structural and nonstructural proteins from infected cells. We utilized a subgenomic WNV replicon and a reporter virus particle (RVP) system to determine whether specific PTMs on viral proteins influence the viral life cycle.

Methods: We used site-directed mutagenesis to generate ubiquitin-null (Lys to Arg), phospho-null (Ser to Ala), and phospho-mimetic (Ser to Asp) substitutions at modified residues. To determine the effect of structural protein substitutions, we used an RVP system to generate infectious mutant viruses and measured reporter activity in target cells to determine infectivity. To investigate the role of PTMs in RNA replication, we introduced substitutions into a subgenomic replicon encoding WNV nonstructural proteins and a GFP reporter, then measured viral RNA using qPCR and the GFP reporter using fluorescence microscopy.

Results: We identified several modifications on WNV proteins that significantly affect infectivity and RNA replication. We made a ubiquitin-null substitution in a conserved residue on WNV envelope that resulted in a 40-fold reduction in infectivity of mutant reporter viruses. Interestingly, this mutant did not have defects in RNA packaging or virion secretion from packaging cells. These data indicate a requirement for ubiquitination of WNV envelope protein to infect target cells. While testing the impacts of phosphorylation on non-structural proteins, we identified a phospho-null mutation in the helicase domain of NS3 that is required for RNA replication. Further, we identified two inhibitory phospho-mimetic mutations in NS5 that interfere with RNA replication. Together, these data suggest that phosphorylation of WNV nonstructural proteins can alternately enhance or inhibit viral replication.

Conclusions: Our data demonstrate PTMs on viral proteins influence different stages of the viral life cycle. Modification of viral proteins represents an additional layer of regulation resulting from interactions between virus and host proteins, as viral genes do not encode the enzymatic domains required to make these modifications. Future studies will define host factors mediating these modifications and the mechanisms by which viral PTMs influence viral infection.

Session 2
Adaptive Immunity



O4 **Restored hepatitis C virus-specific CD4+ T cells associated with viral control after childbirth express cytokine, transcription factor, and chemokine receptor signatures of a Th1-biased Tfh lineage**
Christopher Phelps, Nationwide Children's Hospital, USA

O5 **Convergent co-evolution of multiple human HCV bNAb lineages with diverse VH-genes**
Clinton O. Ogega, Johns Hopkins University School of Medicine, USA

O6 **Structural studies of flavivirus-antibody complexes and comparison with hepatitis C virus**
Richard J. Kuhn, Purdue University, USA

Restored hepatitis C virus-specific CD4+ T cells associated with viral control after childbirth express cytokine, transcription factor, and chemokine receptor signatures of a Th1-biased Tfh lineage

Christopher Phelps[1], Christopher Walker[1, 2], and Jonathan Honegger[1, 2]

[1] Center for Vaccines and Immunity, The Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH 43206, USA
[2] Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH 43210, USA

Chronic HCV infection is characterized by dysfunctional HCV-specific CD8+ T cells, rare or transient CD4+ T cell responses, and persistent high-level viremia. However, a surprising recovery of detectable antiviral CD4+ T cells and marked decrease in viremia occurs in some women after pregnancy. Though the lineage of T helper responses restored after childbirth has not been determined, preliminary viral sequence data suggest both enhanced antibody and CD8+ T cell selection pressure exist in those with viral control. We hypothesized that spontaneous recovery of both Th1 and T follicular (Tfh) biased HCV-specific CD4+ T cells contribute to enhanced viral control postpartum. We studied PBMCs from 14 women with chronic HCV. Seven women had > 10-fold decreases in viremia by 3 months postpartum (3PP) (controllers) and the remainder did not (non-controllers). We assessed the function of HCV-specific CD4+ T cells by intracellular cytokine staining after stimulation with peptide pools corresponding to HCV proteins NS3-4. We stained for IL-2, IFN gamma, TNF alpha, IL-17, and IL-21 to differentiate Th1-, Th17-, and Tfh-like functional biases. To further assess the phenotype of these cells, we used HCV-specific class II tetramer (tet) staining with lineage-defining transcription factor (TF) and chemokine receptor (CR) staining to differentiate Th1, Th17 and Tfh phenotypes. At 3PP, HCV-specific CD4+ T cells in both controllers and non-controllers produced combinations of all five cytokines after stimulation. IL-2 was most common followed by TNF, IFN gamma, IL-21, and IL-17. The proportion of HCV-specific CD4+ cells producing multiple cytokines was higher in controllers than in non-controllers (median 40% vs 19% p = 0,04 Mann-Whitney (MW)). Increased polyfunctionality correlated with viral control (p = 0,048 Spearman (SP)). TF mean fluorescent intensity (MFI) on tet+ cells was significantly increased for both Tbet (p = 0,001 Wilcoxon) and BCL6 (p = 0,001 Wilcoxon) when compared to bulk CD4+ T cells but did not differ between controllers and non-controllers. When staining for lineage-defining CRs, tet+ cells were, on average, 60% CXCR5+/CXCR3+/CCR6- (Tfh1), and 25% CXCR5-/CXCR3+/CCR6- (Th1). The proportions of Tfh1 cells within tet+ populations were higher in controllers compared to non-controllers (median 74% vs 53%, p = 0,026, MW), while the proportions of Th1 cells were higher in non-controllers (p = 0,007 MW). Higher proportion of tet+ Tfh1 cells correlated with viral control (p = 0,007 SP). Together, these data point to a postpartum HCV-specific CD4+ T cell restoration driven by TFs favoring Th1/Tfh phenotypes. While these cells may support viral control by activating cytotoxic T cells via Th1 cytokines, greater CXCR5 expression on HCV-specific CD4+ T cells in controllers points to a potential key role for increased migration to sites of germinal center reactions and enhanced B cell help.

Convergent co-evolution of multiple human HCV bNAb lineages with diverse VH-genes

Authors: Clinton O. Ogega1,2, Nicole E. Skinner3,4, Kaitlyn E. Clark1, Nicole Frumento1, Harry T. Paul1, Desiree A. Wright1, Ariadne Sinnis-Bourozikas1, Pamela J. Bjorkman5, Stuart C. Ray1, Andrew I. Flyak5, Justin R. Bailey1

1Division of Infectious Diseases, Department of Medicine, Johns Hopkins University School of Medicine; Baltimore, MD; 2Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine; Baltimore, MD; 3Division of Infectious Diseases, Department of Medicine, The Ohio State University Wexner Medical Center, Columbus, OH; 4Center for Vaccines and Immunity, The Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, OH; 5Division of Biology and Biological Engineering, California Institute of Technology; Pasadena, CA

Background and Aims: Characterization of broadly neutralizing antibody (bNAb) development, genetic features, and targeted epitopes is paramount in guiding selection or structure-based design of hepatitis C virus (HCV) vaccine antigens. The majority of human bNAbs isolated to date utilize VH1-69 along with D2-15 gene segments, suggesting that there is a very restricted path to human bNAb induction that could be difficult to replicate with a vaccine, and difficult to optimize in animal models lacking VH1-69 and D2-15 gene homologs. However, most human bNAbs have been isolated from chronically infected individuals using a limited set of HCV envelope (E2) antigens. Here, we used an antigenically diverse set of E2 proteins to exhaustively characterize longitudinal E2-specific BCR sequences and monoclonal antibodies (mAbs) from an 'Elite Neutralizer' (EN) with broadly neutralizing plasma who spontaneously cleared multiple HCV infections.

Methods: By screening early-infection plasma from 63 HCV-infected participants using an antigenically diverse panel of HCV pseudoparticles, we identified an EN with broadly neutralizing plasma antibodies who naturally cleared multiple HCV infections. We utilized single B cell sorting with a cocktail of three antigenically diverse soluble E2 proteins to generate 259 monoclonal B cell cultures and isolate 55 E2 reactive mAbs. We measured mAb neutralizing breadth, analyzed sequence features, performed epitope mapping using a library of E1E2 alanine mutants, and determined x-ray crystal structures of E2-bNAb complexes. We also performed E2-specific B cell receptor sequencing (BCR-seq) at longitudinal timepoints, tracking co-evolution of multiple bNAb lineages over time.

Results: Somatic hypermutation of E2-specific B cells increased throughout the course of infection. Binding and neutralization assays revealed that 81/259 cultured B cells (31%) were cross-reactive with multiple E2 variants, and 29/55 mAbs (53%) were bNAbs. Epitope mapping and crystal structures demonstrated that these bNAbs targeted conserved front layer and novel epitopes on the E2 protein. Unexpectedly, these bNAbs used a diverse set of VH and D gene segments, showing that VH1-69 or D2-15 gene usage is common but not essential for a broad neutralizing activity. Remarkably, BCR-seq and bNAb sequence analysis revealed that multiple front layer-specific bNAb lineages using different VH genes acquired the same somatic mutations over time, many of which are contact residues in the E2-bNAb structures.

Conclusions: In this study, we used a B cell sorting strategy to identify bNAbs targeting novel epitopes and bNAbs with diverse VH and D gene usage that evolved convergently to target the highly conserved front layer of E2. These findings indicate that a wide range of B cell evolutionary pathways can lead to the development of bNAbs in humans, providing reassurance that animal models without VH1-69 or D2-15 homologs can provide useful information for vaccine development, and increasing optimism that a vaccine capable of inducing bNAbs in humans is within reach.

Structural studies of flavivirus-antibody complexes and comparison with hepatitis C virus

Richard J. Kuhn1,2, Madhumati Sevvana1,2, Pavlo Gilchuk3,4, Andrew S. Miller1,2, Thomas Klose1,2, and James E. Crowe, Jr.3,4

1Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA; 2Purdue Institute of Inflammation, Immunology, and Infectious Disease, Purdue University, West Lafayette, IN 47907, USA; 3Department of Pediatrics (Infectious Diseases), Vanderbilt University Medical Center, Nashville, TN, USA.; 4Vanderbilt Vaccine Center, Vanderbilt University Medical Center, Nashville, TN, USA.; 4Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA.

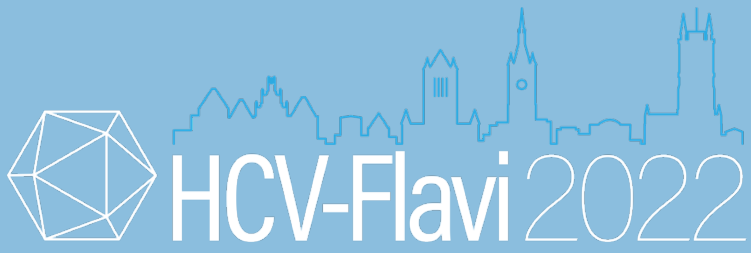
Background and aims: We have been studying flavivirus – antibody complexes using cryo-electron microscopy. Extensive analyses of the assembly and maturation of the virus particles has previously been described although the dynamic properties of these viruses remains to be completely described. A comprehensive analysis of the structural landscape of the humoral immune response against flaviviruses is crucial for understanding the role of antibodies in controlling virus infection. Here, we compare the available structural data of several flavivirus antibody complexes and discuss the mapped epitopes, the stoichiometry of antibody binding and mechanisms of neutralization.

Methods: Neutralizing monoclonal antibodies were prepared as Fab fragments and complexed with purified Zika virus strain H/PF/2013. Zika virus was grown in Vero cells and purified as previously described. Complexes were prepared by taking various molar ratios of Fab to E protein epitopes to achieve saturation. The complex was blotted onto lacey carbon grids and flash frozen. Micrographs were collected using a dose of 30.0 e-/Å2 on an FEI Titan Krios electron microscope equipped with a Gatan K2 Summit detector using a nominal magnification of 81,000 in the “super-resolution” mode. Density-guided real-space refinement of the ZIKV (PDB ID: 5IRE) - antibody complexes were performed using the programs COOT and PHENIX.REFINE.

Results: The structures of several new flavivirus – antibody complexes will be shown and interpreted. Antibodies against flaviviruses have been shown to have multiple diverse interactions and have revealed multiple mechanisms for antibody-induced virus neutralization. The presentation will compare multiple flaviviruses and their antibody complexes to demonstrate mechanisms of action with some common and some unique binding modes.

Conclusions: Although several effective flavivirus vaccines have been developed, the exact mechanisms for protection remain to be fully explained. We have used structural tools to probe the mechanisms by which human antibodies engage a variety of flaviviruses. This fundamental knowledge of antibody-mediated neutralization may be useful in the design of immunogens for future vaccines. Importantly, the design of an HCV vaccine may require such structural insights of virus and protective epitopes and this topic will also be addressed.

Session 4
Vaccine Development



O7 **Investigating factors that limited immunogenicity in vectored hepatitis C virus vaccine trial.**
Kimberly E. Rousseau, Johns Hopkins University School of Medicine, USA

O8 **Vesicular stomatitis virus (rVSV) vectored HCV vaccine candidate induces potent cross-neutralizing antibodies.**
Maurice Labuhn, Twincore Center of Experimental and Clinical Infection Research, Germany

O9 **Live-attenuated YF17D-vectored COVID-19 vaccine protects from lethal yellow fever virus infection**
Ji Ma, Rega Institute - KULeuven, Belgium

O10 **Robust CD8+ and CD4+ T cell responses with an HCV vaccine adjuvanted with TLR4 agonists**
Abdolamir Landi, University of Alberta, Canada

O11 **Structure of the hepatitis C virus E1E2 glycoprotein complex**
Kwinten Sliepen, University of Amsterdam, The Netherlands

O12 **Structural analysis of a native-like secreted form of the HCV E1E2 glycoprotein and immunological analysis in mice and macaques**
Thomas Fuerst, University of Maryland, USA

O13 **Inactivated genotype 1a, 2a or 3a HCV vaccine candidates induced broadly neutralizing antibodies in mice**
Garazi Pena Alzua, University of Copenhagen, Denmark

O14 **Repeated exposure to heterologous, antibody sensitive hepatitis C viruses is associated with induction of potent and broadly neutralizing antibodies**
Nicole Frumento, Johns Hopkins University School of Medicine, USA

Investigating factors that limited immunogenicity in prophylactic HCV vaccine trial

Kimberly E. Rousseau1, Kimberly Page2, Stefania Capone3, Antonella Folgori3, Michael R. Wierzbicki4, Andrea L. Cox1

1 Johns Hopkins University School of Medicine, Baltimore, MD, USA; 2 University of New Mexico, Albuquerque, NM, USA; 3 ReiThera, Rome, Italy; 4 The EMMES Company, Rockville, MD, USA

Background and Aims: A placebo-controlled clinical trial assessed the efficacy of a prime/boost strategy with chimpanzee adenovirus 3 (ChAd3) and modified vaccinia Ankara vectors in preventing chronic hepatitis C virus (HCV) in people at-risk for infection through injection drug use. This prophylactic HCV vaccine regimen suppressed peak viremia relative to placebo but did not prevent chronic infection. The same vaccine regimen tested in volunteers not at risk for infection resulted in a greater percentage of participants responding to vaccination (100 % vs. 78 %) and a three-fold higher magnitude of T-cell responses. Here, we compare baseline immune characteristics of people who inject drugs (PWID) to healthy donors to understand whether baseline immune suppression or pre-existing immunity to vector or HCV limited efficacy.

Methods: Plasma and peripheral blood mononuclear cells collected from trial participants were assessed via enzyme-linked immunosorbent assay and interferon gamma enzyme-linked immunosorbent spot assays for antibodies (Ab) or T-cells targeting HCV structural and non-structural regions. Pre-vaccination plasma was also assayed for cross-reactive neutralizing Ab (nAb) targeting the ChAd3 vector and for the levels of 23 cytokines and chemokines.

Results: HCV-specific T-cells and Abs were undetectable at baseline, indicating no immunologic evidence of repeated subclinical exposure to HCV. Further, HCV core- or envelope-specific responses, proteins not included in the vaccine formulation, remained undetectable at trial completion in participants who remained HCV RNA negative. Median cross-reactive ChAd3 nAb titers at baseline in trial participants was not significantly different from healthy control populations. However, in participants with a cross-reactive ChAd3 nAb titer of > 150, we found no statistically significant increases in median T-cell responses at any tested timepoint. No measured cytokine or chemokine was differentially expressed pre-vaccination between those who generated HCV-specific T-cell responses and those who did not.

Conclusions: Pre-existing cross-reactive nAb to vector limited HCV-specific T-cell induction in those with a titer > 150. However, those with low or undetectable ChAd3 nAb titers did not mount responses of the same magnitude as healthy volunteers in previous trials. Future studies will further assess the baseline immune status of the PWID cohort to determine if global immunometabolic dysregulation limited T-cell induction.

Vesicular stomatitis virus (rVSV) vectored HCV vaccine candidate induces potent cross-neutralizing antibodies

Maurice Labuhn, Dorothea Bankwitz, Mandy Doepke, Corinne Ginkel, Gert Zimmer, Thomas Pietschmann
Twincore Center of Experimental and Clinical Infection Research, Germany

Background & Aims: More than 60 million people are chronic HCV carriers. Each year around 300.000 people die due to HCV-associated liver diseases. Therefore, HCV infection causes a considerable global disease burden and the need for a prophylactic vaccine is undiminished. Multiple lines of evidence indicate that neutralizing antibodies are important for HCV immune protection. Recently, we surveyed the susceptibility of a large, genetically diverse set of HCV strains against patient-derived polyclonal antibodies. We identified six functionally distinct viral neutralization clusters represented by six reference viruses. Here we hypothesized that the envelope proteins (E1/E2) of these viruses exhibit both, differential susceptibility to antibodies and divergent immunogenicity. Therefore, we aimed to evaluate the immunogenicity of these HCV-E1E2 variants expressed by vesicular stomatitis virus (rVSV) vectors.

Methods: We cloned the HCV-E1E2 sequences of six reference viruses into the VSV genome replacing the VSV-G surface protein. We prepared rVSV-HCV-E1E2 vector particles and immunized both, wildtype mice and transgenic mice liver-specifically expressing the human HCV entry factors CD81 and Occludin. We conducted ELISA binding and neutralization assays using sera after nine weeks of immunization.

Results: All rVSV-HCV-E1E2-vectors produced evenly high particle titers (1,2 x 107 to 1,9 x 107 particles per ml cell culture supernatant). In mice, all constructs induced comparably potent cross-binding ELISA titers assayed against 13 different HCV-E1E2 variants. Strikingly, neutralization assays revealed major differences between the experiment groups. Sera of three out of six groups showed only a minor cross-neutralization of the HCV reference virus panel (mean of less than 1,5-fold reduction of infectivity compared to control sera). Notably, the immunization with one of the six rVSV-HCV-E1E2 vaccine constructs induced robust cross-neutralizing antibodies across all tested reference viruses (7,3-fold reduction of infectivity compared to the control group [a 9,8-fold reduction in the sera of wildtype mice; a 5,4-fold reduction in the sera of receptor transgenic mice, respectively]).

Conclusions: These results qualify rVSV-HCV-E1E2 vectors as powerful platform for induction of cross-neutralizing antibodies. Moreover, this work highlights profound strain-dependent immunogenicity showing that viral determinants control the efficacy of antiviral immune responses induced by rVSV-HCV-E1E2 vectors. The rVSV-HCV-E1E2 vector characterized here represents a promising starting point for development as HCV vaccine candidate.

Live-attenuated YF17D-vectored COVID-19 vaccine protects from lethal yellow fever virus infection

Ji Ma a, Dominique Van Looveren a, b, Thomas Vercruysse a, b, Xin Zhang a, Robbert Boudewijns a, Sander Jansen a, Michael Bright Yakass a, Lorena Sanchez-Felipe a, Bert Malengier-Devlies c, Birgit Weynand d, Tania Roskam d, Mahadesh Prasad Arkalagud Javarappa a, Johan Neyts a, Hendrik Jan Thibaut a, b, Kai Dallmeier a

[a] KU Leuven Department of Microbiology, Immunology and Transplantation, Rega Institute, Laboratory of Virology, Molecular Vaccinology and Vaccine Discovery, Leuven, Belgium; b KU Leuven Department of Microbiology, Immunology and Transplantation, Rega Institute, Laboratory of Virology and Chemotherapy, Translational Platform Virology and Chemotherapy, Leuven, Belgium; c KU Leuven Department of Microbiology, Immunology and Transplantation, Rega Institute, Immunity and Inflammation Research Group, Immunobiology Unit, KU Leuven, Leuven, Belgium; d KU Leuven Department of Imaging and Pathology, Translational Cell and Tissue Research, B-3000 Leuven, Belgium.

Background and Aims: A dual vaccine protecting against both severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and yellow fever virus (YFV) would be of great extra benefits for those living in YFV-endemic areas who are struggling with limited access to SARS-CoV-2 vaccine supply and continuous YFV emergence. Our live-attenuated YF17D-vectored SARS-CoV-2 vaccine candidate YF-S0 has previously demonstrated high protective potential against YFV (Sanchez-Felipe, et al. Nature 590.7845 (2021): 320-325.). In addition, like other viral vector vaccines, concerns regarding the impact of pre-existing anti-YFV immunity need to be addressed before further evaluations.

Methods: The immunogenicity and protective efficacy of YF-S0 against YFV and SARS-CoV-2 in the presence of strong pre-existing YFV immunity have been evaluated in both mouse and hamster models.

Results: Here, we show that a single dose YF-S0 induces strong humoral and cellular immunity against both YFV and SARS-CoV-2 spike in mice and hamsters. Immunity thus induced results in full protection from YFV challenge in either model; in mice against lethal intracranial YF17D challenge, and in hamsters against vigorous viscerotropic infection and virus-induced liver disease when a highly pathogenic hamster-adapted YFV-Asibi strain was used for challenge. Importantly, strong pre-existing immunity against the YF17D vector did not interfere with subsequent YF-S0 vaccination in mice or hamsters; nor with the protective efficacy of YF-S0 conferred against SARS-CoV-2 strain B1.1.7 (variant of concern Alpha) infection in hamsters.

Conclusions: Our findings warrant the development of YF-S0 as dual vaccine with the potential to contain both COVID-19 and YFV in certain geographic areas.

Contact: kai.dallmeier@kuleuven.be

Robust CD8+ and CD4+ T cell responses with an HCV vaccine adjuvanted with TLR4 agonists

A. Landi, J. Law, M. Logan, J. Kundu, D. Hockman, J. Johnson, K. Crawford, L. Tyrrell & M. Houghton; Li Ka Shing

B.

Applied Virology Institute, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, T6G 2E1, Canada.

Background: Regardless of the available curative HCV antivirals, development of a prophylactic vaccine to prevent persistent hepatitis C virus (HCV) infection in at-risk populations is still a necessity and in line with WHO recommendations for global health. Here we present an improved version of our previously studied vaccine, which is also complemented with a cellular immune component. It comprises HCV envelope glycoproteins (E1E2) and a cocktail of short chemically synthesized peptides (4TPs) that have been selected based on conserved regions of different HCV genotypes that contain numerous CD4+ and CD8+ T cell epitopes restricted by multiple HLA alleles for a high population coverage. Our adjuvanted E1E2-4TPs vaccine has been able to induce efficient neutralizing antibodies in mice, but hitherto, only limited T cell responses using older adjuvants. We now report very strong CD8+ T cell response against the 4TPs component of our vaccine, when adjuvanted with proprietary SLA-LSQ or SLA-SE developed by the Access to Advanced Health Institute (AAHI, Seattle, USA). These CD8+ T cell responses are accompanied with robust CD4+ T cell responses to the 4TPs as well as strong broadly cross-neutralizing antibodies to E1E2.

Methods: A mixture of adjuvanted E1E2 and 4TPs with either Alum-OH/MPLA, AddaAS03, SLA-LSQ, or SLA-SE was used to immunize CB6-F1 mice intramuscularly. Three injections were administered on day 0, 14, and 42 and mice were euthanized on day 56. Blood was collected at different time-points and spleens were collected on day 56. The level of E1E2-specific IgG was measured in sera, which was also used in neutralizing assays to block HCV pseudoparticles entry. To evaluate cellular responses, splenocytes were stimulated in vitro with overlapping peptide pools and the presence of polyfunctional T cells, expressing IFN-gamma, TNF-alpha, and IL-2 was investigated in an intracellular cytokine assay using flow cytometry.

Results: Our results demonstrated that AAHI's adjuvants present equivalent or stronger humoral immunity than other adjuvants but are the only ones generating very strong CD8+ T cell responses against 4TPs along with a very high CD4+ T cell response against both E1E2 and 4TPs. Detected polyfunctional CD4+ T cells were able to simultaneously produce three Th1-type cytokines including IFN-gamma, TNF-alpha, and IL-2, while CD8+ T cells were able to produce IFN-gamma and TNF-alpha (Fig. 1). A statistical analysis of our T cell data confirms a p-value of less than 0.001 with a power of above 99%, indicative of an extremely high level of reproducibility.

Conclusions: This study reports adjuvanted HCV vaccine candidates that elicit neutralizing antibodies as well as very strong CD4+ and CD8+ T cell responses to epitopes highly conserved among the hepacivirus genus. These will be tested in a phase 1 clinical trial shortly.

Structure of the hepatitis C virus E1E2 glycoprotein complex

Alba Torrents de la Peña[1,*], Kwinten Sliepen[2,*], Lisa Eshun-Wilson[1,*], Maddy Newby[3], Joel D. Allen[3], Sylvie Koekkoek[2], Ian Zon[2], Ana Chumbe Mendoza[2], Max Crispin[3], Janke Schinkel[2], Gabriel C. Lander[1], Rogier W. Sanders[2,4], Andrew B. Ward[1]

1. Department of Structural Biology and Computational Biology, The Scripps Research Institute, La Jolla, California, USA; 2. Department of Medical Microbiology, Amsterdam Institute for Infection and Immunity, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands; 3. School of Biological Sciences, University of Southampton, Southampton, UK; 4. Weill Medical College of Cornell University, New York, New York, USA*: co-first authors

The HCV envelope E1 and E2 glycoproteins are essential for viral entry and comprise the primary antigenic target for neutralizing antibody responses. The molecular mechanisms of E1E2 assembly, as well as how the E1E2 heterodimer binds broadly neutralizing antibodies (bNAbs), remain elusive. High-resolution structure determination of the full-length E1E2 heterodimer has been hindered by intrinsic flexibility, conformational heterogeneity, disulfide bond scrambling and abundant glycosylation.

Using an optimized expression and purification scheme by co-expressing the AR4A bNAb produced a promising sample for structure determination. Subsequently, we used cryo-electron microscopy to solve the structure of membrane-extracted full-length E1E2 heterodimer in complex with AR4A, AT1209 and IGH505 bNAbs at ~3.5 Å resolution. We resolve the epitopes of these bNAbs and reveal how the interface between the E1 and E2 ectodomains is stabilized by hydrophobic interactions and glycans. This structure deepens our understanding of the HCV glycoprotein and delivers a blueprint for the rational design of novel vaccine immunogens and anti-viral drugs.

Structural analysis of a native-like secreted form of the HCV E1E2 glycoprotein ectodomain in complex with neutralizing antibodies

Metcalf, M.C.[1], Wang, R.[1], Guest J.D.[1], Pozharski, E.[1,3,5], Law, M.[4], Pierce, B.G.[1,2], Toth, E.A.[1], Fuerst, T.R.[1,2], Ofek, G.[1,2]

[1]Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD 20850, USA; [2]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742, USA; [3]Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201, USA; [4]Department of Immunology and Microbiology, The Scripps Research Institute, La Jolla, California, USA; [5]Maryland Center for Advanced Molecular Analysis, University of Maryland, Rockville, MD 20850, USA

Background and Aims: The Hepatitis C virus (HCV) is a major global health burden, as it 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. The main antigenic target for HCV immune recognition is the heterodimeric transmembrane E1E2 surface glycoprotein. While the E2 subunit, which is responsible for interacting with various cellular receptors, has been characterized structurally, little structural information is currently available on the native E1E2 heterodimer. Here, we describe the cryoEM structural analysis of a native-like secreted form of the E1E2 heterodimer ectodomain in complex with three neutralizing antibodies.

Methods: Grids were prepared consisting of secreted E1E2 ectodomain in complex with antibodies AR4A, HEPC74, and IGH520. Images were collected using a Glacios transmission electron microscope at 200 kV using a Gatan K3 detector. The resulting data was processed in cryoSPARC and modelling was assisted using Alphafold2.

Results: A structure of secreted E1E2 in complex with the antibodies was obtained to ~3.9 Å resolution. The structure reveals the three-dimensional heterodimeric organization of the E1 and E2 subunits of the glycoprotein. The interface between E1 and E2 is defined and reveals the presence of N-linked glycans. Details of antibody interactions with E1E2 are illuminated, including determinants for AR4A heterodimer-specific recognition.

Conclusions: The findings presented in this study provide a structural model for a native-like secreted form of the E1E2 heterodimer, including expanded structural details for E2 and E1. The structure lays the foundation for E1E2 heterodimer-based rational vaccine development.

Presented by Thomas

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

Ruixue Wang[1], Saori Suzuki[2], Johnathan Guest[1,3], Brigitte Heller[2], Maricar Alemda[2], Alexander Andrianov[1], Alexander Marin[1], Roy Mariuzza[1,3], Zhen-Yong Keck[4], Steven Foung[4], Hanne Anderson[5], Yunus Abdul[1], Liudmila Kulakova[1], Brian Pierce[1,3], Eric Toth[1], Alexander Ploss[2], Thomas Fuerst[1,3]

[1]Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, Maryland, 20850, USA.; [2]Department of Molecular Biology, Princeton University, NJ 08540, USA.; [3]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland, 20742, USA.; [4]Department of Pathology, Stanford University School of Medicine, Stanford, California, 94305, USA.; [5]BioQual, Rockville, Maryland, 20850, USA.

Background and Aims: Development of an effective vaccine for hepatitis C virus 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 in the context of a vaccine. In previous studies, we showed that mice immunized with sE2 harboring the mutation H445P exhibited an increase in breadth of neutralization. Here, we describe the antigenicity and immunogenicity of these designs, including a soluble, 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 (Fos/Jun) to replace the hydrophobic E1 and E2 transmembrane domains which mediate assembly of native E1E2 (sE1E2.LZ). In anticipation of prospective vaccine studies in humans, we adapted a synthetic structural homologue with no human sequence homology (SynZip) as an alternate scaffold (sE1E2.SZ). We further analyzed the 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.

Results: Mice immunized with mbE1E2, sE1E2.LZ, and wild-type 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 neutralization systems. We found that the two sE1E2 scaffolds, sE1E2.LZ and sE1E2.SZ, enabled proper folding of the antigenic domains as measured by binding to a panel of human monoclonal antibodies that target conformational epitopes in E2 and the E1E2 complex. In macaques immunized with mbE1E2, sE1E2.SZ, and sE1E2.SZ harboring the H445P stabilizing mutation, broadly neutralizing antibodies were detected in all cases, albeit at lower levels than those exhibited 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.

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.

Inactivated genotype 1a, 2a or 3a HCV vaccine candidates induced broadly neutralizing antibodies in mice

Garazi Peña Alzua1, Anne Finne Pihl1, Anna Offersgaard1, Shan Feng1, Ulrik Fahnøe1, Mansun Law2, Jannick Prentoe1, Jan P. Christensen3, Jens Bukh1 and Judith Margarete Gottwein1*

1 Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Copenhagen University Hospital-Amager and Hvidovre, 2650 Hvidovre, Denmark and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark; 2 Department of Immunology and Microbiology, The Scripps Research Institute, La Jolla, CA 92037, USA; 3 Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark

Background and Aims: A prophylactic vaccine is needed to control the hepatitis C virus (HCV) epidemic, with genotypes 1-3 causing >80% of worldwide infections. Vaccine development is hampered by HCV heterogeneity, immune-escape mechanisms including protection of conserved neutralizing epitopes, and suboptimal efficacy of HCV cell culture systems. We aimed to develop cell culture-based whole inactivated genotype 1-3 HCV vaccine candidates, an attractive approach to present natively folded HCV envelope proteins for elicitation of protective neutralizing antibodies.

Methods: High-yield genotype 1a, 2a and 3a HCV were developed by serial passage of previously developed TNcc, J6cc, and DBN3acc in Huh7.5 cells and engineering of acquired mutations detected by next generation sequencing analysis. HCV neutralizing epitope exposure was determined in cell-based neutralization assays using human monoclonal antibodies AR3A and AR4A, and polyclonal antibody C211. BALB/c mice were immunized with processed and UV inactivated high-yield genotype 1a, 2a or 3a viruses formulated with AddaVax, a homologue of the licensed adjuvant MF-59. Serum IgG was purified from the immunized animals and evaluated for neutralization capacity by cell-based HCV neutralization assay and for E1/E2 binding capacity by ELISA.

Results: Compared to the original viruses, high-yield viruses had up to 1000-fold increased infectivity titers (peak titers were 6-7 log10 focus forming units (FFU)/ml) and up to 2470-fold increased exposure of conserved neutralizing epitopes. Vaccine induced IgG broadly neutralized genotype 1-6 HCV with EC50 values of 43-131 microgram/ml (mean 87 microgram/ml). Further, purified IgG bound genotype 1-3 E1/E2 complexes, while immune sera showed endpoint titers of up to 32,000.

Conclusions: We developed high-yield genotype 1-3 HCV as basis for inactivated vaccine candidates that induced efficient broadly neutralizing antibodies in mice supporting further preclinical development.

Repeated exposure to heterologous, antibody sensitive hepatitis C viruses is associated with induction of potent and broadly neutralizing antibodies

Nicole Frumento[1], Alexis Figueroa[1], Tingchang Wang[2], Muhammad N. Zahid[3], Shuyi Wang[4,5], Guido Massaccesi[1], Georgia Stavrakis[1], James E. Crowe, Jr. [6,7,8], Andrew I. Flyak[9], Hongkai Ji[2], Stuart C. Ray[1], George M. Shaw[4,5], Andrea L. Cox[1], Justin R. Bailey[1]

[1]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [2]Departmen of Biostatistics, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA; [3]University of Bahrain, Department of Biology, College of Science, Sakhir Campus, Bahrain; [4]Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA; [5]Department of Microbiology, University of Pennsylvania, Philadelphia, PA, USA; [6]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA; [7]Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN, USA; [8]Vanderbilt Vaccine Center, Vanderbilt University Medical Center, Nashville, TN, USA; [9]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, USA

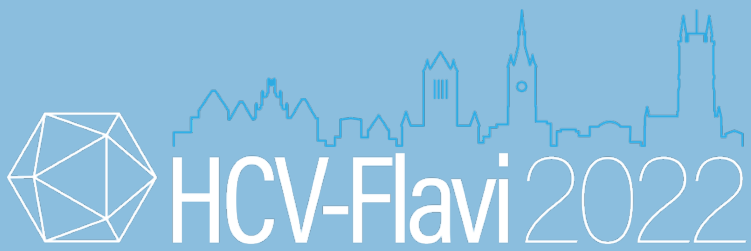
Background and Aims: Development of a prophylactic hepatitis C virus (HCV) vaccine that elicits antibodies with strong neutralizing activity could be key to HCV eradication. However, the genetic and antigenic properties of HCV envelope (E1E2) proteins capable of inducing potent broadly neutralizing antibodies (bNAbs) against HCV in humans have not been defined. Here, we investigated the development of bNAbs in longitudinal plasma of HCV-infected persons with persistent infection or spontaneous clearance of multiple reinfections.

Methods: We measured neutralizing breadth and potency of plasma antibodies at multiple timepoints in participants with spontaneous clearance of multiple reinfections (n = 6), clearance of primary infection followed by persistent reinfection (n = 2), persistent, sequential infections with genetically distinct viruses (n = 3), or persistent, chronic infection with a single viral lineage (n = 17). We also identified antigenic differences and similarities among E1E2 proteins of infecting viral strains by measuring their sensitivity to a panel of monoclonal antibodies. We evaluated the relationship between development of bNAbs and exposure to multiple genetically distinct infections, duration of viremia, genetic distance between primary infection and reinfection viruses, and antigenic similarity between primary infection and reinfection viruses. We used these data to develop a rigorous model identifying key features of stimuli capable of inducing potent bNAbs in humans.

Results: We identified four major bNAb-types (HEPC146, AR4A, HEPC74, and HEPC108) commonly induced upon reinfection. We found that the breadth and potency of the antibody response increased upon exposure to multiple genetically distinct infections and with longer duration of viremia. Surprisingly, exposure to infecting strains from multiple HCV subtypes was not associated with enhanced neutralizing breadth. Rather, repeated exposure to antigenically-related, antibody-sensitive E1E2 proteins was associated with induction of potent bNAbs.

Conclusions: We have identified key features of the stimuli associated with the induction of potent anti-HCV bNAbs in humans, casting doubt on the commonly-held assumption that a vaccine must incorporate antigens from multiple genotypes to induce bNAbs. These data reveal that a prime-boost vaccine strategy with genetically distinct, antibody-sensitive viruses is a promising approach to induce potent bNAbs in humans.

Session 5
ANTIVIRAL TREATMENT & HCV ELIMINATION



GHENT • ICC
JULY 6-9, 2022

O15 **Virological characterization of treatment failures and retreatment outcomes in patients infected with "unusual" HCV genotype 1 subtypes**
Slim Fourati, Hôpital Henri Mondor, Université Paris-Est, France

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O17 **Novel indolediketopiperazine derivatives targeting the replication of Flaviviridae viruses**
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Carlota Fernandez Antúnez, University of Copenhagen, Denmark

Virological characterization of treatment failures and retreatment outcomes in patients infected with “unusual” HCV genotype 1 subtypes

Slim Fourati, Hôpital Henri Mondor,

Université Paris-Est, France

Background and Aims: Among so-called “unusual” HCV genotypes, genotype 1 (GT1) subtypes non-1a/1b are highly prevalent in regions of Sub-Saharan Africa. Some unusual GT1 subtypes have been shown to be less sensitive to NS5A inhibitor-containing regimens than GT-1a or GT-1b. In this study, we characterized the resistance-associated substitution (RAS) profiles in DAA-targeted regions in patients infected with unusual GT1 subtypes who failed to achieve SVR after NS5A inhibitor-based therapy and assessed their response to retreatment.

Method: This retrospective French national study included HCV-infected patients who experienced a virological failure after NS5A inhibitor-containing therapy referred to our National Reference Center between 2015 and 2022. RASs were initially identified by means of Sanger sequencing of the NS3, NS5A and NS5B regions of HCV genome. Shotgun metagenomics was then used for in-depth characterization of full-length genome sequences.

Results: Among 521 patients who failed to achieve SVR after NS5A inhibitor-containing therapy, 272 (52.2%) were infected with genotype 1, including 131/272 (48.2%) with subtype 1a, 93/272 (34,2%) with subtype 1b and 48/272 (17.6%) with GT1 subtype non-1a/1b. Among those infected with subtype non-1a/1b, 41/48 patients (85.4%) were born in Africa (1c, n=2; 1d, n=4; 1e, n=12; 1f, n=1; 1g, n=2; 1i, n=2; 1l, n=18), including 19/41 (46.3%) in Cameroun. The median age of the study population was 61 years (IQR: 55-68) and the male/female ratio was 1. Only 4/48 patients (8.3%) were HIV co-infected and 9/48 (18.8%) had cirrhosis. The received treatment regimens were SOF/LDV±RBV (n=33), SOF/VEL (n=2), G/P (n=3), and various others (n=10). NS5A sequences at treatment failure were available for 40/48 (83.3%) patients, showing ≥2 and ≥3 dominant NS5A RASs in 26/40 (65.0%) and 12/40 (30.0%) patients, respectively. The most frequent NS5A RASs were L31M (n=11; 27.5%), L28M (n=8; 20.0%), A92T (n=7; 17.5%) and Y93H (n=5; 12.5%). NS5B RASs (C316Y) were found in one patient (1/40; 25.0%). NS3 sequences at treatment failure were available for 24/48 (50.0%) patients. The most frequent NS3 RASs were Q80K (n=4; 16.7%), T54S (n=4; 16.7%), I132V (n=3; 12.5%). To date, 27 of the 48 patients have completed DAA retreatment (triple combination DAAs, n= 17; SOF + NS5A inhibitor; n=10) and follow-up. Among them, 25 achieved SVR, while 2 failed SOF + NS5A inhibitor retreatment, with an overall SVR rate of 92.6% (100% for triple therapy, 80% for SOF + NS5A inhibitor). Deep sequencing of full-length HCV genomes is ongoing and results will be presented.

Conclusion: We report the largest cohort of patients infected with unusual GT1 subtypes failing DAA therapy. These patients were mostly born and infected in Africa. Treatment failure was associated with at least 2 or 3 NS5A RASs, including RASs known to be pre-existent in these subtypes. Retreatment with a triple combination of sofosbuvir, an NS5A inhibitor and an NS3 protease inhibitor was successful in 100% of cases. Our results emphasize the need to identify these subtypes prior to therapy, especially in Africa where they are common, in order to guarantee access to first-line triple DAA therapy in patients who need it

Targeting the capsid protein – a strategy for the generation of a broadly acting Flaviviridae inhibitor

Natalia Ruetalo[1], Johanna Dürrwald[1], Marcel Benz[1], Linda Wiltzer-Bach[1], Anja Schöbel[2], Eva Herker[2], Janis Müller[3], Jan Münch[3], Barbara Schnierle[4], Renate König[4], Veronika von Messling[4], Marcos V. Gondim[5], Kenji Schorpp[5], Kamyar Hadian[5], Sundy Yang[6], David A. Jans[6], Marcel Günther[7] Stefan Laufer[7], Michael Schindler[1.5]

[1]Institute for Medical Virology and Epidemiology, University Hospital Tübingen, Germany; [2]Institute of Virology, Philipps University Marburg, Germany; [3]Institute of Molecular Virology, University of Ulm, Germany; [4]Paul-Ehrlich-Institute, Langen, Germany; [5]Helmholtz-Center Munich, Neuherberg, Germany; [6]Department of Biochemistry and Molecular Biology, Monash University, Australia; [7]Pharmaceutical Chemistry, University of Tübingen, Germany

Background and Aims: Flaviviruses pose a constant threat to emerge as epidemics or pandemics. As such, Dengue virus (DENV) is one of the world's fastest-growing infectious diseases. Currently, there is no antiviral therapy against insect-borne flaviviruses. Hence, our goal is to provide novel therapeutic options against this viral family.

Methods: FACS-FRET was used to screen for flavivirus capsid multimerization inhibitors. The hit compound C10 was further analyzed regarding antiviral activity against several members of the Flaviviridae family, using cell-based infection assays. Cytotoxicity was tested in cell lines, and in vivo in zebrafish and mice. Flaviviral capsid proteins were expressed and purified from *E. coli* cultures and biophysically characterized. Cross-linking assays were performed to study the interaction between the capsid proteins and C10. Mass spectrometry and crystallography are being used to map the binding surface. Finally, structure-activity relationship (SAR) studies were conducted to optimize the compound.

Results: C10 shows excellent antiviral activity against Flaviviridae family members including DENV, West Nile, Zika, Yellow-Fever, Tick-borne encephalitis virus, and HCV. In contrast, it shows low or no activity against influenza A, respiratory syncytial virus, measles virus, human immunodeficiency virus, human cytomegalovirus, and hepatitis B virus. In cell-based assays, C10 has a remarkable therapeutic index with a CC50 > 50µM and an IC50 between 10-200nM. In addition, C10 was not toxic neither in zebrafish nor in mice. In vitro experiments showed the ability of C10 to establish covalent interactions with HCV and DENV capsid proteins, inducing the formation of dimers, trimers, and higher molecular weight species. The oligomer formation starts below the IC50 and increases in a dose-dependent manner. The cross-linking effect was not observed using other viral capsid or unrelated proteins.

For SAR studies, a set of 45 C10-derivatives were designed and synthesized. All compounds were tested against HCV and DENV in Huh7.5 cells and evaluated in cross-linking assays. A high correlation between the crosslinking ability of each compound and its activity in cell-based assays was observed. The activity and toxicity of the top hit derivative C45 were further evaluated. C45 is ~3-4 times better than C10 in reducing the infectivity of released viral particles, having a similar CC50. PK/ADME/Tox experiments in mice revealed that C10 and C45 exert low toxicity in vivo and reach plasma and organ levels higher than the IC50.

Conclusions: C10 and C45 are broadly active antiflaviviral compounds, which target the capsid protein interfering with its proper formation. Both compounds are highly active in cell-based assays and show low toxicity in vivo, offering an excellent therapeutic window and therefore being promising candidate molecules for further development.

Novel indolediketopiperazine derivatives targeting the replication of Flaviviridae viruses

George Mpekoulis,[1] Efseveia Frakolaki,[1] Erofili Giannakopoulou,[2] Vanesa Madan,[3,4] Vassilios Myrianthopoulos,[2] Ralf Bartenschlager,[3,4] Grigoris Zoidis[2] and Niki Vassilaki[1]

[1] Molecular Virology Laboratory, Hellenic Pasteur Institute, Athens, Greece; [2] Department of Pharmacy, Division of Pharmaceutical Chemistry, University of Athens, Athens, Greece; [3] Department of Infectious Diseases, Molecular Virology, University of Heidelberg, Heidelberg, Germany; [4] German Center for Infection Research, Heidelberg partner site

Targeting the Flaviviridae RNA-dependent RNA polymerase (RdRp) by active-site metal ion chelators have been proposed as a successful and broadly effective therapeutic strategy. We have previously identified novel substituted 1,2-annulated indolediketopiperazines as metal chelating inhibitors targeting HCV RNA replication (Zoidis et al. MedChemComm 2016). These were synthesized by introducing the drug-like core structure of indole to the 2,6-diketopiperazine scaffold of flutimide, which is a fully substituted 1-hydroxy-3H-pyrazine-2,6-dione, originally isolated from the fungus Delitschia confertaspora. Here we rationally designed a second series of compounds by introducing various substituents into the core structure of indole, to improve potency against the Flaviviridae RdRp. The compounds were evaluated against different HCV genotypes, DENV-2 and YFV-17D, in cell culture-based subgenomic and genome-length replicon systems. Resistance mutation analysis and in vitro enzymatic assays were performed with the most promising compounds to verify their mechanism of action. First, we confirmed that the hydroxyl group in the imidic nitrogen is crucial for the activity against viral replication.

Its replacement by bulkier groups (carboxy methylene or acetohydroxamic acid), performed to increase the flexibility of the chelating moiety, was detrimental for compound activity. Next, we retained the hydroxyl group on the imidic nitrogen and inserted further substitutions at positions 4, 6, 7, and 8 of the heterocyclic scaffold. The most favourable substitution was nitro at position 6 (compound ZF66), conferring EC50 1.6 μM against HCV 1b and 2.5 μM against HCV 1a, with high selectivity index (~109). ZF41, which carries an acetohydroxamic acid group on the imidic nitrogen, and ZF104, methyl-substituted at position 4, were the most effective against DENV and YFV. Interestingly, ZF66 had a high genetic barrier to resistance. Analysis of resistance mutations by NGS suggested that the compound targets HCV RdRp. This was confirmed by an in vitro enzymatic assay with recombinant NS5B RdRp. These results combined with the crucial role of the metal-chelating moiety for the antiviral activity and the in silico data, confirm the predicted mode of action of the synthesized compounds that involves chelation of the divalent metal ions in the RdRp catalytic pocke

Recapitulating hepatitis E virus-host interactions in human liver and intestinal organoids

Pengfei Li1, Kuan Liu1,2, Robert A. de Man1, Luc J. W. van de Laan2, Maikel P. Peppelenbosch1, Qiuwei Pan1

1Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands; 2Department of Surgery, Erasmus MC-University Medical Center, Rotterdam, The Netherlands.

Background and Aims: Hepatitis viruses naturally have narrow host and tissue tropisms, which is a fundamental impediment for the development of robust experimental models. The advent of organoid technology provides a unique avenue for moving the field forward. Although hepatitis E virus (HEV) is a major cause of acute hepatitis, extrahepatic manifestations have been widely reported in patients including infections in the intestine. We aim to establish human liver and intestinal organoid-based HEV models for mapping virus-host interactions and discovering new therapeutics.

Methods: Human fetal and adult intrahepatic organoids as well as intestinal organoids were isolated and cultured from different donor tissues. Reverse genetics systems were used to produce subgenomic and full-length genotype 1 (GT1) and GT3 HEV genome. Liver organoids with cholangiocyte-phenotype were differentiated into hepatocyte-like phenotype by previous established protocol. Human liver and intestinal organoids were directed to polarized columnar cells in transwell culturing system. A library of 94 safe-in-man broad-spectrum antiviral agents were screened in liver organoids based HEV model.

Results: We demonstrate that 3D cultured organoids from human liver and intestine, are highly permissive to the replication of GT1 and GT3 HEV. By inoculation with infectious HEV particles, staining of viral proteins and replicating double-strand viral RNA, and testing the infectivity of produced viral particles, we demonstrate that liver organoids support the full life cycle of HEV infection. Genome-wide transcriptomic analysis reveals a robust host response triggered by HEV replication in liver organoids, in particular antiviral interferon response. Similarly, HEV replication in intestinal organoids elicited robust antiviral interferon response showing the induction of many interferon-stimulated genes. By directing organoid cells towards polarized monolayers in transwell system, we observed predominantly apical secretion of HEV particles in liver and intestinal organoid monolayers. Drug screening identified homoharringtonine as a potent HEV inhibitor, which are also effective against the ribavirin resistance variant harboring G1634R mutation.

Conclusions: We successfully established innovative HEV models based on human liver and intestinal organoids. With this, the understanding of HEV-host interactions is catalyzed and antiviral drug discovery is boosted. Currently, we are attempting to construct the “gut-liver axis” model by integrating intestine-liver organoids for studying HEV extrahepatic spreading.

Contact: Pengfei Li, p.li@erasmusmc.nl

Resistance to NS5A-inhibitors and enhanced viral fitness compromise the efficacy of pangenotypic antiviral regimens against hepatitis C virus genotype 3 in cell culture

Carlota Fernandez-Antunez, Ulrik Fahnøe, Lotte Mikkelsen, Jens Bukh and Santseharay Ramirez.

Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital and Department of Immunology and Microbiology, University of Copenhagen.

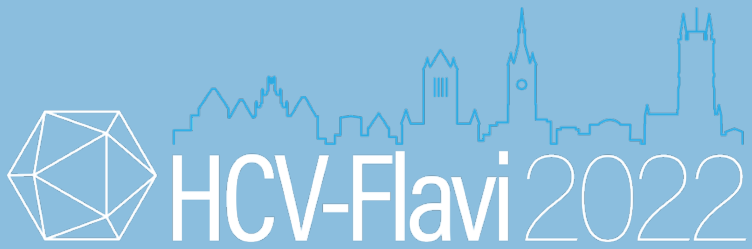
Background and Aims: Hepatitis C virus (HCV) elimination efforts rely on the use of direct-acting antiviral (DAA) based therapies, since no vaccine is yet available. Success in re-treating patients that experience DAA failure with emergence of antiviral resistance is crucial for achieving HCV elimination. Thus, it is essential to study the efficacy of the most recent pangenotypic regimens in the context of pre-existance of multiple resistance associated substitutions (RAS). We aimed at studying how resistance to protease-inhibitor glecaprevir, NS5A-inhibitors velpatasvir and pibrentasvir, and NS5B-inhibitor sofosbuvir influences treatment outcome with pangenotypic regimens sofosbuvir/velpatasvir or glecaprevir/pibrentasvir for genotype 3 in cell culture, since higher DAA treatment failure rates have been observed for this HCV genotype.

Methods: Virus variants with decreased DAA susceptibility (resistance) of the genotype 3a prototype strain S52, previously adapted to efficient growth in human hepatoma Huh7.5 cells, were selected in long-term escape experiments. Sofosbuvir and/or velpatasvir or glecaprevir and/or pibrentasvir escape viruses were then treated for 50 days with a combination of glecaprevir/pibrentasvir or sofosbuvir/velpatasvir, respectively. We determined the phenotypic and genotypic resistance profile of all viruses in short-term drug concentration-response assays and next-generation sequencing. Competition experiments between viruses were performed to assess viral fitness.

Results: DAA resistance was associated with the emergence of known RAS in the DAA targets for all viruses, and persistence of RAS after successive treatment failure correlated with substitutions outside the DAA target. NS5A-inhibitor resistance was sufficient to promote treatment failure to the otherwise highly efficient glecaprevir/pibrentasvir and sofosbuvir/velpatasvir regimens. Moreover, viruses showed increased NS5A-inhibitor resistance after treatment failure. Enhanced fitness correlated with pibrentasvir treatment failure and with accelerated escape from glecaprevir/pibrentasvir treatment.

Conclusions: The presence of NS5A-inhibitor resistance at baseline is sufficient to compromise the efficacy of pangenotypic regimens for HCV genotype 3, whereas enhanced viral fitness can accelerate viral escape from treatment. Thus, both factors seem to lower the barrier to resistance of these regimens.

Session 6
VIRAL REPLICATION



O20 **Hepatitis C virus RNA is 5’ capped with flavin adenine dinucleotide**
Jeppe Vinther, University of Copenhagen, Denmark

O21 **A pan-Flavivirus capsid interactome ATLAS identifies novel sub- and pan-flaviviral activities**
Pietro Scaturro, Leibniz Institute for Experimental Virology, Germany

O22 **A Hepatitis C virus genotype 1b isolate with high replication efficiency in cell culture and its adaptation to infectious virus production in vitro and in vivo**
Paul Rothhaar, Heidelberg University, Germany

O23 **Hepatitis E virus replication is controlled by a novel mechanism of polyprotein processing**
Morgan Herod, University of Leeds, UK

O24 **Interdomain interaction stabilized by divalent cation coordination within hepatitis E virus open reading frame 1 protein provides structural features critical for viral replication**
Robert LeDesma, Princeton University, USA

O25 **CRISPR activating the genome to identify novel anti-viral restriction factors of flavivirus replication**
Michael R. Beard, University of Adelaide, Australia

Hepatitis C virus RNA is 5’ capped with flavin adenine dinucleotide

Jeppe Vinther[1], Anna V. Sherwood[1], Lizandro R. Rivera-Rangel[2], Line A. Ryberg[2], Helena S. Larsen[2], Klara M. Andersen[1], Rui Costa[2], Cathrine B. Vågbø[3], Gabriele Indrisiunaite[1], Long V. Pham[2], Agnieszka Podolska-Charlery[1], Julius E.N. Grothen[1], Nicklas W. Langvad[1], Nicolas Fossat[2], Anna Offersgaard[2], Amal Al-Chaer[1], Louise Nielsen[2], Judith M. Gottwein[2], Kenn Holmbeck[2], Sandro Bottaro[4], Santseharay Ramirez[2], Jens Bukh[2], Troels K.H. Scheel[2]

[1]Section for Computational and RNA Biology, Department of Biology, University of Copenhagen; Ole Maaløes Vej 5, DK-2200 Copenhagen N, Denmark; [2]Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Copenhagen University Hospital, Hvidovre and Department of Immunology and Microbiology, University of Copenhagen; Copenhagen, Denmark; [3]Proteomics and Modomics Experimental Core (PROMEC), Norwegian University of Science and Technology, and the Central Norway Regional Health Authority; Trondheim, Norway; [4]Section for Biomolecular Sciences, Department of Biology, University of Copenhagen; Copenhagen, Denmark

Background and Aims: RNA viruses have evolved elaborate strategies for protection of their genomes against innate immune recognition and degradation, including 5’ capping. However, so far no RNA 5’ cap was identified for hepatitis C virus (HCV). Based on previous observations that HCV replication depends on enzymes of the flavin adenine dinucleotide (FAD) synthesis pathway, the universal conservation among HCV isolates of 5’A on the negative strand, and partial conservation for the positive strand, we hypothesized that HCV RNA is 5’ capped with FAD.

Methods: To investigate RNA FAD capping, we modified the high-throughput sequencing based CapZyme-seq methodology to use the FAD specific de-capping enzyme AtNUDX23. We further optimized mass-spectrometry (MS) methodology for FAD detection. HCV replicons were used to assess FAD dependency of replication, and *in vitro* replication initiation assays were used to study NS5B RNA-dependent RNA polymerase *de novo* initiation.

Results: We demonstrate that the cellular metabolite FAD is used as noncanonical initiating nucleotide by the viral NS5B polymerase resulting in a 5’ FAD cap on the HCV RNA. The HCV FAD capping frequency is ~75 %, which is the highest observed for any RNA metabolite cap across all kingdoms of life. FAD capping is completely conserved among HCV isolates for the negative strand and partially for the positive strand. It is also observed *in vivo* on HCV RNA isolated from serum of a chimpanzee and liver and a uPA-SCID human liver chimeric mouse model. The observed replication dependence on the FAD precursor, riboflavin, correlates with strain specific FAD-capping levels. Furthermore, we show that 5’ FAD capping has limited effect on HCV RNA stability but protects RNA from cell-intrinsic innate immune recognition.

Conclusions: These results establish capping with cellular metabolites, such as FAD, as a novel viral RNA capping and immune evasion strategy, which potentially could be used by many viruses and thereby affect viral treatment outcomes and infection persistency.

A pan-*Flavivirus* capsid interactome ATLAS identifies novel sub- and pan-flaviviral activities

Laura Gauglitz[1], Anna Lena Kastner[2], Charlotte Flory[1], Maryna Chaika[1], Mirko Cortese[3], Andreas Pichlmair[2,4] and Pietro Scaturro[1]

[1]Leibniz Institute for Experimental Virology, Systems Arbovirology, Hamburg, Germany; [2]Technical University of Munich, School of Medicine, Institute of Virology, Munich, Germany; [3]The Telethon Institute of Genetics and Medicine, Naples, Italy; [4]German Center for Infection Research (DZIF), (Munich Partner Site), Munich, Germany

Arboviruses are a prime reason for mortality worldwide. Among them flaviviruses represent a significant burden given their ability to trigger epidemics, establish persistence in endemic areas and the complete lack of effective antiviral drugs. Despite a relatively high genetic similarity and overall conserved replication strategies, flaviviruses evolved finely tuned and divergent mechanisms of host exploitation, resulting in extraordinarily distinct tropisms and pathogeneses.

The ability of viruses to avoid or evade host antiviral responses and to exploit cellular machineries is essential for replication and successful transmission. However, it is not fully understood how evolution in different reservoir hosts has shaped replication and pathogenesis of different flaviviruses. Here, we employed affinity-purification coupled to mass-spectrometry (AP-LC-MS/MS) to systematically evaluate the ability of capsid proteins of 12 prototypic flaviviruses to interact with the human proteome. This extensive protein-protein interaction ATLAS allowed the identification of novel host factors and cellular pathways specifically targeted by capsid proteins, providing a unique perspective on broadly conserved as well as unique host-binding specificities across the entire Flavivirus genus.

Among these, we identified cellular proteins with known functions in neuronal development and synaptic plasticity. Further characterization of their binding profile across different flavivirus capsids identified novel *bona fide* pan-viral capsid binders, as well as binding specificities unique to the neurovirulent clade of genus. Using a combination of functional, biochemical and imaging-based approaches, we uncovered distinct effects on virus growth across unrelated RNA(+) viruses, and highlighted a profound impact of viral infection on subcellular redistribution and homeostatic functions of these proteins.

Our integrative study provides a comprehensive framework to categorize and characterize novel, yet unidentified molecular targets, and a rational framework to streamline the identification of critical host and viral determinants associated with distinct flaviviruses.

A Hepatitis C virus genotype 1b isolate with high replication efficiency in cell culture and its adaptation to infectious virus production in vitro and in vivo

Paul Rothhaar[1], Christian Heuss[1], Rani Burm[2], Ji-Young Lee[1], Philipp Ralfs[1], Ralf Bartenschlager[1], Arvind H. Patel[3], Frederik Graw[1], Thomas Krey[4], Vibor Laketa[1], Philip Meuleman[2], Volker Lohmann[1]

[1]Heidelberg University, Heidelberg, Germany.; [2]Ghent University, Gent, Belgium.; [3]University of Glasgow, Glasgow, UK.; [4]University of Lübeck, Lübeck, Germany.

Background & Aims: Hepatitis C virus (HCV) is highly diverse and grouped into eight genotypes (gts). Infectious cell culture models are limited to a few subtypes, that do not include the highly prevalent gt1b, hampering the development of prophylactic vaccines.

Methods: A consensus gt1b genome (termed GLT1) was generated from an HCV infected liver-transplanted patient. Subsequently, HCV replication and infection assays were performed in hepatoma cells using replication enhancing mutations, SEC14L2 expression and a Phosphatidylinositol-4 kinase III alpha (PI4KA) inhibition strategy. The isolate was adapted to cell culture through contiguous long-term passaging of GLT1-replicating cells and media. Furthermore, in vivo infection studies in human liver chimeric mice were performed.

Results: GLT1 replicated to an outstanding efficiency in Huh7 cells upon SEC14L2 expression, use of replication enhancing mutations or PI4KA inhibition. RNA replication levels almost reached JFH-1, but full-length genomes failed to produce detectable amounts of infectious virus. Long-term passaging led to the adaptation of a genome carrying 21 mutations and concomitant production of high levels of infectious virus (GLT1cc). During the adaptation, genome replication and particle production efficiency were enhanced while cell entry competence of HCV pseudoparticles (HCVpp) was not affected. Furthermore, GLT1cc retained the ability to replicate in human liver chimeric mice, which was critically dependent on a mutation in domain 3 of NS5A. In the course of infection, only one mutation in E2 reverted to wildtype, which facilitated assembly in cell culture but potentially affected CD81 interaction in vivo.

Conclusion: Overall, GLT1cc is the first efficient gt1b infectious cell culture model, paving the road to a rationale-based establishment of new infectious HCV isolates, and represents an important novel tool for the development of prophylactic HCV vaccines. Furthermore, the patient's background of receiving two liver transplantations will allow for evolutionary analysis and studies on fitness determinants governing HCV replication in vivo and in vitro.

Hepatitis E virus replication is controlled by a novel mechanism of polyprotein processing

Danielle M. Pierce, Frazer J.T. Buchanan, Abigail Cox, Khadijah Abualsaoud, Joseph C. Ward, Mark Harris, Nicola J. Stonehouse and Morgan R. Herod

School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, U.K.

Background and Aims: Hepatitis E virus (HEV) is a leading cause of acute viral hepatitis. It can be transmitted faecal-orally between humans and zoonotically to humans from animal reservoirs such as pigs. Zoonotic transmissions are of particular concern and are associated with increased mortality in some groups. The HEV pORF1 polyprotein is necessary and sufficient to support viral genome replication and, based on sequence homology to related viruses, is predicted to contain seven domains. By analogy to other positive-sense RNA viruses, it is likely that pORF1 undergoes proteolysis to produce individual functional proteins. However, both the molecular mechanism of proteolysis and the nature of the corresponding products remain obscure.

Methods: Using a combination of *in vitro* translation, proteolysis and sub-genomic replicon assays we study the processing of pORF1 and the importance for HEV replication.

Results: Our data suggest that in contrast to related RNA viruses, HEV pORF1 has no detectable proteolytic activity *in vitro* and no auto-catalytic proteolysis of pORF1 could be detected using any fragment of the polyprotein. However, in the presence of the cellular enzyme thrombin, we show that pORF1 undergoes specific proteolysis to produce distinct protein products. Using mutagenesis we show that this proteolysis occurs at defined sites which match the thrombin recognition consensus and are highly conserved. Mutagenesis prevents viral genome replication, as does pharmacological inhibition of thrombin. Finally, we are able to demonstrate by immunoblot and immunofluorescence that thrombin is present within cells that support HEV replication.

Conclusions: We propose that HEV exhibits a novel strategy for genome replication that is dependent on cellular enzymes to mediate polyprotein proteolysis. We speculate this mechanism could be important for controlling cellular tropism and zoonosis.

Interdomain interaction stabilized by divalent cation coordination within hepatitis E virus open reading frame 1 protein provides structural features critical for viral replication

Robert LeDesma[1], Abhishek Biswas[1], Stephanie Maya[1], Stefania Gili[2], John Higgins[2], Alexander Ploss[1,]

[1]Department of Molecular Biology, Lewis Thomas Laboratory, Princeton University, Princeton, NJ 08544, USA.; [2]Department of Geosciences, Princeton University, Princeton, NJ, 08544, USA.; *Correspondence should be address to A.P. (aploss@princeton.edu)

Background and Aims: Hepatitis E virus (HEV) is a positive sense, single stranded RNA virus of the *Hepeviridae* family responsible for over 20 million infections causing approximately 70.000 deaths annually. HEV encodes three gene products, the open reading frame (ORF) 1 polyprotein encodes the viral replicase, ORF2 the capsid and ORF3 a phosphoprotein essential for viral egress. It is currently unknown how the different domains of ORF1 function within a defined structural context, leading to the impetus of our study.

Methods: Human hepatoma cells transfected with *in vitro* transcribed HEV reporter replicons allowed us to interrogate the effect of point mutations on viral replication across several human-tropic genotypes of HEV. The A.I. driven structural prediction algorithm AlphaFold provided us with a novel structural model of ORF1 from which we developed testable hypotheses. Further, inductively-coupled plasma mass spectrometry facilitated the investigation of divalent metal ion binding capacity of wild-type (WT) or mutated ORF1 protein, and confocal microscopy allowed us to determine the subcellular localization of native or mutated ORF1.

Results: Our data suggest that ORF1 functions as a single multidomain protein which does not appear to be subject to proteolytic processing. To support such a model, targeted mutational analysis and comprehensive scanning mutagenesis performed on a domain traditionally designated as a putative papain-like cysteine protease (pPCP) revealed six cysteines forming a $CxC[x]_{11}CC[x]_{8}CxC$ motif that is highly conserved across all known HEV genotypes, with each cysteine being essential for viral genome replication. While one of these cysteines has been previously implicated as a part of a catalytic dyad, our data suggests its role lies in divalent metal ion coordination contributing to the predicted overall structure of ORF1. We further identified a novel long-range interaction between the pPCP and the preceding enigmatic Y-domain via a divalent metal ion binding domain; the conserved interacting residues are necessary for the proper structural conformation of ORF1 as a single multidomain protein whose subcellular localization is perturbed when these interdomain interactions are genetically disrupted. Consistent with these presumed functions in establishing the overall structure of ORF1 the “pPCP” domain can only rescue viral genome replication *in trans* when expressed in the context of the full-length ORF1 protein but not as an individual subdomain.

Conclusions: Our work provides a comprehensive model of the structure and function of HEV ORF1, which can conceivably contribute to the development of novel therapeutics for this understudied human viral pathogen. Our work highlights novel domain-domain interactions between the upstream Y-domain and the metal-coordinating structural domain of HEV, previously (and incorrectly) called the PCP.

CRISPR activating the genome to identify novel anti-viral restriction factors of flavivirus replication

Emily N. Kirby (1)*, David C. Bersten (1), Bastien Llamas (2), and Michael R. Beard (1)

1. Department of Molecular and Biomedical Sciences, School of Biological Sciences, The University of Adelaide, Adelaide, SA, 5005, Australia; 2. Department of Ecology and Evolutionary Biology, School of Biological Sciences, The University of Adelaide, SA, 5005, Australia; *. Presenting Author

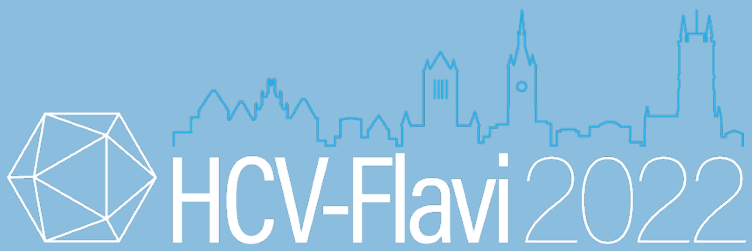
The Flaviviruses include the human pathogens Dengue Virus (DENV), Zika Virus (ZIKV) and West Nile Virus (WNV), all which elicit a significant global health burden. Host cellular restriction factors play a critical role in limiting all stages of the viral lifecycle and thus identification and characterisation of proteins with anti-viral potential is essential to further our understanding of Flavivirus biology with potential for the development of effective novel anti-viral strategies.

In contrast to genome wide CRISPR KO screens, CRISPR/dCas9 activator (CRISPRa) screens use the activity of human and viral transcriptional activators to recruit transcriptional machinery to the proximal promoter of a target gene, resulting in targeted gene expression. We employed a CRISPRa screen to identify novel host restriction factors for the WNV variant, Kunjin Virus (KUNV) in Huh7.5 cells expressing the CRISPRa library. Our screen selected for cells resistant to KUNV induced cytopathic cell death, suggesting expression of anti-viral restriction factors. Guide RNAs (sgRNAs) were PCR amplified from surviving cell DNA and submitted for Next Generation Sequencing to identify enrichment of sgRNA's in comparison to uninfected controls.

Bioinformatics analysis (CaRPools) for amplified sgRNAs, allowed this strategy to identify enrichment of previously described anti-viral factors, such as Interferon Inducible Protein 6 and Interferon Lambda 2, validating our approach. In addition, we identified novel candidates such as Sterolin- 2 (ABCG8), an ATPase Binding Cassette protein which acts as a critical regulator of intracellular cholesterol levels by modulating cholesterol secretion. CRISPRa activation of ABCG8 was also inhibitory of other closely related flaviviruses such as DENV, ZIKV and YFV, but not the human coronavirus 229E or Herpes Simplex- 1. Interestingly, addition of interferon (IFN)-alpha to ABCG8 CRISPRa cells resulted in a significant increase in interferon stimulated gene expression relative to a non-targeting control, suggesting that ABCG8 and cholesterol homeostasis is an indirect regulator of type -I IFN signaling pathways.

This work highlights the capabilities of CRISPRa as a significant genome editing technology, allowing for identification of novel anti-viral proteins and host pathways critical for the inhibition of viral replication, which may aid in development of anti-viral therapies.

Session 7
VIRAL TRANSLATION AND ASSEMBLY



O26 **The cargo adaptor protein CLINT1 is phosphorylated by the Numb-associated kinase BIKE and mediates dengue virus infection**
Sirle Saul, Stanford University, USA

O27 **Core protein promotes clustering and intracellular redistribution, but not biogenesis of lipid droplets during HCV infection**
Angeliki-Anna Beka, Institut Pasteur, France

The cargo adaptor protein CLINT1 is phosphorylated by the Numb-associated kinase BIKE and mediates dengue virus infection

Sirle Saul[1], Stanford Schor[1], Szuyuan Pu[1], Vlad Nicolaescu[2], Siavash Azari[1], Mardo Kõivomägi[3], Marwah Karim[1], Patricia Cassonnet[4], Gregory Neveu[1], Andrew Yueh[5], Caroline Demeret[4], Jan M. Skotheim[3], Yves Jacob[4], Glenn Randall[2], and Shirit Einav[1, 6 #]

[1]Department of Medicine, Division of Infectious Diseases and Geographic Medicine, and Department of Microbiology and Immunology, Stanford University, CA, USA; [2]Department of Microbiology, University of Chicago, IL, USA; [3]Department of Biology, Stanford University, CA, USA; [4]Department of Virology, Molecular Genetics of RNA Virus Genetics (GMVR), Pasteur Institute, National Center for Scientific Research, and Paris Diderot University, France; [5]Institute of Biotechnology and Pharmaceutical Research, Taiwan; [6]Chan Zuckerberg Biohub, San Francisco, CA, USA

Background and aims: The signaling pathways and cellular functions regulated by the four Numb-associated kinases (NAKs) are largely unknown. We previously reported that AAK1 and GAK control intracellular trafficking of RNA viruses, and recently revealed a requirement for BIKE in early and late stages of dengue virus (DENV) infection. However, the downstream targets phosphorylated by BIKE in this process have not yet been identified. We therefore aimed to identify substrates of BIKE and define their roles in DENV infection.

Methods: We screened for BIKE interactions with the human proteome by a Barcode Fusion Genetics-Yeast 2-Hybrid (BFG-Y2H) approach and retrieved existing interactomics data generated by affinity-purification mass spectrometry (BioPlex). Mammalian cell-based protein-protein interaction assays (PCAs) and co-immunoprecipitation (IP) experiments were used to validate putative interactions. Candidate phosphorylation substrates were identified by Phos-tag™ gel analysis in Huh7 cells depleted of BIKE and confirmed by a radioactive *in vitro* kinase assay. The specific residue that BIKE modifies was defined by LC-MS/MS analysis. DENV replication and specific lifecycle stages were measured using standard assays in Huh7 cells ectopically expressing or depleted of CLINT1. Live cell imaging was used to monitor cotrafficking of tetracysteine-tagged DENV particles stained with FlAsH with CLINT1.

Results: Thirty six and twelve candidate BIKE interactors were identified via the BFG-Y2H and BioPlex screens, respectively. CLINT1, a cargo-specific adaptor implicated in bidirectional Golgi-to-endosome trafficking, emerged as a predominant hit in both screens. CLINT1 and 18 other putative BIKE interactors were confirmed by PCAs and co-IPs. Our experiments indicated that BIKE catalyzes phosphorylation of a threonine 294 (T294) CLINT1 residue both *in vitro* and in cell culture. Our findings revealed that CLINT1 phosphorylation mediates its binding to the DENV nonstructural 3 protein and subsequently promotes DENV assembly and egress. Additionally, using live-cell imaging we revealed that CLINT1 cotraffics with DENV particles and is involved in mediating BIKE's role in DENV infection. Finally, our data suggest that additional BIKE interactors implicated in the host immune and stress responses and the ubiquitin proteasome system might also be candidate phosphorylation substrates of BIKE.

Conclusions: These findings reveal cellular substrates and pathways regulated by the understudied NAK enzyme BIKE, a mechanism for CLINT1 regulation, and control of DENV infection via BIKE signaling, with potential implications for cell biology, virology, and host-targeted antiviral design.

Core protein promotes clustering and intracellular redistribution, but not biogenesis of lipid droplets during HCV infection

Angeliki-Anna Beka[1,2], Emeline Simon[1,2], Dmitry Ershov[3], Damien Batalie[1], Brigitte Blumen[1], Houda Tabbal[1], Stephanie Aicher[1], Hugo Varet[3], Claire Gondeau[4], Philippe Roingeard[5] and Annette Martin[1]

[1] Institut Pasteur, Université Paris Cité, CNRS UMR3569, Paris, France ; [2] Université Paris Cité, ED BioSPC 562, Paris, France ; [3] Institut Pasteur, Université Paris Cité, Image Analysis Hub, Paris, France ; [4] Institute for Regenerative Medicine and Biotherapy, INSERM U1183, Montpellier, France ; [5] Université François Rabelais, Faculté de Médecine, INSERM U966, Tours, France

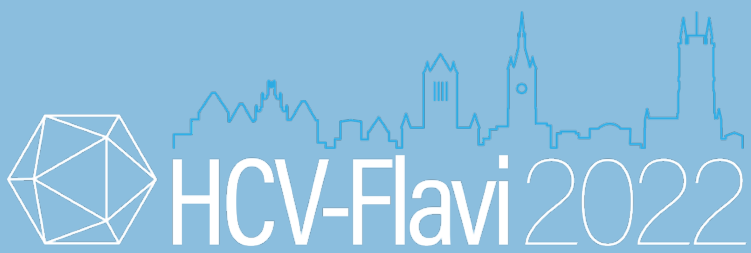
Hepatitis C virus (HCV) core protein associates at the surface of cytosolic lipid droplets (LDs), a step that is critical for virion morphogenesis. How HCV hijacks LDs to promote viral particle assembly and whether this process results in important hepatocyte deregulations are not fully deciphered. Our aims were to (i) investigate how HCV infection impacts LD biogenesis and dynamics, and (ii) determine the spatial and temporal interplay between core proteins of various HCV genotypic origins and LDs.

A time-course monitoring of LDs in hepatoma cells through quantitative co-localization approaches using confocal and electron microscopy showed that the global LD content did not significantly differ between noninfected cells and cells infected with a highly infectious HCV 2a-JFH1 derivative (Jad). Together with the progressive enlargement and marked redistribution of LDs resulting in compact aggregates during the course of infection, these data indicate that Jad infection does not promote LD biosynthesis, but rather LD clustering. Using an in-house Python computational program, we substantiated a significant increase in the rate of LD close contacts in infected hepatocytes. Furthermore, we found that Jad core associated with largest LDs and accumulated preferentially at LD contact sites.

To examine whether these observations were common to all HCV strains, intergenotypic recombinant viruses derived from Jad and encoding core proteins of various genotypes were produced and shown to be highly and equally infectious in hepatoma cells. We noted incomplete wrapping of the LD surface by core proteins of subtypes 1a, 3a, 4a, 4f at late time-points of infection, compared to full wrapping by Jad core, highlighting diverse rates of core recruitment and/or unloading from LDs, without notably affecting HCV infectious titers. Importantly, all core proteins associated with largest LDs, as Jad core, while infection with recombinant viruses led to various degrees of LD enlargement. Furthermore, in cells transfected with genome-length HCV RNAs encoding mutated core unable to associate to LDs, the enlargement, aggregation and redistribution of LDs in close proximity of replication factories were not observed.

Altogether, these data show that functional HCV core is the driver of LD intracellular redistribution and LD clustering, possibly by physically bridging neighboring LDs, destabilizing their surface tension and favoring their coalescence. These processes are affected by core sequence to variable degrees, although not in direct link with HCV subtypes. To investigate underlying mechanisms, we have undertaken to identify host LD-associated factors either interacting with core or whose expression is deregulated by core in infected cells.

Session 8
PATHOGENESIS



O28 **An atlas of the human liver circadian transcriptome and its carcinogenic perturbation by hepatitis C virus infection.**
Atish Mukherji, Université de Strasbourg, France

O29 **Cellular and molecular determinants preceding the progression to severe dengue in children and adults via virus-inclusive single cell RNAseq approach**
Luca Ghita, Stanford University School of Medicine, USA

O30 **The magnitude and kinetics of immune responses associated with severe dengue progression in humans via proteomic single-cell profiling of PBMCs**
Makeda L. Robinson, Stanford University School of Medicine, USA

O31 **IL-15-induced activation of liver damaging bystander T cells is insensitive to PD-1-mediated inhibition in viral hepatitis.**
Hoyoung Lee, Korea Virus Research Institute, Republic of Korea

O32 **Is contemporary Zika getting more on your nerves?**
Maïlis Darmuzey, KU Leuven and Université de Liège, Belgium

O33 **Impact of HCV genotypic variability on hepatocyte pathway deregulations in link with steatosis and HCC**
Angeliki-Anna Beka, Institut Pasteur, France

O34 **Contribution of the Cellular Lipid Kinase PI4KA to HCV-induced Liver Pathogenesis**
Cong Si Tran, University of Heidelberg, Germany

An atlas of the human liver circadian transcriptome and its carcinogenic perturbation by hepatitis C virus infection

Atish Mukherji[1,*], Frank Jühling[1,*], Michio Imamura[2,3], Fahmida Rasha[4], Naoto Fujiwara[4], Alexandre Haller[5], Philippe Baltzinger[5], Carla Eller[1], Katharina Herzog[1], Chloé Gadenne[1], Clara Ponsolles[1], Marine A. Oudot[1], Laurent Mailly[1], Catherine Schuster[1], Xiaodong Zhuang[6], Jacinta Holmes[7], Ming-Lun Yeh[8], Hiromi Abe-Chayama[3,9], Angelo Sangiovanni[10], Massimo Iavarone[10], Massimo Colombo[11], Steven K.H. Foung[12], Jane A McKeating[6], Irwin Davidson[5], Ming-Lung Yu[8], Raymond T. Chung[13], Yujin Hoshida[4], Kazuaki Chayama[3,14,15], Joachim Lupberger[1,§], and Thomas F. Baumert[1,16,17,§]

[1]Université de Strasbourg, Inserm, Institut de Recherche sur les Maladies Virales et Hepatiques UMR_S1110, Strasbourg, France. ; [2]Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan.; [3]Research Center for Hepatology and Gastroenterology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. ; [4]Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, USA. ; [5]Department of Functional Genomics and Cancer, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université de Strasbourg, Illkirch, France. ; [6]Nuffield Department of Medicine, University of Oxford, Oxford OX3 7FZ, UK. ; [7]University of Melbourne, St Vincent's Hospital, Melbourne, Australia. ; [8]Hepatobiliary Division, Department of Internal Medicine, School of Medicine and Hepatitis Research Center, College of Medicine, and Center for Liquid Biopsy and Cohort Research, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 80708, Taiwan. ; [9]Center for Medical Specialist Graduate Education and Research, Hiroshima, Japan.; [10]Division of Gastroenterology and Hepatology, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, University of Milan, Italy. ; [11]Liver Center and General Medicine, IRCCS San Raffaele Hospital, Milan, Italy. ; [12]Department of Pathology, Stanford University School of Medicine, California 94305, USA. ; [13]Gastrointestinal Division, Hepatology and Liver Center, Massachusetts General Hospital, Boston, MA 02114, USA.; [14]Collaborative Research Laboratory of Medical Innovation, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. ; [15]RIKEN Center for Integrative Medical Sciences, Yokohama, Japan. [16]Service d'hépato-gastroentérologie, Hôpitaux Universitaires de Strasbourg, Strasbourg, France. ; [17]Institut Universitaire de France, Paris, France. ; *contributed equally, §supervised jointly.

Background and Aims: Mammals are endowed with an inbuilt timing system known as the circadian clock (CC), a critical regulator of physiology. Studies with animal models confirm an essential role for the CC to regulate metabolism, immune and endocrine functions and its disruption leads to diverse pathologies including metabolic diseases and cancer. However, the role of a disrupted liver CC and the development of chronic liver disease in patients is largely unknown due to our limited knowledge on diurnal gene expression in human liver. Despite significant strides, the molecular basis of hepatitis C virus (HCV)-induced liver disease and hepatocellular carcinoma (HCC) development is poorly known. Here, we aimed to investigate the role of the liver CC in disease biology by identifying the circadian transcriptome and epigenome of human hepatocytes and its perturbation during chronic HCV infection.

Methods: To unravel the human liver circadian transcriptomic atlas well as their epigenetic profile under physiological conditions and following HCV-infection, we performed RNA- and ChIP-sequencing of livers from control and virus-infected humanized liver chimeric mice (HLCM) sampled at six timepoints within a 24 hour period. These genome-wide analyses enabled us to determine diurnal transcriptomic and epigenetic changes in human hepatocytes. By applying MetaCycle and dryR we identified the rhythmic genes and biochemical pathways under physiological conditions and their perturbation following HCV-infection. To substantiate the clinical impact of our findings, we investigated the virus-induced perturbation of the circadian clock in four independent cohorts of patients with chronic hepatitis C.

Results: Here, we unraveled the atlas of human liver circadian transcriptome and epigenome. We identified more than 2000 human specific, rhythmically expressed genes including transcription factors, chromatin modifiers, and key enzymes. We show that HCV infection, a major cause of liver disease and HCC, perturbs the human liver clock, leading to an activation of key pathways mediating steatosis, fibrosis and cancer. HCV-disrupted circadian pathways remained deregulated in patients following HCV cure and advanced liver disease. Altogether, our genome-wide analyses uncovered the molecular basis of human liver-specific rhythmic gene networks and its perturbation leading to chronic liver disease and HCC.

Conclusions: Our combined transcriptome and epigenomic analyses uncovered the molecular basis of human liver-specific rhythmic gene networks and its perturbation leading to chronic liver disease and HCC. We show that the virus-induced perturbation of liver clock plays a significant role in HCC development and may provide opportunities for cancer prevention and biomarkers to predict HCC risk

Cellular and molecular determinants preceding the progression to severe dengue in children and adults via virus-inclusive single cell RNAseq (viscRNA-seq) approach

Luca Ghita[1, ¶], Zhiyuan Yao[1, ¶], Yike Xie[2, ¶], Veronica Duran[1], Makeda L. Robinson[1], Jerome Samir[3], Olga Lucia Agudelo Rojas[4], Ana Maria Sanz[4], Malaya Kumar Sahoo[5], Rosa Margarita Gelvez[6], Nathalia Bueno[6], Fabio Luciani[2,3,7], Benjamin A. Pinsky[1,5], Maria Isabel Estupiñan Cardenas[6], Luis Angel Villar Centeno[6], Elsa Marina Rojas Garrido[6], Fernando Rosso[4,8], Stephen R. Quake[9, 10, 11], Fabio Zanini[2,8,+], and Shirit Einav[1,10, 12,.]

[1] Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA; [2] School of Clinical Medicine, University of New South Wales, Sydney, NSW, Australia; [3] Kirby Institute for Infection and Immunity, University of New South Wales, Sydney, NSW, Australia; [4]Clinical Research Center, Fundación Valle del Lili, Cali, Colombia.; [5]Department of Pathology, Stanford University School of Medicine, Stanford, California, USA; [6]Centro de Atención y Diagnóstico de Enfermedades Infecciosas (CDI), Bucaramanga, Colombia.; [7]Cellular Genomics Futures Institute, University of New South Wales, Sydney, NSW, Australia; [8]Department of Internal Medicine, Division of Infectious Diseases, Fundación Valle del Lili, Cali, Colombia.; [9] Department of Bioengineering, Stanford University, Stanford, CA, USA; [10] Chan Zuckerberg Biohub, San Francisco, CA, USA; [11]Department of Applied Physics, Stanford University, Stanford, CA, USA; [12]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA; ¶: these authors contributed equally; +: these authors also contributed equally

Background and Aims: A fraction (5-20%) of symptomatic dengue patients, particularly children, progresses in days to severe dengue (SD). Yet, SD pathogenesis in humans is incompletely characterized, and there are no effective measures to predict or prevent it. To help address these gaps, we mapped the immune cell targets of DENV in the human blood and the transcriptomic landscape preceding the progression to SD across ages at a high resolution.

Methods: PBMCs were derived from 24 children enrolled in our Colombia dengue cohort (8 progressed after enrollment to SD (SDp), 12 had uncomplicated course, 4 were healthy controls). We optimized our previously reported viscRNA-Seq platform, enabling simultaneous coverage of host and viral transcriptomes from 193,727 cells. 16 immune cell subtypes were defined by unsupervised clustering and subject to comparative analyses between virus RNA (vRNA) harboring (VHC) and bystander cells, and differential cell abundance and gene expression between SDp and nonprogressors by single-cell or patient-level statistics. Alterations in cell-cell communications were explored using the OmniPath database of known physical or paracrine interactions. Our dataset of 10 DENV-infected adults from the same cohort was integrated in the analysis.

Results: vRNA was detected primarily in B cells followed by monocytes in children and adults. Patient-derived B cells harbored positive and negative-strand vRNA and infected hepatoma cells in co-culture, indicating viral replication. VHCs showed transcription signatures consistent with naïve B cells and suppressed antigen presentation and interferon signaling relative to bystander cells from the same samples. VHCs in children showed signatures consistent with increased inflammation and impaired viral sensing relative to adults. Cell type abundance was altered in SDp children with reduced non-classical monocytes and cytotoxic NK cells and increased proliferating plasmablasts, and these changes robustly predicted progression to SD. Myeloid cells in SDp exhibited upregulation of pro-inflammatory genes and downregulation of antigen presentation and interferon response, and these alterations were more prominent in children than adults. NK cells in SDp showed immune activation (stronger in children than adults), whereas an adaptive, possibly exhausted NK cell population emerged in both age groups. The cell-cell communication network in SDp children was altered with cDCs and monocytes playing a central role in coordinating immune cell migration and proinflammatory signals, and NK cells showing possible impairment in orchestrating the innate-adaptive immune junction.

Conclusion: These results provide insight into the pathogenesis of natural DENV infection and reveal determinants in the host response that may explain increased disease severity in children, with implications for SD prediction and prevention.

The magnitude and kinetics of immune responses associated with severe dengue progression in humans via proteomic single-cell profiling of PBMCs

Makeda L. Robinson[1*], David Glass[2*], Veronica Duran[1], Olga Lucia Agudelo Rojas[3], Ana Maria Sanz[3], Malaya Kumar Sahoo[4], Rosa Margarita Gelvez[5], Nathalia Bueno[5], Benjamin A. Pinsky[1,4], Maria Isabel Estupiñan Cardenas[5], Luis Angel Villar Centeno[5], Elsa Marina Rojas Garrido[5], Fernando Rosso[3,6], Holden Maecker[7], Sean Bendall[4], and Shirit Einav[1,8,9].

[1] Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA; [2] Fred Hutchinson Cancer Research Center, Seattle, WA, USA; [3]Clinical Research Center, Fundación Valle del Lili, Cali, Colombia. ; [4]Department of Pathology, Stanford University School of Medicine, Stanford, California, USA; [5]Centro de Atención y Diagnóstico de Enfermedades Infecciosas (CDI), Bucaramanga, Colombia.; [6]Department of Internal Medicine, Division of Infectious Diseases, Fundación Valle del Lili, Cali, Colombia.; [7]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA; [8] Chan Zuckerberg Biohub, San Francisco, CA, USA; [9]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA

Background and Aims: Severe dengue (SD) affects 4-6 million people annually. Antibody-dependent enhancement is a risk factor, yet other factors underlying SD are largely uncharacterized. Moreover, it remains unknown why SD is more common in children than adults and why it manifests with bleeding and shock (DHF/DSS) in some and with organ impairment (OI) in others. We aimed to investigate the immune response associated with disease progression across age groups and SD categories.

Methods: PBMC samples were collected at presentation, hospitalization and convalescence from patients enrolled in our Colombia dengue cohort. Samples from 100 individuals (42 children, 58 adults; 63 uncomplicated dengue (D), 22 severe dengue progressors (SDp), 15 controls) were analyzed by mass cytometry (CyTOF) using a 39-antibody panel. Data was analyzed by the FlowSOM R package.

Results: Longitudinal analysis of the abundance and protein expression profile of 29 cell populations (clustered from ~9 million cells) revealed distinct patterns in D and SDp.

Innate immune cell activation was greater in SDp than D early during infection, with overexpression of Ki-67, CD38, CD64, and CD141 on myeloid cells and HLA-DR, CD38 and CD69 on NK cells. While this activation declined in several days in D, it persisted throughout the disease course in SDp. Adaptive immune responses were also activated early and persisted throughout the disease course in SDp with expansion of plasma cells overexpressing Ki-67 and IgG and T cell subtypes overexpressing Ki-67 and CD38. Contrastingly, in D, this activation developed gradually and peaked on day 8. Beyond activation, signatures of immune suppression were also detected in SDp, but not D patients. These included reduced abundance of myeloid subtypes, NK, and EMRA$^+$ CD8 T cells, and reduced HLA-DR expression on myeloid cells. These coincided with a dramatic increase in the abundance of Tregs showing a hyper-suppressive profile (increased CTLA-4, PD-1, CD38 and Ki-67 expression) and increased PD-L1 expression in T, NK and B cells, which peaked early in SDp and gradually declined. Profiling distinct age groups pointed to increased plasma cell abundance and proliferation in adult SDp, but NK cell transition from a cytotoxic to inflammatory phenotype, increased activation of EMRA$^+$ CD8 T cells, and reduced antigen presentation capacity in myeloid cells in child SDp. Distinct SD categories showed differential responses with activation of cDC1 and Tregs in OI, vs. activation of plasma cells in DHF/DSS.

Conclusion: These findings propose a model wherein progression to SD is associated with a dysregulated temporal switch between innate and adaptive immune responses and early hypersuppressive immune regulation and reveal age-specific and syndrome-specific determinants with potential relevance for SD prediction and treatment.

IL-15-induced activation of liver damaging bystander T cells is insensitive to PD-1-mediated inhibition in viral hepatitis.

Hoyoung Lee[1], So-Young Kim[1,2], Su-Hyung Park[2] and Eui-Cheol Shin[1, 2]

[1]The Center for Viral Immunology, Korea Virus Research Institute, Institute for Basic Science (IBS), Daejeon 34126, Republic of Korea; [2]Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea

Background and Aim: Previously, it was demonstrated that IL-15-induced bystander activation of memory CD8+ T cells contributes to liver damage during acute hepatitis A virus (HAV) infection (Kim et al. Immunity 2018, 48:161-173). Recently, this immunopathological mechanism of bystander-activated memory CD8+ T cells was also reported in patients with chronic hepatitis C virus (HCV) infection (Huang et al. Hepatology 2022, DOI: 10.1002/hep.32349). However, underlying mechanism regulating bystander activation of memory CD8+ T cells is unknown. In the present study, we investigated the role of inhibitory receptors in bystander activation of memory CD8+ T cells in viral hepatitis.

Methods: We analyzed expression of inhibitory receptors on bystander-activated memory CD8+ T cells from patients with acute HAV infection by using MHC class I multimers. Additionally, we performed in vitro experiments with memory CD8+ T cells to examine the role of PD-1 during T cell receptor (TCR)-independent IL-15-induced activation of memory CD8+ T cells.

Results: Expression of PD-1, TIM-3, TIGIT, CD39 and CTLA-4 on CD8+ T cells was increased in patients with acute HAV infection. Using MHC class I multimers, we found that expression of PD-1 was significantly increased on bystander-activated CD8+ T cells including cytomegalovirus (CMV)-, Epstein-Barr virus (EBV)- and influenza A virus (IAV)-specific CD8+ T cells but to a lower extent compared to HAV-specific CD8+ T cells during acute HAV infection. We demonstrated that the expression of PD-1 was increased by IL-15, a potent proinflammatory cytokine that induces bystander activation of memory CD8+ T cells. Moreover, unlike TCR-mediated stimulation, we showed that IL-15-induced activation and proliferation of memory CD8+ T cells are not inhibited by ligation of PD-1 with PD-L1/2. Furthermore, we demonstrated that TCR-independent IL-15-induced NK-like cytotoxicity of memory CD8+ T cells is not restrained when co-cultured with target cells that highly express PD-L1.

Conclusions: Our finding suggests that IL-15-induced bystander activation of memory CD8+ T cells is insensitive to PD-1-mediated inhibition, thereby providing insight into the regulatory mechanisms of bystander-activated memory CD8+ T cells responsible for liver damage during viral hepatitis.

Is contemporary Zika getting more on your nerves?

Maïlis Darmuzey[1,2], Johan Neyts[1], Suzanne JF Kaptein[1]

1 KU Leuven Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Leuven, Belgium
2 GIGA-I3, Interdisciplinary Cluster for Applied Genoproteomics (GIGA-R), C.H.U. Sart Tilman, University of Liège, Liège, Belgium

Background and Aims: In 2015, a massive Zika virus (ZIKV) outbreak occurred in Brazil. The discovery of the vertical transmission potential of ZIKV and the ability to cause fetal brain abnormalities led to the recognition that ZIKV should be considered a major public health threat. Since the isolation in 1947, ZIKV has evolved in 2 lineages: the African and the Asian lineage. During its evolution, several amino acid changes occurred in the genome. In the Asian lineage, to which the Brazilian ZIKV strains belong, a serine (S) to asparagine (N) substitution at position 139 in the prM protein was identified, which was hypothesized to lead to an increase in neurovirulence in neonates (Yuan et al., 2017). However, our previous results with the Thailand_2014 (139S) and French_Polynesia_2013 (139N) strains did not support this hypothesis (Aubry et al., 2021). In our current study, we studied the pathogenic potential and neurovirulence of different ZIKV strains, carrying 139S or 139N, in fetal brain.

Methods: Based on our previous results, we selected a panel of 8 ZIKV strains containing either a serine or asparagine at position 139 of the prM protein (referred to as the 139S and 139N ZIKV strains, respectively). We infected immunocompetent mouse embryos intraplacentally with these ZIKV strains at embryonic day 10.5 (E10.5) and harvested them 4 or 8 days after infection (E14.5 respectively E18.5). We determined the viral load in the brain and other tissues by RT-qPCR and end-point titration. Brain development was examined by immunohistochemistry.

Results: At E14.5, 48% of the embryos infected with the 139S ZIKV strains presented subcutaneous edema against only 8% of the embryos infected with the 139N ZIKV strains (Figure). Moreover, the viral load at E14.5 was on average significantly higher in brains of embryos infected with the 139S ZIKV strains than in those infected with the 139N ZIKV strains. At E18.5, both the 139S and 139N ZIKV strains led to a microcephalic phenotype, characterized by a reduction in the cortex size and cell number. However, the 139S ZIKV-infected brains displayed a more dramatic phenotype. Interestingly, all embryos infected with the 139S ZIKV strains displayed ventriculomegaly at E18.5. In contrast, 34% of embryos infected with the 139N ZIKV strains did not develop ventriculomegaly (Figure).

Conclusions: Our results with the 139S and 139N ZIKV strains do not support the hypothesis of Yuan *et al.* Our data seem to indicate that the 139S ZIKV strains are more pathogenic and cause more severe brain abnormalities in a higher proportion of the embryos than the 139N ZIKV strains. Hence, our results suggest that the S-to-N change at position 139 in the prM protein resulted in a phenotypic attenuation of the Asian ZIKV strains and thus not in an increase in neurovirulence.

Impact of HCV genotypic variability on hepatocyte pathway deregulations in link with steatosis and HCC

Angeliki-Anna Beka[1,2], Emeline Simon[1,2], Houda Tabbal[1], Stephanie Aicher[1], Damien Batalie[1], Brigitte Blumen[1], Philippe Roingeard[3], Claire Gondeau[4] and Annette Martin[1]

[1] Institut Pasteur, Université Paris Cité, CNRS UMR3569, Paris, France; [2] Université Paris Cité, ED 562 BioSPC, Paris, France ; [3] Université François Rabelais, INSERM U966, Tours, France ; [4] Institute for Regenerative Medicine and Biotherapy, INSERM U1183, Montpellier, France

Certain HCV genotypes have been associated with higher frequencies of liver damage progression toward hepatocellular carcinoma (HCC), e.g. subtype 1b, or liver metabolic disorders, such as steatosis, an abnormal accumulation of lipid droplets in hepatocytes, e.g. genotype 3, in HCV infected patients. Several studies have pointed to the role of HCV core and NS5A proteins in the dysregulation of host cell signaling and metabolic pathways using protein overexpression systems. Using HCV infection systems, our work aims at identifying whether the genotypic origin of these proteins differentially impacts host metabolism or cancer related pathways.

To this end, we produced 17 intergenotypic recombinant viruses within the backbone of a highly infectious HCV-JFH1 (2a) derivative (Jad), which encode core sequences from prototypic or clinical isolates of genotypes 1, 2, 3 and 4. These core intergenotypic viruses proved highly and equally infectious in hepatoma cells, enabling comparative studies in infected cells. Using RNA sequencing, we performed comparative statistical analyses of infected cell transcriptomes to monitor the regulation of (i) individual gene expression levels with respect to noninfected cells, and (ii) pathways, following Gene Set Enrichment Analysis (GSEA) from lists of transcripts identified as reliably modulated by groups of viruses.

First, we found that all recombinant viruses highly impacted hepatoma cell transcriptomes with respect to noninfected cells. When comparing the transcriptomes of cells infected with 6 recombinant viruses encoding core of subtype 3a to those of cells infected with 5 viruses encoding core of either genotype 1, 2 or 4 (non-genotype-3), we found 384 highly differentially regulated genes. Upon GSEA, the majority of the pathways related to subtype 3a core were involved in glucose and lipid metabolism, such as adipogenesis, fatty acid metabolism and bile acid metabolism. Pathways more closely related to carcinogenesis, such as Wnt/beta-catenin pathway, Mitogen-Activated Protein Kinase (MAPK), Janus kinase/signal transducers and activators of transcription (JAK/STAT) and Kirsten rat sarcoma virus (KRas) signaling were found enriched by non-genotype-3 core. We further compared the transcriptomes of cells infected with Jad recombinants encoding core sequences of genotype 1 to those of cells infected with genotype 4 core recombinants and found that the majority of cancer related pathways were enriched in the genotype 1 group.

In conclusion, we show that in spite of high conservation across genotypes, HCV core proteins of various genotypic origins do differentially regulate specific host pathways in infection systems. These findings support the association of genotype 3 strains with increased steatosis rates and genotype 1 strains with higher HCC prevalence.

Contribution of the Cellular Lipid Kinase PI4KA to HCV-induced Liver Pathogenesis

Cong Si Tran, Julia Kersten, Stefan Diehl, Marco Breinig, Jingyi Yan, Gustaf Ahlén, Lars Frelin, Suzanne Faure-Dupuy, Tobias Riedl, Ombretta Colasanti, Christian Heuss, Pascal Mutz, Ralf Bartenschlager, Matti Sällberg, Mathias Heikenwälder, Tanja Poth, Darjus Tschaharganeh, Volker Lohmann

Background and Aims: Phosphatidylinositol-4-phosphate (PI4P) generated by phosphatidylinositol-4-kinase IIIα (PI4KA) plays a direct role in cellular trafficking and provides substrates for the synthesis of other phosphoinositides which are largely involved in signal transduction. Hepatitis C virus (HCV) is known to activate PI4KA. Recent studies showed elevated PI4KA expression in hepatocellular carcinoma, particularly associated with poor prognosis. Therefore changes in PI4KA activity and abundance might be a critical determinant in regulating tumor progression. The aim of this study is to unravel the contribution of PI4KA to liver pathogenesis, focusing on cytoskeletal rearrangements.

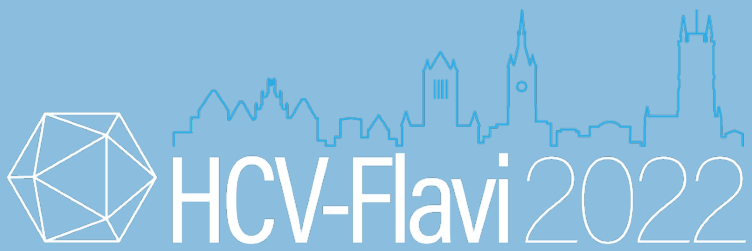
Methods: shRNA-mediated gene silencing, inhibitor treatment, infectious systems or transient expression models were applied in hepatoma cells or primary human hepatocytes (PHH).

Results: In this study we found that PI4KA silencing or inhibitor treatment in hepatoma cells induced changes in cell morphology due to reorganization of cytoskeletal structures. Phosphorylation of paxillin (p-PXN) and cofilin 1 (p-CFL1), two important regulators of the actin cytoskeleton, promoting cell migration and invasion, was reduced under these conditions, resulting in less focal adhesions and lower invasiveness. The activation of PI4KA upon HCV infection or expression of NS3-5B led to opposite phenotypes with increased p-PXN and p-CFL1, elevated numbers of focal adhesions and enhanced cell invasiveness, suggesting PI4P concentration as the driving force. Evaluation of PIP synthesis pathways revealed that silencing of PIK3C2G, a lipid kinase responsible for producing PI(3,4)P2 from PI4P, led to similar phenotypes observed in PI4KA-knockdown cells. Knockdown of PI4KA or PIK3C2G reduced PI(3,4)P2 containing podosome-like structures at cell plasma membrane, dampening AKT2 phosphorylation. HCV infection or expression in contrast stimulated AKT2 phosphorylation. Key findings were validated using immortalized hepatocytes, primary human hepatocytes and mouse models.

Conclusions: In conclusion, our data suggest that elevated PI4KA expression or activity promotes cellular pathways governing cell morphology, actin cytoskeleton dynamics and cell invasiveness, favorable for cancer progression via the signaling molecule PI(3,4)P2 and its downstream mediator AKT2. HCV therefore potentially contributes to liver pathogenesis via activating PI4KA.

Contact: CongSi.Tran@med.uni-heidelberg.de, Volker.Lohmann@med.uni-heidelberg.de

Session 9
INNATE IMMUNITY



O35 **Comparison of HAV and HCV infections in vivo and in vitro reveals distinct patterns of innate immune evasion and activation.**
Ombretta Colasanti, University of Heidelberg, Germany

O36 **An interactome study identifies a novel antiviral factor in Flavivirus infection**
Andrew Isopi, Thomas Jefferson University, USA

O37 **Proteomics approaches identify post-translational modifications that regulate Flavivirus infection**
Holly Ramage, Thomas Jefferson University, USA

Comparison of HAV and HCV infections *in vivo* and *in vitro* reveals distinct patterns of innate immune evasion and activation.

Ombretta Colasanti[1], Rani Burm[2], Hao-En Huang[1], Tobias Riedl[3] ,Suzanne Faure-Dupuy[3], Tengfeng Li[1], Nadine Gillich[4], Cong Si Tran, Marco Binder[4], Ralf Bartenschlager[1,5], Matthias Heikenwälder[4], Philip Meuleman[2], Volker Lohmann[1]

[1]Department of Infectious Diseases, Molecular Virology, Heidelberg University, [2]Laboratory of Liver Infectious Diseases, Ghent University, [3]Division of Chronic Inflammation and Cancer, German Cancer Research Center (DKFZ), Heidelberg, [4]Research Group "Dynamics of early viral infection and the innate antiviral response", Division "Virus-Associated Carcinogenesis", German Cancer Research Center (DKFZ), Heidelberg, [5]German Centre for Infection Research (DZIF), Partner Site Heidelberg

Background and Aims: Despite strong similarities in terms of biology and replication, Hepatitis A (HAV) and Hepatitis C (HCV) viruses cause opposing infection outcomes. Previous reports show that HAV does not induce an innate immune response in infected chimpanzees, in contrast to HCV (Lanford et al., 2011). This lack of response has been imputed to strong counteraction by HAV proteases 3CD and 3ABC (Yang et al., 2007; Qu et al., 2011). We aimed at elucidating these mechanisms *in vivo* and *in vitro.*

Methods: Looking for an *in vivo* model allowing a side-by-side comparison of the virus interplay with the host innate immune response, we infected SCID Alb-uPA humanised mice with patient-derived HAV and HCV. We then moved to appropriate cell culture models, in order to inquire how efficiently HAV and HCV were sensed by the endoplasmic Toll-Like-Receptor 3 (TLR3) and the cytoplasmic Rig-I-Like-Receptors (RLRs). Furthermore, we investigated potential hindering of these pathways by HAV and HCV, detecting proteolytical cleavage of the TLR3 adaptor TRIF and the RLR adaptor MAVS, through transient and stable expression of HAV and HCV viral proteases.

Results: Diverging from literature, we detected higher expression of Interferon stimulated genes (ISGs) in the hepatocytes of HAV infected humanised mice compared to the ones infected with HCV. In cell culture, we found HAV giving rise to an exclusively RLR-mediated innate immune response, whereas the reported proteolytic cleavage of TRIF and MAVS was only limited, and not sufficient to interfere with the signaling. In contrast, HCV efficiently blocked the RLR-mediated sensing, fully cleaving MAVS. However, we found no TRIF cleavage by HCV, but a moderate induction of TLR3. Here, a partial escape mechanism relies on dsRNA secretion, as previously shown (Grünvogel et al., 2018).

Conclusions: Our data indicates that HAV triggers an innate immune response, *in vivo* and *in vitro*, in systems which are devoid of adaptive immunity, and clarifies persistence versus clearance mechanisms in HCV and HAV infections.

An interactome study identifies a novel antiviral factor in Flavivirus infection

Andrew Isopi[1#], Minghua Li[2#], Mark Dittmar[2], Jeff Johnson[3,] Sara Cherry[2*], Holly R. Ramage[1*]

[1]Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, USA ; [2]Department of Pathology, University of Pennsylvania, Philadelphia, USA; [3]Department of Microbiology, Icahn School of Medicine at Mt. Sinai, New York, USA; [#] Co-first authors; *Co-corresponding authors

Background and Aims: During infection, flaviviruses utilize the host cell machinery to carry out replication and subvert cellular defenses. Identification of the host cellular proteins and complexes that physically associate with viral proteins greatly improves our understanding of both viral infection and host antiviral mechanisms. Previously, we combined an affinity purification-mass spectrometry approach (AP-MS) with genetic screening to find host factors that physically interact with flavivirus proteins and influence infection. We identified DDX55, a DEAD-box helicase protein, which interacts with flavivirus capsid and is antiviral during infection. We aimed to define the antiviral mechanism of DDX55 and characterize the importance of the capsid-DDX55 interaction.

Methods and Results: We performed co-immunoprecipitation and immunofluorescence assays to validate the interaction between DDX55 and the capsid protein of West Nile (WNV), Dengue (DENV) and Zika (ZIKV) virus. Next, we treated cells with siRNAs targeting DDX55 and non-targeting controls followed by infection with WNV, DENV or ZIKV. Depletion of DDX55 resulted in increased viral RNA, as measured by quantitative RT-PCR (qRT-PCR) and enhanced infectious virus production from infected cells. Together, these data suggest that DDX55 is antiviral in flavivirus infection and we sought to define this antiviral mechanism. Using crosslinking and immunoprecipitation, we demonstrated that DDX55 interacts with flavivirus genomic RNA. Further, we used reporter constructs to show that DDX55 binds the highly structured flavivirus genomic 3` untranslated region. We infected siControl or siDDX55-treated cells and inhibited viral replication using the inhibitor MK-0608. Next, we used qRT-PCR to measure viral RNA abundance over time and observed increased viral RNA stability in DDX55-depleted cells. These data suggest that the interaction with DDX55 leads to degradation of viral RNA. Finally, we show that the interaction of DDX55 with viral RNA is disrupted in the presence of flavivirus capsid.

Conclusion: Our data suggest a model in which DDX55 is an antiviral factor that binds to viral RNA, resulting in degradation. We propose that the interaction between DDX55 and capsid antagonizes this antiviral function. Together our data demonstrate that proteomic techniques are a powerful tool to uncover novel mechanisms regulating flavivirus infection.

Presentation by Holly Ramage.

Proteomics approaches identify post-translational modifications that regulate Flavivirus infection

Minghua Li[2], Zachary Walter[1], Andrew Isopi[1], Chike Nwaezeapu[1], Sara Cherry[2], Jeffrey Johnson[3], Holly Ramage[1]

[1]Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia USA; [2]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia USA; [3]Department of Microbiology, Icahn School of Medicine, Mt. Sinai, New York USA

Background and Aims: During infection, viruses manipulate host pathways to replicate while evading immune recognition. Little is known about the changes in abundance and post-translational modifications (PTMs) on viral and cellular proteins during pathogenesis. We examined these changes by comparing the proteomes of West Nile virus (WNV)-infected and uninfected cells to identify mechanisms regulating infection.

Methods and Results: Using quantitative mass spectrometry, we identified 115 host proteins with altered abundance during WNV infection. This included a subset of known interferon-induced genes (ISGs); however, our results demonstrate that the majority of the increased non-ISGs are not regulated at the level of transcription. This data suggests that the integration of proteomic and transcriptomic data may provide a more complete understanding of the antiviral programs induced during infection. Next, we identified ~700 host protein phosphorylation sites that are altered during WNV infection. Further analysis to pinpoint changes that likely affect enzymatic activity led to our discovery that two kinases, AMPK and PAK2, are activated during infection to restrict WNV replication. We also identified ~500 ubiquitylation sites that were induced upon infection. There was an increase in ubiquitylation on proteins involved in ER-associated degradation (ERAD), a pathway that is required for WNV, and other flaviviral infections. We found that core ERAD components interact with WNV NS2b/3, suggesting that these proteins are both physically engaged by the virus and modulated during infection. Finally, we analyzed our data to identify sites of modification on viral proteins during infection. We discovered several novel phosphorylation sites in both structural and non-structural viral proteins. Using a WNV replicon system, we found two phosphorylation sites in WNV NS5 that negatively regulate replication.

Conclusions: Altogether, our proteomics data reveal a complex layer of regulation of viral and host protein function via translational and post-translational mechanisms. Future work will focus on deciphering the mechanism by which PTMs affect protein function and viral infection. Moreover, we will determine whether these modifications are specific to WNV infection, are conserved in infection with other flaviviruses and/or are mediated by Type I interferon signaling.

Session 1
VIRAL ENTRY

28TH INTERNATIONAL SYMPOSIUM ON HEPATITIS C VIRUS, FLAVIVIRUSES, AND RELATED VIRUSES

GHENT • ICC

JULY 6-9, 2022

P1 **Identification of SLC35F5 as a Hepatitis E Virus Entry Factor Candidate based on Protein Microarray Analyses**
Volker Kinast, Carl von Ossietzky University Oldenburg and Ruhr-University Bochum, Germany

P2 **The role of complement regulatory protein CD46 as molecular determinant in pestiviral entry**
Alexander Postel, University of Veterinary Medicine Hannover, Germany

P3 **Generation of a chimeric Zika virus expressing Powassan virus prM and E proteins and investigating cellular tropism of circulating Powassan viruses**
Sydney Majowic

P4 **Hepatitis E virus capsid labeling by genetic code expansion to study cell entry**
Rebecca Fu

P5 **Disintegrin and metalloproteinase domain-containing protein 10 is a co-factor for Hepatitis C virus entry**

"Belen Carriqui Madronal"

P6 **TLR4 plays an important role in TMUV proliferation which is enhanced by bacterial LPS**
Wu Zhen

P7 **Low density hepatitis C virus infectious particles are protected from oxidation by secreted cellular proteins**

François-Loïc Cosset

P8 **Infection of polarized bovine respiratory epithelial cells by bovine viral diarrhea virus (BVDV)**
Ang Su

P9 **Characterization of E1/E2 transmembrane domain swap chimeras of hepatitis C virus**
Margherita Fanalista

P10 **Scavenger receptor class B type I expression and lipoproteins modulate the cluster of differentiation 81-protein interactome**
Alina Matthaei

Identification of SLC35F5 as a Hepatitis E Virus Entry Factor Candidate based on Protein Microarray Analyses

Volker Kinast[1,2], Henning Boekhoff[3], George Ssebyatika[4], Thilo Bracht[2], Martin Paetz[3], Florian WR Vondran[5], Ruth Bröring[6], Daniel Todt[2], Barbara Sitek[2], Patrick Behrendt[7], Thomas Krey[4], Jörg D Hoheisel[3], Eike Steinmann[2]

[1]Department of Medical Microbiology and Virology, Carl von Ossietzky University Oldenburg, Oldenburg, Germany; [2]Ruhr-University Bochum, Faculty of Medicine, Department of Molecular and Medical Virology, Germany; [3]Division of Functional Genome Analysis, DKFZ, Heidelberg, Germany; [4]Institute of Biochemistry, Center of Structural and Cell Biology in Medicine, University of Luebeck, Luebeck, Germany; [5]Regenerative Medicine and Experimental Surgery (ReMediES), Department of General, Visceral and Transplantation Surgery, Hannover Medical School, Hannover, Germany; [6]Department of Gastroenterology and Hepatology, Institute of Virology, University Hospital of Essen, Germany; [7]Institute for Experimental Virology, TWINCORE Centre for Experimental and Clinical Infection Research, a Joint Venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Hannover, Germany

Background: The presence of entry factors on host cells critically determines the tropism and pathogenesis of viruses. For many - especially emerging - viruses such as hepatitis E virus (HEV) the entry processes remain obscure. In this project we currently establish a novel approach based on interaction of intact HEV virions and *in situ*-translated membrane-associated host proteins allowing to rapidly identify entry factor candidates.

Methods: PCR products encoding 908 cell surface-associated proteins were spotted on epoxysilane-coated slides followed by *in situ* transcription and translation to generate a cell surface protein microarray. HEV virions and purified HEV ORF2 capsid proteins were incubated on the microarray and specific protein-protein interactions were detected by HEV-specific antibodies and Cyanine-labeling of purified proteins. Follow up studies of one entry factor candidate were performed by utilizing virological, biochemical and genetic state-of-the-art techniques.

Results: Cell surface protein microarray analyses uncovered significantly enriched binding of HEV virions and purified HEV ORF2 capsid proteins to 22 and 38 cell surface proteins, respectively, including an overlap of eight proteins. Amongst them was SLC35F5, an ubiquitous expressed predicted transporter with unknown ligand and function. Ectopic expression of SLC35F5 revealed increased susceptibility to HEV infection without affecting viral replication. In contrast, both RNA interference and CRISPR/Cas9-mediated gene editing of SLC35F5 hampered HEV infection in hepatoma cells.

Conclusion: Here we provide a potential novel microarray-based platform enabling to rapidly identify viral entry factor candidates. First evidence suggests that SLC35F5 physically interacts with HEV virions and play an important role for the HEV entry process. In future the rapidity and straightforward application of this array may allow to screen multiple viruses in parallel helping to identify multiple novel viral entry factors.

The role of complement regulatory protein CD46 as molecular determinant in pestiviral entry

Alexander Postel[1], Elena Leveringhaus[1], Gökce Nur Cagatay[1, 2], Paul Becher[1]

[1]Institute of Virology, University of Veterinary Medicine, Hannover, Germany; [2] now Coriolis Pharma Research GmbH, Martinsried, Germany

Background and Aims: Complement regulatory protein CD46 is used by different pathogens as a receptor to enter their host cells, comprising very distinct viruses like adenoviruses, herpesviruses, some measles virus strains, and the bovine viral diarrhea virus (BVDV). BVDV belongs to the genus *Pestivirus* within the family *Flaviviridae.* Pestivirus infections are of outstanding economic relevance as causing severe diseases or reproductive disorders in livestock. Aim of the study was to unravel the role of CD46 as molecular determinant in the entry mechanism of different pestiviruses.

Methods: CRISPR/Cas9 technology was applied to generate different porcine and bovine CD46 knockout cells. In addition, CD46 specific antibodies were used to block the receptor on bovine MDBK cells. Rescue of MDBK knockout cells was performed by lentivirus-mediated expression of CD46. Different established porcine and bovine cell lines as well as the generated genetically engineered cell lines were characterized with regard to their expression of CD46 and their permissivity to porcine and bovine pestiviruses belonging to seven different pestivirus species.
Results: In contrast to what was supposed previously, it turned out that Classical swine fever virus (CSFV, *Pestivirus C*) and Bungowannah pestivirus (BuPV, *Pestivirus F*) can efficiently infect porcine cells in the absence of porcine CD46. Simultaneous blocking of putative viral Heparan sulfate (HS) binding sites did not diminish the high permissivity of the CD46 knockout cells. Thus, unspecific viral binding to the host cell via HS, as seen in cell culture adapted strains, did not occur. Entry of a more recently discovered porcine pathogen designated Atypical porcine pestivirus (APPV, *Pestivirus K*) turned out to be highly dependent on CD46 like known for BVDV-1 (*Pestivirus A*) and BVDV-2 (*Pestivirus B*). A non-cell culture adapted HoBi-like pestivirus (*Pestivirus H*) isolated within this study revealed to own the same dependence on bovine CD46. Members of the species *Pestivirus G* can efficiently replicate on bovine cells and revealed to be able to enter these cells by using HS. Interestingly, a closely related non-culture adapted strain of this species demonstrated to enter bovine cells in a HS and CD46 independent manner.
Conclusions: CD46 is used as a major factor during entry by diverse pestiviruses. In addition to BVDV-1 and BVDV-2, this study demonstrated that also entry of bovine HoBi-like pestiviruses as well as porcine APPV is highly dependent on CD46. In addition, our data revealed that there must be an unknown entry mechanism independent on CD46 and HS that is used by CSFV and members of several distinct pestivirus species. Future research will focus on the identification of the so far unknown receptor to understand the molecular mechanism of pestiviral entry.
This work was funded by the Deutsche Forschungsgemeinschaft under project number 427829762.

Generation of a chimeric Zika virus expressing Powassan virus prM and E proteins and investigating cellular tropism of circulating Powassan viruses

Sydney A Majowicz, Anoop Narayanan, Aswathy Sebastian, Istvan Albert, Kurt J Vandegrift, and Joyce Jose

Penn State, University Park, PA, USA

Powassan virus (POWV) is a tick-borne flavivirus that causes neuroinvasive disease. There has been elevated detection of POWV; consequently, human cases are increasing. There are no antivirals or vaccines specific to POWV. Defining the mediators of POWV tropism may aid in development of therapeutic antiviral strategies. Several cell-specific receptors have been implicated for mosquito-borne flaviviruses, however, less is known for tick-borne flaviviruses. We aim to identify the mediators of entry and cell specificity for POW viruses; POWV Lineage I (POWV I) and POWV Lineage II (POWV II).

Strains of POWV II were isolated from black-legged ticks (*Ixodes scapularis*) collected in Pennsylvania and growth kinetics and tropism were analyzed in mammalian and insect cell lines (*Vero E6*, *BHK 21*, *HEK 293T*, *Huh 7,5*, and *C636*). Prototype strains of POWV I (LB) and POWV II (Spooner) were included in our analyses. We identified small, medium and large plaque forming isolates which were characterized via sequencing technologies. We generated a chimeric cDNA of Zika virus (ZIKV) and POWV II by substituting the prM and E proteins from ZIKV with those of POWV, facilitating a BSL-2 platform for studying the role of amino acid variations in virus attachment and entry. For site directed mutagenesis, surface-exposed E residues were identified in structural comparisons between tick-borne and mosquito-borne flaviviruses and additional amino acids were selected based on sequence alignment. Over 20 mutant cDNAs were evaluated for ability to produce infectious virus. We further characterized defects in replication and entry using immunofluorescence assay, western blot and qRT-PCR. Mutations critical to entry and virus attachment were made on a cDNA encoding full length POWV II. Full length mutants were analyzed in BSL-3 where viral titer in multiple cell lines was determined. We show that two sets of mutations on E protein, changing from POWV surface exposed amino acid residues to that of ZIKV, inhibit entry. These mutations, NENRK to GYETD at positions E 157-161 and EK to PR at E 170-171, inhibit cell entry. They may interact with cell-type specific receptors required for viral entry and infection. Our chimera provides a novel platform for analysis of POWV structural glycoproteins in a BSL-2 lab setting, guiding more targeted BSL-3 experiments. We are first to report and identify surface-exposed POWV residues critical to receptor-mediated entry and cell-line specificity.

Hepatitis E virus capsid labeling by genetic code expansion to study cell entry

Jun-Gen Hu[1*], Rebecca Fu[1*], Viet Loan Dao Thi[1, 2]

[1]Schaller Research Group, Department of Infectious Diseases, Virology, University Hospital Heidelberg, Heidelberg, Germany, [2]German Centre for Infection Research (DZIF), Partner Site Heidelberg, Heidelberg, Germany.

Background and Aims: Hepatitis E virus (HEV) is a major causative agent of acute fulminant hepatitis. HEV is a non-enveloped virus (nHEV) but gains a membranous envelope from the host cell when budding from cells. This form of the virus is called quasi-enveloped HEV (eHEV). While nHEV is ingested and shed into feces for transmission to another host, eHEV is found circulating in the blood of infected patients. While eHEV entry was shown to depend on endocytic pathways, post-internalization routes of nHEV remain to be determined. In addition, an HEV cell entry receptor has not been identified yet. Integrin αlpha 3 (ITGA3), a cellular membrane protein which is internalized through clathrin-mediated endocytosis, was shown to be involved in nHEV entry. The corresponding β integrin partner that co-facilitates entry with ITGA3 was not described. Here, we want to study and describe nHEV entry and post-internalization pathways.

Methods and Results: We generated a range of integrin α and β knockout cell lines. Together with the application of specific inhibitors and peptides that block integrins, we observed moderate effects on nHEV infection and identified the heterodimer integrin α2β1 as a potential entry factor. In agreement, we used endosomal acidification inhibitors and found that nHEV infection was significantly reduced. In order to corroborate our findings, we want to track HEV particles during entry in real time. We implemented a minimally invasive HEV capsid ORF2 labelling strategy based on genetic code expansion, allowing the incorporation of a non-canonical amino acid with biorthogonal activity, followed by click-labeling with a fluorescent dye. We identified positions in the capsid protein ORF2 that can be Amber suppressed to yield functional ORF2 as evidenced by the detection of full length ORF2 and the formation of infectious nHEV and eHEV particles. We are currently optimising the click labelling process in order to use these particles for our studies. Click-labelled particles will be imaged in conjunction with established cell lines expressing GFP-tagged endosomal markers in co-localisation studies using high resolution microscopy. In addition, we are complementing capsid visualisation by a highly sensitive fluorescent in situ hybridization (FISH) approach against the HEV genome to describe post-entry events. Preliminary analysis showed that nHEV entry completes around 6 hrs post-internalization, suggesting a rather slow process.

Conclusion: Our efforts should lead to a better understanding of nHEV cell entry. Once established, we will also use the established biochemical and imaging pipelines to study eHEV entry.

Disintegrin and metalloproteinase domain-containing protein 10 is a co-factor for Hepatitis C virus entry

Belén Carriquí-Madroñal[1,2], Lisa Lasswitz [1,2], Francisco J. Zapatero-Belinchón[1,2,3,4], Gisa Gerold[1,2,3,4]

[1] Institute for Biochemistry & Research Center for Emerging Infections and Zoonoses. University of Veterinary Medicine Hannover, 30559 Hannover, Germany
[2] Institute for Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research, 30625 Hannover, Germany
[3] Department of Clinical Microbiology, Virology, Umeå University, SE-90185 Umeå, Sweden
[4] Wallenberg Centre for Molecular Medicine (WCMM), Umeå University, SE-90185 Umeå, Sweden

Background and Aims: Hepatitis C virus (HCV) entry requires four host factors: scavenger receptor class B type I (SR-BI), CD81, claudin-1 and occludin. Another key factor involved in post binding events of HCV entry is the epidermal growth factor receptor (EGFR). One of the EGFR transactivation mechanisms implies matrix metalloproteinases and more specifically the disintegrin and metalloproteinase (ADAM) family. Previous studies described ADAM10 as the main sheddase of EGF with a key role in regulating EGFR transactivation. By quantitative affinity enrichment proteomics, we identified ADAM10 and EGFR as interaction partners of CD81. Here, we aim to assess the role of ADAM10 in HCV infection.

Methods: To assess the impact of ADAM10 on HCV entry, we knocked down ADAM10 mRNA using a combination of three siRNAs in human hepatoma cells. In parallel, we pharmacologically inhibited ADAM10 protease activity. We then generated an ADAM10 knockout hepatoma cell line using the CRISPR/Cas9 technique. The knock down and knockout efficiency was assessed by flow cytometry. Treated or edited hepatoma cell lines were infected using cell culture derived HCV (HCVcc). We also tested the impact of ADAM10 inhibition on EGFR activation by flow cytometry quantification of phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2), a downstream effector protein phosphorylated upon EGFR activation.

Results: Inhibition, silencing or editing of ADAM10 lowers its cell surface levels. Upon ADAM10 inhibition, RNA silencing and knockout, we observe reduced susceptibility to HCVcc (Figure 1). We also show that inhibition of ADAM10 causes a decrease in the intracellular levels of phosphorylated ERK1/2 indicative of reduced EGFR signalling.

Conclusions: Our study identifies ADAM10 as a component of the HCV entry complex including CD81, SR-BI and EGFR and as entry co-factor, promoting HCV uptake by activation of EGFR signaling.

TLR4 plays an important role in TMUV proliferation which is enhanced by bacterial LPS

Zhen Wu [a, b], Anchun Cheng [a], Shun Chen [a, *]

a. Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China. ; b. KU Leuven Department of Microbiology, Immunology and Transplantation, Rega Institute, Laboratory of Virology, Molecular Vaccinology and Vaccine Discovery, Leuven, Belgium.; * Correspondence addressed to: shunchen@sicau.edu.cn

Background and Aims: Egg drop disease syndrome, which is caused by Tembusu virus (TMUV), has emerged as a major Asian avian health problem during the last decade. Under natural conditions, flaviviruses are mainly transmitted by arthropods, such as mosquitoes and ticks, but based on an analysis of the prevalence of avian flavivirus, TMUV, temperature changes are not consistent with other flaviviruses transmitted by mosquitoes, and animals are still infected during the cold season. TMUV transmitted through airborne and direct contact implies that the virus and commensal microbes have more interplay in the respiratory and digestive tracts during infection than mosquito-borne viruses. However, it is unclear whether bacteria and their products may be interacting directly with TMUV to impact virion replication.

Method: Using the RNA interference, an anti-TLR4 antibody treatment and a TMUV single-round infection virus particle model, the TLR4 protein, which serves as a TMUV entry cell determining factor, was uncovered. Using an established TMUV disease model by oral gavage combined with an antibiotic treatment, we revealed that a decrease in cultural intestinal bacteria significantly reduced local TMUV proliferation in the small intestine.

Results: We can conclude the following several aspects: 1. TMUV binds LPS receptors competitively with LPS, thereby inhibiting host inflammatory signal transduction; 2. LPS promotes TLR4-mediated TMUV attachment; 3. The knockdown of TLR4 expression reduces the ability of LPS to promote virus adsorption; and 4. reduced LPS sources and intestinal commensal bacteria decrease viral proliferation in vivo (Fig. 1).

Conclusions: TLR4 served as the determining factor for TMUV attachment and is uncovered for the first time. We propose a model that can explain the complex and important role played by LPS and TLR4 in modulating TMUV infection. More importantly, the role of LPS in flaviviruses infection is highlighted. Especially in flaviviruses transmit through the air and the digestive tract.

Low density hepatitis C virus infectious particles are protected from oxidation by secreted cellular proteins

Chloé Mialon[1#], Christelle Granier[1#], Johan Toesca[1#], Maureen Ritter[1], Natalia Freitas[1], Thomas Bourlet[1], Eve-Isabelle Pécheur[2], François-Loïc Cosset[1$], Solène Denolly[1$].

[1]CIRI – Centre International de Recherche en Infectiologie, Univ. Lyon, Université Claude Bernard Lyon 1, Inserm, U1111, CNRS, UMR5308 ENS de Lyon, F-69007, Lyon, France.

[2]Univ Lyon, Université Claude Bernard Lyon 1, CNRS 5286, INSERM 1052, Centre Léon Bérard, Centre de Recherche en Cancérologie de Lyon, 69008, Lyon, France.
 # co-first authors; $ co-last authors

Background and Aims*:* HCV particles secreted from cells are stable at 37°C, whether the producer cells media contain serum or not. Yet, we found that intracellular HCV particles harvested after freeze-thawing of producer cells are highly unstable upon resuspension in a serum-free medium, indicating that either HCV particles gain intrinsic stability during their secretion and egress from producer cells or, alternatively, that a factor secreted from cells can stabilize intrinsically unstable HCV particles. Here, we aimed at investigating either possibility and at studying the mechanism of stabilization of HCV particles.

Methods and Results*:* We showed that after purification via ultracentrifugation and resuspension in a serum-free medium, secreted HCV infectious particles are quickly and specifically degraded at 37°C, in comparison to other hepatotropic viruses. We also found that cell-secreted factors including human serum albumin and transferrin could protect particles from this loss of infectivity. Moreover, we showed that such protection mainly impacted low-density particles (d<1.10), suggesting a specific alteration of lipidated particles. Indeed, since we demonstrated that neither HCV RNA nor surface glycoproteins were altered, we concluded that virion lipids are sensitive to oxidation, resulting in a loss of infectivity. Our results further indicate that HCV particles are sensitive to oxidation, leading to a loss of their fusion capacity.

Conclusions: Altogether, our results indicate that HCV is highly sensitive to oxidation and highlight a specific protection mechanism evolved by HCV to prevent oxidation-mediated degradation of its lipidated particles by using proteins secreted by hepatocytes.

Infection of polarized bovine respiratory epithelial cells by bovine viral diarrhea virus (BVDV)

Ang Su[1], Yuguang Fu[2], Jochen Meens[3], Wei Yang[1,4], Fandan Meng[1,5], Georg Herrler[1*†] and Paul Becher[1*†]

[1] Department of Infectious Diseases, Institute of Virology, University of Veterinary Medicine Hannover, Foundation, Hannover, 30559, Germany; Ang.Su@tiho-hannover.de (A.S.); yangwei0727@hotmail.com (W.Y.); Georg.Herrler.iR@tiho-hannover.de (G.H.); Paul.Becher@tiho-hannover.de (P.B.); [2] State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China; fuyuguang@caas.cn (Y.-G.F.); [3] Institute of Microbiology, University of Veterinary Medicine Hannover, Foundation, Hannover, 30559, Germany; Jochen.Meens@tiho-hannover.de (J.M.) ; [4] College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, China; [5] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China; mengfandan11111@hotmail.com (F.-D.M.); [†] Paul Becher and Georg Herrler contributed equally to this work

Bovine viral diarrhea virus (BVDV) which belongs to the genus *Pestivirus* within the family *Flaviviridae* is affecting cattle populations all over the world. Infection by BVD virus causes immunosuppressive effects, severe respiratory diseases, gastrointestinal and reproductive failure in cattle which may result in dramatic economic losses in the farming system. Very little is known about the initial stage of infection, especially about the mechanism how BVD virus overcomes the epithelial barrier in the airways. This information is important to understand how the virus spreads to the blood circulation. In order to investigate how BVDV infects the respiratory tract, we established polarized but not differentiated culture systems for bovine airway epithelial cells (BAEC). Polarized airway cells that had not yet differentiated into specialized BAEC were susceptible to infection by BVDV. Infection was most efficient, when the virus was applied to the basolateral plasma membrane of the polarized airway cell culture compared to infection via the apical compartment. CD46 is the one known receptor for BVDV infection. Here, we found that CD46 was preferentially expressed on the apical surface of polarized airway epithelial cells. In CD46 blocking assay, CA17 can block BVDV infection well while it can only block the basolateral infection partially by BVDV.

More research need to be facilitated that how BVDV could overcome the epithelial barrier and to spread to the submucosal cell layers

[*]Correspondence: Paul.Becher@tiho-hannover.de; Gerog.Herrler@tiho-hannover.de

Characterization of E1/E2 transmembrane domain swap chimeras of hepatitis C virus

Margherita Fanalista[1*], Christina Holmboe Olesen[1*], Jens Bukh[1], Jannick Prentoe[1]

[1]Copenhagen Hepatitis C Program (CO-HEP), Copenhagen University Hospital, Hvidovre and University of Copenhagen, DK; [*]Authors contributed equally

Background and Aims: Hepatitis C virus (HCV) E1 and E2 glycoproteins form a heterodimer, E1/E2, and are essential mediators of HCV entry into hepatocytes as well as an important vaccine target. The C-terminal transmembrane domain of E1 (TME1) and E2 (TME2), exhibit protein-protein intramembrane interaction motifs which, together with the ectodomains, form the heterodimerization interface of E1/E2. However, given the lack of a structural model, TM assembly dynamics remain largely unknown. The aim of the study was to probe TME1 and TME2 interaction in the context of infectious HCV particles to provide novel insights on the E1/E2 interface.

Methods: We generated HCV recombinants, in which either TME1, TME2 or both (TME1E2) of the H77 (gt 1a) sequence were swapped into JFH1-based HCV recombinants with Core-NS2 from isolates TN(1a), J6(2a), S52(3a), ED43(4a) or SA13(5a). HCV RNA transcripts were transfected into Huh7.5 cells and supernatants collected every 24 hours. Viral particles from the collected supernatants were quantified by 48h infection of Huh7.5 cells and titration of HCV focus forming units. The attenuated S52 TME1 swapped recombinants were adapted in Huh7.5 cells and when virus spread to ~80% of the cells, HCV RNA was extracted, and the envelope protein sequences determined.

Results: H77 TME2 had no effect on SA13 infectivity, whereas TME1 caused attenuation that was rescued for TME1E2, supporting a model of TM interactions that is independent of ectodomain interactions. However, H77 TME1 swap also attenuated TN, whereas swapping TME2 and TME1E2 completely attenuated the virus. Yet a third pattern was observed for J6, S52 and ED43, for which swapping TME1, TME2 or TME1E2 all lead to dramatic attenuation. Adapting $S52_{H77\text{-}TME1}$ in cell culture (both S52 with TME2 or TME1E2 were non-viable) lead to the emergence of putative adaptive mutations, which could potentially restore virus infectivity. F345S/C in the E1 stem was found for both adapted $S52_{H77\text{-}TME1}$ cultures suggesting a fundamental role of the ectodomain in TME1E2 interactions.

Conclusions: The H77 TME1, TME2 and TME1E2 swapping into different HCV recombinants had a broad range of effects on virus infectivity, suggesting isolate or genotype-specific TME1E2 interactions. The results demonstrate that TME1E2 interactions were generally not independent from E1/E2 ectodomain interactions with a potential specific role of the E1 stem and with general implications for understanding E1/E2 heterodimerization and the contribution of TMs in rational vaccine design.

Scavenger receptor class B type I expression and lipoproteins modulate the cluster of differentiation 81-protein interactome

Alina Matthaei[1], Janina Bruening[1], Dorothea Bankwitz[1], Birthe Reinecke[1], Mandy Doepke[1], Richard Brown[2], Chris Lauber[1], and Thomas Pietschmann[1#]

[1]Institute for Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research; a joint venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Hannover, Germany; [2]Division of Veterinary Medicine, Paul Ehrlich Institute, 63225 Langen, Germany

Background and aims: Hepatitis C virus (HCV) cell entry is a complex process involving numerous virus-host factor interactions, viral trafficking to tight junctions, internalization and escape from endosomes. The tetraspanin cluster of differentiation (CD)81, the high-density lipoprotein (HDL) receptor scavenger receptor class B type I (SR-BI) and the tight junction proteins Occludin (OCLN) as well as Claudin1 (CLDN1) are essential HCV entry factors. Lipoproteins, such as HDL and oxidized low-density lipoproteins (oxLDL), up- and downregulate infection efficiency, respectively. However, it is not clear how these factors orchestrate cell entry, and if lipoproteins influence HCV cell entry via remodeling of protein-protein interaction networks of key entry factors. Here, we aimed to dissect the influence of SR-B1 and lipoproteins on the CD81 protein-protein interactions.

Methods: We established work flows for label-free quantitative mass spectrometry analysis of the CD81-interactome to define the CD81 interaction partners in primary human hepatocytes (PHH) and Huh-7.5 cells. We analyzed CD81 interaction partners in mock treated or SR-B1 silenced Huh-7.5 cells. We pre-treated Huh-7.5 cells with lipoproteins and analyzed changes of the CD81 interactome, used RNA interference and inhibitor treatment of hepatoma cells to explore the functional relevance of CD81-binding proteins.

Results: The steady state CD81 proteome was similar to previously published studies and included 24 proteins. Ten of these proteins, including SR-B1, were described as CD81-interactors in previous studies. Silencing of SR-B1 decreased the diversity of the CD81 interactome, with only solute carrier family 44 member 1 (SLC44A1) remaining. Treatment with oxLDL reduced the number of CD81 protein-protein interactions, whereas HDL treatment changed CD81 interaction partners. Modulation of SLC44A1 expression by RNA interference and treatment with the inhibitor hemicholinium-3 suggest that SLC44A1 influences HCV cell entry.

Conclusions: These data suggest that SR-B1 expression influences the CD81 interaction network and that lipoproteins modify CD81 protein-protein interactions. Additionally, virus particle-associated lipoproteins may change CD81 interactions, thus mobilizing the protein for subsequent HCV cell entry stages.

Session 2
ADAPTIVE IMMUNITY

28TH INTERNATIONAL SYMPOSIUM ON HEPATITIS C VIRUS, FLAVIVIRUSES, AND RELATED VIRUSES

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Broadly neutralizing human monoclonal antibodies targeting the Hepatitis E virus ORF2 protein

George Ssebyatika[1], Katja Dinkelborg[2,4], Luisa J Ströh[3], Elina Muriel Guzman[1], Lucas Hüffner[2], Prossie Lindah Nankya[1], Heiner Wedemeyer[4,5], Thomas Pietschmann[2,5,6], Patrick Behrendt[2,4,5], Thomas Krey[1,3,5,6,7]

1 Center of Structural and Cell Biology in Medicine, Institute of Biochemistry, University of Lübeck, Germany. ; 2 TWINCORE, Centre for Experimental and Clinical Infection Research, a Joint Venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Hannover, Germany; 3 Institute of Virology, Hannover Medical School, Hannover, Germany; 4 Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Germany; 5 German Center for Infectious Disease Research (DZIF); Partner Sites Hannover-Braunschweig and Hamburg-Lübeck-Borstel-Riems, Germany; 6 Excellence Cluster 2155 RESIST, Hannover Medical School, Hannover, Germany; 7 Centre for Structural Systems Biology (CSSB), Hamburg, Germany

Background and Aim: Hepatitis E virus (HEV) is a positive sense single stranded RNA virus that exists as quasi-enveloped species in blood or naked particles in bile and feces. It is the leading cause of acute viral hepatitis worldwide with 20 million infections estimated to occur annually (WHO HEV Fact sheet, 2021). While mostly asymptomatic and self-limiting in healthy individuals, HEV infection may cause acute liver failure in pregnant women and chronic hepatitis in immunosuppressed individuals. To date there is no approved antiviral therapy available, except the off-label application of ribavirin to treat chronic HEV infection, which is limited due to the associated side effects and treatment failure. Therefore, safe and effective alternative strategies to treat HEV infections in vulnerable patient groups are urgently needed.
Here, we identified human neutralizing anti-HEV monoclonal antibodies (mAb) that constitute a novel therapeutic option for treatment of HEV-infected patients.

Methods: We isolated and sequenced HEV pORF2-specific memory B cells from HEV convalescent individuals using multi-colour FACS. Antibody fragments were expressed in insect cells and their neutralization potential tested in a GT3 based cell-culture infection model. The best antibodies were further analysed virologically and biochemically as intact IgG molecules, and structurally characterized in complex with a HEV gt3 P-domain.

Results: Overall, > 10% of the selected antibodies potently inhibited (IC_{50} < 0,5 µg/ml) HEV infection in a dose-dependent manner in vitro. The most potent nAbs cross-reacted with P-domains from the 4 human-infective genotypes as well as a rat HEV P-domain, suggesting broad reactivity. A fraction of these bnAbs recognized exclusively a non-glycosylated P-domain, but not a secreted, glycosylated P-domain, demonstrating a glycan-sensitive binding mode. Crystal structures of relevant P-domain/antibody complexes revealed binding of the glycan-sensitive bnAbs to a conserved conformational epitope located within antigenic site C3 at the tip of the P-domain dimer. Importantly, the identified epitope explained the observed glycan sensitivity and indicated that these antibodies are able to overcome the putative decoy role of the highly abundant, glycosylated pORF2 dimer to block antibody-mediated neutralization.

Conclusion: We have identified and expressed human antibodies that broadly neutralize HEV infection. Our most potent bnAbs target a conserved, glycan-sensitive conformational epitope at the tip of the P-domain. Based on recent experiences with e.g., SARS-CoV-2 or Ebola virus, our human mAbs may constitute a promising therapeutic option for treatment of HEV infection.

Contribution of HEV soluble ORF2 protein to viral replication and host immune response

Philipp Ralfs[12], Brantley Holland[1,2], Eduardo Salinas[1,2], Jingting Zhu[3], Charuta Ambardekar[3], Bill Bremer[3], Heather Blasczyk[3], Minghang Wang[3], Christopher M. Walker[3], Zongdi Feng[3], and Arash Grakoui[12]

1Emory University School of Medicine, Emory University, Atlanta, GA 30329; 2Yerkes National Primate Research Center, Atlanta, GA 30329; 3Abigail Waxner Research Center, Nationwide Children's Hospital, Columbus, OH 43205

Background and Aims: The hepatitis E virus (HEV) is a small positive sense RNA virus that is a major cause of acute viral hepatitis globally. HEV encodes two forms of capsid protein: a cytoplasmic form (ORF2c) is essential for virion structure, and a secreted glycosylated form (ORF2s) that accumulates at high titer in serum and can mask neutralizing epitopes. Here, we explored the contribution of ORF2s to HEV replicative fitness, and its role in generating anti-ORF2 antibodies and T cell responses during primary and secondary infections in a nonhuman primate model.

Methods: Rhesus macaques (RM) were challenged by intrahepatic inoculation of infectious wildtype HEV (ORF2swt) RNA and a variant lacking ORF2s expression (ORF2smut). HEV faecal viral shedding and HEV-specific antibody and T-cell responses in the blood of infected RMs were analyzed. In addition, RMs were re-challenged with ORF2swt to analyse the protective immune responses elicited by ORF2smut infection.

Results: The replication of ORF2smut virus was delayed by approximately 2 weeks when compared with ORF2swt and peak titers were nearly 10-fold lower. No reversion of the three ORF2s silencing mutations was detected in the ORF2smut genomes, indicating genetic stability. The delay in replication and lower peak titer was unexpected as the viruses replicate similarly in cell culture. However, serum anti-ORF2 antibodies were transiently detected in ORF2smut infected RM, whereas they were abundant and long-lasting in ORF2swt infected RM. In addition, frequency of HEV-specific CD8+ T cells was higher in ORF2smut infected RMs, potentially compensating for the attenuated HEV-specific antibody responses. Moreover, HEV-specific, memory immune responses among RMs were strikingly different following HEV re-infection. Unlike ORF2swt, ORF2smut infected RMs were more susceptible to re-infection as evidenced by fecal viral RNA and by massive expansion of HEV-specific CD8+ T cells.

Conclusions: These findings indicate ORF2s may be dispensable for viral replication in vivo but is required for long-lived antibody responses to mediate protection against HEV re-exposure.

Distinct B cell receptor repertoire signatures distinguish spontaneous clearance of hepatitis C virus and development of broadly neutralizing antibodies

Nicole E. Skinner[1,2], Clinton O. Ogega[3], Harry T. Paul[3], Nicole Frumento[3], Kaitlyn E. Clark[3], Srinivasan Yegnasubramanian[4], Andrea L. Cox[3,4], James E. Crowe, Jr.[5,6,7], Stuart C. Ray[3,4], and Justin R. Bailey[3]

[1]Center for Vaccines and Immunity, The Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, OH; [2]Division of Infectious Diseases, Department of Medicine, The Ohio State University Wexner Medical Center, Columbus, OH; [3]Division of Infectious Diseases, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, United States; [4]Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD; [5]Department of Pathology, Microbiology and Immunology, [6]Department of Pediatrics, [7]Vanderbilt Vaccine Center, Vanderbilt University Medical Center; Nashville, TN, United States

Background and aims: Early development of broadly neutralizing antibody (bNAb) responses against hepatitis C virus (HCV) is associated with spontaneous clearance of infection. Consequently, humoral immunity to HCV is usually studied by comparing B cell responses during persistent and spontaneously cleared infection. However, HCV clearance can be bNAb-independent, and the presence of bNAbs does not ensure clearance. Therefore, we hypothesized that the molecular features characterizing B cell responses associated with HCV clearance are distinct from the features associated with HCV neutralization.

Methods: To disentangle clearance and neutralization-associated features of anti-HCV B cell responses, we performed RNA sequencing of the B cell receptors (BCR-seq) of HCV E2-reactive B cells in people with cleared or persistent HCV, including subjects with high and low plasma neutralizing breadth in both the clearance and persistence groups.

Results: We found differential skewing of the BCR repertoire depending on whether the same subjects were grouped by neutralization capacity or clearance status. This was manifested in differences in BCR repertoire parameters such as CDR3 length and V gene usage, but also in distinct patterns of repertoire convergence. Not only were specific *IGHV* mutations enriched based on group assignation, but public clonotypes with identical V gene, J gene, and CDR3 sequence were identified among subjects grouped by clearance or neutralization status. Furthermore, unbiased analysis of the public clonotypes identified distinct CDR3 motifs enriched in each grouping. Experiments to validate these results by production of mAbs from public clonotypes identified in the BCR-seq analysis are underway with preliminary positive results.

Conclusions: These results reveal distinct clearance and neutralization-associated BCR repertoire signatures and suggest that both clearance status and neutralization capacity must be considered when defining effective anti-HCV humoral immune responses.

A Zika virus-specific IgM elicited during pregnancy exhibits isotype-dependent ultrapotent neutralization.

Tulika Singh[1,2], Kwan-Ki Hwang[1], Andrew S. Miller[3], Rebecca L. Jones[1], Cesar A. Lopez[4], Camila Giuberti[5], Morgan A. Gladden[1], Itzayana Miller[1,6], Helen S. Webster[1], Joshua A. Eudailey[1,6], Kan Luo[1], Tarra Von Holle[1], Robert J. Edwards[1], Sarah Valencia[1], Katherine Burgomaster[7], Summer Zhang[8], Jesse F. Mangold[1], Joshua J. Tu[1], Maria Dennis[1], S. Munir Alam[1], Lakshmanane Premkumar[4], Reynaldo Dietze[5,9], Theodore C. Pierson[7], Eng Eong Ooi[8], Helen M. Lazear[4], Richard J. Kuhn[3], Sallie R. Permar[1,6]*, Mattia Bonsignori[1,10]*

[1] Duke Human Vaccine Institute, Duke University School of Medicine, Durham, NC USA; [2] Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, CA USA; [3] Department of Biological Sciences, Purdue Institute of Inflammation, Immunology, and Infectious Disease, Purdue University, West Lafayette, IN USA; [4] Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC USA; [5] Núcleo de Doenças Infecciosas, Universidade Federal do Espírito Santo, Vitoria, Espírito Santo, Brazil; [6] Department of Pediatrics, Weill Cornell Medicine, New York, NY USA; [7] Viral Pathogenesis Section, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD USA; [8] Duke-National University of Singapore Medical School, Singapore; [9] Global Health & Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal; [10] Translational Immunobiology Unit, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD USA

Background and aims: Flavivirus infections are characterized by prolonged IgM responses. Zika virus (ZIKV)-specific serum IgM has been recently shown to contribute to ZIKV neutralization in vivo. However, ZIKV neutralizing IgM monoclonal antibodies (mAb) have not been studied. Here, we isolated a potently neutralizing human mAb, DH1017.IgM, in its native pentameric form, demonstrated protection from lethal challenge in mice and investigated the role of its multivalency in modulating neutralization.

Methods: DH1017.IgM was produced from an EBV-transformed B cell isolated from a ZIKV-infected pregnant woman who delivered a healthy baby. Neutralization was measured in focus reduction assays on Vero cells. ADE was measured in RVP-based assays on K562 and THP-1 cells with a fluorescent readout. Cryo-EM data were collected on a Titan Krios microscope. Five-week-old male *Ifnar1*$^{-/-}$ mice were inoculated with a lethal dose of ZIKV (1 x 10^3 FFU) sub-q in the footpad on day 0. On days -1 and +1, 100 µg of antibody was delivered i.v. via the retro-orbital route. Viremia was monitored by qRT-PCR for 11 days and survival was monitored for 15 days.

Results: DH1017.IgM neutralized ZIKV with FRNT50 = 12 pM (range: 4 – 31 pM) with no cross-reactivity with serotypes 1-4 Dengue viruses. DH1017.IgM neutralized >40-fold and >10.000 more potently than when expressed as an IgG (DH1017.IgG) or a Fab (DH1017.Fab), respectively. At equimolar concentrations, DH1017.IgG mediated ADE in both K562 and THP-1 cells whereas DH1017.IgM did not. DH1017.IgM controlled viremia to the limit of detection (3,2 – 3,6 Log_{10} viral copies/mL) and protected mice from lethal challenge. Human IgM was maintained *in vivo* at detectable levels up to 4 days post challenge, or 3 days after the last administration. A cryo-EM density map (5.3 Å) of DH1017.Fab in complex with Zika virion showed that DH1017.IgM binds to a novel discontinuous epitope involving primarily DII of all three E monomers in the asymmetric unit and the interface of DII and DI on chains A and C. Each asymmetric unit contained two epitope footprints, at the i2f and q2f axes of symmetry, respectively. At the i2f axis, the epitope footprint can be approached from two angles and one bound Fab excludes the other i2f site related by two-fold symmetry

from being bound, resulting in an occupancy of 1.5 Fab per asymmetric unit. The arrangement of the epitope footprints on the virion surface and computational modeling are compatible with an IgM decavalent mode of epitope recognition.

Conclusions: DH1017.IgM is an ultrapotent ZIKV-specific neutralizing antibody that protected mice from lethal challenge and bound to a novel discontinuous epitope. DH1017.IgM ultrapotency depended upon its isotype. A multivalent mode of antigen recognition, a solution not available to IgG, can contribute to such dependency and suggests a unique functional niche of IgM antibodies in protection against ZIKV.

Antibody neutralization and potential effector functions directly after primary HCV is associated with the protection against reinfection

Ana Chumbe[1], Marloes Grobben[1], Joan Capella-Pujol[1], Ian Zon[1], Sylvie Koekkoek[1], Jelle Koopsen[1], Kwinten Sliepen[1], Astrid Newsum[2], Thijs van de Laar[3,5], Maria Prins[2,4], Marit J van Gils[1], Janke Schinkel1, and on behalf of the MOSAIC (MSM Observational Study of Acute Infection with hepatitis C) study group

[1] Department of Medical Microbiology, Amsterdam UMC, University of Amsterdam, Netherlands.; [2] Department of Infectious Diseases, Research and Prevention, Public Health Service of Amsterdam, Amsterdam, The Netherlands.; [3] Laboratory of Medical Microbiology, OLVG Lab BV, Amsterdam, The Netherlands. ; [4]Division of Infectious Diseases, Department of Internal Medicine, Amsterdam Infection and Immunity Institute, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands.; [5]Department of Donor Medicine Research, Laboratory of blood-borne infections, Sanquin Research, Amsterdam, the Netherlands.

Upon spontaneous clearance or after treatment of a primary hepatitis C virus (HCV) infection, reinfection is a serious risk, especially among HIV+ men who have sex with men (MSM) with high-risk behavior. HIV+ MSM at risk for acute HCV infection can be identified by the validated HCV-MOSAIC risk score. To study correlates of protective immunity, we analysed the antibody responses or lack thereof in MSM with continued risk behaviour who did or did not become reinfected following successful treatment of primary infection.

HCV non-reinfected (NR) participants of the MOSAIC cohort with at least 2 years of follow up following primary clearance and documented HCV risk score were selected. As a control group, reinfected (R) participants, matched for calendar time of primary HCV infection and follow-up duration were selected. Sera from two time points were used; (T1) 3-6 months after primary infection and (T2) 3-6 before reinfection or at a comparable follow-up moment since primary infection in the matched NR group. A panel of HCV pseudoparticles was used to assess neutralization breath and potency. A bead-based immunoassay (Luminex xMAP technology) with beads bearing membrane bound E1E2 envelopes was used to evaluate HCV-specific IgA, IgG (IgG1, IgG2, IgG3 and IgG4) and IgM binding and potential antibody-dependent effector functions (FcγRIIa, FcγRIIIa and C1q binding).

Age at the time of infection, CD4 T cell counts at T1, duration of infection or duration of treatment showed no significant differences between R and NR groups (Mann Whitney test, $p>0.05$ in all cases). Reported risk behaviour was higher among R than in NR gropus, median risk score at T1 of 3.4 versus 1.4, respectively (Mann Whitney test, $p=0.0003$). We found higher neutralization potency and breadth as well as higher IgG1 binding directly after primary infection in the NR group, also when adjusted for risk score. We also observed an increased binding of FcγRIIa, FcγRIIIa and C1q in the NR group directly after primary infection, suggesting higher antibody dependent cellular phagocytosis (ADCP), antibody dependent cell mediated cytotoxicity (ADCC) and complement dependent cytotocicity (CDC) activities in this group, also when adjusted for risk score. At the second time point, we only detected higher C1q binding in the NR group.

In conclusion, antibody responses shortly after primary infection were associated with the protection against reinfection in MSM with continued risk behaviour. Most likely memory B cell rather than circulating antibodies may be playing a role in long-term immunity, as the associations were only found shortly after primary infection but not later during follow-up. These results may guide future development of vaccines and new therapeutics to protect against HCV (re)infection.

Long-term evolution of rodent hepacivirus under neutralizing antibody pressure in chronically infected Lewisrats

Caroline E. Thorselius[1], Raphael Wolfisberg[1], Ulrik Fahnøe[1], Troels K. H. Scheel[1], Kenn Holmbeck[1], Jens Bukh[1]

[1] Copenhagen Hepatitis C Program (CO-HEP), Denmark

Background and aims: Hepatitis C virus (HCV) vaccine research is severely impeded by the lack of small immunocompetent animal models amenable to experimental challenge. Rodent hepacivirus (RHV) shares important characteristics with HCV, including hepatitis with lymphocyte infiltration, steatosis, and chronicity upon infection of rats, which makes it an attractive surrogate model for vaccine candidate evaluation and to investigate the role of the adaptive immune response during infection. To gain further insight into antibody development and virus evolution, we here inoculated rats with RHV and followed infection for 80 weeks.

Methods: Blood was sampled regularly from nine inbred Lewis rats upon intrahepatic inoculation with in vitro transcribed RHV genomic RNA or infection with cell culture derived RHV (RHVcc) during an 80-week experiment. Viral load was quantified by RT-qPCR and RHV-specific neutralizing antibodies (nAbs) were determined utilizing a novel cell culture-based neutralization assay. Viral evolution was followed over time by whole-ORF deep sequencing of circulating RHV variants.

Results: All nine Lewis rats became persistently infected. Mutations in the envelope proteins E1 and E2 appeared concomitantly with emerging nAbs during the chronic phase of infection. As seen in HCV infection, the mutations clustered in specific regions of E1 and E2. nAb titers against the inoculum virus, RHVcc, peaked to later decline in most rats. Ongoing experiments will clarify if this reflects that Abs emerging at later time points reacts solely with the mutated virus and replaced the first wave of nAbs or if nAb titers dropped per se.

Conclusions: RHV causes chronic infection in rats and the elicited neutralizing antibody response is coincident with emergence of viral variants with clustered E1 and E2 mutations. The waning neutralizing activity of sera following viral evolution suggests possible viral immune escape, immunosupression or shift in antibody reactivity to match emerging variants dissimilar to the inoculum RHVcc. Further, as frequently observed for HCV, the nAbs of the chronic phase are unable to control concurrent infection, although they appear to lower viral titers and force mutations in the viral envelope. The characteristics of humoral immunity in RHV infection thus closely resemble the dynamics of HCV natural infection immunity making RHV well-suited to explore HCV vaccine conceptualization.

Antibody neutralization and selection pressure in the postpartum control of hepatitis C virus

John Gridley[1], Jonathan R. Honegger[2], Jennifer Kohout[3], Stephanie Smith[4], Christopher M. Walker[2], Arash Grakoui[1]

[1]Emory Vaccine Center, Atlanta, Georgia, USA.; [2]The Ohio State University College of Medicine, Columbus, Ohio, USA.; [3]Abigail Wexner Research Institute, Columbus, Ohio, USA. ; [4]Inova Children's Hospital, Falls Church, Virginia, USA.

Background and Aims: While the early induction of neutralizing antibodies during acute hepatitis C virus (HCV) infection has been associated with viral control, envelope glycoprotein sequence diversification enables evasion of such responses in chronic infection. An unusual sharp decline in viremia has been observed in some chronically infected women following childbirth. This study aims to test the hypothesis that enhanced neutralizing antibody responses have a direct role in postpartum suppression of viral replication.
Methods: Samples taken from "controllers" (women with >= 1 $\log_{10}$ postpartum viral load reduction, n=17) and "non-responders" (<0.5 $\log_{10}$ decreased viremia, n=15) were assessed at the third trimester (T3) and three months postpartum (3P). Plasma antibody neutralization responses were characterized using the HCVpp system. Serum antibody binding to JFH HCV E2 and total IgG quantification were measured by ELISA. In a subset of women (8 controllers, 9 non-controllers), circulating HCV E2 sequences were determined by RT-PCR amplification and Illumina sequencing at T3 and 1-2 years postpartum. E2 sequences were assessed for dominant non-synonymous codon shifts (from < 20% to > 80% of the quasispecies) arising within or outside of HLA haplotype-specific predicted class I epitopes.
Results: Plasma antibody binding to JFH E2 rose more from T3 to 3P in controllers than in non-responders and this increase retained significance after normalization for total IgG increases only in controllers (p=0.035 vs p=0.287). Controllers also displayed a greater postpartum increase in ability to neutralize multiple HCVpp genotypes than non-responders (H77 p=0.017 vs p=0.151, J6 p=0.003 vs p=0.359). HCV E2 amino acid substitutions outside HVR1 and outside predicted class I epitopes were more frequent in controllers than non-controllers (median 2 vs 0, p = 0.007, Mann-Whitney) and were enriched in the exposed front layer of E2 (E2 38-70).
Conclusions: Spontaneous suppression of HCV replication after childbirth was associated with enhanced capacity for heterologous HCVpp neutralization, greater E2 antibody binding, and selection of viral variants with substitutions outside of CD8+ T cell epitopes within the front layer of E2, potentially altering CD81 binding. These findings point to a previously unknown direct role of restored B cell responses in natural postpartum anti-HCV immune recovery and support HCV vaccines design efforts that aim to elicit robust B cell responses targeting the E2-CD81 domain interface.

In vivo evaluation of HCV escape for broadly neutralizing AR5A antibody in a humanized mouse model

Rodrigo Velázquez-Moctezuma[1], Rani Burm[2], Lieven Verhoye[2], Mansun Law[3], Jens Bukh[1], Philip Meuleman[2] and Jannick Prentoe[1]

[1]Copenhagen Hepatitis C Program (CO-HEP), Copenhagen University Hospital, Hvidovre and University of Copenhagen, DK; [2]Laboratory of Liver Infectious Diseases, Ghent University, BE; [3]Department of Immunology and Microbiology, The Scripps Research Institute, US

Background and Aims: Despite 30 years of effort, a hepatitis C virus (HCV) vaccine remains elusive. Key obstacles include high viral diversity and mutagenic rate, which suggest that vaccine escape could be a major issue. However, HCV escape from broadly neutralizing antibodies (bNAbs) has been primarily studied *in vitro* using cell culture adapted HCV and little is known about escape *in vivo*. Here, we analyzed the barrier to resistance of the bNAb, AR5A, in the human liver chimeric mouse model (humanized mice) for *in vivo* adapted genotype 2 HCV, $J6_{A876P}$ and HVR1-deleted $J6_{\Delta HVR1/A876P}$, either with or without the AR5A resistance substitutions L665S and S680T.
Methods: Viruses were cultured in Huh7.5 cells, and neutralization was carried out using FFU reduction assays. Mice were infected by intraperitoneal injection with or without prior loading with AR5A antibody. Mouse infections were monitored by plasma HCV RNA titration and samples were sequenced by direct sequencing of the envelope protein sequences.
Results: A876P conferred increased fitness to all viruses *in vitro* and L665S reduced AR5A susceptibility and virus viability. S680T compensated for L665S fitness loss and further increased AR5A resistance in an L665S dependent manner. In humanized mice, all viruses spread immediately with HCV RNA levels above 10^6 IU/ml in weeks 1-16, indicating that AR5A escape mutants $J6_{L665S/S680T/A876P}$ and $J6_{\Delta HVR1/L665S/S680T/A876P}$ were fully viable *in vivo*. Sequence analysis revealed no changes at week 2. However, at week 8 we observed S449P for virus with HVR1, N430D for virus without HVR1 and finally M702L for viruses with L665S, which we showed in reverse genetics studies to further compensate the fitness reduction caused by L665S. Finally, in animals loaded with AR5A we confirmed resistance *in vivo* as $J6_{\Delta HVR1/L665S/S680T/A876P}$ replicated immediately. For $J6_{\Delta HVR1/S680T/A876P}$ without L665S, two of four animals were fully protected whereas the infection of the remaining two animals was severely attenuated. In addition, sequence analysis revealed that the $J6_{\Delta HVR1/S680T/A876P}$ virus acquired L665S at week 2 indicating rapid escape from AR5A.
Conclusion: Our findings show strong correlation between *in vitro* and *in vivo* models of escape while also demonstrating how the humanized mouse model adds additional depth to these important studies, permitting us to predict the appearance of vaccine-resistant variants.

Engineered hepatitis C virus broadly neutralizing antibodies with enhanced breadth and potency

Laura Radić[1], Joan Capella-Pujol[1], Ian Zon[1], Sylvie Koekkoek[1], Vera Spek[1], Ana Chumbe Mendoza[1], Rogier W. Sanders[1], Janke Schinkel[1], Kwinten Sliepen[1]

[1]Department of Medical Microbiology, Amsterdam Infection & Immunity Institute, Amsterdam UMC, University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands.

Despite the availability of efficient direct-acting antivirals against hepatitis C virus (HCV), the development of novel effective prophylaxis and next-generation therapeutics is imperative to curb the ongoing epidemic. Broadly neutralizing antibodies (bNAbs) are able to neutralize a large proportion of circulating HCV strains. These bNAbs can protect animals against HCV infection and might even be useful as therapeutics for curing an established infection. Several bNAbs have been isolated from patients that spontaneously cleared the virus, which are categorized according to their distinct epitopes on the HCV E1E2 envelope glycoprotein complex. Here, we present a panel of engineered HCV antibody-based constructs, designed to reduce the likelihood of viral escape by utilizing distinct mechanisms of binding to E1E2. We strategically combined antibody specificities to achieve modes of action unavailable to conventional HCV bNAbs, showing the importance of avidity and cooperativity in novel and broad HCV targeting antibody formulations. Some of our candidates demonstrate enhanced breadth and improved potency in neutralization assays against a panel of HCV pseudoparticles representing six genotypes. These novel designs provide generalizable avenues for improving HCV bNAbs and might be promising as candidates for therapeutic purposes or prophylaxis. Understanding how these antibodies neutralize the virus could also aid vaccine immunogen design.

The diagnostic potential of activated CD8+ T cells during acute COVID-19

Alexandra Vujkovic[a,c], My Ha[c,d], Tessa de Block[b], Lida van Petersen[b], Isabel Brosius[b], Caroline Theunissen[b], Sabrina van Ierssel[f], Esther Bartholomeus[c,d], Wim Adriaensen[g], Maartje van Frankenhuijsen[b], Erika Vlieghe[f], Benson Ogunjimi[c,d], Kris Laukens[c], Pieter Meysman[c], Koen Vercauteren[a]

[a] Clinical Virology Unit, Institute of Tropical Medicine, Antwerp, Belgium; [b] Department of Clinical Sciences Institute of Tropical Medicine, Antwerp, Belgium; [c] Antwerp Unit for Data Analysis and Computation in Immunology and Sequencing, University of Antwerp, Belgium; [d] Antwerp Centre for Translational Immunology and Virology, Vaccine & Infectious Disease Institute, University of Antwerp, Belgium; [f] Department of General Internal Medicine, Infectious Diseases and Tropical Medicine, University Hospital Antwerp, Belgium; [g] Clinical Immunology Unit, Institute of Tropical Medicine, Antwerp, Belgium

Background and aim: In a cell-mediated immune response to viruses, the T cell is crucial. Antigenic peptides attached to major histocompatibility complex (MHC) molecules are recognized by the T cell receptor (TCR). Given enough sequencing power, a biased TCR repertoire should be detected in T cells directed towards a specific antigen in a disease context. The aim in this study was to explore the activated CD8+ T cell repertoire as a potential diagnostic tool for COVID-19.

Methods: TCR RNA sequencing was performed on all chains (TCRalpha/beta/gamma/delta) of HLADR+/CD38+ (activated) and HLADR-/CD38- (non-activated) FACS-sorted CD8+ T cell subsets of hospitalized COVID-19 patients (n = 30) and healthy controls (n = 30). Among the healthy controls, volunteers had never been exposed (n = 10; pre-pandemic), presumably not been exposed (n = 10; sampled during the pandemic first wave and having negative SARS-CoV-2 spike serology) or previously been exposed (n = 10; SARS-CoV-2 PCR-positive >80 days before sampling) to SARS-CoV-2. TCRalpha/beta clusters[1] were matched[2] to a database of >140.000 known SARS-CoV-2 associated TCRalpha/beta sequences. TCR cluster database matches were considered as SARS-CoV-2 specific.

Results: The retrieved amount of SARS-CoV-2-specific TCRs (normalized to all TCRs) was significantly higher in activated vs. non-activated T cell subsets during acute COVID-19 ($p < 0{,}001$) and did not longer differ after recovery (in previously SARS-CoV-2 exposed controls) (fig. 1A). Moreover, a logistic regression classifier using SARS-CoV-2-specific TCR count measures (depth and breadth) of activated CD8+ T cells in COVID-19 patients vs. all healthy controls generated a receiver operating characteristic (ROC) area under the curve (AUC) of 0,85 ± 0,12 after five-fold cross validation (fig. 1B, individual TCR counts expressed as depth are presented in fig. C).

Conclusion: We demonstrate the possibility to infer disease state by matching TCR sequences of activated CD8+ T cells against a database of known SARS-CoV-2-specific TCR sequences. This is the first time TCR sequencing has been used to successfully diagnose an acute viral infection and discriminate it from historic exposure.

References

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Longitudinal neutralizing antibody dynamics after infection with severe acute respiratory syndrome coronavirus 2 or vaccination against coronavirus disease 2019

Alexander P. Underwood, Christina Sølund, Carlota Fernandez-Antunez, Signe Lysemose Villadsen, Lotte S. Mikkelsen, Alekxander Binderup, Signe Bollerup, Anni Assing Winckelmann, Anna-Louise Sørensen, Santseharay Ramirez, Nina Weis & Jens Bukh.

Background and aims: Since ultimo 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has caused millions of deaths. Recently, it has been shown that a correlate of protection from coronavirus disease 2019 (COVID-19) are the levels of neutralizing antibodies induced either by infection, vaccination or both. The aim of this study was to longitudinally map the neutralizing titers of individuals previously infected with SARS-CoV-2, those vaccinated against COVID-19 and those with a mix thereof as a means of understanding the length of protection offered.
Methods: Using a whole SARS-CoV-2 virus isolate (D614G variant), neutralizing titers were longitudinally determined (>12 months) from 2-fold serially diluted plasma from 157 individuals previously infected with SARS-CoV-2, with a range in disease severity, and 130 individuals vaccinated against COVID-19, with various vaccine regimens. Overtime, the majority of previously infected individuals became vaccinated, and some of the uninfected-vaccinated individuals became infected allowing analyses of neutralizing titers across many different outcomes. Select individuals were tested for breadth of neutralization to delta and omicron SARS-CoV-2 isolates.
Results: Both infection with SARS-CoV-2 and vaccination against COVID-19 induce neutralizing antibody responses with peak titers at 1 month post symptom onset or completed vaccination regime that rapidly wane to a level that is sustained for over a year. Individuals with severe infection sustained significantly higher neutralizing titers than those with mild infection or vaccinated. Nine months after vaccination (prime-boost), neutralizing titers had dropped to nearly background levels. Infection, reinfection or vaccination/revaccination after infection/vaccination induced a significant boost to neutralizing titers, which corresponded to significant increases in breadth of neutralization to both delta and omicron variants. This boost was also found to significantly increase the sustained level of neutralizing antibodies in plasma over time.
Conclusions: The severity of disease caused by SARS-CoV-2 infection directly reflects the level of neutralizing antibodies induced and sustained over time. Infection appears to induce higher levels of sustained neutralizing antibodies than vaccination alone. However, Vaccination or reinfection of previously infected individuals and re-vaccination (2^{nd} booster) or infection of vaccinees dramatically boost the level of sustained neutralizing antibodies.

CD19+CD24hiCD38hi B regulatory cells exhibit regulatory capacity and modulate T cell response in hepatitis E virus infection

Anurada tripathy

Antibodies as well as memory B cells are the potential correlates of a protective immune response against hepatitis E virus (HEV) infection. We have evaluated the role of IL-10 expressing Bregs in HEV infection. A total of 108 acute hepatitis E patients, 55 hepatitis E recovered individuals and 128 HEV naïve healthy controls were enrolled. The percentages of peripheral $CD19^{+}$, immature $CD19^{+}CD24^{hi}CD38^{hi}$, mature $CD19^{+}CD24^{int}CD38^{int}$ and memory $CD19^{+}CD24^{hi}CD38^{-}$ B cells were analyzed by flowcytometry. Intracellular cytokine staining for IL-10 and TGF-β, HEV-rORF2p specific T cell response (IFN-γ expression) pre/post IL-10/IL-10R blocking and $CD19^{+}IL\text{-}10^{+}$ B cells-depletion based assays were carried out to assess the functionality of Bregs. The percentage of HEV-rORF2p specific immature B cell phenotype was significantly higher in acute hepatitis E patients compared to hepatitis E recovered individuals and controls. Significantly higher IL-10 expression on B and HEV-rORF2p stimulated B cells of acute hepatitis E patients compared to controls indicated that Bregs are functional and HEV-rORF2p specific.

Enhanced IFN-γ expression on $CD8^{+}$ T cells upon IL-10/IL-10R blocking and also post $CD19^{+}IL\text{-}10^{+}$ B cells depletion suggested that $CD3^{+}CD8^{+}IFN\text{-}\gamma^{+}$ T cells corroborate the regulatory potential of Bregs via IL-10 dependent mechanism. We have identified HEV specific functional, immature $CD19^{+}CD24^{hi}CD38^{hi}$ B cells having IL-10 mediated regulatory activities and a potential to modulate IFN-γ mediated T cell response in Hepatitis E. The prognostic/pathogenic role of Bregs in recovery from severe hepatitis E needs evaluation.

Correlates of humoral immune protection in acute resolving and chronic HCV infection

Dorothea Bankwitz

Up to one third of individuals in contact with hepatitis C virus (HCV) naturally clear the infection within six months after exposure. Vigorous and broad T cell responses associate with natural viral clearance. Moreover, neutralizing antibodies are key effectors of infection- and of vaccine-induced immunity. Quantification of antibodies´ breadth and potency is critical for understanding mechanisms of protection.
Here, we utilized a well characterized and standardized HCVcc panel to quantify breadth and potency of neutralizing antibodies from 19 individuals that spontaneously resolved HCV and from 41 individuals that progressed to chronic HCV infection. The high-risk subjects analyzed in our study were infected with diverse genotypes.
Neutralizing antibody responses during the acute phase of the infection were generally modest in potency and breadth compared to antibodies present in long-term infected individuals. Of note, the potency and breadth of antibodies during the acute phase of infection were significantly greater in patients with self-limiting infection as opposed to those contracting a chronically progressing infection. We investigated whether antibodies generated during self-limiting infection target specific viral epitopes that differ from those that dominate in patients with chronic HCV infection. In addition, we correlated neutralization data with clinical data to identify parameters that may predict spontaneous HCV clearance.

Session 3
EPIDEMIOLOGY AND DIAGNOSIS

28TH INTERNATIONAL SYMPOSIUM ON HEPATITIS C VIRUS, FLAVIVIRUSES, AND RELATED VIRUSES

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Single-cell level analysis of the cell-intrinsic antiviral response to hepatitis E virus infection in induced pluripotent stem cell-derived hepatocytes

Ann-Kathrin Mehnert[1], Rebecca Menhua Fu[1], Sarah Prallet[1], Viet Loan Dao Thi[1,2]

[1] Schaller Research Group, Department of Infectious Diseases, Virology, University Hospital Heidelberg, Heidelberg, Germany
[2] German Centre for Infection Research (DZIF), Partner Site Heidelberg, Heidelberg, Germany

Contact: ann-kathrin.mehnert@med.uni-heidelberg.de

Background and Aims:
Hepatitis E virus (HEV) is one of the major causes of acute viral hepatitis worldwide. Depending on the genotype (GT), HEV infection can also result in fulminant courses (GT1) or chronic hepatitis (GT3). Due to difficulties to culture the virus *in vitro*, the HEV life cycle remains poorly understood. We and others previously proposed induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (HLCs) as a more physiological cell culture model compared to conventional hepatoma cells. We showed that HLCs are permissive for HEV infection and observed a robust type III interferon (IFN) and IFN-stimulated gene (ISG) induction. However, viral replication was not dampened by this response, supporting the finding that HEV cannot be cured by high doses of IFN. These observations are intriguing as HEV appears to have developed mechanisms to persist in the presence of sustained antiviral signaling.
Methods:
The human iPSC.C3A line was differentiated to HLCs based on our previous study (Wu & Dao Thi, 2018, Gastroenterology). We used CRISPR/Cas9 ribonucleoprotein complex delivery to knock out the *IFN-lambda receptor 1* gene in iPSC.C3A cells, allowing us to distinguish between the antiviral response branches upstream and downstream of IFN production. Viral RNA and host IFN/ISG mRNAs were quantified by RT-qPCR and visualized by RNA fluorescence *in situ* hybridization (FISH) at early and late time points post infection in HLCs and the hepatoma cell line HepG2/C3A.
Results:
Upon infection with HEV GT3, we could visually localize parts of the antiviral response on a single-cell level. We identified HEV-infected cells by detection of viral positive-sense RNA genomes. At day 3 post infection, we observed nuclear translocation of IRF3 and a rise in IFN-lambda transcripts in HEV-infected cells. We are currently performing the same analysis to localize ISGs via FISH and IF. We also found that HEV infection could not be further enhanced by specifically inhibiting the pathways upstream and downstream of IFN production. Our results indicate that HEV replication, once fully established, can persist within a cell that shows an antiviral phenotype. This may suggest antagonism mechanisms against effector proteins downstream of the antiviral response pathways but not the induction of a response itself.
Conclusions:
Using RNA FISH, we visually localized the antiviral response upon HEV infection in HLCs on a single-cell level. We are complementing this by single-cell RNA sequencing to analyze the limiting potential of an early response, which may not be detectable with current methods. This will further help understand how HEV persists in the presence of sustained antiviral signaling following establishment of replication. It will also allow identification of the ISGs induced upstream and downstream of IFN. Comparison of the response induced by GT1 and GT3 could then contribute to a better understanding of acute and chronic HEV infections.

Novel zebrafish-based *in vivo* model of Zika virus infection unveils NS4A as a key viral determinant of neuropathogenesis

Aïssatou Aïcha Sow, Priyanka Jamadagni, Kessen Patten and Laurent Chatel-Chaix

Centre Armand-Frappier Santé Biotechnologie, Institut National de la Recherche scientifique, Laval, Québec, Canada

Infection of pregnant women by Zika virus (ZIKV) can cause neurodevelopmental defects in newborns known as congenital Zika syndrome, which includes microcephaly. Murine models studying ZIKV neurovirulence have several limitations in terms of cost, time, ethics, cell imaging, and genetic manipulation. Thus, alternative animal models more conducive to the study of early development of the ZIKV-infected brain *in vivo* are required.

Zebrafish is a powerful and cost-effective tool for studying human neurological diseases due to a neuroanatomy comparable to that of mammals. Moreover, zebrafish is permissive to several human viruses. Optically transparent, this model is ideal for imaging labelled neural cell populations in whole animals. Considering this, we aimed to develop a zebrafish-based *in vivo* model of ZIKV infection to study viral neuropathogenesis.

Eighty percent of zebrafish larvae infected with ZIKV infectious particles exhibited developmental defects ranging from curved spinal cord to ovoid morphology. TUNEL assays on whole animals showed an increased apoptosis in the brain following ZIKV infection. This correlated with a decrease in head size and in neural progenitor cell abundance, as well as drastic mobility impairments. Importantly, these defects were reversed when the larvae were treated with the flaviviral polymerase inhibitor NITD008, which decreased viral loads more than 150-fold, unambiguously demonstrating that ZIKV replicates in zebrafish. Whole animal immunostaining of viral proteins revealed infection foci in the hindbrain and in the spinal cord, strongly supporting that ZIKV replicates in the central nervous system. Furthermore, expression of viral protein NS4A alone recapitulated morphological defects, demonstrating that this viral protein is a key determinant in ZIKV neurovirulence.

Overall, our data unveil the zebrafish larva as a model for ZIKV infection with neurological phenotypes comparable to the defects observed in humans. This model will enable rapid antiviral drug testing *in vivo* and a better understanding of host determinants required for ZIKV neuropathogenesis given its flexibility for genetic manipulations.

Interplay between hepatitis C virus and peroxisomes.

Esther Martin de Fourchambault[1], Nathalie Callens[1], Yasmine Sebti[2], Santseharay Ramirez[3], Jens Bukh[3], Jean Dubuisson[1], Yves Rouillé[1]

[1] Center for Infection and Immunity of Lille, INSERM U1019-CNRS UMR9017, Université de Lille, Institut Pasteur de Lille, Lille, France; [2] INSERM UMR1011, Université de Lille, CHU Lille, Institut Pasteur de Lille, Lille, France; [3] Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

Background and Aims: Despite the introduction of effective treatments for hepatitis C in clinics, issues remain regarding the pathogenesis induced by chronic hepatitis C virus (HCV) infection. HCV is known to disturb the metabolism of infected cells, especially lipid metabolism and redox balance, but the mechanisms leading to HCV-induced pathogenesis are still poorly understood. Peroxisomes are small versatile organelles involved among other functions in lipid metabolism and regulation of reactive oxygen species. Their metabolism and turnover are altered by several viral infections, such as herpesviruses or some flaviviruses, and their functions appear to be impaired in the liver of chronically infected hepatitis C patients (Lupberger *et al.* 2019).

Methods: Potential HCV-interacting host cell proteins were identified using a proximity- biotinylation assay performed with a replicon containing the peroxidase APEX2 and confirmed by western blot and immunofluorescence. Peroxisome morphology was investigated by confocal microscopy and image analysis. Peroxisome-free Huh-7 cells were generated by inactivating the PEX3 gene using CRISPR/Cas9. HCV was titrated by TCID50.

Results: We identified ACBD5, a peroxisome membrane protein, as a protein located near HCV replication sites. Confocal microscopy confirmed the relocation of peroxisomes near HCV replication complexes and indicated that their morphology is altered in 20-35% of infected Huh-7 cells. We characterized the kinetics of peroxisomes alteration up to 16 days post-infection (dpi) in Huh-7 cells infected with the DBN3a (gt3a) and JFH1 (gt2a) strains. Their average volume increased from 0,15 ± 0,01 μm^3 in control cells to 0,33-0,35 ± 0,02 μm^3 at 16 dpi, while their average number per cell dropped from 547 ± 94 to 242 ± 59 with the DBN3a strain and 204 ± 35 with the JFH1 strain. To assess their importance in the HCV life cycle, we titrated infectious particles secreted at 4 dpi by peroxisomes-free cells and found that the absence of peroxisomes had no impact on infectious titers.

Conclusion: These results indicate that peroxisomes are morphologically altered in HCV-infected cells, but not essential for short-term HCV infection. We are currently studying the importance of peroxisomes in long-term infection and the impact of HCV on peroxisome functions.

Chronic HCV Infection Fuels HIV Reservoir Persistence in CD4-T Cells: Beneficial Effects of Direct Acting Antivirals

Samaa T. Gobran[*1, 2], Jessica Dion[2], Laurence Raymond Marchand[2], Amélie Pagliuzza[2], Nicolas Chomont[1, 2], Marina Klein [3, 4], Julie Bruneau[5], Petronela Ancuta[1,2], Naglaa H. Shoukry[2, 7]

[1]Département de Microbiologie, Infectiologie, et Immunologie, Université de Montréal, Faculté de Médicine, Montréal, [2]Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Montreal, [3]Department of Medicine, Division of Infectious Diseases, McGill University Health Centre, Montréal, [4]Department of Medicine, McGill University, Faculty of Medicine and Health sciences, Montreal, [5]Département de médecine de famille et de médecine d'urgence, Université de Montréal, Faculté de Médicine, Montréal, [7]Département de médecine, Université de Montréal, Faculté de Médicine, Montréal, Canada

Background: Hepatitis C virus (HCV) co-infection is serious comorbidity in people living with Human Immuno-deficiency Virus (HIV), as reflected by higher HIV-DNA reservoir size in CD4 T-cells of HCV/HIV co-infected individuals compared to HIV mono-infected subjects. HCV cure with Directly Acting Antivirals (DAA) leads to alterations in HIV-DNA/RNA levels thus raising new questions on the relationship between the two viruses.

Methods: Memory CD4-T cells from chronic HCV patients and uninfected controls (n = 20 per group) were infected with HIV-1 strains (HIVNL4.3BaL, HIVTHRO) in vitro. HIV integration and replication were measured by real-time nested PCR and HIV-p24 ELISA/flow cytometry analysis, respectively. Real-time nested PCR and RT-PCR were used to examine HIV-DNA reservoir size and the HIV-RNA/DNA ratio in CD4+ T-cells of HCV/HIV co-infected patients before DAA, at the end of DAA, and three months post-DAA (n = 10).

Results: CD4 T-cells from chronic HCV-infected individuals compared to uninfected controls were more susceptible to HIVNL4.3BaL infection, as demonstrated by soluble HIV-p24 expression ($p = 0,029$), and integrated HIV-DNA levels ($p = 0,056$); this coincides with superior expression of the HIV co-receptor CCR5 ($p = 0,007$) and positively correlated with HCV plasma viral loads ($r = 0,5$, $p = 0,046$). Furthermore, there was a significant reduction in integrated HIV-DNA levels ($p = 0,0013$) and HIV RNA ($p = 0,0029$) following DAA treatment of HCV/HIV co-infected individuals. Interestingly, DAA-mediated HCV cure reduced HIV-RNA/DNA ratio, a surrogate marker of HIV transcription ($p = 0,0121$).

Conclusions: Our preliminary data support a model in which chronic HCV infection is associated with increased CD4 T-cells permissiveness to HIV infection thus, supporting their potential contribution to viral reservoir persistence during ART. Our results also highlight the beneficial impact of DAA on reducing HIV reservoir size in HCV/HIV co-infected subjects.

Impact of physiologically prolonged hypoxia on Zika virus infection and consequences on cellular disturbance

Marianne Macquart, Jade Cochard, Mathilde Couteaudier and Philippe Chouteau

Background & Aims: Zika Virus (ZKV) is a mosquito-transmitted virus which belongs to the *Flaviviridae* family. ZKV is a multi-organ-associated virus and provokes important neurological disorders suggesting that the intracellular ZKV replication is combined with cellular disturbance. Nevertheless, very little is known about the intracellular impact of the viral replication. Of note, in vitro studies published so far related to virus-host interactions present major restrictions since cells are cultured in a supra physiological oxygen tension (21% O_2, normoxia) whereas ZKV-target organs naturally evolve in situ in a low oxygen environment (hypoxia). Thereby, we investigated the impact of physiologically prolonged hypoxia (1% O_2) on in vitro ZKV infection and evaluated the cellular consequences of the infection.
Our results showed that compared to cells cultured in normoxia, intra- and extracellular viral loads were reduced in the natural and prolonged hypoxic environment. These features were associated with a reduction of the intracellular ZKV envelop protein that was found accumulated nearby the nucleus in abnormal pseudo-vacuolar vesicles suggesting a viral assembly default together with a disruption of the cellular equilibrium. Two central transcription factors (HIF1□ and 2□) are essential to ensure the cellular homeostasis in a natural hypoxic environment. In normoxic conditions, these proteins are post-translationally hydroxylated and degraded by the proteasome. HIF1□ and 2□ hydroxylation is absent in hypoxia allowing the nuclear translocation of these proteins. In ZKV-infected hypoxic-cells HIF1□ and 2□□were found dramatically down-regulated in a transcriptional-independent manner suggesting that ZKV was responsible for these proteins degradation thus contributing for the cellular disturbance. To explore the mechanism involved in these degradations, HIF1□ and 2□□hydroxylation mutants were expressed in normoxic cells. While HIF1□ and 2□□were stabilized in mock-infected cells, ZKV-infected normoxic-cells exhibited a dramatic down-expression of both HIF1□ and 2□□mutants indicating that ZKV is involved in both HIF1□ and 2□□degradations in a manner independent of the canonical-proteasome pathway.
Naturally hypoxic cell cultures infected by ZKV are thus subjected to profound cellular disturbance. These disorders could be in relation with defect of viral assembly and/or impairment of HIFs functions that may explain ZKV-associated pathophysiological features. Although additional experiments are now necessary to decipher the precise mechanisms involved in these observations, we report here the first demonstration of this close relationship.

Hepatitis C Virus Genotype 1a Transmission Dynamics in the UK Population

E. Carol McWilliam Leitch*, Christopher Davies, Emma C. Thomson, Sema Nickbakhsh and John McLauchlan

MRC-University of Glasgow Centre for Virus Research, Glasgow, UK.

*Current address: The Roslin Institute, University of Edinburgh, UK.

Background: HCV transmission dynamics are important in surveillance including monitoring the impact of prevention methods and intervention with direct-acting antivirals (DAAs). Additionally, they can illuminate aspects of the origin and historical geographic spread of viruses.

Methods: Phylogenetic methods were applied retrospectively to 419 full-length coding regions of HCV genotype (Gt)1a genomes from UK residents. Maximum likelihood trees were parsed with patient metadata (residential region, birthplace, ethnicity, risk factor) and empirical data assessing potential associations were generated by Bayesian General Linear Models. Time-correlated trees and population history were inferred in Beast 1.10.4. Transmission dynamics within monophyletic clades were investigated. Discrete transition events between locations were reconstructed in the phylogeny via asymmetric continuous-time Markov chain models, with SPREAD3 software to visualise transmission and identify Bayes factor support. Markov jump analyses and rewards allowed quantification of regional transmission and time spent in each region.

Results: Gt1a sequences clustered according to residential region with strong Bayes factor support. Bayesian MCMC analysis inferred a mean substitution rate of 6.8×10^{-4} substitutions/site/year for the dataset and the MRCA was 1814. The population expanded exponentially from 1850 to 2000 but declined after 2006. Each of the 11 monophyletic clades displayed unique transmission dynamics. As an exemplar, the MRCA of lineage A1a1 was 1933. Bayes factors supported this lineage as originating in the East Midlands, being transmitted to the West Midlands and London around the 1950s and spreading to other regions from the 1990s onwards (figure). Markov jump analyses and rewards suggested A1a1 most frequently spread within and between the West Midlands and London and was detected most often in London. Overall, London (4) and Scotland (3) constituted major hubs for 7 of the 11 lineages.

Conclusions: Population expansion of HCV Gt1a commenced in the UK from the mid-19th century, a timeframe that coincides with the invention of the hypodermic needle (1844). Residential region of HCV-infected individuals was the most influential epidemiological factor in the spread of the 11 Gt1a lineages. Moreover, each lineage displayed distinctive transmission dynamics and hubs, for example lineage A1a1 as described above. Overall, our approach establishes an analytical pipeline for determining any future HCV outbreaks and potential spread of emergent DAA-resistant lineages. Further, this approach could be used to monitor the transmission of other viral infections.

Investigating Phylogenetic Dynamics of Hepatitis C Virus Genotype 3a in the UK Population

E. Carol McWilliam Leitch*, Christopher Davies, Emma C. Thomson, Sema Nickbakhsh and John McLauchlan

MRC-University of Glasgow Centre for Virus Research, Glasgow, UK.

*Current address: The Roslin Institute, University of Edinburgh, UK.

Background: HCV genotype (Gt)3a is thought to originate in the Indian subcontinent and is the second most prevalent genotype circulating in the UK. Here, we investigated the origin and historical geographic spread of Gt3a. We examined also recent transmission dynamics of this genotype within the UK to improve HCV surveillance techniques.

Methods: Phylogenetic analyses were applied retrospectively to 380 full-length coding regions of HCV Gt3a genomes from UK residents. Maximum likelihood trees with embedded patient metadata were inferred and MCMC General Linear Models (GLM) were used to provide empirical evidence for associations. Time-correlated MCC trees, including genome region partitioning and population history were inferred in Beast 1.10.4. Monophyletic clades of UK origin were identified and transmission within each lineage was investigated using continuous-time Markov chain models and SPREAD3 software. Quantification of regional transmission and time spent in each region was examined using Markov jump analyses and rewards.

Results: The Gt3a full coding region had a mean substitution rate of 2.9 x 10^{-4} substitutions/site/year (s/s/y), ranging from 1.8 x 10^{-4} s/s/y (NS3) to 4.8 x 10^{-4} s/s/y (E2). The MRCA of the dataset was 1545 (Figure). The basal clades consisted of sequences from UK residents predominantly (91%) of Indian or Pakistani ethnicity whereas the core cluster constituted sequences mainly from individuals of White British ethnicity (84%) and contained 7 lineages. Lineages from European individuals were embedded within these British lineages. The viral population expanded exponentially between 1600 and 1850, remained constant until 2005, decreasing thereafter. Within the 7 lineages constituting mainly individuals of White British ethnicity, GLM analysis suggested UK residential distance was the most important epidemiological factor in transmission. For example, lineage E originated in Northwest England circa 1820 but was not transmitted out of this region until the 1960s. Markov jump analysis indicated Yorkshire, Northwest England and the West Midlands were the main hubs of transmission of this lineage.

Conclusions: Gt3a strains from UK residents had a South Asian origin. This genotype was probably introduced into native UK populations during the colonial period of the Indian subcontinent's history then spread from the UK to mainland Europe. Individual UK lineages may have initially only spread locally prior to the mid-20th century when factors such as increased travel, injecting drug use and universal use of hypodermic needles contributed to more widespread transmission. Our approach establishes a pipeline for investigating outbreaks of HCV infection including the potential emergence of transmissible DAA-resistant strains through treatment failure and could be easily modified to examine spread of other viruses.

MS-TRACE: molecular surveillance of transmission networks for hepatitis C elimination in men who have sex with men

Jelle Koopsen[1], Maarten Pater[1], Christoph Boesecke[2], Martin Daeumer[3], Stephan Grunwald[4], Hayette Rougier[5], Joël Gozlan[5], Colin Russell[1], Karine Lacombe[5,6], Patrick Ingiliz[7,8], Marc van der Valk[9,10], Janke Schinkel[11]

1. Department of Medical Microbiology and Infection Prevention, Laboratory of Applied Evolutionary Biology, Amsterdam University Medical Centers, University of Amsterdam, the Netherlands
2. University of Bonn, Bonn, Germany
3. Institute of Immunology and Genetics, Kaiserslautern, Germany
4. Center for Infectiology, Berlin, Germany
5. Saint-Antoine Hospital, AP-HP, Paris, France
6. Sorbonne University, INSERM IPLESP, AP-HP, Paris, France
7. Department of Gastroenterology and Hepatology, Charité University Medical Center, Berlin, Germany
8. Zentrum für Infektiologie Berlin-Prenzlauer Berg, Berlin, Germany
9. Division of Infectious Diseases, Amsterdam Infection and Immunity Institute, Amsterdam University Medical Centers, University of Amsterdam, the Netherlands
10. Stichting HIV Monitoring, Amsterdam, the Netherlands
11. Department of Medical Microbiology and Infection Prevention, Section of Clinical Virology, Amsterdam University Medical Centers, University of Amsterdam, the Netherlands

Background and Aims: Although hepatitis C virus (HCV) incidence has been declining after the introduction of highly effective direct-acting antiviral medication (DAAs), incidence among men who have sex with men (MSM) remains high. Elimination of HCV as a public health threat by 2030 could be impeded by continuing international transmission and the emergence of variants that harbor intrinsic resistance to current treatment options. Here we introduce MS-TRACE, a genomic surveillance tool that tracks the evolution, transmission, and resistance of HCV among MSM.

Methods: HCV viral genomes from MSM with an acute or chronic infection are eligible for inclusion in MS-TRACE. HCV genomes with rich epidemiological metadata such as transmission route and demographic data of the person that was sampled are included. Such detailed epidemiological metadata is necessary for interpretation of the phylogenetic results. Full length genomic sequences are preferred, but shorter fragments (such as NS5A/NS5B) can also be included for monitoring resistance. Viral genomes of genotypes 1, 2, 3, and 4, the most common genotypes among MSM, are included.

Results: MS-TRACE comprises of a HCV viral genome database annotated with clinical, demographic, and behavioral metadata, a bioinformatic pipeline, and a web-bases platform to interact with the data. MS-TRACE is built upon several open-source bioinformatic software packages to process the viral genomes and epidemiological metadata. Full-length sequences and metadata enter a bioinformatic pipeline that aligns sequences against genotype specific reference genomes, constructs maximum-likelihood phylogenies, and infers transmission clusters. In addition, shorter genomic fragments (NS5A/NS5B) are included in the database, specifically to monitor resistance against currently available DAAs. Phylogenies are enriched with epidemiological metadata such as country-level geographical data, HIV-status, pre-exposure prophylaxis usage, and injecting drug use. This is then visualized using *Nextstrain* software to allow for interaction with the data.

Conclusions: Here we introduce MS-TRACE, an HCV genomic surveillance platform focused on achieving and monitoring micro-elimination among MSM. It was designed to empower public health professionals to improve their understanding of local HCV transmission in a broader, international context. HCV genetic surveillance enables identifying key targets for rapid interventions, awareness campaigns, and testing strategies. MS-TRACE is available at https://mstrace.nl/ .

Hepatitis C virus transmission dynamics in a global cohort of men who have sex with men with recently acquired infections

Jelle Koopsen[1], Gail Matthews[2,3], Juergen Rockstroh[4], Tanya Applegate[2], Sanjay Bhagani[5], Andri Rauch[6], Jason Greberly[2], Rachel Sacks-Davis[8], Patrick Ingiliz[9], Gregory Dore[2,3], Janke Schinkel[10]*, Marc van der Valk[11,12]*, on behalf of REACT Study Group

1. Department of Medical Microbiology and Infection Prevention, Laboratory of Applied Evolutionary Biology, Amsterdam University Medical Centers, University of Amsterdam, The Netherlands
2. Kirby Institute, UNSW Sydney, Australia
3. St Vincent's Hospital, Sydney, Australia
4. University Clinic Bonn, Bonn, Germany
5. Royal Free Hospital, London, United Kingdom
6. Department of Infectious Diseases, Inselspital, Bern, Switzerland
7. Toronto Centre for Liver Diseases, Toronto General Hospital, Toronto, Canada
8. Burnet Institute, Melbourne, VIC, Australia
9. Zentrum für Infektiologie Berlin-Prenzlauer Berg, Berlin, Germany
10. Department of Medical Microbiology and Infection Prevention, Section of Clinical Virology, Amsterdam University Medical Centers, University of Amsterdam, The Netherlands
11. Division of Infectious Diseases, Amsterdam Infection and Immunity Institute, Amsterdam University Medical Centers, University of Amsterdam, The Netherlands
12. Stichting HIV Monitoring, Amsterdam, the Netherlands

Background: Micro elimination of hepatitis C virus (HCV) among men who have sex with men (MSM) could be compromised by continuous external introductions and the emergence of clusters harboring clinically significant resistance associated substitutions. We analyzed whole genome HCV genetic sequences from MSM who participated in a large international acute HCV treatment trial to investigate international clustering and RAS prevalence and transmission.

Methods: MSM with a recently acquired HCV infection in the REACT trial, a multicentre international, phase IV non-inferiority trial examining the efficacy of short course vs. standard course therapy, were eligible for inclusion. We obtained whole genome sequences of 128 HCV infections from MSM recruited at 24 international sites in 8 different countries. We inferred maximum-likelihood phylogenies for HCV genotypes separately and transmission clusters were determined. Using a Bayesian coalescent approach, we constructed time-scaled phylogenies to estimated cluster introduction dates. A Bayesian Skygrid approach was used to estimate the effective population size over the past 50 years. We determined the RAS prevalence and extent of RAS transmission in the study population.

Results: The majority of recent HCV infections were part of international networks that arose in the late 1990s – early 2000s. Sequences obtained in the same country clustered frequently and in 36% of recent subclusters we showed evidence of international transmission. European MSM were more likely to be in a cluster than non-European MSM (odds ratio 11.9 [CI: 3.6 - 43.4], $p<0.0001$). The effective population size of HCV in Europe decreased rapidly in recent years. Clinically significant RAS prevalence was low and transmission of highly resistant viruses was not observed.

Conclusion: In recent years, after widespread availability of antiviral treatment, international HCV transmission still occurs among MSM, which complicates micro elimination among MSM. RAS-enriched clusters and prevalent RAS transmission are currently not a threat to the elimination goals.

An 8-gene machine learning model improves clinical prediction of severe dengue progression

Sirle Saul,

Stanford University, USA

Each year 3–6 million people develop life-threatening severe dengue (SD). Clinical warning signs for SD manifest late in the disease course and are nonspecific, leading to missed cases and excess hospital burden. Better SD prognostics are urgently needed. We integrated 11 public datasets profiling the blood transcriptome of 365 dengue patients of all ages and from seven countries, encompassing biological, clinical, and technical heterogeneity. We performed an iterative multi-cohort analysis and identified eight differentially expressed genes (DEGs) between non-severe patients and SD progressors. Using these eight DEGs, we trained an XGBoost machine learning model on public data to predict progression to SD. All model parameters were “locked” using public data, after which we validated the model in an independent, prospectively enrolled cohort of 377 dengue patients in Colombia. We measured expression of the DEGs in whole blood samples collected prior to SD progression. We then compared the accuracy of the locked 8-gene XGBoost model to clinical warning signs in predicting SD. The 8-gene XGBoost model accurately predicted SD progression in the independent validation cohort with 86.4% (95% CI 68.2–100) sensitivity and 79.7% (95% CI 75.5–83.9) specificity. Given the 5.8% proportion of SD cases in this cohort, the 8-gene model had a positive and negative predictive value of 20.9% (95% CI 16.7–25.6) and 99.0% (95% CI 97.7–100.0), respectively. Compared to clinical warning signs at presentation, which had 77.3% (95% CI 58.3–94.1) sensitivity and 39.7% (95% CI 34.7–44.9) specificity, the 8-gene model led to an 80% reduction in the number needed to predict (NNP) from 25.4 to 5.0. Importantly, the 8-gene model accurately predicted subsequent SD in the first three days post-fever onset and up to three days before SD progression, when SD prediction remains clinically difficult. The model has potential to be translated to a point-of-care prognostic assay to reduce dengue morbidity and mortality without overwhelming limited healthcare resources.

NS5A domain I antagonises PKR to facilitate the assembly of infectious hepatitis C virus particles

Shucheng Chen, Mark Harris

School of Molecular and Cellular Biology, University of Leeds, Leeds, United Kingdom

HCV NS5A is a highly phosphorylated, multifunctional, protein comprised of three domains (I, II and III). Domains I and II have been shown to function in genome replication whereas domain III has a role in virus assembly.
Previously, we demonstrated that domain I of NS5A in genotype 2a (JFH1) also played a role in virus assembly, exemplified by the phenotype of an alanine substitution at P145 (Yin *et al*, 2018), which was not required for RNA replication. In the current study we extended this analysis to identify other conserved and surface exposed residues proximal to P145 that exhibited a similar phenotype. Specifically, these were C142, shown to be disulphide-bonded to C190 in NS5A domain I, and E191. Alanine substitutions of these residues did not exhibit a genome replication defect, but produced no infectious virus. Additionally, the size and distribution of lipid droplets in cells infected with these mutants was altered compared to wildtype (WT).
In parallel, to investigate the mechanism underpinning this role of domain I, we assessed the involvement of the interferon-induced double-stranded RNA-dependent protein kinase (PKR). Intriguingly, in PKR knockout cell lines, C142A and E191A exhibited levels of infectious virus production and lipid droplet size and distribution that were indistinguishable from WT. Protein interaction studies revealed that these mutants were defective in binding to PKR (unlike WT and C190A). In addition, in vitro pulldown experiments confirmed that WT NS5A domain I interacted with PKR. These data suggest a novel interaction between NS5A domain I and PKR that functions to evade an antiviral pathway that blocks virus assembly. Ongoing studies investigating the roles of both domain I and PKR in assembly of infectious HCV particles will be presented.

Optimizing a cell culture model for Hepatitis E virus infectivity assessment

Tatjana Locus[1*], Michael Peeters[1], Ellen Lambrecht[2], Thomas Vanwolleghem[3] and Steven Van Gucht[1]

[1:] *Viral Diseases, Infectious Diseases in Humans, Sciensano, Ukkel, Belgium*

[2:] *Technology and Food Unit, Flanders Research Institute for Agriculture, Fisheries and Food, ILVO, Melle, Belgium*

[3:] *Viral Hepatitis Research Group, Laboratory of Experimental Medicine and Pediatrics, University of Antwerp, Antwerp, Belgium*

Background and Aims: Hepatitis E virus (HEV) is a major cause of viral hepatitis worldwide. Most infections in Western countries are caused by genotype 3 (gt3). Foodborne transmission is considered to be a significant route of infection since pigs and wild boars were identified as the main source of human HEV gt3 infection [1]. However, methods for detecting infectious particles in food products are not well standardized. Furthermore, a paucity of permissive *in vitro* and *in vivo* infection models precludes exhaustive HEV infectivity studies. In order to assess the infectivity of HEV, an efficient, easy and robust cell culture model was designed.

Methods: Cells were seeded in maintenance medium (*i.e.* DMEM or MEM with 5-10% Fetal bovine serum (FBS)) three days before being inoculated with cell culture derived gt3 47832c HEV strain. After an incubation of one hour, cells were washed three times with PBS (Phosphate buffered saline) and supplemented with infection medium (*i.e.* MEM with 10% FBS and 2% Dimethyl sulfoxide (DMSO)). HEV infection read-outs consisted of an HEV ORF2 immunofluorescence staining of target cells and RT-qPCR HEV RNA determination in supernatants.

Results: In order to optimize previously proposed models [2], ten different cell lines were compared. Hepatocyte cell line HuH7-S10-3 showed the highest percentage of infected cells by ORF2 immunostaining, while lung carcinoma cell line A549-D3 displayed a significant increase in RNA detection by RT-qPCR (p-value < 0,05). The effect of infection medium composition (*i.e.* DMSO and FBS addition) was evaluated. The addition of 2% DMSO increased infection in both cell lines, but only when medium was supplemented with 5-10% FBS (p-value < 0,05). Finally we determined the kinetics of infection and found ORF2 to be expressed within five days in A549-D3 cells, corresponding with a significant increase in RNA detection (p-value < 0,05). Infection in HuH7-S10-3 cells was noticeable only after seven days.

Conclusions: The A549-D3 cell infection model using ORF2 staining and RNA determination by RT-qPCR at day five after inoculation as read out for infectivity was chosen as the model of choice because of the reproducible and relatively fast turnaround time of the assay.

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* : Corresponding author: Tatjana.Locus@sciensano.be

Validation of a reporter cell line for flavivirus inhibition assays

Tatiana Rezende, Gabriella Macera, Leo Heyndrickx, Sandra Coppens, Kevin Ariën, Koen Bartholomeeusen

Institute of Tropical Medicine, Antwerp, Belgium

Flaviviruses are responsible for a large portion of clinically relevant arbovirus infections and their differential diagnosis is important for prognosis and therapeutic strategies. Neutralization assays are the gold standard for determining neutralizing antibody titers against viruses in the serum of patients. Neutralization assays and testing of antiviral compound activity is often based on quantification of the reduction in cytopathic effect of the virus of interest by microscopic assessment of CPE or plaques. As different viruses can infect different cells or don't produce cytopathic effects separate cell lines would be used to analyze even related viruses. The positive single-stranded RNA flaviviruses produce double stranded vRNA replication intermediates and are potent inducers of IFNb gene expression following detection by the infected cell.

We report the construction and validation of a single reporter cell line for flavivirus antiviral assays. The Hec1a endometrial adenocarcinoma cell line was modified by stable incorporation of a Luciferase reporter gene driven by an IFNb gene promoter (Hec1a-IFNLuc). Infection of the cell line with several clinically relevant flaviviruses: WNV, YFV, ZIKV, TBEV (Hypr and Neudörfl strains), DENV-1 through DENV-4, and JEV showed consistent and titration-dependent activation of the reporter luciferase gene.
The use of the reporter cell line for diagnostic purposes was validated in serum neutralization assays. These were performed for YFV, TBEV and ZIKV and results compared to the gold standard CPE assay. Propensity for cross neutralization was determined for YFV vs. ZIKV. The potential use of the reporter cell line for antiviral compound screening was further examined using the antiflaviviral adenosine analog, NITD008. We determined its inhibitory activity against DENV-2, YFV, ZIKV and TBEV and verified it relied on suppression of active viral replication.
Together these data indicate the potential use of the reporter cell line as a convenient pan-flavivirus diagnostic and antiviral therapy screening tool and suggest its potential use for other viruses capable of infecting the cell line and stimulating IFNb gene expression.

Phylogeographic analysis of dengue virus type 1 and 2 in Africa

Philippe SELHORST[a,d,*], Sebastian LEQUIME[e], Sam PROESMANS[g], Pascal Lutumba[h,i], Freddy Katshongo[j], Kadrie RAMADAN[b], Isabel MICALESSI[b], Veerle VANLERBERGHE[f], Marjan Van ESBROECK[b], Kevin K. ARIËN[a,c]

Affiliations:
[a] *Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium*
[b] *Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium*
[c] *University of Antwerp, Antwerp, Belgium*
[d] *The Outbreak Research Team, Institute of Tropical Medicine, Antwerp, Belgium*
[e] *Institute for Evolutionary Life Sciences, Groningen, The Netherlands*
[f] *Department of Public Health, Institute of Tropical Medicine, Antwerp, Belgium*
[g] *Department of Internal Medicine, Infectious Diseases & Tropical Medicine*
[h] *University of Kinshasa, Kinshasa, Democratic Republic of Congo*
[i] *Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of Congo*
[j] *Institut Supérieur des techniques médicales, Kinshasa, Democratic Republic of Congo*
**corresponding author*
pselhorst@itg.be +32(0)32476546 Nationalestraat 155 2000 Antwerpen BELGIUM

Background.
Recent decades have seen an increase in the disease burden and spread of Dengue virus (DENV). However, the origin and spread of DENV circulating on the African continent remains poorly characterized.

Methods.

Serum samples (n = 29) were collected in 2016 during an undifferentiated fever study in the Democratic Republic of Congo (DRC, ClinicalTrials.gov NCT02656862) as well as from febrile travelers returning from Africa. DENV-1 or -2 diagnosis was made using RT-qPCR after which whole genome sequencing was performed using Oxford Nanopore's MinION platform. To reconstruct the evolutionary history of the newly acquired African DENV genomes, a phylogeographic, time-scaled Bayesian analysis was performed using a curated panel of 365 DENV-2 and 726 DENV-1 sequences downloaded from NCBI and VIPR including all known African sequences.

Results.
All new DENV-2 genomes (n = 11) belonged to the Cosmopolitan genotype whereas the newly sequenced DENV-1 genomes (n = 18) were either genotype III or unknown. African DENV-2 sequences scatter across all DENV-2 Cosmopolitan clades and most were imported over the last decade from Indonesia, India, China and Malaysia. However, a West Africa clade was introduced in the 70s from Indonesia and has since spread to Eastern and Central Africa.
African DENV-1 genomes occur across all genotypes. The DENV-1 sequences from our DRC study were introduced from Angola and belong to an unknown African genotype introduced from India into Nigeria in the 1940-60's.

Conclusion
Overall strong geographical clustering can be observed with African sequences stemming from the same geographical regions indicating limiting mixing of DENV after each introduction in Africa.

Pregnancy zone protein as a promising biomarker for HEV-related acute liver failure

Jian Wu[1,2], Ze Xiang[3], Hongcui Cao[1]

[1]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qingchun Rd, Hangzhou City, Zhejiang Province, 310003, China;
[2]Department of Clinical Laboratory, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Gusu School, Nanjing Medical University, 242 Guangji Road, Suzhou215008, Jiangsu, China;
[3]Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310009, China;

Objective. In this study, we aimed to develop such accurate prognostic biomarkers for initial risk stratification of HEV-ALF.
METHODS. We performed four tandem mass tag (TMT)-labeled quantitative proteomic and targeted proteomics parallel reaction monitoring (PRM) studies on cross-sectional cohort 1 and 2 including 20 acute hepatitis E and 20 HEV-ALF patients respectively.
RESULTS. Pregnancy zone protein (PZP) was identified by TMT and PRM quantitative proteomics, and verified in retrospective cohort 1, which showed that PZP levels of the HEV-ALF survival group were significantly higher than those of the dead group ($P < 0.001$), and the survival time of the high PZP group was significantly longer than that of the low PZP group ($P = 0.003$). The decreasing PZP levels were also correlated with the increasing number of failed organs in HEV-ALF patients. Compared with PZP levels at admission, levels at discharge increased significantly in the improvement group ($P < 0.001$), and decreased significantly in both the fluctuation and deterioration groups (both $P \leq 0.001$). The area under the curve of PZP to predict the 30-day survival rate of HEV-ALF patients was 0.815. The ePLT score was significantly superior to the models for end-stage liver disease, King's College Hospital and Child–Pugh scores (all $P < 0.05$). PZP-TRFIA method was successfully constructed to provide the possibility for rapid detection for patients with hepatitis E, which contribute to reduce the mortality of HEV-ALF.
CONCLUSIONS. PZP is a promising prognostic biomarker for HEV-ALF patients, and ePLT is a high-performance prognostic score for HEV-ALF, which contribute to clinical decision-making in the management of HEV-ALF
Keywords: HEV-related liver failure (HEV-ALF); proteomics; pregnancy zone protein (PZP); prognostic predictor; ePLT score; PZP-TRFIA

Serological and molecular investigation of hepatitis E virus in pigs reservoirs from Cameroon reveals elevated seroprevalence and presence of genotype 3

NJOUOM Richard 1

1 = Department of Virology, Centre Pasteur of Cameroon, Yaounde´, Cameroon,

Background and aims: Hepatitis E virus (HEV) is an emerging and zoonotic pathogen of humans and animals, which is responsible for significant morbidity and mortality especially in developing countries. HEV affects approximately 20 million persons annually worldwide, causing over 70.000 deaths. HEV belongs to the Hepeviridae family that contains several viral species divided into two genera: Orthohepevirus with four species (Orthohepevirus A–D) and Piscihepevirus with one species (Piscihepevirus A). Eight genotypes exist within Orthohepevirus A and these HEV strains infect humans and multiple mammals' species. Genotype 1 and 2 are restricted to humans; genotype 3 is found among humans, swine, rabbits, deer and mongooses; genotype 4circulates between humans and swine; genotype 5 and 6 are found in wild boars; and genotype 7 and 8 were recently identified in dromedary and Bactrian camels, respectively. In Africa, contaminated water causes serious epidemic outbreaks. Other sources of infection such as animal transmission cannot be excluded since genotype 3 responsible for the zoonotic transmission of HEV has already been reported in some African countries. The information on HEV infection in animals in this continent remains underreported and pigs have been established as reservoirs of HEV. In Cameroon, minimal attention has been paid to HEV epidemiology in human and animal populations. The aim of this study is to determine the seroprevalence of HEV infection in pigs in Center and Littoral regions of Cameroon and to determine the molecular characterization of identified viruses.

Methods: A total of 453 serum and stool samples were randomly collected from pigs in slaughterhouses in Obala, Douala and Yaounde. All samples were examined for the presence of anti-HEV IgG and IgM antibodies using ELISA assays. IgM positive stool samples were tested for HEV RNA using an RT-PCR assay, followed by a nested PCR assay for sequencing and phylogenetic analysis.

Results: Overall, 216 samples (47.7%, 95% CI: 43.1%-52.3%) were positive for at least one of the serological markers of HEV infection. Amongst these, 21.0% were positives for anti-HEV IgM, 17.7% for anti-HEV IgG, and 9.1% for both. A total of eight stool samples (5.9%) were positive for HEV RNA by nested RT-PCR. Phylogenetic analysis showed that the retrieved sequences clustered within HEV genotype 3.
Conclusions: This study shows a high prevalence of anti-HEV antibodies and the circulation of genotype 3 in the swine population in Cameroon. Subsequent studies will be needed to elucidate the zoonotic transmission of HEV from pigs to humans in Cameroon

Genetic diversity and transmission dynamics of hepatitis C virus in Croatia

Petra Simicic[1], Anamarija Slovic[2], Leona Radmanic[1], Adriana Vince[3], Snjezana Zidovec Lepej[1]

[1] University Hospital for Infectious Diseases Zagreb, Croatia
[2] University of Zagreb, Centre for Research and Knowledge Transfer in Biotechnology, Croatia
[3] University of Zagreb, School of Medicine, Croatia

Background and Aims:
Implementation of successful interventions for hepatitis C virus (HCV) requires detailed understanding of molecular epidemiology and local viral transmission patterns. The aim of this study was to analyse distribution and transmission dynamics of the most common HCV genotypes and subtypes circulating in Croatia.

Methods:
This study included 300 patients with chronic hepatitis C receiving clinical care at the University Hospital for Infectious Diseases, Zagreb. Direct Sanger sequencing of the NS3, NS5A and NS5B region was performed and maximum likelihood trees were reconstructed in MEGA v.10.2.6 under 1000 bootstrap replicates. Identification of transmission clusters was performed using ClusterPicker v.1.2.3. with a genetic distance and bootstrap support threshold of 4,5 % and 70 %, respectively. Transmission cluster associations for continuous variables were analysed by the Mann-Whitney test while categorical variables were compared by Pearson's chi squared test or Fisher's exact test using Statistica v.13.5. A p-value < 0,05 was considered significant.

Results:
Majority of patients were infected with HCV genotype 1 (189/300, 63,0 %) followed by subtype 3a (111/300, 37 %) and had a median age of 45,0 years (IQR: 40,0 – 57,0). Phylogenetic tree reconstructions showed two distinct clades within the subtype 1a - clade I (68/109, 62,4 %) and clade II (41/109, 37,6 %). Phylogenetic analysis across all regions demonstrated that 27 (27/300, 9,0 %) HCV sequences had a presumed epidemiological link with another sequence and classified into 13 transmission clusters. Clustered individuals were significantly younger (median age (IQR) = 37,0 (33 - 41) years) (p < 0,001). Transmission clusters were the most common among patients infected with subtype 3a (17/111, 15,3 %) (p = 0,008). Among subtype 1a sequences, clustering was observed exclusively in clade I (8/68, 11,8 %) (p = 0,024). Sequenced obtained from patients who reported intravenous drug use (IDU) as a main risk factor for HCV acquisition more commonly formed transmission clusters compared to other reported HCV infection routes (14,7 % (14/95) vs 6,3 % (13/205), p = 0,018).

Conclusions:
IDU- and subtype 3a- dominated transmission clusters suggests that despite the numerous preventative measures against the spread of HCV infection, needle-sharing and similar behaviors are still common in IDU population. Prevention of HCV among this population will remain a major challenge in the foreseeable future.

The relationship between the transmission mode and selection pressure in HCV subtype 3a and 3b Chinese infections

Ru Xu [1,2#], Xia Rong [1,2,3#]John McLauchlan[4*], Yongshui Fu [1,2,3,5*]

[1] Institute of Clinical Blood Transfusion, Guangzhou Blood Center, Guangzhou, Guangdong, China;
[2] The Key Medical Laboratory of Guangzhou, Guangzhou, Guangdong, China;
[3] School of Laboratory Medicine and Biotechnology, Southern Medical University, Guangzhou, Guangdong, China;
[4] MRC-University of Glasgow Centre for Virus Research, Glasgow, UK.
[5] Zhujiang Hospital of Southern Medical University, Guangzhou, Guangdong, China;
[#] Ru Xu and Xia Rong should be considered joint first authors.
*Correspondence: fuyongshui@gzbc.org; john.mclauchlan@glasgow.ac.uk ;

Abstract:
Genotype 3 (GT-3) represents 22–30% of all infections and is the second most common genotype among all HCV genotypes. It has two main subtypes, 3a and 3b, presenting epidemiological differences in transmission groups. This report generated 56 3a and 64 3b whole-genome sequences to conduct an evolutionary kinetics and selective force analysis with reference sequences from various countries. Three Hypervariable Regions were found in both 3a and 3b subsets. Evolutionary analysis showed that HCV 3a worldwide might have been transmitted from an ancestor of the Indian subcontinent to South Asia, Europe, and North America and then became endemic in China. In China, 3a may be transmitted by intravenous drug users (IDUs) and become endemic in the general population, while 3b may have originated from IDUs, then underwent mutual transmission between blood donors (BDs) and IDUs, and ultimately formed an independent endemic in IDUs. Furthermore, the spread of 3a and 3b sequences from BD and IDU populations exhibit different selective pressures: the proportion of PPSs in E1 and E2 in IDUs was higher than in BDs, whereas the number of PPSs was higher in 3b and IDUs. These results potentially provide infor-mation about the inter-actions between transmission route, host immune pressure, and can help us formulate an HCV 3a and 3b prevention strategy in China.

Keywords: Hepatitis C virus; Evolution; Selection; Transfusion route; Origin

The changing distribution pattern of hepatitis C virus (HCV) genotypes -- a 15-year study from Guangdong blood donors, China

Xia Rong

Background: Hepatitis C virus (HCV) is a major cause of cirrhosis, chronic liver disease and hepatocellular carcinoma. HCV exhibits a high degree of genetic variability, and currently, HCV is phylogenetically divided into eight major genotypes (gt1-8) and 90 subtypes. In recent years, direct-acting antiviral（DAA）for HCV treatment have been proven to cure more than 95 percent of people infected with HCV, but there are still many people who do not get treatment because the infection is not detected or because of economic reasons, and there is currently no effective vaccine for HCV. About 1.5 million people are newly infected with HCV each year, according to the latest WHO report. About 10 million people in China are infected with HCV, which poses a serious threat to public health. For years, curbing the spread of HCV infection has been one of the major tasks of infectious disease prevention and control in China and the world.

Aims: To obtain the changes and progress of HCV epidemiology, in particular, the dynamic distribution of major prevalent genotypes, during the period from 2004 to 2019 in Guangdong province, and to provide evidence for effective and efficient HCV elimination strategies.

Materials and methods: From 2004 to 2019, we tested the HCV antibody in volunteer blood donors, conducted qualitative nucleic acid test on those who were antibody positive, and amplified the NS5B and E1 sequences of HCV RNA positive samples for phylogenetic analysis to determine the genotype. The study was divided into three periods, namely, 2004-2007,2008-2011 and 2012-2019. We compared and statistically analyzed the prevalence of HCV genotypes, geographic information (Guangdong and non-Guangdong) and population distribution patterns (gender and age) related to the main epidemic genotypes obtained in the three periods to track changes in HCV prevalence and subtype distribution from 2004 to 2019.

Results: A total of 1578 HCV RNA positive samples were obtained, and 1425 (90.3%) were successfully genotyped.
1. Changes in HCV subtype prevalence from 2004 to 2019: From 2004, the proportions for HCV 6a in blood donors was gradually increased from 34.75% to 44.60% ($P<0.05$), while the proportions of 1b diminished from 41.10% to 37.40%. Subtype 2a comprised 7%–8% of the HCV-positive donors during 2004–2011, but its proportion dropped to 4%–5% in 2012–2019 ($P<0.05$). We did not observe statistical differences in the distributions of other subtypes during these periods. The ranges of percentages fluctuated for subtypes 1a, 3a, and 3b at 0.47%–1.72%, 5.26%–8.33%, and 4.41%–7.20%, respectively (Table 1).
2. Changes of main epidemic genotypes in Guangdong and non-Guangdong regions: we observed a remarkable upward trend for 6a and a significant downward trend for 1b during 2004–2019 in non-Guangdong blood donors (Figure 1.b). In contradistinction, the proportions for these HCV subtypes were relatively stable during this same period in Guangdong blood donors (Figure 1.a).
3. Changes of HCV gene subtypes in different genders and ages: When the HCV-subtype distributions were analyzed by sex and age groups separately, we noted a significantly elevated trend for 6a but a downward trend for 2a in males and older blood donors (Figure 2.a & 3.b). The significantly downward trend for 1b proportions, however, was only exhibited in the older blood donors (Figure 3.b).
Conclusions：In this study, we describelong-term (2004-2019) trends and distribution of HCV genotypes among blood donors in Guangdong Province. The new HCV subtype 6a replaced 1b as the dominant subtype. We have also noted a significant increase in the proportion of HCV 6a strains among non-Guangdong blood donors in recent decades, indicating the expansion of HCV 6a in China. These trends are helpful to understand the transmission mode of HCV and formulate prevention and control measures.

Prevalence of viral hepatitis in a high-risk cohort of people who inject drugs

Marie-Annett Bernard 1, Tina Senff 1, Andreas Walker1, Norbert Scherbaum2, Jörg Timm1

1 Institute of Virology, Heinrich Heine University, Medical Faculty, Düsseldorf, Germany
2 LVR-Hospital Essen, Department of Addictive Behaviour and Addiction Medicine, Medical Faculty, University of Duisburg-Essen, Essen, Germany
**Character count 2490*

Introduction: Transmission of blood-borne viral infections is common in persons with high-risk behavior such as people who inject drugs (PWID). Accordingly, prevalence rates of infections with hepatitis C virus (HCV), hepatitis B virus (HBV) and human immunodeficiency virus (HIV) are higher when compared to the general population. Here we aimed to comprehensively analyze persistent viral infections in a large cohort of PWID and test if there is evidence for recent transmissions of HCV based on sequence analysis.

Methods: Samples from 698 PWID on opioid substitution treatment or admitted to hospital for addiction treatment were collected at a single center in Essen, Germany. The prevalence of serological markers for HBV, HCV, and HIV was tested by CMIA and the virus concentration was determined by nucleic acid amplification test. HCV genotyping was performed by sequence analysis of the NS5A region followed by phylogenetic analysis. The succeeding cluster analysis was performed using a calculated genetic distance cut-off and bootstrapping.

Results: The average age of the cohort is 38 (range 18-68) and 78% is male. 75% of the cohort is anti-HCV positive (47% HCV-RNA positive, 28% HCV-RNA negative), and 4.8% tested positive for HIV. Regarding the hepatitis B status 35.5% is anti-HBc positive (2.1% HBsAg positive), 34.4% is anti-HBs only positive and 30.1% is negative for HBV markers. Notably, anti-HBc positive PWID were significantly older than PWID carrying only anti-HBs, consistent with effective HBV immunization in the younger age groups ($p<0.0001$). The anti-HBc prevalence is significantly smaller in anti-HCV negative PWID (11.3%) than anti-HCV positive PWID (40.7%; $p<0.001$). The most common HCV genotypes were GT1a (42.4%), GT3a (40.8%) and GT1b (11.5%). Through phylogenetic and cluster analysis of the HCV sequences we identified a total of 21 clusters, 15 of which had a root bootstrap value of ≥ 95 indicating a strong reproducibility.

Conclusion: In this high-risk group for HCV infection there is evidence from phylogenetic analysis for recent transmissions. Although HBV vaccine effectiveness was confirmed in this cohort, still, nearly one third of the cohort is not immune despite being at risk for HBV infection. v

The surveillance of DENV, CHIKV and ZIKV virus infection by nucleic acid in the blood donations in China

Ru Xu[1,2,] Xia Rong[1,2,3], Yongshui Fu[1,2,3]

1 Guangzhou Blood Center, Guangzhou, Guangdong, China
2 The Key Medical Laboratory of Guangzhou, Guangzhou, Guangdong, China

3 Department of Transfusion Medicine, School of Biotechnology, Southern Medical University, Guangzhou, Guangdong, China

Mosquito-borne diseases have brought a serious threat to global public health. Due to approximately 80% of individuals infected with DENV, ZIKV and CHIKV present no symptoms or clinical signs after infection, these three viruses were under-estimation and under-reporting by official passive surveillance and reporting systems. Currently, little is known about the co-circulation and resulting co-infections of DENV, ZIKV and CHIKV in mainland China. Here, we aim to the determine the prevalence of these three arboviruses blood donations by nucleic acid testing in China. 64,555 unpaid blood donations (BDs) were collected from Guangdong, Guangxi, Yun nan, Hainan, Sichuan and Heilongjiang Province in China. No ZIKV and DENV infection were detected, only one donor from Hainan Blood Center in Hainan Province was positive for Chikungunya virus RNA. Our results indicated that the absence of active DENV, ZIKV epidemic and a limited necessity for the implementation of NAT testing in blood screening in China. The fact that one Chikungunya virus infection in Hainan may be a concern in transfusion medicine, further epidemiology studies should be conducted in the future.

Validation of a glycomics-based test associated with risk of HCC development in cirrhosis

Xavier Verhelst[1], Leander Meuris[2], Anja Geerts[3], Annelies Van Hecke[2], Nico Callewaert[2], Hans Van Vlierberghe[1]

1. UZ-Gent
2. Vib-Ugent
3. Ugent

Introduction
Cirrhosis is the main risk factor for the development of hepatocellular carcinoma (HCC). Six-monthly
screening with ultrasound is advocated for the surveillance of cirrhotic patients. We recently showed that a glycomics-based test (GlycoCirrhoTest [GCT]) can provide additional information regarding the risk of HCC development in cirrhotic patients.

Aim
The aim of this study is to provide an independent clinical validation of the GCT for the assessment of the risk of HCC development in cirrhosis.

Methods
Validation study on serum samples of patients with established compensated cirrhosis (CHILD Pugh A&B) in a tertiary liver center. Serum N-glycan profiling was performed and GCT was calculated at baseline using DNA sequencer assisted fluorophore assisted capillary electrophoresis. During the follow up period, patients were screened for the presence of HCC every 6 months with ultrasound and alpha foeto protein (AFP) measurements.

Results
A total of 198 cirrhotic patients were followed during a median follow up time of 7 years. Twenty-nine patients developed HCC and one died during follow up. At baseline, the mean GCT value was significantly higher in patients who developed HCC within 3 and 5 years compared to patients who did not develop HCC (Welch's t-test, p-value 3 years: 0.034, 5 years: 0.022). Hazard ratio for HCC development at 5 years based on GCT was 2.9 (95% CI, 1.2 – 7.0). Applying the same cut-off as from the proof-of-concept study (0.2), the negative predictive value of GCT for HCC development was 98.9%. GCT is based on changes in serum protein glycosylation related to cirrhosis nodularity and malignant transformation.

Conclusions
This independent validation study confirms that GCT is a glycomics-based test that provides additional information for risk assessment of HCC development in cirrhosis. This information could be used to develop personalised HCC screening programs in cirrhotic patients according to the value of GCT. Moreover, refocusing of the screening resources to the reduced number of cirrhosis patients who truly are at elevated risk for developing HCC may result in earlier detection of more HCC cases, for instance by making contrast-enhanced MRI screening cost-effective.

Session 4
VACCINE DEVELOPMENT

28TH INTERNATIONAL SYMPOSIUM ON HEPATITIS C VIRUS, FLAVIVIRUSES, AND RELATED VIRUSES

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Molecular determinants of mouse adaptation of rat hepacivirus

Raphael Wolfisberg[1], Kenn Holmbeck[1], Eva Billerbeck[2], Mariana N. Batista[2], Ulrik Fahnøe[1], Caroline E. Thorselius[1], Louise Nielsen[1], Charles M. Rice[2], Jens Bukh[1], Troels K. H. Scheel[1,2]

[1] Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital and Department of Immunology and Microbiology, University of Copenhagen, Denmark; [2] Laboratory of Virology and Infectious Disease, The Rockefeller University, New York, USA

Background and Aims: Hepatitis C virus (HCV) vaccine efforts and studies of pathology, immune responses and viral evasion are impeded by the lack of robust immunocompetent animal models. HCV related hepaciviruses were discovered in a number of other species and could provide useful infection models. Of particular interest is Norway rat hepacivirus (NrHV), which shares HCV-defining characteristics, including liver tropism, chronicity, and pathology in rats. To exploit a broader set of genetic variants and research tools, we previously adapted NrHV to prolonged infection in laboratory mice. Here, we characterized the molecular determinants of mouse adaptation.

Methods: To allow functional studies of NrHV mouse adaptation, we generated complete consensus clones and used these for intrahepatic RNA inoculation of mice and rats. We used a recently developed cell culture system to study neutralizing antibodies and to assess the effect of mouse adaptive mutations *in vitro*.

Results: Through reverse genetic studies in immune deficient and wild-type mice, we identified four mutations in the E1-E2 envelope proteins responsible for mouse adaptation, including one disrupting a glycosylation site. The adapted variant led to mouse serum titers of up to 10^8-10^9 genome equivalents (GE)/mL, similar to persistently infected rats. Infection was cleared after around 5 weeks compared to 1-3 weeks for non-adapted variants. In contrast, the four mutations led to attenuated infection in rats and they partially reverted accompanied by an increase in serum viral load. The mouse adaptive mutations further attenuated infection after viral RNA transfection of rat hepatoma cells in culture. This demonstrated that the identified mutations are indeed mouse adaptive rather than generally fitness enhancing, and that species determinants and not immune interactions were responsible for attenuation in rats. Unlike persistent infection in rats, acute resolving NrHV infection in mice was not associated with the emergence of neutralizing antibodies.

Conclusions: This study identified specific envelope mutations responsible for NrHV mouse adaptation, suggesting species specific interactions during entry. Persistence in mice beyond five weeks was not obtained with these mutations. Mouse adapted infectious clones will be of utility for reverse genetic studies and titrated serum pools for vaccination studies.

Virus-like-particle immunizations elicit cross-reactive antibody responses which recognize divergent pathogenic flaviviruses and promote both neutralization and infection enhancement

Eduardo Montoya-Diaz[1], Robin K. V. Kleinert[1], Yvonne Krebs[1], Veronika von Messling[1], Christian K. Pfaller[1], Richard J.P. Brown[1]

[1]Department of Veterinary Medicine, Paul-Ehrlich-Institute, 63225 Langen, Germany

Background: Mosquito-transmitted flaviviruses cause substantial morbidity and mortality in endemic regions. Antibody-dependent enhancement (ADE) of infection is reported to amplify flavivirus-induced pathogenesis, mediated by pre-existing immunity acquired from prior infections. Whether cross-reactive ADE can be induced by experimental flavivirus vaccine candidates and compromise their safety remains unclear.

Aims: Flavi-virus like particles (flavi-VLPs) expressing the structural proteins of four pathogenic flaviviruses (JEV, WNV, YFV and ZIKV) were evaluated for their ability to induce immunogen-specific and cross-reactive responses in mice. Their ability to induce neutralizing responses, versus those that promote ADE, were also assessed.

Methods: Plasmid constructs encoding the structural proteins (SP, prM and E) of JEV, WNV, YFV and ZIKV were individually synthesized and transfected into HEK293T cells for flavi-VLP production. Purified flavi-VLPs were checked for correct E protein processing and used to immunize BALB/c mice following a homologous prime-boost regimen. Mouse sera were used in an immunoperoxidase monolayer assay (IPMA) to evaluate flavi-VLP-induced antibody binding and cross-reactivity. Live-virus infection (YFV and ZIKV) of K562 cells after incubation with mouse immune sera determined whether flavi-VLP-induced immunity promoted neutralization or ADE.

Results: Induction of immunogen-specific binding antibodies was observed for all Flavi-VLPs immunizations, and titers were significantly increased after booster immunizations. Despite substantial amino acid heterogeneity between the structural proteins of the four flaviviruses, Flavi-VLP induced antibodies exhibited broad cross-reactivity between ZIKA, YFV, WNV and JEV. To assess whether flavi-VLP induced antibodies promoted neutralization or ADE of ZIKV and YFV, live-virus infections of K562 cells were performed in the presence of immune sera. K562 cells are permissive for flavivirus infection via classical entry routes and also express the Fcγ receptor, mediating uptake of IgG opsonized virions. In this system, Flavi-VLP induced antibodies promoted both neutralization and ADE of infection.

Conclusions: Flavi-VLPs were highly immunogenic, inducing cross-reactive antibodies that mediated both neutralization and ADE. Current investigations seek to determine whether uncleaved prM on the surface of Flavi-VLPs, or highly conserved epitopes in the envelope protein promote ADE. These data highlight the potential for inducing cross-reactive ADE and suggest specific immunofocusing is required when designing flavivirus vaccine candidates.

Adaptation of Hepatitis C virus for the generation of a HCV mouse model

Sheldon J[1], Yuan Q[1], Winkler M[1], Verhoye L[7], Labuhn M[1] Frericks N[1], Sake S[1], Reinecke B[1], Matthaei A[1], Kohn M[1], Zhang Y[1], Brown R[4], Carpentier A[1], Gödecke N[2], Behme S[2], Rox K[3], Vondran F[5], Pöhlmann S[6], Meuleman P[7,] Wirth D[2], Pietschmann T[1].

[1]Twincore EVIR, Hannover, Germany, [2]HZI Division Model Systems for Infection and Immunity, Braunschweig, Germany, [3]HZI Division of Chemical Biology, Braunschweig, Germany, [4]PEI, Langen, Germany, [5]MHH, Hannover, Germany, [6]DPZ, Göttingen, Germany, [7]UGhent, Ghent, Belgium.

Background and Aims: The WHO currently estimates 58 million people have chronic HCV infection, with approximately 1.5 million new infections occurring each year. Despite effective treatment, viral transmission remains high and a prophylactic vaccine is unavailable. The absence of an immune competent HCV animal model limits vaccine research and development. HCV has a narrow species tropism, and only naturally infects humans. However, replication is error-prone giving rise to a vast spectrum of variants, facilitating viral adaptation to changing environments. Here we exploit this property for the generation of a virus population with high replication fitness in primary mouse hepatocytes (PMH).

Method: We used a step-wise adaptation procedure culturing HCV between human (Huh-7.5) and mouse liver cells expressing HCV entry factors (MLT-5H) and eventually culturing in PMH from entry factor transgenic mice (hOC^{hep}) with blunted IFN signalling. Viral populations and a clone of the mouse-adapted population (MadHCV) were compared in mouse and human cells. Finally, MadHCV was used to infect hOC^{hep} $IFNAR^{-/-}$ mice.

Results: The HCV adaptation led to increased infectious virus productions in the MLT-5H, hOC^{hep} PMH + ruxolitinib and hOC^{hep} $IFNAR^{-/-}$ PMH by more than 3 logs compared to the parental virus. In contrast, infection of primary human hepatocytes, human pluripotent stem cell-derived hepatocytes, and primary macaque hepatocytes remained similar to parental HCV, suggesting that adaptation was specific to mouse. The MadHCV population remained susceptible to telaprevir and interferon and depended on the expression of human hCD81 and hOccludin for infection. The MadHCV clone had similar replication and infection kinetics to the population. Finally, in vivo infections resulted in 2/4 mice with an increase in viremia and detectable HCV antibodies after 3 weeks of infection compared to 0/4 mice in the heat inactivated control group, reinfection of the mouse sera from week 3 resulted in detectable viremia for up to three weeks post infection.

Conclusion: This step-wise adaptation has enabled us to broaden the HCV tropism to infect human entry factor transgenic mice with blunted IFN signalling. The MadHCV clone provides new opportunities for development of an immune competent mouse model for HCV, which in turn should facilitate HCV vaccine research and development.

Broad Immunogenicity of a Nucleoside-modified mRNA Lipid Nanoparticle Vaccine for Hepatitis C Virus Prophylaxis

Erin K. Reagan[*1], Elena N. Atochina-Vasserman[1], Kaitlyn Clark[2], Desiree A. Wright[2], Justin R. Bailey[2], George M. Shaw[1], Katharine J. Bar[1], Drew Weissman[1]

[1]Infectious Disease, University of Pennsylvania Perelman School of Medicine, Philadelphia, United States; [2]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, United States

Purpose: Despite the existence of effective curative therapy for hepatitis C virus (HCV) infection, the world has not met WHO's call to decrease HCV incidence by 90% before 2030. An effective vaccine is needed to meet this goal.
Background: In HCV, recent studies show neutralizing antibodies protect from challenge and clear established infection in animal models and are implicated in spontaneous clearance in humans. Modified-mRNA lipid nanoparticle-encapsulated (mRNA-LNP) vaccines have been shown to reliably establish potent humoral and cellular responses. Here, we investigate whether a novel mRNA-LNP vaccine induces neutralizing antibodies in animals with intended extension to humans.
Methods: We designed immunogens which code for HCV proteins using viral sequences derived from an individual who developed broadly neutralizing antibodies and spontaneously cleared HCV infection. mRNA immunogens were synthesized by *in vitro* transcription, purified to remove contaminants, and tested for production of viral proteins *in vitro*. Produced mRNA immunogens were then encapsulated in LNPs and male and female mice, 10 per group, were immunized intramuscularly with multiple mRNA-LNP vaccine constructs or empty LNPs as a control.
Results: Serum was collected and evaluated for anti-E1E2 antibody responses by ELISA. In addition to binding the autologous viral strain, the top mRNA-LNP vaccine construct elicited robust responses against a broad panel of antigenically diverse HCV variants. Serum from immunized mice was also evaluated for neutralizing antibodies against HCV pseudoparticles, where the top vaccine construct neutralized both the autologous and heterologous strains tested.
Conclusion: We present the design, synthesis, *in vitro* evaluation, and *in vivo* testing of an mRNA-LNP vaccine encoding HCV viral proteins. Our study demonstrated that a novel mRNA-LNP HCV vaccine was strongly immunogenic, eliciting responses against a broad selection of antigenically diverse HCV viral variants, and is being considered for future clinical trials to evaluate safety and immunogenicity in humans.
Disclosure of Interest: E.K.R., K.C., D.A.W., J.R.B., and D.W. receive research support from a biopharmaceutical company. E.K.R., J.R.B., G.M.S., and D.W. are inventors on patents describing the use of a nucleoside-modified mRNA vaccine for HCV prophylaxis (US Provisional Patent Application No. 20200222528, filed 27 April 2018 and No. 63218685, filed 6 July 2021). In accordance with the University of Pennsylvania policies and procedures and our ethical obligations as researchers, D.W. is named on additional patents that describe the use of nucleoside-modified mRNA and targeted LNPs as platforms to deliver vaccines. These interests have been fully disclosed to the University of Pennsylvania, and approved plans are in place for managing any potential conflicts arising from licensing these patents.

The development of a pan-genotypic viral vectored T cell vaccine against hepatitis C

Rebecca Strain[1,2], Claire Hutchings[2], Matthew Edmans[2], Senthil Chinnakannan[2], Paul Klenerman[2], Eleanor Barnes[2,3]

[1] Presenting author ; [2] Peter Medawar Building for Pathogen Research, Nuffield Department of Medicine, University of Oxford, OX1 3SY; [3] Jenner Institute, Nuffield Department of Medicine, University of Oxford, OX3 7DQ, United Kingdom

Background: The significant genetic diversity of hepatitis C virus (HCV) poses a major challenge for the development of an effective prophylactic vaccine. A previous promising vaccine candidate using chimpanzee adenovirus (ChAd) vectors and modified vaccinia virus Ankara (MVA) vectors encoding the non-structural (NS) proteins of HCV genotype 1b (Gt1b) failed to prevent chronic infection, despite producing high magnitude HCV specific T cells. The inability of T cells to cross recognise dominant epitope variants may have contributed to vaccine failure. To overcome this challenge, we have developed viral vectors encoding conserved segments of HCV genotypes 1–6 (ChAd-Gt1-6 and MVA-Gt1-6), ancestral 'Bole1a' HCV NS sequence (ChAd-Bole1a-NS), and Gt3a NS sequence (MVA-Gt3a-NS). We compared the immunogenicity and cross-reactivity of these vaccines in mouse head-to-head *in vivo* experiments.

Methods: Vaccines were compared following a single dose (prime) and in heterologous prime-boost. In prime experiments, inbred C57BL/6 or transgenic HLA-A*02:01 mice were vaccinated intramuscularly (IM) with ChAd-Gt1-6 or ChAd-Bole1a-NS ($1x10^8$ IU). In prime-boost experiments, HLA-A*02:01 mice were vaccinated IM with ChAd-Gt1-6 or ChAd-Bole1a-NS ($1x10^8$ IU) followed 8-weeks later with MVA-Gt1-6 or MVA-Gt3a-NS ($5x10^7$ PFU) respectively. HCV specific T cell magnitude, breadth and function was assessed 14-days post-vaccination using Gt-1a, -1b and -3a peptides in *ex vivo* IFN-gamma ELISpot and intracellular cytokine staining (ICS) assays.

Results: In prime only studies, both ChAd-Gt1-6 and ChAd-Bole1a-NS generated functional T cell responses. However, ChAd-Gt1-6 induced a broader response than ChAd-Bole1a-NS. The response to ChAd-Bole1a-NS was predominantly targeted to the NS3 helicase peptide pool and was specific to Gt-1 with limited cross reactivity to Gt-3a (Fig.1A). In contrast, the response to ChAd-Gt1-6 was targeted across multiple peptide pools and was cross reactive to all genotypes assessed, with a significantly higher response to Gt3a compared to ChAd-Bole1a-NS (p= 0,035). Prime-boost greatly increased the magnitude of the response to both vaccines (Fig.1B). When ChAd-Bole1a-NS vaccine was boosted with the heterologous MVA-Gt3a-NS vaccine the breadth of response was comparable to that induced by ChAd-Gt1-6 followed by MVA-Gt1-6, though with differences in specificity within the different genotypes (Fig.1B).

Conclusion: Viral vectors encoding different HCV genotypes used in prime-boost, or viral vectors encoding the same HCV immunogen consisting of conserved regions of genotypes 1-6 are two novel approaches to generate cross-genotypic HCV vaccines. Both strategies generate high magnitude T cell responses targeting multiple HCV genotypes when used in prime-boost regimens. Phase-I human clinical trials would be required to select the optimal pan-genotypic strategy.

The effect of glycan shift on antibodies elicited by chimeric sHBsAg-based virus-like particles carrying HCV E2 412-425 epitope

Anna Czarnota[1], Anna Offersgaard[2], Ania Owsianka[3], Jens Bukh[2], Judith Margarete Gottwein[2], Arvind H. Patel[3], Krystyna Bieńkowska-Szewczyk[1], Katarzyna Grzyb[1]

[1]Laboratory of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology of the University of Gdańsk and Medical University of Gdańsk, Poland; [2]Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Copenhagen University Hospital–Hvidovre and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; [3]MRC-University of Glasgow Centre for Virus Research, Glasgow G61 1QH, UK

Background: Hepatitis C Virus (HCV) developed several immune evasion strategies including variability of the amino acid sequence and epitope shielding via heavy glycosylation of the envelope proteins. In our previous studies, we showed that chimeric virus-like particles (VLPs) carrying highly conserved epitope I of the HCV E2 glycoprotein (sHBsAg_412-425) elicited broadly neutralizing antibodies (bnAbs). However, many reports have identified escape mutations for such bnAbs arising due to a shift of the N-glycosylation site from N417 to N415. This shift effectively masks the recognition of epitope I by antibodies raised against the wild-type glycoprotein. In this study, we aimed at overcoming such epitope I immune evasion by designing a vaccine eliciting antibodies with affinity to HCV glycan shift escape variants.

Results: We designed sHBsAg-based VLPs carrying epitope I with amino acid change N417S (sHBsAg_N417S). Both sHBsAg_N417S and the original sHBsAg_412-425 VLPs were recognized by the epitope I-specific monoclonal antibody (mAb) AP33 with similar, high efficiency. Immunogenicity studies revealed that both sHBsAg_412-425 and sHBsAg_N417S VLPs were immunogenic, eliciting antibodies recognizing peptides encompassing epitope I regardless of the N417S change. However, sHBsAg_N417S immune sera binding to the mammalian cell-expressed E1E2 heterodimers was strongly dependent on the presence of the N417S change. Those results were further supported by neutralization assays against cell culture infectious HCV (HCVcc) carrying N417S change. Additionally, E1E2 alanine scanning and competitive ELISA assays allowed us to identify similarities between sHBsAg_412-425 immune sera and mAb AP33 binding pattern.

Conclusions: Our study emphasizes the impact of the N417S change on epitope I antigenicity and reveals a high degree of similarity between mAb AP33 and antibodies elicited by sHBsAg_412-425 VLPs. Additionally, sHBsAg_N417S immune sera binding and neutralization showed strong dependency on the presence of the N417S change, suggesting interplay between E1E2 glycosylation status and epitope I or global E2 glycoprotein conformation. These results suggest that vaccines generating antibodies against epitope I are highly desirable but should be accompanied by an antigen eliciting an immune response against the epitope comprising a glycan shift change.

Binding of soluble hepatitis C virus glycoproteins to germline VH1-69-derived broadly neutralizing antibodies

Joan Capella-Pujol[1], Marlon de Gast[1], Laura Radić[1], Ian Zon[1], Ana Chumbe Mendoza[1], Sylvie Koekkoek[1], Wouter Olijhoek[1], Rogier W. Sanders[1], Kwinten Sliepen[1],
[1]Department of Medical Microbiology, Amsterdam Infection & Immunity Institute, Amsterdam UMC, University of Amsterdam, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands.

An effective preventive vaccine for hepatitis C virus (HCV) is a major unmet need. Such a vaccine should probably induce broadly neutralizing antibodies (bNAbs) to effectively counteract the extreme genetic variability of the virus. The E1E2 glycoprotein on the viral membrane is the main target for bNAbs. antigenic region (AR) 3, which is located on the E2 subunit, is an important epitope for a many bNAbs. AR3 bNAbs usually require little somatic hypermutation (SMH) to acquire breadth, which makes AR3 a desirable target for vaccine design. Germline vaccine strategies aim to specifically target the naïve precursor B cells that are capable of developing bNAbs. Most AR3 bNAbs utilize the *V_H1-69* gene and share structural features. Identifying key signatures of binding of these bNAbs is key to a successful germline-targeting vaccine design. In this work, we analyzed a subset of *V_H1-69*-derived AR3 bNAbs (i.e. AR3C-like) and their germline predecessors. We used a panel of recombinant E1E2 proteins based on different primary HCV strains to test binding and performed neutralization assays using HCV pseudoparticles. We found that some inferred germline AR3C-like bNAbs can bind to recombinant E1E2 and neutralize the sequence-matched virus. This indicates that certain HCV strains are able to activate some germline HCV bNAbs and these sequences are thus a promising starting template for generating a germline-targeting immunogen. More in-depth analyses of the inferred germline AR3C and HEPC74 bNAbs highlighted the importance of the complementarity-determining regions of the heavy chain 3 (CDRH3) for binding and allowed us to identify polymorphisms in the *V_H1-69* gene that unexpectedly and strongly altered antibody binding. Finally, we show that our recombinant immunogens displayed on two-component nanoparticles are capable of activating in vitro B cells carrying inferred germline AR3C and HEPC74 as their B cell receptor when multivalently displayed on nanoparticles. This work offers novel insights into the binding characteristics of germline AR3 bNAbs and provides promising starting templates for generating HCV germline-targeting immunogens.

Chimeric recombinant vaccine against hepatitis E virus and hepatitis B virus infection

Laura Collignon[1], Lieven Verhoye[1], Elodie Beaumont[2], Ali Farhoudi[1], Freya Van Houtte[1], Ann-Sofie Vercouter[1], Karin Weening[1], Philippe Roingeard[2], Philip Meuleman[1]

[1] Laboratory of Liver Infectious Diseases, Department of Diagnostic Sciences, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium; [2] INSERM Unit 1259 MAVIVH, University and CHRU of Tours, Tours, France

Background: The hepatitis E virus (HEV) has become a global health burden, infecting more than 20 million individuals each year. Certain populations have a higher risk of developing a severe HEV infection (e.g. pregnant women, transplant recipients, food industry workers and veterinarians). Moreover, new HEV strains, infectious to humans or resistant to treatment, are being identified. A prophylactic vaccine against HEV would be beneficial in these high-risk groups and in endemic regions. Hepatitis B virus (HBV) is another major human pathogen; fortunately, HBV infection can be prevented with an efficient subunit vaccine of recombinant surface antigen (HBsAg) subviral particles.

Methods: We developed a chimeric recombinant vaccine in which a fragment of the HEV capsid protein (p239) was linked to the N-terminus of HBsAg. In order to obtain sufficient coverage of the vaccine, two variants were made, based on the HEV-1 and HEV-3 sequence.

Results: Upon co-expression with wild-type HBsAg, the chimeric HEV-HBV proteins were efficiently incorporated into subviral particles. These particles are the active component of our dual vaccine against HEV and HBV infection. Vaccination of mice with these chimeric particles induced production of anti-HEV and anti-HBV antibodies. The induced antibodies were able to neutralise HEV-3 virus both *in vitro* and *in vivo*.

Conclusion: Subviral particles of HBsAg and HEV p239 are able to elicit a humoral immune response in mice and induce antibodies that are neutralising against HEV-3. Further research will reveal the cross-neutralisation capacity of the vaccine-induced antibodies.

Intergenotypic HCV JFH1-based Core-NS2 variants with genetic stability in vivo and in vitro: important tools in evaluation of virus neutralization

Laura Collignon[1,3], Kenn Holmbeck[1], Ashley Just[1], Lieven Verhoye[2], Ulrik Fahnøe[1], Thomas H. R. Carlsen[1], Jannick Prentoe[1], Troels K. H. Scheel[1], Judith M. Gottwein[1], Philip Meuleman[2], Jens Bukh[1]

[1] Copenhagen Hepatitis C Program (CO-HEP), Denmark; [2] Laboratory of Liver Infectious Diseases, Ghent University, Belgium; [3] Presenting author

Background and aims: Evaluation of humoral responses including to vaccines would greatly benefit from genetically stable viruses of HCV genotypes (GT) 1-6, of use both in vivo and in vitro. JFH1-based Core-NS2 recombinants with cell culture adaptive mutations have been essential in neutralization and cross-reactivity studies.
Here, we assessed their infectivity in human-liver chimeric mice to identify genetically stable virus variants infectious in vivo and in vitro.

Methods: Humanized mice were infected with culture derived HCV Core-NS2 recombinants and genetic stability was assessed through high-throughput sequencing. Mouse-selected mutations were introduced into the genome of parental genotype-specific variants. Infectivity and genetic stability of these new virus variants were assessed in Huh7.5 cells. Selected variants were used to infect humanized mice to determine if engineered mutations resulted in enhanced infectivity in these mice.

Results: We demonstrated that the cell culture adapted viruses are capable of sustained replication in humanized mice. However, most variants acquired one or more mutations not previously observed in vitro. Of note, all viruses except J8, acquired E1/E2 mutations over time. During first passage in Huh7.5 cells, GT 1-2 variants (strains H77, TN, J4, DH1, J8, S83) showed improved infectivity (0,5 to 2 log10) in cell culture, whereas other variants had similar (GT 4 strain ED43 and GT 6 strain HK6a) or reduced (GT 3 strain S52 and GT 5 strain SA13; 0,5 to 1 log10) infectivity compared to the parental strains. Sequencing revealed that all introduced mutations were retained in cell culture. Interestingly, variants with improved infectivity did not acquire additional cell culture adaptive mutations and are therefore genetically stable in cell culture. Variants with reduced infectivity acquired multiple cell culture mutations. Humanized mice, infected with genetically stable variants, showed enhanced infection with significant increases in HCV RNA serum titers, compared to parental strains.

Conclusions: JFH1-based Core-NS2 recombinant viruses of GT 1-6 are infectious both in vitro and in vivo. Those with mouse-selected mutations of GT 1-2 showed increased infectivity both in vitro and in vivo, and these can therefore easily be passaged between cell culture and humanized mice. Although the effect of E1/E2 mutations on neutralization should be evaluated, these versatile cross platform viruses will serve as important tools enabling evaluation of antibody-based regimens first in vitro and subsequently in vivo.

Hepatitis C virus modified E2 mRNA-LNP candidate vaccine enhances protective immune response

Vijayamahantesh Vijayamahantesha, Tapas Patraa, Keith Meyera, Mohamad-Gabriel Alamehc, Erin K. Reaganc, Drew Weissmanc, and Ranjit Raya,

a)Departments of Internal Medicine and bMolecular Microbiology & Immunology, Saint Louis University, Missouri, MO 63104; and cDepartment of Medicine, University of Pennsylvania, Philadelphia, PA 19104

Background and Aims: Hepatitis C virus (HCV) is characterized by a high number of chronic cases owing to an impairment of innate and adaptive immune responses. CD81 facilitates HCV entry by interacting with the E2 envelope glycoprotein. In addition, E2 binding to CD81on immune related cells may also influence host response outcome to HCV infection.
Methods: We modified the front layer of the soluble E2 (sE2) sequence (F442NYT), including mutations and adding potential N-linked glycosylation site, to reduce CD81 binding and evaluated immunogenicity. Protective response to challenge infection of mRNA-lipid nanoparticle (LNP) as a candidate vaccine and resistance to challenge infection of a recombinant vaccinia virus expressing HCV proteins was investigated in BALB/c mice as a preclinical trial.
Results: The modified sE2 protein, unlike wildtype sE2, induced higher levels of pro-inflammatory cytokines, repressed anti-inflammatory responses in primary monocyte-derived macrophages, and stimulated CD4+T cell proliferation. Immunization of mice with an E1/sE2F442NYT nucleoside modified mRNA-lipid nanoparticle (LNP) vaccine generated an improved Th1 response, IgG1 to IgG2a antibody isotype switching, an increase in neutralizing antibodies against HCV pseudotype virus, and protective efficacy against vaccinia challenge model (expressing HCV E1-E2-NS2aa134) in mice, as compared to immunization with an E1/unmodified sE2 mRNA-LNP vaccine.
Conclusions: Our results clearly suggested that HCV sE2 exhibits immunoregulatory activity which inhibits induction of robust protective immune responses. Selection of engineered sE2 antigen in a mRNA-LNP platform amenable to nucleic acid sequence alterations holds promise for a future multi-genotype HCV vaccine.

Identification and Characterization of Immunodominant Epitopes on Hepatitis C Viral Envelope protein associated with infection outcome

Haneen Faris-Gadban[1], Anastasia Brodov[1], Michal Webner[1], Haim Ashkenazy[2], Tal Pupko[2], Jonathan Gershoni[2] Meital Gal-Tanamy[1]

[1]Molecular Virology Lab, The Azrieli Faculty of Medicine, Bar-Ilan University. [2]The George S. Wise Faculty of Life Sciences, Tel-Aviv University.

Background and aims: Hepatitis C virus (HCV) is a leading cause of liver disease despite the availability of effective HCV treatments, and therefore there is still a need for developing anti-HCV vaccine. Approximately 25% of infected patients clear the virus spontaneously (SC) while the remaining 75% will develop a chronic disease (CI). It is widely accepted that neutralizing antibodies play a key role in viral clearance and have a wider neutralization breadth in SC compared to CI patients. Here, we aimed to identify and explore immunodominant epitopes on the HCV-E2 envelope protein that are associated with specific infection outcome.

Methods: To identify neutralization epitopes we undertook an unbiased approach by screening a random phage display library presenting various peptides to detect binders to antibodies in sera obtained from SC and CI patients. By repetitive rounds of biopanning followed by next generation sequencing of the binding peptides, we identified enriched peptide sequences that were aligned to HCV E2. The most significantly enriched epitopes were synthesized as peptides that were tested for binding to neutralizing antibodies in SC and CI sera using ELISA and neutralization assays. To evaluate the potential of the identified epitopes to induce broad neutralizing antibody response, ICR-mice where immunized with the different peptides, and the collected sera from these mice were tested for binding to and neutralizing HCV.

Results: by bioinformatic analysis, we identified epitopes that are abundant more in CIs and others that are abundant more in SCs. The specificity of the epitopes was validated by binding and neutralization assays using a wider panel of sera from SCs and CIs. Among these we identified the well-known immunodominant epitopes on the viral E2 glycoprotein: epitope I (a neutralizing epitope) and epitope II (an interfering epitope). We found that epitope I is unique to SCs and contributes to efficient HCV-neutralization as it binds neutralizing antibodies that are more abundant in SC compared to CI sera, while epitope II is unique to CIs. Furthermore, mice vaccinated with peptides representing epitopes unique to SCs showed higher potency of viral neutralization and neutralization breadth compared to peptides representing epitopes unique to CIs.

Conclusion: This study identifies neutralization epitopes that are associated with viral clearance and induce broad and effective antibody immune response as linear peptide and therefore is expected to lead to rational design of anti-HCV peptide-based vaccine.

Two-component Vaccine Consisting of Virus-Like Particles Displaying Hepatitis C Virus Envelope Protein 2 Oligomers

Jannick Prentoe[1], Christoph M. Janitzek[2], Rodrigo Velázquez-Moctezuma[1], Andreas Soerensen[1], Thomas Jørgensen[3], Stine Clemmensen[3], Vladislav Soroka[3], Susan Thrane[2], Thor Theander[2], Morten A. Nielsen[2], Ali Salanti[2], Jens Bukh[1], Adam F. Sander[2,4]

[1]Copenhagen Hepatitis C Program (CO-HEP), Copenhagen University Hospital, Hvidovre and University of Copenhagen, DK; [2]Centre for Medical Parasitology, Copenhagen University Hospital, Rigshospitalet and University of Copenhagen, DK; [3]ExpreS²ion Biotechnologies, DK; [4]AdaptVac Aps, DK

Background and Aims: Hepatitis C virus (HCV) is the cause of significant disease worldwide and despite effective treatments, a vaccine is urgently needed. Inducing (broadly) neutralizing antibodies ((b)NAbs) is hindered by sequence diversity and low immunogenicity of the envelope glycoprotein vaccine candidates, most notably soluble E2 (sE2). Thus, we tested whether bNAb induction could be boosted by employing a two-component approach using virus-like particles (cVLPs; component 1), displaying monomeric or oligomeric forms of soluble HCV sE2 of the isolate Con1 (genotype 1b; component 2).

Methods: Prime-boost-boost immunization studies were performed in BALB/C mice and total anti-E2 IgG content of mouse sera was quantified by ELISA. The neutralizing capacity of vaccine-induced IgG was tested in cell culture infectious HCV (HCVcc) neutralization assays, with isolates representing diverse HCV genotypes. Vaccines were antigenically characterized using human monoclonal NAbs AR1B, AR2A and AR3A. These antibodies were also used to assess mouse serum epitope specificities by competition ELISA.

Results: Vaccines consisting of cVLPs displaying monomeric or oligomeric sE2 induced significantly higher levels of NAbs against HCVcc J4 (Genotype 1b; p=0.0065) compared to corresponding sE2 vaccines without cVLPs. Additionally, cVLP-displayed oligomeric sE2 induced higher levels of bNAbs against HCVcc H77 (genotype 1a; p=0.04) and J6 (genotype 2a; p=0.06) than cVLP-displayed monomeric sE2, and also induced bNAbs capable of neutralizing an HCVcc panel of genotypes 1-5. Finally, antigenic characterization revealed that AR2A (NAb of low potency) was less antigenic in cVLP-coupled oligomeric sE2 than in cVLP-coupled monomeric sE2. Interestingly, we observed a significant reduction in AR1B (p=0.02; genotype 1-specific) and AR2A (p=0.004) blocking antibodies in mice immunized with cVLP-coupled oligomeric sE2 than for cVLP-coupled monomeric sE2 and similar levels of bNAb AR3A blocking antibodies (p=0.33).

Conclusions: cVLP-coupling boosts induction of HCV NAbs. Also, a cVLP-coupled oligomeric sE2 vaccine induced high levels of bNAbs with a reduced focus on AR1B and AR2A epitopes, making it a promising HCV vaccine candidate.

HCV vaccine is effective against viruses escaping broadly neutralising monoclonal antibodies

John Law, Hoa Le, Anson Wong, Juthika Kundu, Michael Logan, Darren Hockman, Amir Landi, Catalina Mesa Prince, Kevin Crawford, Mark Wininger, Drew Nahirney, Janelle Johnson, D. Lorne Tyrrell and Michael Houghton. Li Ka Shing

Institute of Virology, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB

WHO targets to eliminate viral hepatitis by 2030. While effective antivirals can now cure hepatitis C virus (HCV)-infected patients, increases in the annual infection incidence fueled by the opioid crisis, has outpaced the rate of treatment in many countries. Therefore, an effective prophylactic HCV vaccine is urgently needed. Both humoral and cellular responses play critical roles in protection against HCV infection. We are developing a combination vaccine containing a 2nd generation gpE1/gpE2 envelope glycoprotein heterodimer (derived from a Fc fusion precursor) to elicit broadly cross-neutralizing antibodies along with a cocktail of short synthetic peptides containing highly conserved CD4+ & CD8+ T cell epitopes to broaden cross-protective cellular immunity. In support of the protective role of neutralizing antibody, many cross-neutralizing monoclonal antibodies (Mab) capable of preventing infection by many clades of HCV have been identified. While these antibodies target epitopes on various domains of gpE1 and/or gpE2 conserved among many genotypes of HCV, viral escape mutants rendering these Mabs ineffective have been produced in cell cultures. In this study, we investigated if our vaccine induced polyclonal anti-gpE1/gpE2 antisera is capable of preventing in vitro infection of these escape viruses. We have selected escape mutants from Mabs AP33, AR3a, HC33.1, HC84.26 and AR5a. These Abs target key conserved neutralizing epitopes. We show that our vaccine polyclonal antisera retains the ability to neutralize infection against all of these escape viruses. Furthermore, the vaccine polyclonal antisera is capable of neutralising viruses containing single and multiple escape mutations. These data confirm our previous findings that gpE1/gpE2 contains numerous linear and discontinuous broadly neutralising epitopes (J.Wong et al JVI 2014) and, that our vaccine may be expected to exhibit protection against most of the global clades of HCV.

Selection and characterization of an AR3 binding camel-derived VHH nanobody

Soerensen A.[1], Skottrup P.D.[2], Bukh J.[1], Prentoe J.[1].

1 Copenhagen Hepatitis C Program (CO-HEP), Copenhagen University Hospital, Hvidovre and University of Copenhagen, Denmark.; 2 Novo Nordisk A/S, Global Research Technologies, Research Bioanalysis, Denmark.

Background and Aims: Camelid nanobodies (VHHs) are ideal for biological research, as they have distinct properties and often outperform conventional antibodies. In a viral vaccine perspective VHH may bind otherwise cryptic epitopes and can be used as crystallization chaperones specifically effective at locking a specific conformation. For hepatitis C virus (HCV), the envelope proteins E1/E2 are flexible and the E2 have recently been shown to contain an A and B conformation relevant for neutralization and therefore potentially relevant in a vaccine design. Here, we screen a non-immunized camel VHH phage display library by subtractive panning against envelope proteins E1/E2 from HCV isolate H77.

Methods: VHH genes were amplified from PBMCs of a healthy *Camelus bactrianus and inserted into pFab74 phagemid vector,* as described previously by Skottrup et al. Plasmids of H77 E1/E2 or vector-only were transiently expressed in 293T cells, then extracted with DDM and the crude proteins were immobilized with lectin to allow panning and ELISA. After 3 rounds of subtractive panning by depleting with vector-only extract and then positive panning with H77 E1/E2, individual clones were picked and verified by monoclonal phage ELISA and sequencing. A single phage-VHH clone was purified with PEG precipitation and epitope estimated by competition ELISA against AR1-5 antibodies. Binding breadth was determined by ELISA to E1/E2 from TN, J6, S52, ED43, SA13, HK6a (GT1a-6a). Binding data was complemented by folding in Alphafold 2.1.0 on Colab notebook and docking with Haddock 2.4 to H77 E2c (PDB:4MWF).

Results: We isolate a single VHH that competes with AR3A in competition ELISA and binds to E1/E2 from genotypes 1a-6a. This is the first reported anti-HCV VHH isolated from a naïve phage display library and the first from *Camelus bactrianus*. By utilizing Alphafold and Haddock for in silico modelling of binding we demonstrate that the interaction is likely mediated through CDR1 engaging with E431 and S432, the CDR2 with K446 and S432, and the CDR3 with T425, L427, L441, F442 and W529. The epitope overlaps with the epitope of many broadly neutralizing HCV antibodies and further wet-lab structural studies will provide useful information for further candidate vaccine design for HCV.

Conclusions: We report the selection of the first VHH from a naïve phage display library that competes with AR3A in competition ELISA and binds to E1/E2 from HCV genotypes 1a-6a.

Selection of a new class of anti-HCV E1/E2 Fabs

Soerensen A.[1], Olesen C.H.[1], Lohse B.[2], Chen Z.[3], Purcell R.H.[3], Farci P.[3], Bukh J.[1], Prentoe J.[1]

1 Copenhagen Hepatitis C Program (CO-HEP), Copenhagen University Hospital, Hvidovre and University of Copenhagen, Denmark; 2 Department of Drug Design and Pharmacology, University of Copenhagen, Denmark; 3 Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA.

Background and Aims: Identifying novel hepatitis C virus (HCV) envelope protein epitope targets for the induction of robust B-cell responses is key in developing an effective vaccine. Giang and Law defined a comprehensive E1/E2 epitope clustering nomenclature of hitherto non-overlapping antigenic regions (AR), termed AR1-5. Here, we screen a chronic HCV patient (pt. H; genotype 1a) derived phage display library against E1/E2 from the same patient (isolate H77) as well as E1/E2 from a heterologous genotype 3a isolate, S52.

Methods: The library was constructed by bone marrow aspiration as described previously by Schofield et al. E1/E2 was transiently expressed in 293T cells, detergent-extracted and immobilized with lectin. Following 3-5 rounds of E1/E2 panning, individual clones were picked and verified in monoclonal phage ELISA. Unique clones were converted into Fabs, and these were characterized for breadth of binding against genotype 1-6 E1/E2, competition ELISA against AR1-5 antibodies and neutralization of H77 and S52 HCVcc. Binding data was complemented by folding Fabs in Alphafold 2.1.0 on a Colab notebook and docking them to published E2 structures with Haddock 2.4.

Results: H77 and S52 panning led to significant library enrichment and 144 clones were picked, resulting in 13 unique E1/E2-specific clones. All 13 encoded similar heavy chain variable domains from the VH1-69 germline (GL) family, which has previously been reported to be a dominant germline family for E2-specific HCV neutralization. Light chain (LC) pairing was diverse and could be sorted into three distinct classes. Representative Fabs from each class (Fab#2, B1_9 and B1_11) bound to E1/E2 from genotype 1-6 and neutralized both full length H77 and S52 in cell culture (HCVcc). Two classes, namely LC from VK1-39/VK1-33 (similar to AR3A-D antibodies) and VK3-15/VK3-20 (similar to HEPC3/74 antibodies), were described before. The last LC class paired with VK4-1, which has not yet been reported for anti-HCV VH1-69 antibodies. Interestingly, Fab#2 from the VK4-1 LC class competed with both AR2A and AR3A binding, located on the back layer and front layer of E2, respectively. Docking experiments complemented this observation by predicting the characteristic interaction of other VH1-69 mAbs by the hydrophobic tip from HCDR2 with AR3, but also predicted the long LCDR1 of VK4-1 to interact with residues adjacent to AR2 and within distance to block the interaction of AR2 binding mAbs.

Conclusions: We identify 3 classes of VH1-69 Fabs based on varying LC pairing from a patient derived phage display library. The VH1-69 Fab#2 of the novel LC GL pairing, VK4-1, blocks both AR2A and AR3A and displays cross-genotype reactivity and neutralization. This LC-dependent cross-AR2/3 reactivity of Fab#2 is highly relevant for devising novel HCV vaccine immunogens.

The generation of stem cell-like memory cells early after BNT162b2 vaccination predicts the durability of vaccine-induced memory CD8⁺ T-cell responses

Sungmin Jung,[1] Jae Hyung Jung,[1] Ji Yun Noh,[1,2] Woo-Joong Kim,[1] Soo-Young Yoon,[3] Hee Jin Cheong,[2] Woo Joo Kim,[2] Su-Hyung Park,[1] Joon Young Song,[2] Eui-Cheol Shin[1,4]

[1] Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea; [2] Division of Infectious Diseases, Department of Internal Medicine, Korea University Guro Hospital, Korea University College of Medicine, Seoul 08308, Republic of Korea; [3] Department of Laboratory Medicine, Korea University Guro Hospital, Korea University College of Medicine, Seoul 08308, Republic of Korea; [4] Center for Viral Immunology, Korea Virus Research Institute, Institute for Basic Science (IBS), Daejeon 34126, Republic of Korea

Background and Aims: Vaccines have yet to be developed for the prevention and control of many infectious diseases, including those caused by HCV and HIV. Recently, the mRNA vaccine platform has been developed and successfully employed against COVID-19. In this study, we aimed to examine the phenotypic characteristics of mRNA vaccine-induced CD8⁺ T cells and identify which parameters determine the longevity of such vaccine-induced memory CD8⁺ T cells.

Methods: Peripheral blood from 40 healthcare immunized with prime and boost doses of the BNT162b2 vaccine were serially collected up to 6 months post-boost vaccination. Activation and differentiation of vaccine-induced CD8⁺ T cells were examined using MHC class I (MHC-I) multimers, and correlations between early differentiation and the durability of CD8⁺ T-cell responses were assessed.

Results: The frequency of MHC-I multimer⁺ cells was robustly increased by BNT162b2 but reduced 6 months post-boost to 2,4 – 54,6% (20,0% on average) of the peak. MHC-I multimer⁺ cells dominantly exhibited phenotypes of activated effector cells 1 week post-boost and gradually acquired phenotypes of long-term memory cells, including stem cell-like memory T (T_{SCM}) cells. Importantly, the frequency of T_{SCM} cells 1 week post-boost significantly correlated with the 6-month durability of CD8⁺ T cells.

Conclusions: Our results indicate that mRNA vaccines successfully elicit a CD8⁺ T-cell response, and early generation of T_{SCM} cells determines the longevity of vaccine-induced memory CD8⁺ T-cell responses.

Combined DNA vaccination with chimeric HBV-HCV virus-like particles and NS3/4A protease induces a potent humoral and cellular responses against HCV

Anna Czarnota[1], Aleksandra Raszplewicz[1], Alicja M Chmielewska[1], Krystyna Bieńkowska-Szewczyk[1], Katarzyna Grzyb[1]

[1]Laboratory of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology of the University of Gdańsk and Medical University of Gdańsk, Poland

Background: A successful vaccine against HCV should elicit both, humoral and cellular immune responses. In our previous studies we characterized a bivalent vaccine candidate against hepatitis B (HBV) and hepatitis C (HCV) virus based on chimeric virus-like particles (VLPs) in which the highly conserved epitope of HCV E2 glycoprotein (residues 412-425) was inserted into the hydrophilic loop of HBV small surface antigen (sHBsAg). We proved that sHBsAg_412-425 VLPs were able to elicit cross-neutralizing antibodies but not T-cell response against HCV. Therefore, the goal of this study was to design a vaccine candidate that would engage both arms of adaptive immune response and hopefully provide protection from the HCV infection. Here, we propose a DNA vaccine based on minicircles (MCs) technology as an antigen delivery method alternative to conventional plasmid vectors.

Methods: In this study we designed two minicircles MCs carrying DNA sequences coding for sHBsAg_412-425 VLPs and NS3/4A HCV protease. Mice were immunized twice with MCs-adjuvant mixture on day 0 and 28. Three weeks after the last immunization, cellular and humoral immune responses were analyzed. T-cell response was analyzed by gamma interferon ELISpot assay. Antibody response was measured by direct solid-phase ELISA using 412-425 synthetic peptide and E1E2 HCV complex.

Results: Our results showed that combined immunization with both MCs induced a potent T-cell response against HCV NS3/4A and sHBsAg protein. Interestingly, the analysis of antibody response indicated that binding patterns of sHBsAg_412-425 MC immune sera to 412-425 peptide and native E1E2 glycoprotein complexes were comparable to those elicited by sHBsAg_412-425 VLPs.

Conclusions: In conclusion we show that combined DNA immunization with sHBsAg_412-425 VLPs and NS3/4A protease is effective in inducing a potent T-cell and antibody responses, both of them crucial for the successful protection against HCV.

Establishment of high-titer HCV and SARS-CoV-2 production in a scalable packed-bed bioreactor for inactivated vaccines inducing neutralizing antibodies in animals

Anna Offersgaard 1, Carlos Rene Duarte Hernandez 1, Anne Finne Pihl 1, Shan Feng 1, Pavel Marichal-Gallardo 2, Kenn Holmbeck 1, Nandini Prabhakar Venkatesan 3, Xiangliang Lin 3, Carlota Fernandez-Antunez 1, Garazi Peña Alzua 1, Rui Costa 1, Katrine Top Hartmann 4, Long V. Pham 1, Yuyong Zhou 1, Karen A. Gammeltoft 1, Ulrik Fahnøe 1, Troels K. H. Scheel 1, Uffe Vest Schneider 5, Henrik Elvang Jensen 4, Jan Pravsgaard Christensen 6, Santseharay Ramirez 1, Udo Reichl 2, Yvonne Genzel 2, Jens Bukh 1, and Judith Margarete Gottwein 1.

1 Copenhagen Hepatitis C Program (CO-HEP), Department (Dept.) of Infectious Diseases, Copenhagen University Hospital-Amager and Hvidovre and Dept. of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen (UCPH), Denmark; 2 Bioprocess Engineering, Max Planck Institute for Dynamics of Complex Technical Systems, Germany; 3 Esco Aster Pte Ltd., Singapore; 4 Dept. of Veterinary and Animal Sciences, UCPH, Denmark; 5 Dept. of Microbiology, Copenhagen University Hospital-Amager and Hvidovre, Denmark; 6 Dept. of Immunology and Microbiology, Faculty of Health and Medical Sciences, UCPH, Denmark

Background and aims: Hepatitis C virus (HCV) incidence remains high despite available treatments, and a vaccine is urgently needed. Inactivated vaccines are available against several viral pathogens, and inactivated vaccines were among the first available and most widely used for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The manufacturing of inactivated vaccines, however, requires efficient virus production. We investigated the scalable packed-bed CelCradle bioreactor for high-titer HCV and SARS-CoV-2 production and confirmed immunogenicity of inactivated bioreactor-derived virus preparations in animals.

Methods: The CelCradle bioreactor (0,5 L reactor volume) was used to grow a previously described high-titer HCV genotype 5a/2a recombinant in Huh7.5 cells, which were seeded and infected in serum-containing medium and maintained in serum-free medium (SFM) during harvesting. A SARS-CoV-2 with the spike protein mutation D614G virus was grown in VeroCCL81 cells maintained in SFM. Purified and UV inactivated HCV or beta-propiolactone inactivated SARS-CoV-2 and an MF59-like adjuvant was used for immunization of mice (HCV and SARS-CoV-2) or hamsters (SARS-CoV-2 only). For both vaccines, induction of neutralizing antibodies was evaluated in cell-based assays. For the SARS-CoV-2 vaccine, protection of hamsters from disease was evaluated by airway viral titers and lung pathology.

Results: HCV and SARS-CoV-2 CelCradle virus yields were highest with two daily harvests, for HCV reaching 5,9 x 10^10 focus forming units and 33 microgram Core from one CelCradle culture (20 harvest days). A temperature shift to 33 °C at the time of infection further improved SARS-CoV-2 production, reaching 3,0 x 10^10 50% tissue culture infectious doses and 3,7 milligram spike subunit 1 from one CelCradle culture (6 harvest days). Neutralizing antibodies were induced upon immunization with the HCV vaccine (mean EC_{50} of purified serum IgG of 65 microgram / mL following 4 immunizations) and the SARS-CoV-2 vaccine (mean ID_{50} dilution of immune sera of 583 in mice and 2100 in hamsters following 3 and 2 immunizations, respectively). Importantly, the SARS-CoV-2 vaccine protected hamsters from disease upon challenge.

Conclusions: The scalable CelCradle bioreactor was suitable for virus production of inactivated vaccine candidates inducing neutralizing antibodies, and for SARS-CoV-2 conferring protection against virus challenge, in small animal models.

Corticosteroids and cellulose purification improve, respectively, the in vivo translation and vaccination efficacy of self-amplifying mRNAs

Zifu Zhong[1], Séan Mc Cafferty[1,2], Lisa Opsomer[1], Haixiu Wang[3], Hanne Huysmans[1], Joyca De Temmerman[1,4], Stefan Lienenklaus[5], João Paulo Portela Catani[1], Francis Combes[1,2], Niek N. Sanders[1,2]

[1] Laboratory of Gene Therapy, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, 9820 Merelbeke, Belgium; [2] Cancer Research Institute (CRIG), Ghent University, 9000 Ghent, Belgium; [3] Laboratory of Immunology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [4] Department of Pathology, Bacteriology and Poultry diseases,; Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820, Merelbeke, Belgium; [5] Institute for Laboratory Animal Science, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Hannover, Germany

Synthetic mRNAs are an appealing platform with multiple biomedical applications ranging from protein replacement therapy to vaccination. In comparison with conventional mRNA, synthetic self-amplifying mRNAs (sa-mRNAs) are gaining interest because of their higher and longer-lasting expression. However, sa-mRNAs also elicit an innate immune response, which may complicate their clinical application. Approaches to reduce the innate immunity of sa-mRNAs have not been studied in detail. Here we investigated, *in vivo*, the effect of several innate immune inhibitors and a novel cellulose-based mRNA purification approach on the type I interferon (IFN) response and the translation and vaccination efficacy of our formerly developed sa-mRNA vaccine against Zika virus. Among the investigated inhibitors, we found that corticosteroids and especially topical application of clobetasol at the sa-mRNA injection site was the most efficient in suppressing the type I IFN response and increasing the translation of sa-mRNA. However, clobetasol prevented formation of antibodies against sa-mRNA-encoded antigens and should therefore be avoided in a vaccination context. Residual dsRNA by-products of the in vitro transcription reaction are known inducers of immediate type I IFN responses. We additionally demonstrate a drastic reduction of these dsRNA by-products upon cellulose-based purification, reducing the innate immune response and improving sa-mRNA vaccination efficacy.

Key words: self-amplifying mRNA, type I IFN, mRNA purification, cellulose, Zika vaccine

[*]**Correspondence should be addressed to** Prof. Niek N. Sanders, Niek.Sanders@ugent.be, Tel: 0032 (0)9 264 78

Rationally designed attenuated HCV variants for vaccine development

Roba Dabour[1], Shaked Bergman[2], Michal Werbner[1], Ateret Davidovitz[1],Tamir Tuler[2] and Meital Gal-Tanamy[1]

[1]Molecular Virology Lab, Azrieli Faculty of Medicine in the Galilee, Bar-Ilan University, Safed, Israel.[2]Department of Biomedical Engineering, Tel-Aviv University, Ramat Aviv, Israel.

Background & Aims: Hepatitis C virus (HCV) is a leading cause of liver disease and no vaccine is currently available fo HCV. It is now clear that both humoral and cellular immune responses play a significant role in HCV infection clearance and therefore an effective vaccine should induce both arms of the adaptive immune response. **Live attenuated vaccines that contains weakened viruses are considered verry effective since they** replicate the natural infection without causing serious diseases but still induce efficient B and T cell anti-viral immunity. Understanding how viruses adapt various genomic strategies to reduce their fitness have essential implications in developing new vaccines. In this study we aim to rationally design and generat WT attanated HCV variants by introducing synonymous mutations to disrupt HCV mRNA structure and weaken the virus.

Methods: We utilized novel bioinformatics tools to analyze HCV genomes from databases for identifng 'silent' patterns of HCV mRNA folding, and utilized this information to design HCV variants containing synonymous mutations that affect this structure. By this approach we designed HCV mutants that varies in number and positions of inserted mutations, constructed synthetic HCV genomes containing these mutations and produced the mutant viruses. To evaluate the effect of the synonymous mutations on viral fitness, we measured the ability of the HCV mutants to replicate and spread in Huh7.5 cells. Moreover, we evaluated the mutants' pathogenesis by measuring their effect on expression of host genes related to oncogenic pathways, and examined their invasion properties in Transwell and ECM degradation assays.

Results: We designed and generated 8 HCV variants, that differ in number and positions of inserted synonymous mutations, ranging between 28 to 298 mutations in each mutant and grouped into three levels of attenuation: strong, mid and weak. Upon infection, we observed an overall reduction of HCV fitness in mutants compared to WT HCV, where the viral RNA levels, number of cells in each viral focus and cell infection percentage were compromised. The level of fitness varied between the HCV variants, correlating with the level of viral attanuation. The pathogenesis of the mutants varied also with correlation to the level viral fitness, with minimal effect on oncogenic gene expression and invasion phenotypes for the most attenuated viruses.

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.

Optimization of a bivalent HBV-HCV prophylactic vaccine

Elodie Beaumont, Elsa Gomez-Escobar, Julien Burlaud-Gaillard, Emmanuelle Roch, Philippe Roingeard

INSERM Unit 1259 MAVIVH, University and CHRU of Tours, Tours, France

Background and aims: Prophylactic vaccination constitutes the best long-term hope for controlling the global hepatitis C epidemic. We therefore developed an original vaccination concept based on full-length hepatitis C virus (HCV) genotype 1a E1 or E2 glycoproteins fused to the heterologous hepatitis B virus (HBV) S envelope protein and self-assembling into subviral envelope particles. Immunization of rabbits with these particles induced the production of anti-E1 and anti-E2 antibodies capable to neutralize HCV *in vitro*. However, neutralization of genetically-distant genotypes remains a challenge and different strategies are ongoing to increase the cross-neutralizing properties of the induced antibodies.
Methods: We first took into consideration the important HCV genetic variability and developed novel particles bearing E1 or E2 proteins from genotype 3a and 4a HCV isolates to evaluate the neutralizing properties of antibodies induced by immunization with different combinations of particles. We also considered the role of the apolipoprotein E (apoE) in the folding of the HCV envelope proteins, and therefore in the modulation of the HCV sensitivity to antibody-mediated neutralization. For this, we generated new vaccine particles bearing envelope proteins complexed with apoE to mimic precisely the epitopes at the HCV envelope proteins/apoE interface, and evaluated the impact of apoE on the folding and immunogenicity of the HCV envelope proteins.
Results: We showed that immunization with a cocktail of particles bearing E2 proteins from genotypes 1a, 3a and 4a significantly increased the broad neutralizing properties of the induced antibodies, as compared to immunization with particles containing only E2 from genotype 1a. However, the gain of adding particles bearing E1 in a cocktail of particles had low impact in this multi-genotype strategy. This may be due to the simultaneous presentation of many different immunogens to the immune system, which may result in the frustration of the affinity maturation process of B cells. We then demonstrated that the presence of apoE on the surface of particles improved the folding of the HCV envelope proteins and the neutralizing capacity of the anti-E2 antibodies. However, the incorporation of the E2-S protein was significantly decreased in our initial vaccine candidate bearing apoE.
Conclusions: All these investigations allowed a significant optimization of our bivalent HBV-HCV vaccine, but some issues still remain to be addressed. To reduce the conflicting selection forces responsible for the frustration of the affinity maturation process of B cells, we are currently testing a new vaccination strategy based on sequential immunizations with particles bearing HCV envelope proteins from different genotypes. Moreover, immunizations with a new set of vaccine particles containing a better E2/apoE ratio are currently under investigation.

Mechanisms and Determinants of Hepatitis C Virus Species Tropism

Melina Winkler[1], Julie Sheldon[1], Natascha Gödecke[2], Qinggong Yuan[1], Nicola Frericks[1], Luisa Ströh[3], Dagmar Wirth[2], Thomas Krey[4], Thomas Pietschmann[1]

[1]TWINCORE Centre for Experimental and Clinical Infection Research, Hannover, Germany; [2]Helmholtz Centre for Infection Research, Braunschweig, Germany; [3]Hanover Medical School, Hannover, Germany; [4]University of Lübeck, Lübeck, Germany

Background and Aims: Chronic infections with hepatitis C virus (HCV) progressing to liver cirrhosis and hepatocellular carcinoma remain a major health burden despite the availability of direct acting antivirals, mainly due to undiagnosed cases and reinfection after cure. A preventive vaccine may help eradicate chronic hepatitis C. However, a vaccine is not available, in part because pre-clinical testing of vaccine efficacy is difficult due to the lack of an immune competent small animal permissive to HCV infection. Here, we generated a molecular clone of a mouse adapted HCV population and dissected it to identify the mutations most relevant for the tropism towards mice.
Methods: We generated a mouse adapted HCV population by repeatedly passaging the lab strain Jc1 in human hepatoma cells, an engineered mouse cell line expressing human entry factors for HCV, and finally primary mouse hepatocytes from receptor transgenic mice. We generated a molecular clone based on the consensus sequence of the mouse adapted virus population. Subsequently, we characterized its phenotype using full-length and replicon constructs in different human and murine cells.
Results: Our results indicate that the full-length molecular clone is as replicative fit as the viral population in primary mouse hepatocytes from receptor transgenic mice. Our data suggests that synonymous mutations are dispensable for enhanced fitness in mouse liver cells. Introduction of the adaptive mutations into a replicon does not increase replication fitness in Huh-7.5 cells or in an engineered mouse liver tumor cell line. Furthermore, we found that the glycoprotein mutations render the virus more susceptible to neutralizing antibodies and that reversion of the mutations restores neutralization resistance while maintaining the replication phenotype of the original clone.
Conclusions: Coding mutations across the HCV polyprotein are necessary and sufficient for the extended tropism of HCV towards mouse liver cells. Enhanced neutralization susceptibility due to glycoprotein mutations is rescued by reverting these mutations. The reversion does not eliminate enhanced infection of receptor transgenic primary mouse hepatocytes.

Scale-up and production of a recombinant Fc-tagged hepatitis C virus glycoprotein E1/E2 antigen for phase 1 clinical trials

Janelle Johnson, Darren Hockman Michael Logan, Ayo Oludiran-Ayoade, Jeanine Koshelek, Kevin Crawford, Elzbieta Dudek, Drew Nahirney, Chao Chen, Joydeb Kundu, Nicole Chomin, Juthika Kundu, Catalina Mesa Prince, Jason Wong, Lia Firth, Louise Tan, Simran Brar, Amir Landi, John Law, Michael Houghton

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Background: A vaccine that elicits hepatitis C (HCV) broad neutralizing antibodies (bNAbs) is still urgently needed in the global effort to eradicate HCV. Although highly effective in curing HCV, the cost and availability of direct acting antiviral (DAA) drugs remains a significant global challenge. In addition, DAAs do not prevent re-infection with HCV or completely eliminate the risk of hepatocellular carcinoma in patients with advanced cirrhosis, and mutations that confer resistance to DAAs can occur in the virus. [1–3] Previously, a subunit glycoprotein (gp)E1/gpE2 (E1E2) vaccine candidate was tested in phase 1 clinical trials and shown to produce HCV bNAbs in humans[4]. To aid in scale-up of this vaccine candidate, we developed an Fc-tagged E1E2 vaccine candidate that could be purified by affinity chromatography[5]. This Fc-tagged E1E2 vaccine candidate was shown to elicit a similar or improved bNAb response in animal models compared to wild type (untagged) E1E2. In addition to antibody responses, we have shown that when the vaccine candidate is combined with specific HCV peptides and appropriate adjuvants, cross reactive T-cell responses are elicited. A scaled-up production, purification and formulation of this vaccine candidate is in progress at the Li Ka Shing Institute of Virology, University of Alberta under good manufacturing practices (GMP) to perform phase 1 clinical trials.
Methods: Kilogram quantities of CHOK1 cells expressing Fc-tagged E1E2 are produced, lysed, clarified and subjected to depth filtration. E1E2 is purified from the filtered lysate using protein A affinity chromatography, as well as additional affinity and ion exchange chromatography steps to remove host DNA and host cell proteins. The bulk drug product (BDP) (purified E1E2) is then formulated with HCV NS peptides and adjuvant so as to initiate pre-clinical toxicity studies in a few months.
Results: Analysis of BDP from process development runs shows visible E1 and E2 by SDS-PAGE (stained gels and Western blot) as well as consistent peak profiles on HPLC. BDP also exhibits immunoreactivity with known HCV neutralizing monoclonal Abs that target E2 and E1E2-specific residues.
Conclusion: A scaled-up production process under GMP conditions produces E1E2 antigen that is pure and retains native structure and importantly, contains bNAb epitopes lacking in E2 alone. This E1E2 antigen will be used in upcoming phase 1 clinical trials.

Self-amplifying RNA as a tool to tackle emerging flavivirus outbreaks: lessons learned from the COVID-19 pandemic

Aster Vandierendonck[1], Itishri Sahu[1], Sean McCafferty[1], Sophie Valembois[1], Katrien Poelaert[1], Leonie wyffels[1], AKM Ashiqul Haque[1]

[1] Ziphius Vaccines NV, Heidestraat 19 Z-03, B-9820 Merelbeke, Belgium

Background Outbreaks of multiple Flaviviruses have afflicted humankind for centuries due to their rapid spread predominantly by arthropod vectors. Global warming has caused epidemics to emerge in previously unaffected regions. The SARS-CoV-2 outbreak and subsequent race in vaccine development can provide strategies to the scientific community to avoid a flavivirus pandemic. The COVID-19 battle led to the first-time authorization of messenger ribonucleic acid (mRNA) based vaccines that employ *in vitro* transcribed (IVT) synthetic mRNA molecules to instruct host cells to produce the viral antigen in a natural way. The mRNA platform has proven to carry several advantages over other vaccination strategies, including its flexibility due to relatively easy sequence engineering and its inability to integrate into the host's genome. In recent years, mRNA vaccines have been generated against multiple flaviviruses, including thick-borne encephalitis virus (TBEV) and Dengue virus. However, most of these vaccines target structural protein epitopes to induce neutralizing antibody responses, which is complicated by the potential induction of antibody-dependent enhancement (ADE). ADE is a phenomenon in which sub-neutralizing concentrations of anti-viral IgGs enhance infection of Fc gamma receptor positive cells, thereby increasing the risk of an exacerbated pathogenesis upon infection after vaccination.

Methods To avoid vaccine-induced ADE, next-generation vaccination strategies should aim to stimulate T-cell-mediated immunity against multiple antigens, including flaviviral non-structural proteins. The self-amplifying (sa) RNA platform provides such opportunity, as it carries the option for single-vector delivery of multiple and/or complex polypeptides by incorporation of multiple subgenomic promotors. saRNA vaccines also encode for components of an alpha-viral derived RNA-dependent RNA polymerase (RDRP). Upon cytoplasmic delivery of the saRNA, the RDRP is capable of amplifying the original RNA strand and generating high levels of sub-genomic RNA encoding for the viral antigen(s). This mechanism leads to higher antigen abundance inside the host cell for longer periods of time, which can drive equivalent or more potent immune responses at lower doses compared to those achieved by non-replicating mRNA vaccines.

Results Ziphius recently demonstrated that its saRNA platform can induce T-cell mediated immunity following a low dose of a dual-antigen anti-SARS-CoV-2 saRNA vaccine, thereby protecting against SARS-CoV-2 variants displaying a highly mutated structural Spike protein.

Conclusion As only few research groups have characterized saRNA vaccines against flaviviruses (i.e. Zika virus and TBEV), Ziphius advocates the further acceleration of the development of new multi-antigenic saRNA vaccines with reduced risk for ADE to avoid flavivirus outbreaks.

Contact via aster.vandierendonck@ziphius.org

Session 5
ANTIVIRAL TREATMENT & HCV ELIMINATION

28TH INTERNATIONAL SYMPOSIUM ON HEPATITIS C VIRUS, FLAVIVIRUSES, AND RELATED VIRUSES

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JULY 6-9, 2022

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Alekxander Binderup, University of Copenhagen, Denmark

P71 **Dengue virus infection and dissemination in Aedes aegypti mosquitoes is significantly reduced upon exposure to JNJ-A07, a potent DENV inhibitor, in the blood meal**
Leen Delang, Rega Institute - KULeuven, Belgium

P72 **Efficacy of HCV Protease Inhibitors against SARS-CoV-2 and Characterization of Nirmaltrevir Resistance in Vitro**
Karen Anbro Gammeltoft

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P86 **Antioxidant and xanthine oxidase inhibitory activities of the medicinal plant Centaurium erythraea and its possible use against liver damages and pathologies associated with chronic hepatitis c**
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P87 **Antioxidant and xanthine oxidase inhibitory activities of the medicinal plant Centaurium erythraea and its possible use against liver damages and pathologies associated with chronic hepatitis c**
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P88 **Persistence of hepatitis E virus in the cerebrospinal fluid despite apparently successful ribavirin therapy.**
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Screening of the antiviral activity of existing drugs against tick-borne encephalitis and yellow fever viruses in human cell lines

Alekxander Binderup, Andrea Galli, Nicolas Fossat, Carlota Fernandez-Antunez, Lotte Mikkelsen, Lizandro René Rivera-Rangel, Troels Scheel, Ulrik Fahnøe, Jens Bukh and Santseharay Ramirez

Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital and Department of Immunology and Microbiology, University of Copenhagen.

Background and Aims: The tick-borne encephalitis virus (TBEV) and yellow fever virus (YFV) of the Flaviviridae family cause high morbidity in humans, with approximately 15.000 and 300.000 worldwide infections every year, respectively. Despite availability of effective vaccines, there has been a significant increase in the number of human infections during the last decades and no antiviral drugs are currently approved for treatment. Since nucleos(t)ide analogues (nucs) can have broad activity, also across the Flaviviridae family, this study aimed at exploring the antiviral activity of nucs with repurposable potential against TBEV and YFV in relevant human cell lines.

Methods: To assess the antiviral activity of drugs, the drug effective concentration 50% (EC50) was determined in human hepatoma Huh7.5, human medulloblastoma Daoy, and human neuroblastoma SH-SY5Y cells using concentration-response assays based on determination of TBEV (strain Neudoerfl) or YFV (strain 17D) positive cells at 72 hours post infection/treatment. The highest non-cytotoxic concentration (≥ 80% cell viability) was determined with a commercial cell proliferation assay.

Results: The antiviral activity of 14 nucs (remdesivir, uprifosbuvir, sofosbuvir, galidesivir, ribavirin, GS-6620, molnupiravir, valopicitabine, mericitabine, tenofovir alafenamide, tenofovir disoproxil, entecavir, lamivudine and favipiravir) was initially tested in Huh7.5 cells. Remdesivir, uprifosbuvir, sofosbuvir, galidesivir, and molnupiravir were effective against both TBEV and YFV at non-cytotoxic concentrations, with EC50 values between 0,2 and 59,5 µM. In addition, valopicitabine could inhibit TBEV, but not YFV. Remdesivir, uprifosbuvir, sofosbuvir, and galidesivir were also effective against both viruses in Daoy cells (EC50 increased by 0,9- to 6,6-fold compared to Huh7.5), whereas only uprifosbuvir and galidesivir were effective in SH-SY5Y cells (0,7- to 22,2-fold higher EC50 compared to Huh7.5).

Conclusions: In conclusion, we identified several nucs that exhibit promising antiviral activity against TBEV and YFV in relevant human cell lines. To determine which compounds might have clinical significance, studies addressing the barrier to resistance and drug efficacy for preventing disease in relevant animal models should be conducted.

Dengue virus infection and dissemination in *Aedes aegypti* mosquitoes is significantly reduced upon exposure to JNJ-A07, a potent DENV inhibitor, in the blood meal

Leen Delang[1], Ana Lucia Rosales Rosas[1], Lanjiao Wang[1], Suzanne Kaptein[1], Olivia Goethals[2]

[1] Rega Institute for Medical Research, KU Leuven, Leuven, Belgium.
[2] Janssen Global Public Health, Janssen Pharmaceutica, Beerse, Belgium.

Background & aims: Dengue virus (DENV) is the most widespread mosquito-borne virus worldwide; however, no antiviral therapies are available yet. The pan-serotype DENV inhibitor JNJ-A07 has shown potent activity in a mouse model (Kaptein et al, Nature 2021). As a prophylaxis, this drug could prevent human infection after the bite of an infected mosquito. Moreover, infected mosquitoes might ingest the drug during blood feeding. It is currently unknown whether an antiviral drug ingested by mosquitoes could inhibit virus replication, and thus reduce virus transmission to other hosts. Here, we investigated the antiviral activity of JNJ-A07 administered via a DENV-infectious blood meal to *Aedes aegypti* mosquitoes.
Methods: *Aedes aegypti* mosquitoes were infected by infectious blood meal containing DENV-2 supplemented with either JNJ-A07 or DMSO. DENV titers were determined by plaque assay on day 3 and 7 post infection.
Results: We first demonstrated that a high concentration of JNJ-A07 (25 µM) had no detrimental effect on the mosquitoes' lifespan and on egg development. When mosquitoes were exposed to a blood meal containing DENV-2 and JNJ-A07 (25 µM), DENV infection in the mosquitoes was completely blocked (0% vs 62% at day 3 pi). Furthermore, no virus dissemination to mosquito secondary organs was observed at day 7, in contrast to the control group (0% vs 40%). We next evaluated the antiviral effect of lower concentrations of JNJ-A07. At 2 µM and 0,2 µM, JNJ-A07 was still able to stop DENV infection and dissemination in the mosquito. However, lowering the concentration to 0,02 µM did not fully block DENV infection, but was still able to reduce the infection rate (36% vs 64%), showing that the anti-DENV effect of JNJ-A07 in mosquitoes is dose-dependent.
Conclusions: Our results suggest that mosquito exposure to a potent antiviral drug in the blood of treated humans could significantly decrease DENV transmission by mosquitoes to other humans and therefore might impact the magnitude of DENV outbreaks.
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Efficacy of HCV Protease Inhibitors against SARS-CoV-2 and Characterization of Nirmaltrevir Resistance in Vitro

Karen Anbro Gammeltoft[1]*, Yuyong Zhou[1]*, Line Abildgaard Ryberg[1], Alekxander Marcus Binderup[1], Long Pham[1], Carlos R. D. Hernandez[1], Anna Offersgaard[1], Carlota Fernandez-Antunez[1], Ulrik Fahnøe[1], Günther H. J. Peters[2], Santseharay Ramirez[1], Jens Bukh[1], Judith Margarete Gottwein[1]

*Authors contributed equally; [1]Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Copenhagen University Hospital–Hvidovre, 2650 Hvidovre, Denmark and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark ; [2]Department of Chemistry, Technical University of Denmark, 2800 Kongens Lyngby, Denmark

Background and aims: We have previously characterized the efficacy and resistance of clinically relevant direct acting antivirals for hepatitis C virus (HCV) in human hepatoma (Huh7.5) cells, and their potential application in other viral infections. In this study, we investigated the potential effect of HCV NS3 protease inhibitors (PI) against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and their interactions with remdesivir in cell culture based antiviral assays. Further, we studied SARS-CoV-2 escape from treatment with boceprevir and the related novel SARS-CoV-2 protease inhibitor nirmatrelvir.

Methods: Half maximal effective concentrations (EC50) and fold resistance levels were determined in short term treatment assays using African green monkey kidney (VeroE6) cells, Huh7.5 cells, and human angiotensin converting enzyme 2 expressing lung carcinoma (A549-hACE2) cells. Longer term treatments, escape experiments and evaluation of viral fitness were carried out in VeroE6 or A549-hACE2 cells. Putative resistance mutations were identified by next generation sequencing and verified by reverse genetics. The structural consequences of the mutations in the SARS-CoV-2 main protease (Mpro) were investigated using molecular dynamics simulations.

Results: The 13 HCV PI showed differential potencies for treatment of SARS-CoV-2. In VeroE6 cells, simeprevir had the highest (EC50 = 15 µM) and glecaprevir the lowest (EC50 >178 µM) potency. Boceprevir had the highest selectivity index (SI >28). Similar results were obtained in Huh7.5 and A549-hACE2 cells. EC50 of nirmatrelvir was 4,9 µM and 0,07 µM with selectivity indexes of 261 and 18.300 in VeroE6 and A549-hACE2 cells, respectively, with the difference likely caused by differential expression of an efflux transporter. Macrocyclic but not linear PI showed synergism with remdesivir in combination treatment experiments in both VeroE6 and A549-hACE2 cells. Boceprevir and nirmatrelvir resistant variants selected in longer term escape experiments in VeroE6 cells had acquired combinations of substitutions in Mpro, which conferred up to 100-fold resistance to nirmatrelvir. Resistant variants showed relatively high fitness and genetic stability. On the molecular level, the identified substitutions were predicted to interfere with the binding of nirmatrelvir to the Mpro while improving binding of Mpro to its natural substrate.

Conclusions: These findings have implications for monitoring and securing treatment programs with high efficacy against SARS-CoV-2. They further demonstrate the relevance of HCV antivirals or derivatives of such for potentially emerging viruses.

Simplified identification of broad-spectrum hits on the virus RGB palette

Li-Hsin Li[1], Winston Chiu[1], Yun-An Huang[2,3], Madina Rasulova[4], Thomas Vercruysse[4], Hendrik Jan Thibaut[4], Suzanne Kaptein[1], Pieter Leyssen[1], Johan Neyts[1] and Kai Dallmeier[1]

[1] KU Leuven Department of Microbiology and Immunology, Rega Institute, Laboratory of Virology, Antiviral Drug, and Vaccine Research, Leuven, Belgium.; [2] KU Leuven Department of Biology, Leuven, Belgium.; [3]Neuro-Electronics Research Flanders, Leuven, Belgium.; [4] KU Leuven Department of Microbiology and Immunology, Rega Institute, Translational Platform Virology & Chemotherapy (TPVC), Leuven, Belgium.

Background and Aims: Flaviviruses, such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), and dengue virus (DENV), pose a significant health threat to the world. Potent antivirals, ideally with activity beyond specific viruses (i.e. pan-flavivirus inhibitors), are urgently needed. To readily identify such broad-spectrum inhibitors, we need novel approaches in drug discovery. To this end, we developed a multiplex antiviral assay that is based on the visualization of concurrent infections by high content imaging (HCI) using a selection of recombinant flaviviruses tagged with different fluorescent proteins (FPs). Based on brightness and spectral separation of a range of FPs, we combined YF17D/mCherry (Red, R), JEV/eGFP (Green, G), and DENV2/Azurite (Blue, B) for mixed infection experiments on target cells constitutively expressing a far-red FP. This RGB (Red-Green-Blue) color mixing assay can thus be employed to reflect the relative infectivity of three reporter viruses in a multiplex-virus antiviral assay upon treatment, and to readily identify the specificity and the potency of antivirals.
Methods: For assay validation and proof-of-concept for imaged-based antiviral screening, serum containing neutralizing antibodies specific for either DENV2, JEV or YFV as well as a selection of reference compounds, such as interferon (IFN)-alpha (universal inhibitor), the nucleoside analog NITD008 (pan-flavivirus RdRp inhibitor), and the 3-Aryl-indole (DENV-specific inhibitor) were used to validate the multiplex antiviral assay. To facilitate data analysis and to visualize individual inhibition spectra, we developed a kernel based on the RGB paradigm that allows to deconvolute our multidimensional quantitative data into a simple color code per condition tested. Such an approach provides color-coded coordinates in a 3D-plot visualizing broad spectrum activity and/or selectivity towards the individual members of the flavivirus family tested. All assays were run in an automated combined robotics-biosafety containment system.
Results: Both approaches, the individual single virus assays as well as the novel multiplex assay, yielded similar inhibition and sensitivity profiles. Furthermore, RGB color mixing converts values of virus-neutralization (SNT_{50}) or virus inhibition (EC_{50}) into specific color-coded coordinates, revealing the inhibitor's profile against each of the flaviviruses in the multiplex virus mixture and thus its pan-flavivirus potential.
Conclusions: This all-optical multi-virus infection system is amenable to imaged-based antiviral screening and can be run in an automated combined robotics-biosafety containment system to efficiently identify potential pan-flavivirus inhibitors to curb current and future flavivirus outbreaks.

RGB approach. RGB (Red-Green-Blue) color mixing is used to reflect the relative infectivity of three reporter viruses in a multiplex-virus antiviral assay upon antiviral treatment (panel A&B). In brief, color-coded coordinates (R, G, B) in a 3D-plot (panel C) are deconvoluted on a series of RGB palettes (panel D) plus a white-black spectrum (panel E) to interpret the specificity and the potency of the antivirals tested.

Flavitransin, a pan-flavivirus inhibitor that targets the ER co-translational translocation of the viral polyprotein

Marijke Verhaegen, Eva Pauwels, Becky Provinciael, Joren Stroobants, Anita Camps, Eef Meyen and Kurt Vermeire

KU Leuven, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Leuven, Belgium

Flavivirus infections by Dengue and Zika virus impose a significant healthcare threat worldwide. At present no FDA-approved specific antiviral treatment is available, and the safety of a vaccine against Dengue virus is still on debate. We identified a small molecule, named flavitransin, with potent activity against Dengue virus serotype 2 (DENV-2) in Vero cells (IC_{50} = 0,12 µM). Flavitransin also inhibited DENV-2 replication in a concentration-dependent manner in Huh-7 cells (IC_{50} = 0,13 µM), primary monocyte derived dendritic cells (IC_{50} = 0,19 µM) and cells derived from Aedes mosquitos (IC_{50} = 1,4 µM). Furthermore, flavitransin inhibited infection of all four dengue virus serotypes, and showed activity against Zika and Yellow fever virus. Drug profiling by a-time-of-addition assay revealed a post-entry antiviral effect of flavitransin, targeting the viral replication cycle between 4-8 hpi. Immunoblotting analysis revealed that viral polyprotein biogenesis was completely abolished by flavitransin treatment as evidenced by the absence of processed structural and non-structural viral proteins in Dengue virus-infected cells, suggesting an inhibitory effect of flavitransin on viral transcription and/or translation. Moreover, additional experiments with the individual viral proteins in transfected cells indicated that flavitransin directly targets the expression of the structural proteins (pre-membrane and envelope) only. Finally, cell free in vitro protein translation analysis demonstrated a direct inhibitory effect of flavitransin on the co-translational translocation of the N-terminal pre-membrane segment of the Dengue virus polyprotein across the host endoplasmic reticulum membrane, explaining the strong inhibition on viral protein translation and expression. In conclusion, our data demonstrate the discovery of a small molecule anti-flaviviral inhibitor with a unique mode of action by selectively interfering with the ER co-translational translocation of the flavivirus polyprotein.

Beer bitterness compounds of hops inhibit chikungunya virus

Tsvetelina Mandova[1,2], Marielena Vogel Saivich[3], Leonardo La Serra[4], Mauricio Lacerda Nogueira[3], Fernando Batista Da Costa[1]

[1] AsterBioChem Research Team, School of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo, Avenida do Café s/n, 14040-020, Ribeirão Preto, São Paulo, Brazil; [2] Gilson Purification, 22 rue Bourseul, ZI du Poteau, 56890 Saint Ave, France; [3] Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto (FAMERP), São José do Rio Preto, SP 15090-000, Brazil; [4] Virology Research Center, Ribeirao Preto Medical School, University of São Paulo – USP, Brazil

Chikungunya virus (CHIKV) is an arthropod-borne virus that belongs to the genus *Alphavirus* (family Togaviridae). CHIKV causes chikungunya fever, which is mostly characterized by fever, arthralgia and, sometimes, a maculopapular rash. So far there is no any approved antiviral drug or vaccine against CHIKV, highlighting an urgent need for novel therapies. In a cytopathic effect inhibition assay, the bioactive constituents from hops (*Humulus lupulus*, Cannabaceae), mainly acyl phloroglucides known as well as *α*- and *β*-acids, exerted distinct activity against CHIKV, without showing cytotoxic. For fast and efficient isolation and identification of such bioactive constituents, a silica-free countercurrent separation method was applied. A drug-addition experiment was performed to determine the stage of infection and the effect of hops compounds on Vero cells. The co-infection treatment of *β*-acids fraction of 125 µg/mL expressed the strongest activity, compared with the rest of the tested fractions. The post treatment assays showed similar viral inhibition for all five tested fractions close to 80% viral inhibition, except for the fraction where *α*- and *β*-acids were in mixture. All hops compounds demonstrated a promising virucidal activity. The antiviral activity was determined by plaque forming unit assay and a cell-based immunofluorescence assay. We suggested some hypothesis for mechanism of action of acyl phloroglucinols based on their lipophilicity and chemical structure. Therefore, hops compounds could react as a potent activators of protein kinases C (PKC), known inhibitors of chikungunya virus-induced cytopathic effects *in vitro*.

Flavivirus and alphavirus infectivity is significantly reduced by components of the bacterial cell wall

Lana Langendries[1], Sofie Jacobs[1], Rana Abdelnabi[1], Sam Verwimp[1], Suzanne Kaptein[1], Pieter Baatsen[2], Lieve Van Mellaert[3], Leen Delang[1]

[1] KU Leuven, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, 3000 Leuven, Belgium.; [2] VIB-KU Leuven, Electron Microscopy Platform of VIB Bio Imaging Core, 3000 Leuven, Belgium; [3] KU Leuven, Department of Microbiology, Immunology and Transplantation, Rega Institute for Medical Research, Laboratory of Molecular Bacteriology, 3000 Leuven, Belgium.

Background and Aims: Mosquito-borne viruses pose a significant, worldwide threat to human health. Well-known examples are the Zika virus, dengue virus and chikungunya virus. The first site of infection in the host is the mosquito biting site in the skin, which is colonized by a complex microbial community. It has been shown that microbiota in the gastrointestinal tract interact with enteric viruses, but little information is currently available on the impact of host microbiota on flavi- and alphavirus infections. We therefore studied whether host microbiota could influence virus infection.

Methods: Different bacterial cell wall components (lipopolysaccharides (LPS) of some Gram-negative bacteria and peptidoglycan (PG) or lipoteichoic acids of certain Gram-positive bacteria) or complete, heat- or UV-inactivated bacteria were incubated with different flavi- and alphaviruses for 1 hour at 37°C. Following incubation, infectious virus titers were determined by end-point titrations. Immunofluorescent assays were performed to assess whether the toll-like receptor (TLR) 4 - NF-kappaB immune pathway was stimulated by LPS. To this end, cellular localization of NF-kappaB was determined by staining with the NF-kappaB p65 antibody. Transmission electron microscopy was performed on Semliki forest virus (SFV), incubated with LPS or heat-inactivated bacteria.

Results: We identified that pre-incubation of flavi- and alphaviruses with bacterial cell wall components, including LPS and PG, significantly reduced virus infectivity. This inhibitory effect was observed for mosquito-borne viruses of different genera [including Zika virus of the Flavivirus genus and chikungunya virus and Mayaro virus of the Alphavirus genus], showing that this is a broad phenomenon. Interestingly, LPS from different bacterial species showed major differences in inhibition potency, ranging from no inhibition (e.g. LPS *E. coli*) to complete inhibition (e.g. LPS *K. pneumoniae*). A modest inhibitory effect was observed following pre-incubation with heat- or UV-inactivated bacteria, including bacteria residing on the skin. We determined that the antiviral activity of LPS was not caused by cell-dependent effects, nor by activation of the TLR4 immune pathway. Electron microscopy showed that incubation of SFV with LPS of *Serratia marcescens* majorly distorted the morphology of the virus particles (cfr. figure), suggesting a direct, virucidal effect on the virus.

Conclusion: Together, our results indicate that certain bacteria decrease the infectivity of flavi- and alphaviruses. *In vivo* experiments are currently ongoing and will contribute to a better understanding of the early stages of virus infection in the skin, which may enable the search for new strategies to fight mosquito-borne virus infections.

A Long Noncoding RNA, PWAR5 is Key Regulator in Controlling Hepatitis C Virus Propagation

Heejeong Han, Gun-Hee Lee, Dongseob Tark, and Sang-Min Kang*

Laboratory for Infectious Disease Prevention, Korea Zoonosis Research Institute, Jeonbuk National University, Iksan 54531, Republic of Korea.

Background and Aims: Direct-acting antivirals (DAAs) have recently been developed to treat HCV infection with >95% of the SVR rate. However, a minority of patients (approximately 3-5%) have failed treatment, and treatment costs are still expensive. In addition, Previous results have been reported that hepatocellular carcinoma occurs even after DAAs treatment. According to recent reports, the long non-coding RNA (lncRNA) is involved in liver cancer progression and HCV proliferation. However, only few reports have proved, and their biological and physiological roles are unclear. We aimed to develop a new treatment by identifying lncRNA involved in HCV propagation for controlling the virus.
Methods: We quantified the expression of lncRNAs in non-tumor and tumor of HCV-infected patients. We perform Quantitative Real-Time PCR (qRT-PCR) and In situ hybridization (ISH) to evaluate changes in expression of lncRNAs in hepatocellular carcinoma cells and primary human hepatocytes (PHHs) infected with HCV. In addition, we confirmed whether the lncRNA regulates HCV propagation by overexpressing and silencing of lncRNA expression. To verify the role of lncRNA, we implemented Total RNA sequencing and In-silico analysis. We analyzed the expression of lncRNA in HCV-infected liver cancer tissues, normal liver tissues, and liver cancer progression-stage specific tissues.
Results: We found that the Prader Willi/Angelman region RNA 5 (PWAR5) was decreased in HCV-induced liver tumors than in non-tumors. In addition, we showed that the expression of PWAR5 was high in PHHs, while it was low in liver cancer cells. Furthermore, the PWAR5 was decreased after HCV infection in PHHs and hepatocellular carcinoma cells. On the other hand, PWAR5 was no significant change in HBV infection. Overexpression of PWAR5 inhibited HCV RNA and protein expression, but PWAR5 was not involved in HCV entry, RNA replication, and translation step. In order to uncover the function of PWAR5 in HCV propagation, we performed CatRAPID omics and Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis using NGS data. We found that Poly(C)-binding protein 2 (PCBP2) expression was high in liver cancer cells, while it was low in PHHs. Furthermore, expression of PCBP2 was decreased in PWAR5 stable cells than vector stable cells after HCV infection. We performed RNA-pull-down, the PWAR5 interacts with PCBP2. PCBP2 induces RNA replication and translation of HCV by interacting with the internal ribosome entry site (IRES), but PWAR5 blocks this process. Additionally, we found that PWAR5 enhances type I IFN production via suppression of MAVS cleavage due to HCV infection.
Conclusions: We suggested that the modulation of PWAR5 expression could provide new opportunities for the development of innovative therapeutics for viral suppression.

Reverse Inflammaging: Long-term effects of HCV cure on biological age

Carlos Oltmanns[1,2,3,4], Zhaoli Liu[1,2,4], Jasmin Mischke[1,2,3,4], Jan Tauwaldt[1,2,3,4], Yonatan Ayalew Mekonnen[1,2,4,5], Benjamin Maasoumy[2,3], Heiner Wedemeyer[2,3], Anke R.M. Kraft[1,2,3,4], Cheng-Jian Xu* [1,2,4,6], Markus Cornberg* [1,2,3,4]

[1] Centre for Individualised Infection Medicine (CiiM), a joint venture between the Helmholtz Centre for Infection Research (HZI) and Hannover Medical School (MHH). Hannover, Germany.
[2] Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School (MHH), Hannover, Germany
[3] German Centre for Infection Research (DZIF), partner site Hannover-Braunschweig, Germany
[4] TWINCORE, a joint venture between the Helmholtz-Centre for Infection Research (HZI) and the Hannover Medical School (MHH). Hannover, Germany.
[5] Institute for Bioinformatics, University Medicine Greifswald, Greifswald, Germany
[6] Department of Internal Medicine, Radboud University Medical Center, Nijmegen, the Netherlands
shared last authorship

Background and Aims: Chronic hepatitis C virus (HCV) infection can be cured with direct-acting antiviral agents (DAA). However, not all sequelae of chronic hepatitis C appear to be completely reversible after sustained virologic response (SVR). Recently, chronic viral infections have been shown to be associated with biological age acceleration defined by the epigenetic clock. The aim of this study was to investigate whether chronic HCV infection is associated with epigenetic changes and biological age acceleration and whether this is reversible after SVR.
Methods: We included 54 well-characterized patients with chronic hepatitis C at three time points: DAA treatment initiation, end of treatment, and long-term follow-up (median 96 weeks after end of treatment). Genome-wide DNA methylation status from peripheral blood mononuclear cells (PBMC) was generated and used to calculate epigenetic age acceleration (EAA) using Horvath's clock.
Results: HCV patients had an overall significant EAA of 3.12 years at baseline compared with -2.61 years in the age-matched reference group ($p < 0.00003$). HCV elimination resulted in a significant long-term increase in DNA methylation dominated by hypermethylated CpGs in all patient groups. Accordingly, EAA decreased to 1.37 years at long-term follow-up. The decrease in EAA was significant only between the end of treatment and follow-up ($p = 0.01$). Interestingly, eight patients who developed hepatocellular carcinoma after SVR had the highest EAA and showed no evidence of reversal after SVR.
Conclusions: Our data contribute to the understanding of the biological impact of HCV elimination after DAA and demonstrate that HCV elimination can lead to "reverse Inflammaging". In addition, we provide new conceptual ideas for the use of biological age as a potential biomarker for HCV sequelae after SVR.

A universal flavivirus reporter system as a tool in the quest for a broad-spectrum flavivirus antiviral agent

Doortje Borrenberghs [1], Sarah Megens [2], Peggy Geluykens [3], Anil Koul [1], Marnix Van Loock [1] and Olivia Goethals [1]

[1] Janssen Global Public Health, Janssen Pharmaceutica NV, Turnhoutseweg 30, 2340, Beerse, Belgium; [2] Janssen Infectious Diseases discovery, Janssen Pharmaceutica NV, Turnhoutseweg 30, 2340, Beerse, Belgium; [3] Charles River Laboratories Beerse, Discovery, Turnhoutseweg 30, 2340, Beerse, Belgium

Flaviviruses, such as Dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV), Japanese Encephalitis virus (JEV) and Yellow Fever virus (YFV), which threaten half of the human population worldwide, and are predicted to increase over the next decades. Flaviviruses as such have a huge pandemic potential, and subsequently there is an urgent need to develop new screening assays, newer diagnostics, novel therapeutics as well as explore new vaccine opportunities for tackling these emerging infections. For the discovery of novel antivirals, cell-based phenotypic screening has proven to be highly valuable, especially when complemented with the downstream assays using advanced molecular signatures, high content imaging platforms as well as artificial intelligence (AI) and machine learning (ML) technologies for target deconvolutions etc. Interestingly, the conserved genetic signature of the different flaviviruses as well as conserved cellular replication processes provides the opportunity to develop a uniform reporter system that allows the detection of multiple flaviviruses in a singular assay format. Hereto, a flavivirus susceptible stable Vero cell line is created containing (1) a reporter construct, carrying a long terminal repeat (LTR) upstream of an enhanced green fluorescent protein (eGFP), and (2) a construct encoding for NS4B, to tether the construct to the endoplasmic reticulum (ER), and the N-terminal part of NS5, harboring the conserved and uniform NS4B/NS5 cleavage site, fused to the trans-activator of transcription (Tat) protein. Upon infection of this double stable Vero cell line with a flavivirus, the viral protease cleaves off the Tat protein, which will bind the LTR and induces expression of eGFP. Consequently, the amount of eGFP fluorescence correlates with viral replication and can be used as read-out to evaluate the antiviral activity of compounds against multiple flaviviruses in parallel. Validation and optimization of this reporter system with different reference compounds led to the development of seven assays suitable for screening and testing antiviral compounds against DENV-1-4, ZIKV, WNV, and YFV, which makes this universal flavivirus reporter system a valuable tool for the discovery of broad-spectrum flavivirus inhibitors.

Cell culture studies of the barrier to resistance of broad-spectrum antiviral remdesivir against hepatitis C virus

Kuan Wang, Carlota Fernandez-Antunez, Line Abildgaard Ryberg, Ulrik Fahnøe, Jens Bukh and Santseharay Ramirez.

Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital and Department of Immunology and Microbiology, University of Copenhagen.

Background and Aims: Treatment of hepatitis C virus (HCV) was revolutionized with the advent of direct-acting antivirals (DAA), but treatment failure due to multi-DAA antiviral resistance could compromise the efficacy of these treatments, worldwide. Repurposing of broad-spectrum antivirals already available for other viral diseases could be of interest for treating infections with multi-DAA resistant HCV. Remdesivir (RDV) is a broad-spectrum nucleotide analog (nuc) active against several RNA viruses, including HCV in cell culture. This study aimed at investigating the efficacy and barrier to resistance of RDV against HCV genotype 2 (isolate J6/JFH1) in hepatoma Huh7.5 cells.
Methods: The efficacy of RDV (drug effective concentration 50%; EC_{50}) was determined in short-term concentration-response assays and the barrier to resistance in long-term escape experiments. A RDV escape virus was characterized phenotypically (EC_{50} and viral growth kinetics) and genotypically (next-generation sequencing). Viral escape associated substitutions were similarly characterized in reverse genetic experiments.
Results: RDV was highly effective against J6/JFH1, with an average EC_{50} value of 43,5 nanomolar (nM), thus about 12 times more potent than sofosbuvir. A RDV escape virus exhibited decreased RDV susceptibility with a 4,7-fold increase in EC_{50}. Cross-resistance was observed towards another 1'-cyano nuc (GS-6620; 6,3-fold increase in EC_{50}), but not to other nucs, including sofosbuvir (<2,1-fold increase in EC_{50}). The RDV escape virus showed faster growth kinetics compared to the original J6/JFH1 virus.
RDV escape correlated with the emergence of substitutions throughout the viral genome, including E143Q, T179A, and M289V/L in the NS5B polymerase. All 4 substitutions increased RDV EC_{50}, particularly when they were combined. In long-term treatment assays, infections with the original and single T179A, M289V, or M289L mutant viruses were cleared. In contrast, viruses harboring only E143Q or the three substitutions combined escaped, despite exhibiting decreased growth kinetics compared to J6/JFH1.
Conclusions: RDV showed remarkable efficacy and a relatively high barrier to resistance for HCV genotype 2 in cell culture. Substitutions decreasing RDV susceptibility impaired viral fitness. Clinical trials will need to be conducted to evaluate the relevance of remdesivir for the treatment of HCV in patients.

The regulatory T cell population is not normalized following successful treatment of chronic hepatitis C with direct-acting antivirals

So-Young Kim[1,2], Hyung-Don Kim[1,3], Seong Jin Choi[1,4], June-Young Koh[1], Won Kim[5], Dong Hyeon Lee[5], Eui-Cheol Shin[1, 2]

[1]Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea; [2]The Center for Viral Immunology, Korea Virus Research Institute, Institute for Basic Science (IBS), Daejeon, Republic of Korea; [3]Department of Oncology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea; [4]Department of Internal Medicine, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea; [5]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Seoul Metropolitan Government Boramae Medical Center, Seoul National University College of Medicine, Seoul, Republic of Korea ; [6]Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea

Background and Aim: We recently reported that $CD4^+CD25^+Foxp3^+$ regulatory T (T_{REG}) cells can produce TNF in patients with viral hepatitis and demonstrated that they are *bona fide* T_{REG} cells with hypomethylation at the T_{REG} -specific demethylated region (*Gastroenterology 154:1047-1060, 2018*). In the present study, we examined the frequency and the characteristics of TNF-producing T_{REG} cells in the peripheral blood of patients with chronic hepatitis C (genotype 1b, n=29) before and after direct-acting antiviral (DAA) treatment.

Methods: Twenty nine patients with chronic hepatitis C were treated with DAAs (daclatasvir (DCV)/asunaprevir (ASV) for 14 patients; ledipasvir (LDV)/sofosbuvir (SOF) for 15 patients) and sustained virologic response was achieved in all the patients. The frequency of TNF-producing T_{REG} cells among peripheral blood T_{REG} cells was analyzed by intracellular cytokine staining and flow cytometry. We also analyzed the open chromatin landscape of peripheral blood T_{REG} cells from healthy donors and patients with chronic hepatitis C before and after DAA treatment using assay for transposase-accessible chromatin with sequencing (ATAC-seq).

Results: The frequency of TNF-producing T_{REG} cells among peripheral blood T_{REG} cells was significantly increased in patients with chronic hepatitis C compared to healthy donors. In patients with chronic hepatitis C, TNF-producing T_{REG} cells exhibited Th17-like features, including up-regulation of RORγt and CCR6. At week 8 after starting DAA treatment, the frequency of TNF-producing T_{REG} cells among peripheral blood T_{REG} cells was transiently decreased in both DCV/ASV- and LDV/SOF-treated groups although the frequency of T_{REG} cells among $CD4^+$ T cells was not changed. However, the frequency of TNF-producing T_{REG} cells was increased thereafter, and the frequency measured at week 12 after the cessation of DAA treatment was returned to the level prior to DAA treatment. In addition, even after successful DAA treatment, T_{REG} cells from patients with chronic hepatitis C epigenetically differed from T_{REG} cells from healthy donors.

Conclusions: Our findings indicate that inflammatory changes of T_{REG} cells in patients with chronic hepatitis C are not normalized even after successful treatment with DAAs.

Optimization and validation of rat HEV transmission model

Xin Zhang, Niels Cremers, Johan Neyts, Suzanne Kaptein

Background and Aims: Hepatitis E virus (HEV) is the causative agent of hepatitis E in humans and the leading cause of acute viral hepatitis. It is also responsible for chronic hepatitis in immunosuppressed patients worldwide. The virus is faecal-orally transmitted through consumption of contaminated drinking water or infected undercooked meat of pigs. Previously, we reported that athymic nude rats support productive replication of rat HEV when injected intravenously with diluted liver homogenate collected from HEV-infected rats (PMID: 27483350). The virus is also efficiently transmitted when co-housing infected rats with sentinel rats. Here, we report on the establishment of an in vitro assay for the detection of infectious virus in samples. We also further optimized the transmission model as well as characterized the transmission of HEV in this model to understand the dynamics of transmission. The ability of an antiviral agent to block transmission was assessed using ribavirin, which is used to treat hepatitis E (acute and chronic) in patients (PMID: 31284447).

Methods: Faeces from athymic nude rats infected intravenously with rat HEV contained infectious virus as hepatoma (Huh.7) cells became clearly infected when exposed to the faeces suspension (Figure 1A). We therefore used infectious faeces to inoculate athymic nude rats by orally administrating diluted infectious faeces suspension on day 0 and day 2 post-transmission (pt). Ribavirin treatment consisted of once-daily oral gavage with 60 mg/kg till 12 dpt. Faeces and other tissues were analysed for the presence of viral RNA by RT-qPCR. To set up an infectious in vitro assay, diluted organ or faeces homogenates were incubated with 400.000 Huh.7 cells while rotating plates for 1 h at room temperature, followed by an incubation without rotation at 35 °C for 5 h. Next, the inoculums were replaced by fresh medium. Half of the medium was collected for viral RNA detection every 2 - 3 days until 14 days post-infection (dpi). Cells were fixed on 14 dpi for antigen detection.

Results: To reduce the variability due to the irregular uptake of infectious faeces by the rats in the transmission model, rats were fed an equal volume of infectious faeces suspension. Indeed, all rats became positive for rat HEV at the same time point. Moreover, the virus replicated to similarly high levels in the liver and faeces of individual rats at all time-points pi. In addition to the liver and faeces, the spleen and intestines were also positive for viral RNA on 30 dpt. Intestine-derived rat HEV particles were found to be infectious as they were capable of infecting hepatic cells. Treatment with ribavirin prior to oral gavage with the infectious faeces suspension efficiently protected rats from getting infected with rat HEV.

Conclusions:
We demonstrate that the athymic nude rat transmission model is highly suitable for evaluating the protective potential of antiviral molecules against rat HEV.

Chemical screening targeting Conserved Hsp70 Binding Sites Reveals the biological properties of Hsp70 inhibition

Shuhei Taguwa[1,2] Hao Shao[3], Rahmi Dianty [1,2], Chikako Ono[1,4] , Jason Gestwicki[3], Judith Frydman[5] and Yoshiharu Matsuura[1,2]

[1]Laboratory of Virus Control, Center for Infectious Disease Education and Research,; [2]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; [3]Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, USA; [4]Laboratory for Cell Polarity Regulation, RIKEN Center for Biosystems Dynamics Research, Osaka, Japan; [5]Department of Biology and Genetics, Stanford University, Stanford, USA

Background and Aims: The heat shock protein 70 (Hsp70) family of molecular chaperones plays important role in protein homeostasis. Hsp70s are ubiquitous and conserved across all eukaryotes, with homologues present in each major cellular compartment. Given this broad distribution and plurality of Hsp70 functions, it is perhaps not surprising that Hsp70s have been implicated in many diseases, including infectious diseases, cancer, and neurodegeneration. Here we examine the relationship between subcellular localization, genetic interactions, and their functions, especially clarify how dengue virus utilizes hsp70 functions for the propagation.
Methods: To explore the hsp70 biology, we select chemically-similar inhibitors of the Hsp70 chaperone family that were developed during a medicinal chemistry campaign. We define these hsp70 inhibitors according to the following four points 1) anti-dengue activity, 2) subcellular localization, 3) genetic interaction, and 4) anti-cancer activities.
Results: We found that the different analogues of Hsp70 inhibitors showed a range of potencies against dengue propagation in distinct host cells (human and mosquito), which trends to reflect the dramatic differences in their subcellular distributions, where early-stage compounds were found in mitochondria and nucleoli whereas late-stage compounds colocalized with ER and vesicles. Consistent with the localization, a CRISPRi screen revealed distinct chemogenetic interactions of these compounds with different members of the Hsp70 family, such as ER-resident BiP and mitochondrial Hsp70 (mortalin), as well distinct co-chaperones. Though we could not find a strong correlation of anti-dengue with anti-cancer activities, Interestingly, anti-cancer activities tend to improve during the development of hsp70 chemical campaign.
Conclusions: Our finding revealed that dengue virus utilizes distinct hsp70 functions in a different host, which are partially different from those essential for cancer growth.

TBEV-infected human neuronal/glial cells identify antiviral drugs

N Berry[1], V Chaillot[1], M Cochet[1], F Piumi[1], K Gorna[1], O Blanchet[2], A Danckaert[3], N Aulner[3], A Ahmed-Belkacem[4], B Pain[5], M Coulpier[1]

1: UMR1161 Virologie, Anses-INRAE-EnvA, Université Paris-Est, Maisons-Alfort. 2 : CHU Angers, Centre de Ressources Biologiques, BB-0033-00038. 3 : Institut Pasteur Paris, UTechS-PBI / Imagopole. 4 : INSERM U955 Eq18, Créteil. 5 : INRAe USC 1361 SBRI, Lyon.

Many endemic, emerging, re-emerging or potentially emerging RNA viruses (*Flavi*-, *Alpha*-, *Henipa*-, *Rhabdo*-, *Corona*-viruses…) target the human central nervous system (CNS), causing severe neurological disorders, sometimes fatal or leading to debilitating consequences. The *Flaviviruses* genus includes many of these highly pathogenic neurotropic viruses (West Nile virus, Tick-Borne Encephalitis virus-TBEV, Zika virus…). Despite their dangerousness, only a few vaccines exist and there is currently no available antiviral treatment. Whereas many efforts are made for the identification of antiviral molecules, including some with “broad-spectrum” properties, most studies use models that are not physiologically relevant. This probably gives an explanation to the lack of antiviral activity or excessive toxicity often observed in clinical trials, leading to their failure. To overcome this problem, we believe it is important to use pathological models of infection to identify molecules with high predictive value of therapeutic efficiency *in vivo*. Here, we used an *in vitro* two-dimensional (2D) culture of neuronal/glial cells differentiated from fetal human neural progenitor cells which reproduces major hallmarks of TBEV infection in the human brain. We showed that some molecules selected from the literature for their antiviral activity against TBEV or other Flaviviruses when using cell lines (VERO, A549, Huh-7 cells…) are not efficient when testing them in TBEV-infected neuronal/glial cells (hNGCs). On the contrary, we identified several molecules with an anti-TBEV activity in hNGCs that had no antiviral activity in a TBEV-infected cell line. Finally, we confirmed the antiviral activity of some of the molecules previously identified in cell lines. These results thus clearly show that antiviral activity depends on the cellular models used. They call for developing more physiologically relevant 2D models for testing or screening drugs for their antiviral activity. In an attempt to further improve our *in vitro* models of TBEV infection, we are currently using 3D-cerebral organoids and will soon test selected drugs for their efficiency and toxicity in this model. We hope that this will allow us to select compounds with a very high probability to be efficient and non-toxic in the human brain.

Interferon-free hepatitis C treatment increases surrogate of cardiovascular disease risk in Black Veterans

Poonam Mathur, DO, MPH[1], Nivya George, MSc, MS[1], Roman Kaplan, PharmD[2], Amanda Theppote, MD[1], Eleanor Wilson, MD, MHS[1,2]

[1]Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD, US
[2]Baltimore VA Medical Healthcare Center, Baltimore, MD, US

Background and Aims: Sustained virologic response (SVR) after hepatitis C virus (HCV) treatment with either Interferon (IFN)-based or IFN-free regimens with direct-acting antivirals (DAAs) has been shown to reduce cardiovascular disease (CVD) events in majority white populations stratified by ASCVD score. However, the effect of IFN-free therapy on lipid profiles after SVR, as an indirect measure of CVD risk, is unknown in Black patients.

Method: We evaluated HCV-infected Veterans from the Baltimore VA who were treated with DAAs between 2015-2019. We performed a retrospective analysis using linear regression to compare lipid profile changes following SVR between those with early stage (F0-F2) fibrosis and advanced liver disease (ALD, F3-F4 fibrosis), and to assess differences in lipid profiles based on fibrosis stage in patients with HIV (combined effect) and Type II Diabetes Mellitus (DM2).

Results: Of those treated for HCV (n = 1,528), 96% (n = 1,474) achieved SVR. Most patients were Black males (75%) and a minority (2.7%) received statin therapy prior to starting HCV treatment. Of 1,191 patients for whom data was available, lower triglyceride (TG) levels prior to DAA treatment was significantly associated with achieving SVR (median 106 vs. 127, p = 0.031). Among those who achieved SVR, a significant increase in LDl was seen in those with HIV (combined effect of HIV and ALD, p = 0.037) (Figure 1A), a well as a significant increase in change in LDL in the ALD group compared to early stage (Figure 1B). In addition, among those with DM2, ALD was associated with an increase in TG post-SVR (p = 0.045).

Conclusion: In a cohort of mostly Black HCV-infected Veterans, patients with HIV and ALD have a significantly higher increase in LDL after achieving SVR, suggesting that they may have increased risk for CVD after HCV treatment. In addition, lower pre-treatment TG levels significantly impact achievement of SVR, and increases in TG post-SVR were seen in patients with DM2 and ALD. Overall, these findings suggest that patients with ALD should have optimization of glucose control and hypertriglyceridemia prior to DAA treatment, to increase the probability of achieving SVR. In addition, patients with HIV and ALD should have correlates of CVD risk optimized after SVR, in order to reduce the long-term risk of CVD.

Antioxidant and xanthine oxidase inhibitory activities of the medicinal plant *Centaurium erythraea* and its possible use against liver damages and pathologies associated with chronic hepatitis c

Moufida ADJADJ [1,2,*], Meriem DJARMOUNI [2], Abderrahmane BAGHIANI [2] Youssouf BOUDISSA

[1] Department of Medicine, Faculty of Medicine, University Constantine 3, Constantine, Algeria
[2] Laboratory of Applied Biochemistry, Department of Biochemistry. Faculty of Natural and Life Sciences, University Ferhat Abbas Sétif 1, Sétif, Algeria

Background and Aim: Hepatitis C virus (HCV) can induce reactive oxygen species (ROS) production and accumulation leading to strong cellular oxidative stress resulting in a greater imbalance between pro-oxidants and intracellular antioxidant agents. Xanthine oxidase (XO) is one of the enzymatic sources of ROS especially hydrogen peroxide, superoxide anion radical and hydroxyl radical. Serum xanthine oxidase was markedly increased in acute viral hepatitis. Also, the use of natural and synthetic antioxidants like vitamin C and E, gallic acid, quercetin and other polyphenols, has already shown promising results as co-adjuvants in HCV therapy. This study is aimed to evaluate the potential use of *Centaurium erythraea* as therapeutic agent against various liver damages and pathologies associated with chronic hepatitis C disease by assessing its antioxidant and xanthine oxidase inhibitory activities.

Methods: After hydro-methanolic extraction (80% then 50%, v/v in H_2O) of *Centaurium erythraea* phenolic compounds, Folin-Ciocalteu and aluminium trichloride methods were used to determine respectively total polyphenols and flavonoids contents of this extract. After purification of XO from bovine milk, the antioxidant activity was assessed using the XO inhibitory assay.

Results: The phytochemical investigation showed that the plant extract has significant concentrations of total polyphenols and flavonoids. The results of the XO inhibitory assay demonstrated that the extract exerted a very significant XO inhibitory activity in a dose dependent manner.

Conclusion: *Centaurium erythraea* may serve as a natural antioxidant treatment for ROS-induced hepatic damages and related diseases associated with hepatitis C, where inhibition of ROS production by inhibiting XO activity is necessary.

Keywords: Hepatitis C virus, reactive oxygen species, *Centaurium erythraea*, antioxidants, xanthine oxidase inhibitory activity
Contact: *moufidatoxicology@gmail.com*

Persistence of hepatitis E virus in the cerebrospinal fluid despite apparently successful ribavirin therapy.

Sébastien Lhomme[1,2], Amandine Fayard[3], Sonia Mirafzal[4], Romain Carcenac[2], Pauline Boyer[2], Justine Latour[2], Amélie Brebion[5], Jacques-Olivier Bay[3], Cécile Henquell[5], Jacques Izopet[1,2]

[1] Infinity, Université Toulouse, CNRS, Inserm, UPS, Toulouse, France ; [2] CHU Toulouse, Hôpital Purpan, Laboratoire de Virologie, Toulouse, F-31300, France ; [3] CHU Clermont-Ferrand, Service d'Hématologie, 63000 Clermont-Ferrand, France ; [4] CHU Clermont-Ferrand, Service de Neuroradiologie, 63000 Clermont-Ferrand, France ; [5] CHU Clermont-Ferrand, 3 IHP, Laboratoire de Virologie, 63000 Clermont Ferrand, France

Hepatitis E virus (HEV) is a leading cause of acute hepatitis worldwide. HEV infections can also become chronic in immunocompromised patients, including those with hematological disease receiving chemotherapy. Both immunocompetent and immunocompromised patients can suffer from neurological manifestations but how HEV reaches the central nervous system is still not clear. This report describes the case of a 69-year-old chronically HEV-infected woman given a haploidentical allo-hematopoietic stem cell transplantation. After diagnosis, she was treated with 800 mg/day ribavirin. The neurologic disorders appeared 27 days after ribavirin initiation. Her electroencephalogram was slowed and magnetic resonance imaging showed non-specific peri-ventricular leukopathy. There was also HEV RNA in her cerebrospinal fluid (CSF). Ribavirin therapy (86 days) cleared the HEV RNA from her blood and stool but the neurological symptoms persisted and HEV RNA was found in a new sample of CSF. Analysis of the HEV quasispecies by deep sequencing showed differences between the blood and the CSF, together with evolution of the CSF quasispecies. Our data suggest that HEV replicates autonomously within the central nervous system. Physicians should not overlook neurological symptoms due to the virus in apparently cured patients with recent history of HEV infection.

Session 6
VIRAL REPLICATION

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Viral determinants of hepatitis C virus cell culture adaptation and their relevance for enhanced replication fitness

Nicola Frericks[1], Melina Winkler[1], Richard J.P. Brown[2], Csaba Miskey[3], Zoltán Ivics[3], Thomas Pietschmann[1], Julie Sheldon[1]

[1] Institute of Experimental Virology, TWINCORE, Hannover, Germany; [2] Department of Veterinary Medicine, Paul-Ehrlich-Institut, Langen, Germany; [3] Department of Medical Biotechnology, Paul-Ehrlich-Institut, Langen, Germany

Background: Hepatitis C virus (HCV) establishes chronic infections in the majority of exposed individuals. One of the complex mechanisms by which HCV establishes a persistent infection is the continuous selection of the fittest viral genomes from a swarm of genetically diverse viral variants. *In vitro* adaptation of HCV in Huh-7.5 cells resulted in the selection of a virus population (p100pop) exhibiting an enhanced replicative fitness and partial resistance to interferon and a broad spectrum of HCV specific antivirals. Next generation sequencing revealed fixation of numerous non-coding and coding mutations across the entire viral polyprotein.

Aim: We aimed to understand the mechanism(s) of p100 adaptation in order to learn about the principles by which HCV optimizes replication fitness.

Methods: We created various molecular p100 clones to analyze the impact of the different adaptive mutations on replication fitness. We also generated a p100 subgenomic replicon (SGR) and investigated its replication fitness and antiviral resistance by reporter gene assays. We used transcriptional profiling to compare host cellular responses to infection with p100pop and the parental HCV strain, Jc1.

Results: Analysis of viral replication of the molecular p100 clones and p100pop revealed that multiple adaptive mutations in the viral consensus sequence confer enhanced virus fitness in various human hepatoma cell lines, especially in non-permissive Huh-7.5 miR-122 deficient cells. Although the full-length clone and the SGR, harboring NS3-NS5B mutations only, exhibited a replication capacity similar to the control, enhanced viral resistance to cyclosporine A, an inhibitor of another essential HCV host factor, was observed. Gene set enrichment analysis revealed a unique host cellular response upon p100pop infection. Specifically, pathways involved in ER stress signaling and unfolded protein response were highly dysregulated compared to Jc1.

Conclusion: Our results suggest that the observed enhanced replication fitness of p100 is caused by changes in the virus-host interactions rather than an improved replication capacity by mutations occurring in proteins involved in the replication complex formation. Additionally, our RNA sequencing data suggest that the modulation of the host cellular response may contribute to the observed enhanced replicative fitness.

Dynamic hepatitis E virus genomic rearrangements observed *in vivo* as determinants for viral fitness, resistance and immune escape

Michael Hermann Wißing[1], Leonard Knegendorf[1,2], Lena Allweis[3], Anindya Siddhartha[2], Anett Gisa[4], Birgit Bremer[4], Marc Lütgehetmann[3], Patrick Behrendt[2,4,5], Heiner Wedemeyer[4,5], Eike Steinmann[1], **Daniel Todt**[1,6]

[1]Department for Molecular & Medical Virology, Ruhr University Bochum, Bochum, Germany.; [2]Institute for Experimental Virology, TWINCORE Centre for Experimental and Clinical Infection Research, Hannover, Germany.; [3]University Medical Center Hamburg-Eppendorf, Hamburg, Germany.; [4]Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany.; [5]German Center for Infection Research (DZIF), Partner Site Hannover Braunschweig, Hannover, Germany; [6]European Virus Bioinformatics Center (EVBC), Jena, Germany.

Background and Aims: Hepatitis E virus (HEV) infections are usually self-limiting and asymptomatic in healthy individuals but can progress to chronicity and cause fulminant hepatitis in risk group patients. As established for most RNA viruses, HEV exists as divers population in patients. Recent data suggest a very heterogeneous hypervariable region (HVR) within the open reading frame (ORF1), tolerating major genomic rearrangements. Several in-frame insertions of up to 58 amino acids have been identified in chronically infected patients. The most commonly used cell culture strain harbors an insertion of the human ribosomal subunit S17 and displays by far the highest replication capacity reported. Interestingly, when first cultivated, this insertion was only present in a minority of HEV genomes. In this study we aim to identify HVR rearrangements in chronically infected, treatment-resistant patients and monitor intra-host population dynamics. We classify common patterns of insertions to reconstruct their influence on replication kinetics and aim to understand the impact of the viral population composition on treatment outcome.
Methods: We amplified the HVR region of HEV populations in periodically drawn serum samples of chronically infected and treatment-resistant patients and monitored their composition over time via next generation sequencing. We further analyzed their influence on replication and ribavirin sensitivity via a subgenomic reporter replicon and on particle production and entry via our recently described cell culture system. Furthermore, we applied standard cell culture and fluorescence imaging techniques to identify alterations in sub-cellular location of HEV proteins.
Results: Via longitudinal analysis of the viral population of a treatment-resistant patient, we were able to identify three novel insertions. Two insertions of host-derived interferon stimulated gene sequences, namely TRIM22 and SERPINA1 as well as duplications of the HEV genome in the HVR. The exact population dynamics still lack in-depth analysis. These insertions, as well as eight insertions of human origin identified in public databases display increased replication capacity, while not affecting ribavirin sensitivity in the subgenomic replicon system. Furthermore, full length HEV constructs harboring identified insertions produced higher viral titers than the parental HEV strain. Inserted lysine residues were identified as key determinants for replication.
Conclusions: This study links insertions in the hepatitis E viral HVR to an increased replication capacity and identifies an lysine residues as a common feature and determinant for fitness. Further investigation of the mechanism behind the replication advantage and the reason for the selection of those clones *in vivo* could reveal new therapeutic targets for the development of antiviral intervention strategies.

Pestivirus dependency on micro-RNA-17 is controlled by the host cell

Rui Costa[1], Konstantinos Kokkonos[1], Yu Deng[2], Ulrik Fahnøe[1], Louise Nielsen[1], Nicolas Fossat[1], Louise Lohse[3], Graham J. Belsham[4], Jens Bukh[1], Thomas Bruun Rasmussen[2,3], Troels Scheel[1]

[1] Copenhagen Hepatitis C Programme (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2200 Copenhagen N, Denmark; [2] DTU National Veterinary Institute, Technical University of Denmark, Lindholm, DK-4771 Kalvehave, Denmark; [3] Department of Virus & Microbiological Special Diagnostics, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark; [4] Department of Veterinary and Animal Sciences, University of Copenhagen, Grønnegårdsvej 15, DK-1870 Frederiksberg C, Denmark

Background and Aims: A remarkable feature of hepatitis C virus (HCV) is its dependency on binding the liver-specific micro-RNA, miR-122, to the viral 5' UTR for RNA stability, translation, and replication. Curiously, bovine viral diarrhoea virus (BVDV), an important pestivirus and livestock pathogen, similarly interacts with the cellular let-7 and miR-17 families but through binding to the 3' UTR. Replication of BVDV (type 1) was shown to critically depend on miR-17, although viral mutants depending on different, or no miRNAs could be selected.
Methods: Here, we set out to investigate the breadth of miRNA dependency across the *Pestivirus* genus. Using miR-17 seed site mutants of BVDV-2, border disease virus (BDV) and classical swine fever virus (CSFV), important pathogens of cattle, sheep, and swine respectively, we analysed miRNA dependency across cell lines of different species and tissue type.
Results: The miR-17 dependency of BVDV-1 in bovine MDBK kidney cells was recapitulated for all pestiviruses. Surprisingly, however, infection with BVDV and BDV miR-17 seed site mutants was only slightly attenuated in bovine Kop-R oropharynx cells, ovine SFT-R thymus cells and caprine ZZ-R tongue cells. The same was true for BDV infection of porcine PK-15 and SK-6 kidney cells. These observations were not due to differences in miR-17 expression among the cell lines and replication was not supported by an alternative miRNA, given that knock-down of the Argonaute 2 (AGO2) protein, unlike in MDBK cells, did not lead to viral attenuation. For CSFV, using a sub-genomic replicon, we on the contrary found that strict miR-17 dependency was conserved across all cell types in which CSFV replicated. Trans-complementation of the viral seed site mutants with complementary miR-17 mimics rescued viral replication, confirming that absence of replication indeed was due to miR-17 dependency.
Conclusions: We find that pestiviral miR-17 dependency extends throughout the genus. Most interestingly, however, host cell factors controlled to what extent the same virus was dependent on miR-17 in different cell types. The observation that not only viral but also cellular factors control miRNA dependency is novel and may have broader implications also for HCV. These discoveries highlight that there is yet much to be uncovered in the field of RNA interactions in the *Flaviviridae* family.

NS5A domain I antagonises PKR to facilitate the assembly of infectious hepatitis C virus particles

Shucheng Chen, Mark Harris

School of Molecular and Cellular Biology, University of Leeds, Leeds, United Kingdom

HCV NS5A is a highly phosphorylated, multifunctional, protein comprised of three domains (I, II and III). Domains I and II have been shown to function in genome replication whereas domain III has a role in virus assembly.
Previously, we demonstrated that domain I of NS5A in genotype 2a (JFH1) also played a role in virus assembly, exemplified by the phenotype of an alanine substitution at P145 (Yin *et al*, 2018), which was not required for RNA replication. In the current study we extended this analysis to identify other conserved and surface exposed residues proximal to P145 that exhibited a similar phenotype. Specifically, these were C142, shown to be disulphide-bonded to C190 in NS5A domain I, and E191. Alanine substitutions of these residues did not exhibit a genome replication defect, but produced no infectious virus. Additionally, the size and distribution of lipid droplets in cells infected with these mutants was altered compared to wildtype (WT).
In parallel, to investigate the mechanism underpinning this role of domain I, we assessed the involvement of the interferon-induced double-stranded RNA-dependent protein kinase (PKR). Intriguingly, in PKR knockout cell lines, C142A and E191A exhibited levels of infectious virus production and lipid droplet size and distribution that were indistinguishable from WT. Protein interaction studies revealed that these mutants were defective in binding to PKR (unlike WT and C190A). In addition, in vitro pulldown experiments confirmed that WT NS5A domain I interacted with PKR. These data suggest a novel interaction between NS5A domain I and PKR that functions to evade an antiviral pathway that blocks virus assembly. Ongoing studies investigating the roles of both domain I and PKR in assembly of infectious HCV particles will be presented.

Molecular evolutionary analysis reveals complex path to fitness peak of hepatitis C virus in long-term cell-culture adaptation.

Ulrik Fahnøe[1], Anders Gorm Pedersen[2], Anne Finne Pihl[1], Santseharay Ramirez[1], Judith Gottwein[1], Jens Bukh[1]

[1]Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Hvidovre Hospital, and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; [2]Department of Health Technology, Section for Bioinformatics, Technical University of Denmark, Lyngby, Denmark

Background and Aims: Hepatitis C virus (HCV) circulates as a population of variants due to a high inherent mutation rate. Mutations function in concert and their linkage is vital for viral function and adaptation. However, standard sequencing methods, including Next Generation Sequencing (NGS), commonly used to study virus populations, have severe limitations, since the short reads greatly limit our ability to link distant mutations on individual genomes. In the work presented here, we were interested in examining the evolutionary path followed by HCV towards increased fitness, and in understanding how mutations at multiple locations in the genome together contribute to this fitness.
Methods: We used long-term cell culture of the JFH1-based recombinant with Core-NS2 from strain SA13 (genotype 5a) to examine the gradual adaptation of the virus, with a focus on understanding the combined role of multiple mutations acquired throughout the entire genome. For this purpose, we developed an RT-PCR method to amplify the full HCV polyprotein gene, which was then examined by conventional cloning and sequencing of full-length amplicons and by using long-read (full-length) sequencing platforms, such as PacBio.
Results: Here we show how mutations are distributed and linked on individual virus genomes and identify combinations of mutations leading to higher fitness. After 30 passages one population reached a specific infectivity that was increased 10-fold. Analysis showed a highly homogeneous population with unique mutations that could be the result of a selective sweep. For this virus, linkage of key mutations was shown to be essential for fitness gains over the course of the experiment. Based on longitudinal sequencing data we show that the evolutionary path leading to this extremely fit virus cannot be explained by gradual addition of single point mutations gained over time. This was confirmed by experimentally testing the fitness of major evolutionary steps in cell-culture. Instead, it appears that recombination events caused this virus to obtain superior fitness.
Conclusions: We have performed an experimental investigation of how HCV evolves during long-term cell culture and find that one of the evolving populations obtained a substantial increase in fitness in a way that involved recombination of less fit viruses, such that the virus acquired multiple mutations in one evolutionary event. The recombination events essentially allowed the virus to make leaps in fitness-space, thereby reaching a fitness peak that could not easily be reached through a gradual increase of fitness by acquisition of one point mutation at a time.

CD81 is a replication factor for multiple alphaviruses

Lisa Lasswitz[1,2], Francisco J Zapatero-Belinchón[1,2,3,4], Rebecca Moeller[1,2], Kirsten Hülskötter[5], Timothée Laurent[3,6,7], Lars-Anders Carlson[3,6,7], Christine Goffinet[8,9], Graham Simmons[10], Wolfgang Baumgärtner[5], Gisa Gerold[1,2,3,4*]

[1]Insitute for Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany; [2]Department of Biochemistry & Research Center for Emerging Infections and Zoonoses (RIZ), University of Veterinary Medicine Hannover, Hannover Germany; [3]Wallenberg Centre for Molecular Medicine (WCMM), Umeå University, Umeå, Sweden; [4]Department of Clinical Microbiology, Virology, Umeå University, Umeå, Sweden; [5]Department of Pathology, University of Veterinary Medicine Hannover, Hanover, Germany; [6]Department of Medical Biochemistry and Biophysics, Umeå University, Umeå, Sweden; [7]Molecular Infection Medicine Sweden, Umeå University, Umeå, Sweden; [8]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Institute of Virology, Berlin, Germany; [9]Berlin Institute of Health at Charité – Universitätsmedizin Berlin, Berlin, Germany; [10]Vitalant Research Institute, and Department of Laboratory Medicine, University of California, San Francisco, USA.

Presenting author; * Corresponding author

Alphaviruses are enveloped positive-strand RNA viruses that are transmitted to humans by mosquitoes and cause debilitating arthritis or encephalitis. The host factors required for alphavirus replication remain largely elusive. Here, we identify the plasma membrane protein CD81 as replication factor for multiple emerging alphaviruses including chikungunya, Ross River and Venezuelan equine encephalitis virus. Ablation of CD81 by gene silencing or editing reduces virus permissiveness in human and mouse cells and, reciprocally, ectopic expression of human or murine CD81 enhances infection. CD81 promotes chikungunya virus genome replication but is dispensable for virus entry. Imaging experiments suggest that CD81 localizes to viral replication complexes at the plasma membrane. Consistent with these findings, the cholesterol-binding ability of CD81 is critical for chikungunya virus replication, which takes place in cholesterol-rich membrane compartments. Finally, the closest homolog of CD81, CD9 can partially substitute the function of CD81 as chikungunya virus replication factor. Overall, our study uncovers CD81 as first integral membrane protein being a key host factor for human pathogenic alphavirus replication.

Identification of Antiviral Targets in Host Cell Lipid Metabolism

Anja Schoebel[1] and Eva Herker[1]

[1]Institute of Virology, Philipps University of Marburg, Marburg, Germany

Human pathogenic flaviviruses have rapidly emerged around the globe within the last decades, with increasing numbers of infections and a substantial morbidity rate. Although the majority of flavivirus infections pass without serious consequences, a considerable number of patients develops severe neurological or visceral symptoms. While yellow fever virus (YFV) and dengue virus (DENV) cause visceral diseases, including hepatitis and hemorrhagic fever, West Nile virus (WNV), tick-borne encephalitis virus (TBEV), and Zika virus (ZIKV) can lead to neuropathology. For YFV and TBEV an effective vaccine exists, but no potent antiviral treatment for flavivirus infections has been developed yet. Despite the ir different tissue tropisms, flaviviruses commonly reshape intracellular host membranes and rely on lipid metabolic pathways for efficient replication. Here, we systematically analyze the role of lipid metabolic enzymes in flavivirus replication in order to identify potential targets for novel anti-flaviviral treatments. Using RNAi, we specifically targeted key enzymes in phospholipid, neutral lipid, cholesterol, and fatty acid metabolism. We infected Huh7 knockdown cells with YFV-17D, DENV, TBEV, WNV, or ZIKV, and analyzed viral titers using $TCID_{50}$.assays. In addition, we tested a set of selected inhibitors for their antiviral properties. Comparing YFV-17D, DENV, TBEV, WNV, and ZIKV replication in Huh7 knockdown cells, we observed that downregulation of the cytosolic acetyl-CoA acetyltransferase (ACAT2) and the sterol O-acyltransferase 1 (SOAT1) significantly reduced DENV and ZIKV titers at 48 hours post infection. Knockdown of SOAT1 also slightly decreased TBEV and WNV titers. Preliminary data indicate that ZIKV RNA genome copies and capsid protein levels are decreased in SOAT1- and ACAT2-knockdown cells. Immunofluorescence analysis revealed a reduced number of ZIKV double-stranded RNA foci in SOAT1- and ACAT2-depleted cells. In addition, treatment of ZIKV-infected Huh7 cells with a SOAT1-specific inhibitor significantly reduced ZIKV titers, indicating that functional cholesterol synthesis and esterification are critical for effective ZIKV replication. Interestingly, while depletion of SOAT1 also decreased DENV viral RNA copies and capsid protein levels, both were slightly increased in ACAT2-knockdown cells, suggesting that the function of ACAT2 might be important for infectivity or egress of DENV particles.

Catecholamine Biosynthetic and Metabolic pathway as a Novel Determinant of *Flaviviridae* Viruses Replication and Pathogenesis

George Mpekoulis [1], Vassilina Tsopela [1], Anna Chalari [1], Katerina I. Kalliampakou [1], Georgios Panos [1], Efseveia Frakolaki [1], Raphaela S. Milona [1], Diamantis C. Sideris [2], Dido Vassilacopoulou [2], Niki Vassilaki [1]

[1] Molecular Virology Laboratory, Hellenic Pasteur Institute, Athens, Greece ; [2] Biochemistry and Molecular Biology Section, Faculty of Biology, University of Athens, Greece

Background-Aims: The catecholamine biosynthetic enzyme L-Dopa decarboxylase (DDC) has been negatively associated with hepatitis C (HCV) and dengue (DENV) viruses genome replication in the liver, through its binding to phosphatidylinositol 3-kinase that inhibits the kinase (Frakolaki et al. Cells 2019). The biosynthetic function of DDC is also critical for HCV replication (Mpekoulis et al Viruses 2021). Here we examined the importance of catecholamine biosynthesis, metabolism and transport in DENV replication and the effect of DENV on the enzymes/transporters of the pathway. Moreover, the implication of catecholamine oxidation-produced ROS in this relationship and the role of HIF (hypoxia-inducible factor) were studied in comparison to HCV.
Methods: In hepatocyte and monocyte-based infectious and subgenomic replicon models of DENV, we performed *DDC* silencing, chemically inhibited/induced the proteins of the related pathways, or applied catecholamines or DDC substrates.
Results: Production and uptake of biogenic amines impeded DENV replication, while the virus reduced their biosynthesis, metabolism, and transport. The role of DDC was verified by gene silencing, which enhanced virus replication, but not translation, attenuated the negative influence of DDC substrates on the virus and reduced the infection-related cell death. The uptake and not the 181ignalling of dopamine was found responsible for the impact on DENV. Accordingly, viral replication was suppressed by all treatments expected to enhance the accumulation of catecholamines in the cell cytosol. This was confirmed using chemical inducers of catecholamine biosynthesis. In turn, DENV, apart from DDC, downregulated also the expression of the other enzymes of the pathway tyrosine hydroxylase, dopamine β-hydroxylase, monoamine oxidases, and the vesicular monoamine transporter 2. Cellular redox alterations caused by catecholamines were not related to the effect on DENV. In contrast, HCV replication was sensitive to catecholamine-mediated ROS production. Interestingly, HIF that regulates both *DDC* expression and DENV replication was identified as a key mediator of the interaction between them.

Conclusion: We revealed a negative bidirectional relationship between DENV and catecholamine biosynthesis/metabolism, elucidating novel determinants of viral replication and reinforcing published data on the function of bioactive amines in the periphery.

Characterization of a multipurpose NS3 Surface patch coordinating HCV Replicase Assembly and Virion Morphogenesis

Olaf Isken, Minh Tu Pham[#], Ralf Bartenschlager[#] and Norbert Tautz

Institute of Virology and Cell Biology, University of Luebeck, Germany; [#]Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany

Background and Aims: HCV genome replication and virion morphogenesis takes place in dedicated cellular membrane compartments that serve as assembly sites of specialized multi-protein complexes important for these processes. Steps leading to the maturation of these macromolecular protein complexes require a step-wise succession of protein-protein interaction that are tightly controlled in a temporal and spatial fashion to avoid non-functional maturation/assembly. NS3 has a pivotal role in this temporal regulation by providing multipurpose surface areas for the assembly of functional distinct protein complexes.
To define further contributing factors for these sequential multi-protein complex formations, we extended our previous analysis of NS3 surface determinants important for NS3-mediated $NS2^{pro}$ activation onto additional surface residues of the protease and helicase domain to identify further NS3 surface residues critical for RC assembly or virion morphogenesis.
Methods: The effect of the individual NS3 mutants was studied by revers-genetics in two different HCV genotypes. Their impact on RC assembly, genome replication as well as virion morphogenesis was investigated by using replicon assays and full-length genomes. Trans-complementation assays were used to show that specific virion morphogenesis defects cannot be complemented by supplying functional NS3 protein in trans.
Results: By functional interrogating these NS3 surface residues, we show that (i) a subset of these NS3 residues are critical for RC complex formation and HCV genome replication by negatively regulating NS5A hyperphosphorylation and (ii) that a helicase residue in close proximity to these residues is pivotal for virion morphogenesis without affecting genome replication
Conclusions: We propose that NS3 provides distinct surface areas to regulate, in a temporal and spatial fashion, the assembly of dedicated protein complexes during the different steps of HCV life cycle leading to functional replicase assembly and virion formation. Taken together, our present data set provides a basis to further dissect the formation of viral multi-protein complexes required for the individual steps of the HCV life cycle.

High recombination rate of hepatitis C virus revealed by a green fluorescent protein reconstitution cell system

Andrea Galli, Ulrik Fahnøe, Jens Bukh

Copenhagen Hepatitis C Program (CO-HEP), Department of Infectious Diseases, Copenhagen University Hospital, Hvidovre and Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Background and Aims*:* Genetic recombination is an important evolutionary mechanism for RNA viruses and can facilitate escape from immune and drug pressure. Recombinant hepatitis C virus (HCV) variants have rarely been detected in patients, suggesting that HCV has intrinsic low recombination rate. Recombination of HCV has been demonstrated in vitro between non-functional genomes, but its frequency and relevance for viral evolution and life cycle has not been clarified.
Methods*:* We developed a cell-based assay to detect and quantify recombination between fully viable HCV genomes, using the reconstitution of green fluorescent protein (GFP) as a surrogate marker for recombination. Here, two GFP-expressing HCV genomes carrying different inactivating GFP mutations can produce a virus carrying a functional GFP by recombining within the GFP region. Generated constructs allowed quantification of recombination rates between markers spaced 603 and 553 nucleotides apart by flow cytometry and next-generation sequencing (NGS).
Results*:* Viral constructs showed comparable spread kinetics and reached similar infectivity titers in Huh7.5 cells, allowing their use in co-transfections and co-infections. Single cycle co-transfection experiments, performed in CD81-deficient S29 cells, showed GFP expression in double-infected cells, demonstrating genome mixing and occurrence of recombination. Quantification of recombinant genomes by NGS revealed an average rate of 6,1%, corresponding to 49% of maximum detectable recombination (MDR). Experiments examining recombination during the full replication cycle of HCV, performed in Huh7.5 cells, demonstrated average recombination rates of 5,0 % (40,0% MDR) and 3,6% (28,8% MDR) for markers spaced by 603 and 553 nucleotides, respectively, supporting a linear relationship between marker distance and recombination rates. First passage infections using recombinant virus supernatant resulted in comparable recombination rates of 5,9% (47,2% MDR) and 3,5% (28,0% MDR), respectively, for markers spaced by 603 and 553 nucleotides.
Conclusions*:* We developed a functional cell-based assay that, to our knowledge, allows for the first-time detailed quantification of recombination rates using fully viable HCV constructs. Our data indicate that HCV recombines at high frequency between highly similar genomes, and that the frequency of recombination increases with the distance between marker sites. These results have implication for our understanding of HCV evolution and emphasize the importance of recombination in the reassortment of mutations in the HCV genome.
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Longer poly(U) stretches in the 3UTR are essential for replication of the hepatitis C virus genotype 4a clone in vitro and in vivo

Asako Takagi [1], Yutaka Amako [1], Daisuke, Yamane [1], Bouchra Kitab[2, 3], Ahmed El-Gohary[4], Michinori Kohara[1*], Kyoko Tsukiyama-Kohara[2, 3*]

[1] Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Setagaya-Ku, Tokyo, Japan ; [2] Transboundary Animal Diseases Centre, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima-City, Japan; [3]Laboratory of Animal Hygiene, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima-City, Japan
[4]Egypt-Japan University for Science and Technology

The 3′ untranslated region (UTR) of the hepatitis C virus (HCV) genome plays a significant role in replication including the poly(U) tract (You & Rice, 2008). Here we established an HCV clone that is infectious *in vitro* and *in vivo*, from an Egyptian patient with chronic HCV infection and hepatocellular carcinoma (HCC). First, we inoculated the patient plasma into a humanized chimeric mouse. We observed HCV genotype 4a propagation in the chimeric mouse sera at 1.7×10^7 copies/mL after 6 weeks. Next, we cloned the entire HCV sequence from the HCV-infected chimeric mouse sera using RT-PCR, and 5′ and 3′ RACE methodologies. We obtained first a shorter clone (HCV-G4 KM short, GenBank: AB795432.1), which contained 9,545 nucleotides with 341 nucleotides of the 5′UTR and 177 nucleotides of the 3′UTR, and this was frequently obtained for unknown reasons. We also obtained a longer clone by dividing the HCV genome into three fragments and the poly (U) sequences. We obtained a longer 3′UTR sequence than that of the HCV-G4 KM short clone, which contained 9,617 nucleotides. This longer clone possessed a 3′-UTR of 249 nucleotides (HCV-G4 KM long, GenBank: AB795432.2), because of a 71-nucleotide longer poly (U) stretch. The HCV-G4-KM long clone, but not the HCV-G4-KM short clone, could establish infection in human hepatoma HuH-7 cells. HCV RNAs carrying a nanoluciferase (NL) reporter were also constructed and higher replication activity was observed with G4-KM long-NL *in vitro*. Next, both short and long RNAs were intra-hepatically injected into humanized chimeric mice. Viral propagation was only observed for the chimeric mouse injected with the HCV-G4 KM long RNA in the sera after 21 days (1.64×10^6 copies/mL) and continued until 10 weeks post inoculation (wpi; $1.45–4.74 \times 10^7$ copies/mL). Moreover, sequencing of the HCV genome in mouse sera at 6 wpi revealed the sequence of the HCV-G4-KM long clone. Thus, the *in vitro* and *in vivo* results of this study indicate that the sequence of the HCV-G4-KM long RNA is that of an infectious clone.

Keywords: Hepatitis C virus, genotype 4a, infectivity, humanized chimeric mouse, genomics

Contact: kohara-mc@igakuken.or.jp, kkohara@vet.kagoshima-u.ac.jp

Processing and Subcellular Localization of the Hepatitis E Virus Replicase: Identification of Candidate Viral Factories

Karoline Metzger[1,#], Cyrine Bentaleb[1,#], Kévin Hervouet[1], Virginie Alexandre[1], Claire Montpellier[1], Jean-Michel Saliou[2], Martin Ferrié[1], Charline Camuzet[1], Yves Rouillé[1], Cécile Lecoeur[1], Jean Dubuisson[1], Laurence Cocquerel[1] and Cécile-Marie Aliouat-Denis[1,*]

[1] CNRS, INSERM, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – Center for Infection and Immunity of Lille (CIIL), Université de Lille, Lille, France, [2] CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, UMR2014 – US41 – Plateformes Lilloises de Biologie and Santé (PLBS), Université de Lille, Lille, France
[#] These authors contributed equally to this work.

Background & Aim: Hepatitis E virus (HEV) is the major cause of acute hepatitis worldwide. HEV is a positive-sense RNA virus expressing three open reading frames (ORFs). ORF1 encodes the ORF1 non–structural polyprotein, the viral replicase which transcribes the full-length genome and a subgenomic RNA that encodes the structural ORF2 and ORF3 proteins. The present study is focused on the replication step with the aim to determine whether the ORF1 polyprotein is processed during the HEV lifecycle and to identify where the replication takes place inside the host cell.
Methods & Results: As no commercial antibody recognizes ORF1 in HEV-replicating cells, we aimed at inserting epitope tags within the ORF1 protein without impacting the virus replication efficacy. Two insertion sites located in the hypervariable region were thus selected to tolerate the V5 epitope while preserving HEV replication efficacy.
Once integrated into the infectious full-length Kernow C-1 p6 strain, the V5 epitopes did neither impact the replication of genomic nor the production of subgenomic RNA. Also, the V5-tagged viral particles remained as infectious as the wildtype particles to Huh-7.5 cells. Next, the expression pattern of the V5-tagged ORF1 was compared in heterologous expression and replicative HEV systems. A high molecular weight protein (180 kDa) that was expressed in all three systems and that likely corresponds to the unprocessed form of ORF1 was detected up to 25 days after electroporation in the p6 cell culture system. Additionally, less abundant products of lower molecular weights were detected in both in cytoplasmic and nuclear compartments. Concurrently, the V5-tagged ORF1 was localized by confocal microscopy inside the cell nucleus but also as compact perinuclear substructures in which ORF2 and ORF3 proteins were detected. Importantly, using *in situ* hybridization (RNAScope®), positive and negative-strand HEV RNAs were localized in the perinuclear substructures of HEV-producing cells.
Conclusions: Finally, by simultaneous detection of HEV genomic RNAs and viral proteins in these substructures, we identified candidate HEV factories.
Contact: cecile.aliouat@univ-lille.fr

IL-26 inhibits hepatitis C virus replication in hepatocytes

Elodie Beaumont,[1,*] Vincent Larochette,[2,*] Laurence Preisser,[2] Charline Miot,[2,3] Pascale Pignon,[2] Simon Blanchard,[2,3] Björn-Thore Hansen,[4] Jonathan Dauvé,[5] Caroline Poli,[2,3] Minna M. Poranen,[6] Patricia Lamourette,[7] Marc Plaisance,[7] Alain Morel,[2,5] Helmut Fickenscher,[4] Pascale Jeannin,[2,3,†] Philippe Roingeard,[1,†] Yves Delneste[2,3,†]

[1] INSERM unit 1259 MAVIVH, University and CHRU of Tours, Tours, France; [2] Univ Angers, Université de Nantes, CHU Angers, INSERM, CNRS, CRCI2NA, SFR ICAT, F-49000 Angers, France; [3] Laboratory of Immunology and Allergology, University Hospital of Angers, Angers, France; [4] Institute for Infection Medicine, Christian-Albrecht University of Kiel and University Medical Center Schleswig-Holstein, Kiel, Germany; [5] ICO Cancer Center, Angers, France; [6] Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland.; [7] Service de Pharmacologie et d'immunoanalyse, Commissariat à l'Energie Atomique Saclay, iBiTec-S, Gif sur Yvette, France; * These authors contributed equally to this work.; † These authors supervised equally this work.

Background and aims: Interleukin-26 (IL-26) is a proinflammatory cytokine involved in the pathophysiology of chronic inflammatory disorders. It has properties atypical for a cytokine, such as direct antibacterial activity and DNA-binding capacity. In a previous study, we observed an accumulation of IL-26 in fibrotic and inflammatory lesions in the livers of patients with chronic hepatitis C virus (HCV) infection, and showed that infiltrating $CD3^+$ lymphocytes were the principal source of IL-26 (Miot *et al.*, Gut 2015). Surprisingly, immunohistochemical staining for IL-26 was also detected in the cytoplasm of hepatocytes from HCV-infected patients, even though these cells do not produce IL-26, even when infected with HCV. Based on this observation and possible interactions between IL-26 and nucleic acids, we investigated the possibility that IL-26 controlled HCV infection independently of the immune system.
Methods: We evaluated the ability of IL-26 to interfere with HCV replication in hepatocytes, and investigated the mechanisms by which IL-26 exerts its antiviral activity.
Results: We showed that IL-26 penetrated HCV-infected hepatocytes, where it interacted directly with HCV double-stranded RNA replication intermediates, thereby inhibiting viral replication. IL-26 interfered with viral RNA-dependent RNA polymerase activity, preventing the *de novo* synthesis of viral genomic single-stranded RNA.
Conclusions: These findings reveal a new role for IL-26 in direct protection against HCV infection, independently of the immune system, and increase our understanding of the antiviral defense mechanisms controlling HCV infection. Future studies are required to evaluate the possible use of IL-26 for treating other chronic disorders caused by RNA viruses, for which few treatments are currently available, or emerging RNA viruses.

Phosphorylation guides the cytoplasmic relocalisation and function of HuR upon HCV infection

Harsha Raheja, Biju George and Saumitra Das

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore-560012

RNA binding proteins (RBPs) play a crucial role in determining the fate of RNA viruses in the host cells. Viruses hijack these nuclear-cytoplasmic shuttling proteins and modulate them by post-translational modifications for alteration in sub-cellular localisation. Hepatitis C virus is one such virus where RBPs represent an important arm regulating different viral processes. HuR is a predominantly nuclear resident RBP which relocalises to cytoplasm upon HCV infection and regulates the viral life cycle. In cytoplasm, HuR regulates viral replication by assisting the assembly of replication complex on viral 3'UTR. We have deciphered the mechanism of this increased cytoplasmic abundance of HuR upon HCV infection. Using immunofluorescence imaging and mutational studies, we have demonstrated that viral NS3 protein activates PKC-δ, which phosphorylates S318 residue of HuR. This phosphorylation results in increased cytoplasmic export of cellular HuR. Further, we have explored how this relocalised HuR is retained in the cytoplasm. It appears, the reduction of AMPK activity by viral NS5A protein prevents Importin-alpha phosphorylation. This phosphorylation is important for nuclear targeting of cytoplasmic relocalised/newly synthesised HuR. Therefore, HCV mediated inhibition of AMPK activity might contribute to cytoplasmic retention of HuR, which in turn assists viral replication. Results establish the involvement of multiple viral proteins in achieving increased cytoplasmic abundance of HuR. This depicts an orchestrated mechanism regulating the availability of an RBP and its exploitation in guiding viral life cycle. Such regulation of PTM machinery by HCV could be an eye opener for understanding role of other host factors in the context of similar cytoplasmic viruses.

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Beyond entry – additional roles of CD81 in the life cycle of HCV

Maximilian Bunz[1], Michael Ritter[1], Julia Nehls[1,2], Michael Schindler[1,2]

Affiliations: 1 Institute for Medical Virology and Epidemiology, University Hospital Tübingen, 72076 Tübingen, Germany; 2 Institute for Virology, Helmholtz Zentrum Munich, 85764 Neuherberg, Germany

Background and aims: Though progress has been made in terms of understanding the molecular mechanisms of hepatitis C virus (HCV) infection many aspects remain elusive. HCV is dysregulating cellular lipid metabolism upon infection and is furthermore rearranging membranes to shield sites of viral replication from innate immune sensing. Such rearrangements possibly involve major alterations of cellular membranes. To detect such changes, we performed a flow-cytometry based screening assay and noticed a downregulation of CD81 in HCV-expressing cells. The tetraspanin CD81 plays diverse roles in membrane organization events, for example, arranging cell surface receptors in tetraspanin-enriched microdomains. Its role as HCV entry receptor is firmly established. Here, we aimed to elucidate the purpose of this downregulation by the virus and to uncover additional roles of CD81 in the life cycle of HCV.

Methods: To study CD81 roles in the viral life cycle we applied molecular biology techniques to create engineered cell lines and viral genomes. We checked for differences in viral life cycle processes in the absence of CD81 using flow cytometry and microscopy, as well as qRT-PCR and western blotting. Protein-protein interactions were assessed by flow cytometry-based FRET and viral protein expression dynamics by life cell imaging.

Results: Downregulation of CD81 was not abrogated by proteasomal or lysosomal inhibition accompanied by reduced CD81 mRNA levels. Interestingly, CD81 knock-out (CD81KO) cells showed a high variation in expression dynamics, strongly dependent on the viral genomes that were used. Viruses expressing infection reporters via an internal ribosome entry site (IRES) or T2A self-cleaving peptide in front of the polyprotein open reading frame (ORF), were impaired in viral translation and replication. In contrast, viruses expressing fluorescent fusion proteins with E1, E2 or NS5A showed no impairment or even increased replication in CD81KO cells. Further investigations revealed an increased stress response and potentially increased innate immune sensing in CD81KO cells. Furthermore, the RNA-interacting and stress resolving protein STAU1 seems to interact with viral proteins E1, p7, NS4B and NS5B. For E1 and NS5B, this interaction was higher in CD81KO cells. On top, we found that the interaction of CD81 with HCV Core, E1 and NS5A, seems to be dependent on CD81s ability to bind cholesterol.

Conclusions: Our results show that CD81's entanglement with HCV goes beyond its role as entry receptor. Although not yet entirely clear, the differential expression dynamics of different viral constructs in CD81KO cells point to a more indirect role of CD81 in the HCV life cycle, beyond acting as an entry factor. Altogether, our data indicate novel roles of CD81 in cellular stress response and innate immune signaling that HCV carefully balances through distinct viral protein interactions.

Analysis of the functional interaction between rodent hepacivirus and miR-122

Tomohisa Tanaka[1], Naoki Ohtsu[1] and Kohji Moriishi[1, 2]

1, Department of Microbiology, Division of Medicine, University of Yamanashi, JAPAN: 2, Division of Hepatitis Virology, Institute for Genetic Medicine, Hokkaido University, JAPAN

Background and Aims: Norway rat hepacivirus-1 (NrHV-1) is one of rodent homologs of HCV and is an important surrogate model for HCV research, because NrHV-1 is the hepacivirus species that can experimentally infect immunocompetent laboratory rodents. miR-122, a liver-specific microRNA, plays an important role in regulating viral translation and RNA stability due to the secondary structure formation of the viral genome. Recently, Wolfisberg et al has established the subgenomic replicon model of NrHV-1 and revealed the participation of miR-122 to NrHV-1 replication (J. Virol., 2019). In this study, we aimed to elucidate the precise functional interaction mapping between NrHV-1 RNA and miR-122.
Methods: Rodent hepatoma cell lines were electroporated with NrHV-1 subgenomic replicon (SGR) RNA or its mutants to evaluate the cellular permissiveness of the viral RNA replication. The level of intracellular replication and translation were evaluated by luciferase assay, colony formation assay or qRT-PCR. The expression of miR-122 and the mutants was transduced by lentiviral vectors.
Results: Rodent-derived hepatoma cell lines Hepa1-6 and McA-RH7777, but not AML12, permitted efficient RNA replication of NrHV-1 SGR, as reported previously. However, NrHV-1 SGR were found to replicate more efficiently in miR-122-transduced AML12 cells than Hepa1-6 and McA-RH7777 cells, which suggest that the level of miR-122 expression provides for the cellular permissiveness of viral replication in AML12 cells. We introduced various mutations through 22-nt sequence of miR-122. The mutations in nt 2-to-8 (the seed sequence) and nucleotides G15 and G16 of miR-122 abrogated the functional interaction with NrHV-1 SGR, while the mutations in the other sequence of miR-122 allowed NrHV-1 SGR to replicate in AML12 cells. Furthermore, we are analyzing the effect of the miR-122 mutants on IRES activity to elucidate the role of miR-122 in NrHV-1 replication.
Conclusions: The interaction model between miR-122 and HCV in which the binding of the seed sequence (GGAGUGU) and G15 and G16 of miR-122 to HCV RNA makes a bulge in middle region and 3'-terminal overhang is entirely conserved in the rodent homolog, suggesting that these hepaciviruses evolutionarily share a common mechanism for liver tropism.

Development and use of a chronological and real-time monitoring system to analyze the intracellular lifecycle of hepatitis C virus and related flaviviruses and virus-induced ER stress

Masahiko Ito[1], Xiaoyu Li[1], Tetsuro Suzuki[1]

[1]Department of Virology and Parasitology, Hamamatsu University School of Medicine, Japan

Background and Aims: In microbiological research, it is important to understand the time course of each step in a pathogen's lifecycle and changes in the host cell environment induced by infection. In this study, we developed a real-time monitoring system that chronologically detects luminescence reporter activity over time without sampling cells or culture supernatants for analyzing HCV and related flaviviruses lifecycle.
Methods: To chronologically monitor viral replication and infection in living cells, bioluminescence activities in cells transfected with HCV replicon RNA harboring green-emitting luciferase or infected with single-round infectious particles were quantified using Kronos Dio where luminescence is measured for a long time at regular intervals.
Results: Subgenomic replicon experiments with hepatitis C virus (HCV) showed 1) there is a transient peak of translation at 3–4 h after viral RNA introduction into cells, 2) replication initiates after an interval of 16–17 h after the translation peak, and 3) cell lines with higher replication capacity tend to have a shorter time to the start of replication and a higher initial velocity of replication. Based on the bioluminescence profiles, we constructed a novel mathematical model of HCV replication kinetics, which is defined by HCV replication rate and cell growth rate as parameters. Our real-time monitoring system enabled us to differentiate pharmacological profiles of a fast-acting direct antiviral, and slow-acting drugs targeting host cellular pathway(s). Further analyses using single-round infectious particles of HCV and related flaviviruses showed that the time-course profiles from virus entry into the cell to translation and replication initiation are characteristic among different viruses. Finally, we showed that this system is also useful for detailed analysis of the induction of endoplasmic reticulum (ER) stress caused by HCV infection.
Conclusions: Experimental measurements in the developed system combined with mathematical modeling could improve our understanding of viral life cycle, action mechanism and efficacy of antiviral drugs and host response after viral infection.

Receptor transport protein 4 (RTP4)-mediated repression of HCV replication in mouse cells

Schwoerer MP, Rodriguez C, Cafiero TR, Suzuki S, Berggren KA, Ploss A

Princeton University Department of Molecular Biology

Background & Aims: The flying fox ortholog of receptor transport protein 4 (RTP4) was recently 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 and to delineate functional differences between murine and human RTP4 that drive their divergent impact on HCV 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] crossed to an RTP4-deficient 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-/- IL2RgNULL recipients which are devoid of functional B-, T- and NK cells. We also attempted to knock out RTP4 in a mouse cell expressing the four HCV human entry factors (OCLN, CLDN1, SCARBI, and CD81), miR122, human CypA, SEC14L2, and mouse ApoE which supports low levels of HCV infection. We are screening cell clones for RTP4 deficiency to test whether this disruption would enhance HCV replication. To map precise regions within mouse RTP4 we created mouse-human domain swap chimeras whose functions have been tested in HCV-infected Huh7 cells.

Results: 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 early timepoints; analysis of later timepoints and of immunodeficient transplant-recipient mice is ongoing. Our domain-swap experiments demonstrated the sufficiency of specific domains of murine RTP4 to inhibit HCV replication, which are currently being fine-mapped.

Conclusions: Disruption of RTP4 in mCD81/EL2[h/h] mOCLN/EL2[h/h] mice does not appear to be sufficient to render animals permissive to HCV infection. Ongoing analysis will reveal whether HCV can be established in mCD81/EL2[h/h] mOCLN/EL2[h/h] RTP4-/- mice in the absence of functional adaptive immunity. Our data indicate that specific regions within murine RTP4 are responsible for RTP4's anti-HCV activity. These data point to RTP4 serving as another factor in the complex set of barriers to HCV infection in mice.

Mutation patterns of within-host variants of dengue virus

Rodrigo C[1], Valencia B[1], Leung P[2], Sigera C[3], Adikari T[1], Riaz N[1], Bull R[1], Weeratunga P[3], Rajapakse S[3], Lloyd A[1], Fernando D[3]

[1]Faculty of Medicine and Health, UNSW Sydney, Australia, [2]University of Copenhagen, Denmark, [3]University of Colombo, Sri Lanka

Introduction: RNA viruses have high mutation rates due to the lack of proof-reading during genomic replication. Understanding within-host viral evolution of these viruses may aid better prediction of their between-host evolution. We recently developed a novel bioinformatics pipeline named “Nano-Q” to identify full genomic length within-host RNA viral variants from Oxford Nanopore Sequencing data. Here we apply this tool to study mutation patterns of dengue virus.
Methods: Near-full length dengue genomes were isolated and sequenced from 18 Sri Lankan patients with DENV2 (cosmopolitan genotype) infection using an Oxford Nanopore GridION sequencer with native barcoding. Within-host variants were reconstructed using the Nano-Q tool. Development and calibration of this tool had been published previously[1, 2]. The mutations of within host viral variants were characterised according to their location, relative frequency, and as synonymous/non-synonymous mutations.
Results: A total of 108 within host viral variants were identified in all 18 subjects (range: 2 – 18). These had a total of 166 mutations that differed from their respective consensus sequences. Of these 76 (45.8%) were non-synonymous mutations and most of these were distributed in the NS3 (n=23), Envelope (n=12) and NS5 (n=12) regions. However, when adjusted for genomic length, more non-synonymous mutations were seen in Capsid (0.018 mutations per position), NS3 (0.012) and NS4B (0.009) regions. Thirty-one non-synonymous mutations (31/76, 40.8%) occurred at a frequency > 5% within the viral quasi-species. Fourteen of these were seen in at least two hosts and two mutations were seen across five different hosts (R245Q, G7180R).
Conclusion: This pilot analysis is the first to examine within-host mutations of dengue virus across the full genome, providing proof of concept that such analyses are feasible. It also shows that despite being an acute infection lasting a few days, the within-host dengue viral populations are not homogenous. Signatures of mutations of minor variants may be useful to characterise the spread of local outbreaks and to understand why disease phenotypes differ across individuals.

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Dissecting hepatitis C virus translation efficiency from a population standpoint

Echeverría, Natalia1,2*; Perbolianachis, Paula1,2; Gámbaro, Fabiana1; Beaucourt, Stéphanie3; Cristina, Juan1; Moratorio, Gonzalo1,2; Moreno, Pilar1,2

Affiliations:

1. Laboratorio de Virología Molecular, Centro de Investigaciones Nucleares, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
2. Laboratorio de Evolución Experimental de Virus, Institut Pasteur, Montevideo, Uruguay
3. Viral Populations and Pathogenesis Laboratory, Institut Pasteur, Paris, France

* Presenting author

Background and Aims: Hepatitis C Virus (HCV) is an RNA virus which recruits the ribosome directly to a structured region of the 5' end of the genome: the internal ribosome entry site (IRES). Although it is a highly conserved region, nucleotide (nt) variations may result in inefficient translation. Hence, this work aims to dissect the translation efficiency of HCV populations to select impaired translation variants. To do so, the relative translation efficiency (RTE) of different viral variants was determined in the context of HCV molecular clones versus bicistronic vectors. In addition, the contribution of intra-host IRES translation heterogeneity was explored as an attempt to decipher if group selection might play a role in it**.**

Methods: IRES variants (subtypes 1a and 3a) obtained from chronically HCV infected patients and previously characterized by in vitro/bicistronic assays, were studied by using chimeric reporter full genome HCV clones (non-replicative). Consequently, viral RNAs were in vitro transcribed and co-transfected with a capped reporter mRNA into Huh7.5 cells to study RTE by bioluminescence assays. The results were then compared to those obtained in vitro. IRES intra-host variability will be recapitulated in similar ex vivo assays in which transfected RNA amounts will resemble each variant frequency within the viral population. Long-term stability assays of low efficiency variants (LEVs) are currently on-going in Huh7.5 cells. 10 serial passages (P) will be done from initially transfected replication competent HCV RNA. RNAs from P5 and P10 will be Illumina sequenced to determine the stability of the mutants.
Results: In all cases, the consensus sequence was present in the population as the main variant (master sequence). Some low frequency (< 10%) and master variants exhibit a statistically significant 40 – 70% reduction in RTE. Key nt positions are mainly responsible for these phenotypes. Comparisons between ex vivo/full genome and in vitro/bicistronic assays show that some variants exhibit dissimilar behavior in terms of RTE between approaches. Cell-culture evolution and group selection experiments were conducted.
Conclusions: Different natural IRES variants exhibit dissimilar RTEs despite only differing in a few nucleotides. Although in vitro/bicistronic assays are widely used for translation efficiency analyses, ex vivo approaches with full genome reporter clones displayed some differences. This highlights how much do cellular and viral genetic contexts play in IRES-driven translation studies. We also showed whether translation efficiency is modulated at the population level. Finally, we suggest the use of naturally selected LEVs as a potential antiviral strategy.

Session 7
VIRAL TRANSLATION AND ASSEMBLY

28TH INTERNATIONAL SYMPOSIUM ON HEPATITIS C VIRUS, FLAVIVIRUSES, AND RELATED VIRUSES

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JULY 6-9, 2022

P109 Packaging defects in pestiviral NS4A can be compensated by mutations in NS2 and NS3
Jonas Fellenberg

P110 Nup98 is subverted from Annulate Lamellae by hepatitis C virus core protein to foster viral assembly
Bertrand Boson

P111 A twist in the tail: miR-122 interactions with the HCV genome reveal insights into virion assembly
Selena Sagan

Packaging defects in pestiviral NS4A can be compensated by mutations in NS2 and NS3

Jonas Fellenberg, Danilo Dubrau, Norbert Tautz

Institute of Virology and Cell Biology, University of Lübeck, Lübeck, Germany

Background and Aims: In pestiviruses, like bovine viral diarrhea virus (BVDV), NS3/4A and NS2-3/4A are selectively active in RNA replication or virion morphogenesis, respectively. Furthermore, we previously demonstrated that different surface interactions between NS3 and NS4A are promoting either RNA replication or virion morphogenesis. For a better understanding of the role of the kink- and acidic-domains of NS4A in those processes we performed a reverse-genetic analysis using alanine mutations.
Methods: The effect of the individual NS4A alanine mutants on RNA replication was validated by the use of bicistronic luciferase reporter replicons. Effects on polyprotein processing were studied by using a replication independent T7 expression system. Finally, we evaluated the packaging competence of the replication competent NS4A mutants in cell culture. Packaging-defective full-length cDNA clones were passaged in MDBK cells to identify second site rescue mutations. The resulting viruses were tested in a *trans*-complementation assay using cell lines expressing packaging competent or packaging defective NS2-3-4A variants, respectively.
Results: NS4A residues critical for polyprotein processing, RNA replication, and/or virion morphogenesis were identified. Three double-alanine mutants displayed a selective effect on virion assembly. These packaging defects could be rescued by second site mutations in several viral proteins. Further analysis revealed that two mutations, one in NS2 and one in NS3, were sufficient to suppress the packaging defects of these NS4A mutants. *Trans*-complementation studies confirmed the rescue ability of these mutations in NS2-3/4A packaging molecules provided *in trans*.
Conclusions: NS4A is functionally important for polyprotein processing, RNA replication and virion morphogenesis. Its function in virion assembly can be selectively eliminated by double mutations in different parts of NS4A. Even viruses carrying combinations of these debilitating NS4A packaging mutations could be restored to wild-type levels through single rescue mutations in NS2 and NS3. These findings indicate a surprisingly high flexibility for the assembly of the pestiviral packaging complex.

Nup98 is subverted from *Annulate Lamellae* by hepatitis C virus core protein to foster viral assembly

Bertrand Boson, Chloé Mialon, Konstanze Schichl, Solène Denolly and François-Loïc Cosset

CIRI – Centre International de Recherche en Infectiologie, Team EVIR, Univ Lyon, Université Claude Bernard Lyon 1, Inserm, U1111, CNRS, UMR5308, ENS Lyon, 46 allée d'Italie, F-69007, Lyon, France.

Background and aims: Nup98, an essential component of the nuclear pore that also participates to *Annulate Lamellae* pore structures localized in the cytosol, was shown to be involved in hepatitis C virus (HCV) assembly, but its precise role remained elusive.
Methods: We combined confocal microscopy and biochemical assays to study the interplay between Nup98, core – the HCV capsid protein – and viral genomes.
Results: Our results show that in HCV-infected cells, core protein is necessary and sufficient to induce re-localization of Nup98 from *Annulate Lamellae* to lipid droplet-apposed areas in which core/NS5A and HCV (+)RNA are clustered to promote viral assembly. This process appears to be independent of the use of karyopherins. Furthermore, we found that Nup98 interacts with HCV RNA and that upon Nup98 down-regulation, the viral (+)RNA genome was specifically excluded from areas that contain active translating ribosomes and the core and NS5A proteins. **Conclusions:** Altogether, these results indicate that Nup98 is recruited by HCV core from *Annulate Lamellae* to viral assembly sites to locally increase the concentration of (+)RNA genome, which may favor its encapsidation into nascent virions.

Working model: In Huh7.5 uninfected cells (top left panel), part of the nucleoporins is stored in cytoplasmic nuclear pore complex-like structures stacked in parallel ER membranes referred as Annulate Lamellae (AL). The infection of Huh7.5 cells by HCV (top right panel) induces the redistribution of AL to ER-derived remodeled membranes called membranous web (MW), which is induced by HCV to achieve the viral replication, translation and assembly within a protected environment. HCV core, probably through its high mobility on ER membranes, interacts with Nup98 from AL and translocates it within the MW and in close proximity of assembly sites, where it interacts with HCV RNA. In absence of Nup98 (bottom left panel), the viral genome is excluded from the replication, assembly and translation areas, leading to a decrease of infectious particles assembly. We propose that HCV hijacks Nup98, via its redistribution induced by core, to maintain the viral genome in the proper environment for its replication, assembly and translation.

A twist in the tail: miR-122 interactions with the HCV genome reveal insights into virion assembly

Marylin Rheault[1], Sophie E. Cousineau[1], and Selena M. Sagan[1,2*],

[1]Department of Microbiology & Immunology, McGill University; [2]Department of Biochemistry, McGill University

Hepatitis C virus (HCV) interacts with the liver-specific microRNA, miR-122. miR-122 binds to two sites in the 5' untranslated region (UTR) of the viral genome and ***promotes*** viral RNA accumulation. Several recent studies have revealed that miR-122 plays at least three roles in the HCV life cycle: 1) it acts as an RNA chaperone or riboswitch, suppressing an energetically-favorable conformation, termed SLII^{alt}, and allows the functional internal ribosomal entry site (IRES, SLII-IV) to form; 2) it promotes genome stabilization, by protecting the 5' triphosphate from recognition by cellular pyrophosphates and subsequent exoribonuclease-mediated decay; and 3) it promotes translation through contacts between the Argonaute (Ago):miR-122 complex at site 2 and the viral IRES. Using full-length *Renilla* luciferase reporter RNAs and HCV cell culture (HCVcc), as well as a combination of viral mutants that account for one or more of miR-122's activities, we examined the importance of each of these three roles in isolation. We found that the stability and translational promotion activities were equally important in the establishment of infection, but that translational promotion predominated in the maintenance phase of HCV replication. Curiously, we found that riboswitch activity appeared to have a minimal role in both the establishment and maintenance phase of viral replication. However, using a series of mutations to stabilize the SLII conformation, we noticed that suppression of SLII^{alt} results in a dramatic decrease in virion production, suggesting that SLII^{alt} is required for efficient virion assembly. Notably, the SLII^{alt} conformation is conserved in genotype 2 isolates, but destabilized in other viral genotypes. Thus, our findings may help to explain the success of genotype 2a, Japanese Fulminant Hepatitis 1 (JFH-1)-based HCVcc models, and provides insight into how miR-122 controls the balance of viral RNAs engaged in translation, replication, and assembly during the HCV life cycle.

Session 8
PATHOGENESIS

P112 PPP2R5D promotes hepatitis C virus infection by binding to viral NS5B and enhancing viral RNA replication
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PPP2R5D promotes hepatitis C virus infection by binding to viral NS5B and enhancing viral RNA replication

Muhammad Ikram Anwar[1, 2, #], Ni Li[2, #], Qing Zhou[2], Mingxiao Chen[2], Chengguang Hu[1], Tao Wu[3], Haihang Chen[2], Yi-Ping Li[2, 4*], Yuanping Zhou[1, *]

[1]Guangdong Provincial Key Laboratory of Gastroenterology, Department of Gastroenterology and Hepatology Unit, Nanfang Hospital, Southern Medical University, Guangzhou, China.
[2]Institute of Human Virology, Zhongshan School of Medicine, and Key Laboratory of Tropical Disease Control of Ministry of Education, Sun Yat-sen University, Guangzhou 510080, China.
[3]Department of Infectious Diseases, Hainan General Hospital, Hainan Affiliated Hospital of Hainan Medical University, Haikou, China.
[4]Department of Infectious Diseases, The Fifth Hospital of Sun Yat-sen University, Zhuhai, China.

[#], These authors contributed equally to this work.
Corresponding Author: Yuanping Zhou, email: yuanpingzhou@163.com, ORCID: 0000-0002-2493-396X; Yi-Ping Li, email: lyiping@mail.sysu.edu.cn, ORCID: 0000-0001-6011-3101.

Abstract

Hepatitis C virus (HCV) infection increased the risk of hepatocellular carcinoma. Identification of host factors required for HCV infection will help to unveil the HCV pathogenesis. Adaptive mutations that enable the replication of HCV infectious clone could provide hints that the mutation-carrying viral protein may specifically interact with some cellular factors essential for the HCV life cycle. Previously, we identified D559G mutation in HCV NS5B (RNA dependent RNA polymerase) important for replication of different genotype clones. To search for the factor that potentially interacts with NS5B and investigates its roles in HCV infection, we transfected hepatoma Huh7.5 cells with wild-type-NS5B and D559G-NS5B of HCV genotype 2a clone, J6cc, and pulled down NS5B-interacting proteins. Mass spectrometry identified a number of cellular proteins, of which protein phosphatase 2 regulatory subunit B'delta (PPP2R5D, the PP2A regulatory B subunit) was one of D559G-NS5B-pulled down proteins and selected for further investigation. Co-IP confirmed that PPP2R5D specifically interacted with NS5B, but not HCV Core and NS3 proteins, and D559G slightly enhanced the interaction. NS5B also colocalized with PPP2R5D in the cell. Knockdown and knockout of PPP2R5D decreased and abrogated HCV infection in Huh7.5 cells, respectively, while transient and stable expression of PPP2R5D in PPP2R5D-knockout cells restored HCV infection to a level close to that in wild-type Huh7.5 cells. Replicon assay revealed that PPP2R5D promoted HCV replication, but the phosphatase activity and catalytic subunit of PP2A were not affected by NS5B. In conclusion, PPP2R5D interacted with HCV NS5B and is required for HCV infection in cultured hepatoma cells by facilitating HCV replication.

Hepatocellular organellar abnormalities following elimination of hepatitis C virus

Haruyo Aoyagi 1, Hiroko Iijima 2, Minami Kikuchi 1, Mami Matsuda 1, Koichi Watashi 1, Ryosuke Suzuki 1, Takahiro Masaki 1,3, Noritomo Shimada 4, Keizo Kato 5, Akihito Tsubota 6, Ayako Mimata 7, Yuriko Sakamaki 7, Shizuko Ichinose 8, Masamichi Muramatsu 1, Kenjiro Wake 9, Masaya Sugiyama 10, Masashi Mizokami 10, Takaji Wakita 1, Hideki Aizaki 1

1 Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan
2 Department of Internal Medicine, Division of Hepatobiliary and Pancreatic Disease, Hyogo College of Medicine, Hyogo, Japan
3 Department of Laboratory Medicine, The Jikei University School of Medicine, Tokyo, Japan
4 Division of Gastroenterology and Hepatology, Ootakanomori Hospital, Chiba, Japan
5 Division of Gastroenterology and Hepatology, Shinmatsudo Central General Hospital, Chiba, Japan
6 Core Research Facilities for Basic Science, Research Center for Medical Sciences, The Jikei University School of Medicine, Tokyo, Japan
7 Research Core, Tokyo Medical and Dental University, Tokyo, Japan
8 Department of Plastic, Reconstructive and Aesthetic Surgery, Nippon Medical School, Tokyo, Japan
9 Liver Research Unit, Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan
10 Genome Medical Sciences Project, National Center for Global Health and Medicine, Chiba, Japan

Background & Aims: Direct-acting antivirals (DAAs) produce a sustained virologic response (SVR) in nearly all chronic hepatitis C (CHC) patients. However, the future development of hepatocellular carcinoma (HCC) in SVR patients is a crucial health concern worldwide. The purpose of this study was to characterize organelle abnormalities in the liver of SVR patients that may be related to pathogenesis and carcinogenesis after achieving SVR.
Methods: Viral genomes in liver biopsy specimens of SVR patients were analyzed by RT-PCR. Ultrastructure findings of hepatocytes in liver biopsy specimens of chronic hepatitis C (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 (NGS) was used to analyze epigenetic alterations in SVR patients.
Results: Hepatitis C virus (HCV) RNA was detected in 5 out of 88 liver samples of SVR patients. HCV RNA was detected in only 3 of the 49 SVR-HCC samples, indicating that HCV is not required for the development of SVR-HCC. In the hepatocytes of CHC patients, we found various organellar alterations (nuclei, mitochondria, endoplasmic reticulum (ER), lipid droplets, and pericellular fibrosis), which were similar to those observed in HCV-infected cells and hepatocytes of human liver chimeric mice. Organellar abnormalities other than ER and pericellular fibrosis improved after achieving SVR, but organelle abnormalities in mitochondria, ER, and pericellular fibrosis were observed in the tissues of SVR patients even if the SVR period is long, and were due to factors other than HCV. There were no specific differences in organelle abnormalities in the liver of between HCC and non-HCC patients in the first year after SVR, but after the second year and later, rough ER dilation/smooth ER increment was significantly greater in the liver of HCC patients. Furthermore, transcriptome analysis of the SVR patients by NGS suggested that genes involved in carcinogenesis by HCV protein are upregulated in SVR-HCC patients than in SVR-non-HCC patients.
Conclusions: Abnormal hepatocellular organelles in SVR-patients indicate a persistent disease state (post-SVR syndrome). Long-term follow-up of patients is recommended after achieving SVR.

EV-derived miRNA signature in patients with HCV and HCV/HIV with different stages of liver fibrosis

Cairoli Victoria[1], Valle Millares Daniel[2], Ryan Pablo[3], Dominguez Lourdes[4], Martín-Carbonero Luz[5], Ameigeiras Beatriz[6], Briz Verónica[2], Fernandez Rodriguez Amanda[2,7], Preciado Mª Victoria[1], Valva Pamela[1]

[1]Instituto Multidisciplinario de Investigaciones en Patologías Pediátricas (IMIPP-CONICET-GCBA), Buenos Aires, Argentina. [2]Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, España. [3] Departamento de enfermedades infecciosas, Hospital Universitario Infanta Leonor, Madrid, España. [4] Unidad VIH. Servicio de Medicina Interna. Instituto de Investigación Hospital 12 de Octubre (i+12), Madrid, España. [5] Instituto de Investigación Hospital La Paz (IdiPAZ), Madrid, España. [6] Unidad hepática, Hospital Ramos Mejía, Buenos Aires, Argentina. [7] Universidad Alfonso X el Sabio, Villanueva de la Cañada, Madrid, España

Background & Aims: Extracellular vesicles (EVs) are essential players in cell communication, and their cargo modulates the receptor cell response. MicroRNAs (miRNAs) proved to modulate the immune response both in physiological and pathological conditions. Hepatitis C (HCV) and Human Immunodeficiency (HIV) viruses' infection could modify EVs miRNA content and therefore, the immune response. We aimed to analyze the significant differentially expressed (SDE) EVs-derived miRNAs between HCV and HCV/HIV infected patients with different stages of liver fibrosis and to explore the associated molecular pathways.
Methods: Plasma from 22 chronic HCV and 31 HCV/HIV patients were analyzed. Total EV-containing RNA enriched with small RNAs was isolated and high-throughput sequenced (1 x 50) to characterize the miRNA cargo. Raw reads were analyzed with Fastqc and trimmed with Cutadapt. Human-miRNA identification was performed with miRDeep2. R package edgeR was used to detect SDE miRNAs between groups. *In silico* miRNA target prediction was performed with DIANA mirPath.
Results: First, HCV patients [54 ys (34 - 71), 54,5 % F ≥ 2] showed 36 SDE miRNAs compared with the HCV/HIV group [50 ys (31 - 71), 22,5 % F ≥ 2], which modulate pathways related to fatty acids biosynthesis, extracellular matrix (ECM) interaction, hippo signaling and viral carcinogenesis. Then, patients were stratified by fibrosis status and differential expression analysis was performed (Figure 1). Patients with F < 2 showed downregulation of hsa-miR-122-5p and hsa-miR-194-5p (log_2FC = -1,4, p < 0,001, log_2FC = -0,6, p < 0,001, respectively), which, in turn, condition the expression of genes involved in ECM-receptor interaction, proteoglycans in cancer and TGF-beta signaling pathways. Finally, SDE miRNAs between patients with F < 2 and F ≥ 2 were studied in each HCV condition. In the HCV F < 2 group, hsa-miR-3615 and hsa-miR-320a-3p were downregulated (log_2FC = -0,9, p < 0,001, log_2FC = -0,6, p < 0,001, respectively) which modulate genes of the TGF-beta and hippo signaling pathways. On the other hand, HCV/HIV F < 2 group showed 9 SDE miRNAs where hsa-miR-122-5p (downregulated) and hsa-miR-328-3p (upregulated) had the strongest differences (log_2FC = -1,3, p < 0,001, log2FC = 1,3, p < 0,001, respectively). Most of these miRNAs regulate genes involved in cancer and fatty acids related pathways.
Conclusions: Differentially expressed EVs-derived miRNAs in HCV and HCV/HIV chronic infection and in different stages of liver fibrosis were observed. The specific miRNA signature of each liver fibrosis stage may elucidate potential mechanisms involved in the clinical evolution of these patients and the identification of biomarkers of unfavorable progression.

Immunological Dynamics in HCV Chronic-Infected Patients Associated with Direct-Acting Antiviral Treatment

Leona Radmanić[1], Petra Šimičić[1], Kristian Bodulić[1], Adriana Vince[1,2], Snježana Židovec Lepej[1]

1University Hospital for Infectious Diseases - Zagreb (Croatia)
2University of Zagreb, School of Medicine, - Zagreb (Croatia)

BACKGROUND AND AIM: Chronic HCV infection (CHC) has been associated with inflammatory liver disease, fibrosis, cirrhosis and hepatocellular carcinoma. The molecular mechanisms of immunopathogenesis of CHC and the role of complex biological response modulator interactions have not been sufficiently investigated. The aim of this study was to analyze the immunological dynamics on the serum concentrations of cytokines and growth factors associated with inflammation in HCV chronic-infected patients which are treatment with direct-acting antiviral drugs and compare with controls.
METHODS: Serum concentrations of 12 cytokines (IL-5, IL-13, IL-2, IL-6, IL-9, IL-10, IFN-γ,TNF-α, IL-17A, IL-17F, IL-4 i IL-22) and 13 growth factors (Angiopoietin-2, EGF, EPO, FGF-basic, G-CSF, GM-CSF, HGF, M-CSF, PDGF-AA, PDGF-BB, SCF, TGF-α, VEGF) were analysed in 56 CHC before and after sustained virological response (SVR) and compared with 15 controls by using bead-based flow cytometry. Statistical analysis was performed using R. Unpaired comparisons were performed with Mann-Whitney test. Statistical significance was set at $p < 0.05$.
RESULTS: Statistically significant difference between the control group and the patients in the initial stage of infection was observed for IL-10, IL-13, IL-22, IL-4, IL-5, IL-9 and TNF-α, while statistically significant difference between the control group and patients after the achievement of SVR was observed for IL-10, IL-13, IL-2, IL-22, IL-4, IL-5, IL-9 and TNF-α. Furthermore, statistically significant difference between the control group and the initial stage of infection was observed for Angiopoietin-2, HGF and SCF, while a statistically significant difference between the control group and after achieving SVR was observed in for Angiopoietin-2, EGF, HGF and SCF.
CONCLUSION: Differences in immunological dynamics of serum biomarkes in HCV patients and controls are significant in order to detect changes which are suggestive of progressive involvement in the hepatic tissue. Serum biomarker levels prior and after DAA treatment indicate that HCV chronic-infected patients had an impaired immune response compared with controls.

Serum concentrations (pg/mL) of growth factors in patients with chronic hepatitis C prior and after antiviral treatment and compared with controls.

Delta variant of severe acute respiratory syndrome coronavirus 2 induces severe neurotropic patterns in K18-hACE2 transgenic mice

Ju-Hee Yang[1], Dongseob Tark[1], Sangmin Kang[1] and Gun-Hee Lee[1*]

1, Laboratory for Infectious Disease Prevention, Korea Zoonosis Research Institute, Jeonbuk National University, Iksan 54531, Republic of Korea

Background and Aims: A highly contagious novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged and quickly spread worldwide, resulting in the pandemic of coronavirus disease 19 (COVID-19). The SARS-CoV-2 variant of concern (VOC) as a variant through frequent recombination and evolutionary adaptation with increased transmissibility, virulence and became the dominant strain in circulation. The major symptoms of infected patients manifested respiratory symptoms, fever, and muscle pain. In addition, recent up to 30 percent of infected patients complain of neurological complications such as headache, nausea, stoke, and loss of smell or taste. However, neurotropism caused by SARS-CoV-2 infection remains largely unknown. This study assesses the neurotropic patterns between early variant (Wuhan, Hu-1) and Delta (B.1.167.2) in mice overexpressing the human angiotensin I-converting enzyme 2 (ACE2) receptor driven by the cytokeratin-18 (K18) gene promoter (K18-hACE2).

Methods: The Wuhan (strain BetaCoV/Korea/KCDC03/2020/NCCP 43326) and Delta variant (hCoV-19/Korea/KCDA119861/2021) of SARS-CoV-2 were received from the Korea disease control and prevention agency (KDCA). Eight-week-old K18-hACE2 male mice were inoculated with 2 x 10^4 $TCID_{50}$/ml SARS-CoV-2 via the intranasal route. Total RNA and protein were extracted from the lung and brain of K18-hACE2 Tg mice and subjected to next-generation sequencing (NGS) and SDS-PAGE followed by western blotting.

Results: Delta variant infected K18-hACE2 mice showed clinical symptoms with a massive weight loss, high lethality, and severe conjunctivitis compared to the Hu-1 variant. Delta variant was detected high levels of viral RNA in the lung and brain, whereas low levels of infectious SARS-CoV-2 in the lung compared to the Hu-1. Histopathological changes in the brain observed infiltration of immune cells through perivascular sites after SARS-CoV-2 infection. By 4 dpi, the delta variant changed the histopathological score involved in severe infiltrates immune cells and recruitment glial cells compared to the Hu-1. Upregulation of gene sets involved in type I interferon and cytokine-mediated signaling was most pronounced and the necrosis-related signaling in the SARS-CoV-2 positive brain.

Conclusions: The K18-hACE2 Tg model of SARS-CoV-2 delta variant infection shares many features of severe COVID-19 involved in lung disease. However, it showed a distinct severe neurotropic pattern as a brain damage sign which necrosis-related signal, recruitment of glial cells, and severe immune infiltration compared to the Hu-1. This study can be helpful to understand clinical symptoms and distribution in vivo according to the SARS-CoV-2 VOC.

Persistent hepatitis C virus infection causes permanent transcriptional alterations after virus elimination.

Victoria Castro[1], Juan Carlos Oliveros[2], Sofía Pérez-del-Pulgar[3], Xavier Forns[3] and Pablo Gastaminza[1,3]

[1] Department of Cellular and Molecular Biology and [2] Bioinformatics Core Facility Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas, Madrid.
[3] Liver Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Consorcio Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Universitat de Barcelona, Barcelona (Spain).

Hepatitis C virus infection (HCV) is a major biomedical problem worldwide, with more than 70 million people chronically infected. Chronic HCV infection causes liver inflammation and fibrosis, which can lead to development severe liver disease, such as cirrhosis or hepatocellular carcinoma (HCC). In recent years, approval of direct acting antiviral (DAA) drug combinations has revolutionized antiviral therapy against HCV. These drugs enable virus eradication in >98% of the treated patients, regardless of the genotype and liver disease status. The high efficacy of these antiviral molecules is such, that some models suggest that elimination of infected cells by reactivated immune responses may be dispensable for virus eradication, in contrast to interferon-based therapies. It is thus formally possible that patients that are declared cured, indeed carry formerly infected cells that display irreversible alterations due to prolonged chronic HCV infection.
Given the difficulty of obtaining liver biopsies from cured individuals and the likely low frequency of formerly infected cells surviving in these patients, we used two different cell culture models for persistent infection, in which HCV infection causes profound alterations of host celltranscriptional profile in proliferating and growth-arrested, partially differentiated cell models. These transcriptional alterations are a manifestation of the infected cell to regain homeostasis in the context of intracellular membrane rearrangements, interference with metabolic processes and persistent stress conditions permitting cell survival even under conditions where the virus has colonized the host cell. In this context, we asked the question of whether all transcriptional alterations are back to their original expression levels in the formerly infected cells after DAA treatment-mediated virus eradication.
Using persistent infection models, we determined alterations in the cellular transcriptome due to persistent HCV infection. Our results show that persistently infected cells were virologically cured by DAA-treatment and that the vast majority of the HCV-regulated host genes return to baseline expression after treatment completion. However, we observed a number of transcripts that remain altered several weeks after treatment completion and treatment withdrawal. Comparison of the results obtained in proliferating and growth-arrested cell culture models suggest that permanent transcriptional alterations may be established by several mechanisms. These results indicate that persistent HCV infection causes permanent molecular sequels in cell culture. We will discuss the biological and potential clinical implications of our findings.

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NLRC5 Restricts Dengue Virus Infection by Promoting the Autophagic Degradation of Viral NS3 through E3 Ligase Cullin-2

Jiawei Hao1, Jinqian Li1,2, Zhenzhen Zhang1, Yang Yang1, Qing Zhou1, Tiantian Wu1, Tongling Chen1, Zhongdao Wu3, Ping Zhang4, Jun Cui5, Yi-Ping Li1, *

1, Institute of Human Virology, Zhongshan School of Medicine, and Key Laboratory of Tropical Disease Control of Ministry of Education, Sun Yat-sen University, Guangzhou, 510080, China.
2, Key Laboratory of Tropical Translational Medicine of Ministry of Education, NHC Key Laboratory of Control of Tropical Diseases, School of Tropical Medicine, Hainan Medical University, Haikou, Hainan, 571199, China.
3, Department of Parasitology, Zhongshan School of Medicine, and Key Laboratory of Tropical Disease Control of Ministry of Education, Sun Yat-sen University, Guangzhou, 510080, China.
4, Department of Immunology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China.
5, MOE Key Laboratory of Gene Function and Regulation, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510006, China.
* Corresponding author, Email: lyiping@mail.sysu.edu.cn

Abstract

NLRC5 has been reported to be involved in antiviral immunity, however the underlying mechanism remains poorly understood. Here, we investigated the functional role of NLRC5 in the infection of a flavivirus, dengue virus (DENV). We found that expression of NLRC5 was strongly induced by virus infection and IFN-β/γ stimulation in different cell lines. Overexpression of NLRC5 remarkably suppressed DENV infection, while knockout of NLRC5 led to a significant increase in DENV infection. Mechanistic study revealed that NLRC5 interacted with viral nonstructural protein 3 (NS3) protease domain and mediated degradation of NS3 through a ubiquitin-dependent selective autophagy pathway. We demonstrated that NLRC5 recruited E3 ubiquitin ligase Cullin-2 (CUL2) to catalyze K48-linked poly-ubiquitination of NS3 protease domain, which subsequently served as a recognition signal for cargo receptor Tollip-mediated selective autophagic degradation. Together, we have demonstrated that NLRC5 exerted an antiviral effect by mediating the degradation of a multifunctional protein of DENV, providing a novel antiviral signal axis of NLRC5-CUL2-NS3-Tollip. This study expands our understanding of the regulatory network of NLRC5 in the host defense against virus infection.

Type I interferons mediate antiviral resistance to Zika virus in human microglia

Aidan T. Hanrath[1,2@], Catherine F. Hatton[1@], Florian Gothe[1], Cathy Browne[3], Jane Vowles[3], Sally Cowley[3], William S. James[3], Sophie Hambleton[1,4], Christopher J. A. Duncan[1,2,3]

1. Immunity and Inflammation Theme, Translational and Clinical Research Institute, Newcastle University, UK
2. Department of Infection and Tropical Medicine, Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, UK
3. Sir William Dunn School of Pathology, University of Oxford, UK
4. Department of Paediatric Immunology and Infectious Diseases, Great North Children's Hospital, Newcastle upon Tyne Hospitals NHS Foundation Trust, UK
[@] Co-lead authors

Background & Aims: Zika virus (ZIKV) is an emerging neurotropic disease of recent global public health concern due to its association with microcephaly in congenitally infected babies. There are currently no effective treatments or vaccines and the underlying pathophysiology is poorly understood. Microglia have been implicated in ZIKV pathogenesis by acting as a viral reservoir and initiating neuroinflammation in the human fetal brain. Conversely, microglia may also play a protective role by orchestrating an antiviral response. Type I interferons (IFN-I) form an important part of the innate immune response to viral infection in the brain. However, ZIKV has evolved several strategies to antagonise IFN-α/β-mediated restriction. Therefore, we sought to address the precise role of IFN-I in the response of microglia to ZIKV.

Materials/methods: We used a validated model of human 'microglia-like' cells derived from induced pluripotent stem cells (iPSCs), incorporating unique patient-derived iPSCs lacking the human IFN-alpha/beta receptor (*IFNAR2*), in addition to IFNAR2-/- and isogenic control iPSC pairs that were generated by CRISPR/Cas9 gene editing. Wild-type and IFNAR-deficient iPS-microglia were challenged with Asian lineage ZIKV. Innate signalling, infection and cytopathic effects were quantified by immunofluorescence, RT-qPCR, immunoblot and live-cell viability imaging.

Results: Wild-type iPS-microglia produced a robust type I interferon response to ZIKV infection which resulted in the production of key interferon-stimulated gene (ISG) products. This IFN-α/β-mediated antiviral state restricted ZIKV infection and resulted in protection of iPS-microglia from cytopathic effects of ZIKV. In contrast, IFNAR2-deficient iPS-microglia failed to induce ISGs, showed significantly increased viral infection and were vulnerable to ZIKV-induced cytopathicity. This phenotype was recapitulated in wild-type cells treated with ruxolitinib, which blocks signalling downstream of IFNAR2, and also by CRISPR-Cas9 mediated knock-out of *IFNAR2* in wild-type cells.

Conclusions: These results show that IFN-I mediates resistance of iPS-microglia to ZIKV, suggesting that IFN-I may be an important component of ZIKV immunity in the brain. Further work is required to elucidate the effector mechanisms of this response and to understand its generalisability to other target cell, such as astrocytes or neural progenitor cells. Furthermore, these data raise the possibility that molecular defects in IFN-I signalling might underlie some extreme clinical phenotypes (e.g. microcephaly) and that therapeutic manipulation of the IFN-I system may bring clinical benefit in ZIKV infections.

Linking Long Noncoding RNA Linc-Pint with Hepatitis C Virus Infection and innate immunity

Mousumi Khatun, Subhayan Sur, Robert Steele, Jinsong Zhang , Ranjit Ray and Ratna B. Ray

Saint Louis University, St. Louis, MO, USA

Background and Aims: Chronic HCV infection of liver often causes cirrhosis, and in some instances HCC. HCV encodes several factors impairing host genes for establishing chronic infection. The long noncoding RNAs (lncRNAs) display diverse effects on biological regulations, although their role in virus replication and diseases is poorly understood. We identified a lncRNA, Linc-Pint, which is significantly down-regulated in HCV-replicating hepatocytes and liver specimens from HCV infected patients. The mechanism by which lncRNA regulates host immune response during HCV infection is poorly understood. Although several lncRNAs are regulated by interferon (IFN), little is known regarding the effect of lncRNAs on IFN signaling pathway. Therefore, HCV will be a good surrogate model to investigate the role of this lncRNA in modulation of interferon signaling for establishment of chronic infection. We hypothesize that inhibition of Linc-Pint plays an important role in modulating host innate immune responses in promoting establishment of chronic HCV infection and liver disease progression.
Methods: We performed promoter bashing and ChIP assay for identification of molecules downregulating Linc-Pint by HCV. RNA-pulldown and LC-MS was used for identifying Link-pint partner. Reporter assay and quantitative RT-PCR were performed for examining IFN signaling.
Results: HCV infection of hepatocytes transcriptionally reduced Linc-Pint expression through CCAAT/enhancer binding protein β (C/EBP-β). Subsequently, overexpression of Linc-Pint significantly upregulated IFN-α and IFN-β expression in HCV-replicating hepatocytes. Using unbiased proteomics, we identified that Linc-Pint associates with DDX24, which enables RIP1 to interact with IFN-regulatory factor 7 (IRF7) of the IFN signaling pathway. Furthermore, we show that IFN-α14 promoter activity was enhanced in the presence of Linc-Pint. Our results demonstrated that Linc-Pint acts as a positive regulator of host innate immune response, especially IFN signaling. HCV-mediated downregulation of Linc-Pint expression appeared to be one of the mechanisms by which HCV may evade innate immunity for long-term persistence and chronicity.
Conclusions: Together, our results suggest that Linc-Pint acts as a positive regulator of host innate immune response. Downregulation of Linc-Pint expression by HCV helps in escaping innate immune system for development of chronicity.
In this study, we have shown that HCV exploits lncRNA long non-coding RNA Linc-Pint in hepatocytes for enhancement of lipogenesis.
Using RNA pull-down proteomics, we identified serine/arginine protein specific kinase 2 (SRPK2) as an interacting partner of Linc-Pint. A subsequent study demonstrated that overexpression of Linc-Pint inhibits the expression of lipogenesis-related genes, such as fatty acid synthase and ATP-citrate lyase. We also observed that Linc-Pint significantly inhibits HCV replication. Furthermore, HCV-mediated enhanced lipogenesis can be controlled by exogenous Linc-Pint expression. Together, our results suggested that HCV-mediated down-regulation of Linc-Pint enhances lipogenesis favoring virus replication and liver disease progression.
We found that SRPK2 is a direct target of Linc-Pint and that depletion of SRPK2 inhibits lipogenesis. Our study contributes to the mechanistic understanding of the role of Linc-Pint in HCV-associated liver pathogenesis.
IMPORTANCE: The mechanism by which lncRNA regulates the host immune response during HCV infection is poorly understood. We observed that Linc-Pint was transcriptionally downregulated by HCV. Using a chromatin immunoprecipitation (ChIP) assay, we showed inhibition of transcription factor C/EBP-β binding to the Linc-Pint promoter in the presence of HCV infection. We further identified that Linc-Pint associates with DDX24 for immunomodulatory function. The overexpression of Linc-Pint reduces DDX24 expression, which in turn results in the disruption of DDX24-RIP1 complex formation and the activation of IRF7. The induction of IFN-α14 promoter activity in the presence of Linc-Pint further confirms our observation. Together, our results suggest that Linc-Pint acts as a positive regulator of host innate immune responses. Downregulation of Linc-Pint expression by HCV helps in escaping the innate immune system for the development of chronicity.

Do the extensive C->U transition biases in the genomes of HCV arise from RNA editing of virus genomes by an APOBEC-like cell defence mechanism?

Peter Simmonds[1] Azim Ansari[1]

[1]Nuffield Department of Medicine, University of Oxford, Oxford, UK.

Background and Aims. Rapid sequence change and high population diversity has been long considered to endow HCV and other RNA viruses an enhanced ability to evade immune responses and antiviral therapy through development of resistance-associated mutations. While these are considered to be enhance the adaptive capability of HCV and contribute to its persistence, we have found that much of the diversity in HCV populations may be driven by host mutational processes that constitute a novel cell-mediated defence against RNA virus infections.

Methods. Alignments of large datasets HCV sequences of consensus genotype 1a, 1b, 2b and 3a were constructed from publicly available sequences. Frequencies of mutations, mutational contexts, homoplasy and RNA structure analyses were preformed using SSE v.1.4 and SPSS v.28.

Results. Most sequence changes away from the consensus were C->U transitions, an observation shared in published analyses of SARS-CoV-2 and proposed to be driven by an APOBEC-like RNA editing process. Using a 5% divergence filter to infer directionality, C->U transition showed a 2.1-3.0 -fold normalised based C->U/U->C transition asymmetry. This pattern of C->U over-representation was observed in 18 of 36 datasets of aligned coding region sequences from a diverse range of mammalian RNA viruses (including *Picornaviridae*, other *Flaviviridae*, *Matonaviridae*, *Caliciviridae* and *Coronaviridae*) with a 2.1x–7.5x normalised C->U/U->C transitions asymmetry occurring at sites with a consistently observed favoured 5' U upstream context. A comprehensive analysis of virus compositional variables and RNA structure metrics identified the presence of genome scale RNA secondary structure (GORS) as the only genomic or structural parameter significantly associated with C->U/U->C transition asymmetries by multivariable analysis (ANOVA). This association may potentially reflect an RNA structure dependence of sites targeted for C->U mutations than is consistent with previously described site specificity of APOBEC 3A editing of RNA sequences. The contribution of excess C->U changes in the longer-term sequence diversification of HCV was investigated through an analysis of the association of each transition and transversion with lineage-defining substitutions that underpin the longer-term evolution of HCV. Remarkably, C->U changes were specifically over-represented at phylogenetically uninformative sites with a high degree of mutation and reversion (homoplasy) within different HCV lineages.

Conclusions. Although mechanisms remain to be functionally characterised, excess C->U substitutions accounted for a substantial proportion of the standing sequence variability of HCV and other structured viruses. These observations provide an intriguing insight into the function of large-scale RNA structure and its role in modulation host defences.

Dose-dependent Hepacivirus infection reveals linkage between infectious dose and immune response

André Gömer[1, 2, *], Julien Delaroque[3, *], Christina Puff[4], Birthe Reinecke[5], Wolfgang Baumgärtner[4], Jessika M.V. Cavalleri[6], Karsten Feige[3], Eike Steinmann[1, **], Daniel Todt[1, 7, **]

[1]Department of Molecular and Medical Virology, Ruhr University Bochum, Bochum, Germany
[2]Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany
[3]Clinic for Horses, University of Veterinary Medicine Hanover, Hannover, Germany
[4]Department of pathology, University of Veterinary Medicine Hanover, Hannover, Germany
[5]Institute of Experimental Virology, TWINCORE Center for Experimental and Clinical Infection Research, Hannover, Germany
[6]Clinical department for internal medicine for horses, Veterinary University of Vienna, Vienna, Austria
[7]European Virus Bioinformatics Center (EVBC), Jena, Germany
[*]Contributed equally
[**]Corresponding authors

Background & aims: More than 70 million people worldwide are still infected with the hepatitis C virus 30 years after its discovery, underscoring the need for a vaccine. To develop an effective prophylactic vaccine, detailed knowledge of the correlates of protection and an immunocompetent surrogate model are needed. In this study, we describe the minimum dose required for robust Equine Hepacivirus (EqHV) infection in equids and examined how this relates to duration of infection, seroconversion and transcriptomic responses.

Methods: To investigate mechanisms of hepaciviral persistence, immune response and immune-mediated pathology, we inoculated eight EqHV naïve horses with doses ranging from 1.3 x 10^0 to 1.3 x 10^6 RNA copies per inoculation. We characterized infection kinetics, pathology and transcriptomic responses via NGS.

Results: The minimal infectious dose of EqHV in horses was estimated at 13 RNA copies, whereas 6 copies were insufficient in causing infection. Peak viremia did not correlate with infectious dose, while seroconversion and duration of infection appeared to be affected. Notably, seroconversion was undetectable in the low-dose infections within the surveillance period (40-50 days). In addition, transcriptomic analysis revealed a nearly dose dependent effect, with greater immune activation and inflammatory response observed in high-dose infections than in low-dose infections. Interestingly, inoculation with 6 copies of RNA that did not result in productive infection was associated with a strong immune response, similar to that observed in the high-dose infections.

Conclusion: We demonstrated that the EqHV dose of infection plays an important role for inducing immune responses – possibly linked to early clearance in high-dose and prolonged viremia in low-dose infections. In particular, pathways associated with innate and adaptive immune responses, as well as inflammatory responses were more strongly upregulated in high dose infections than in lower doses. Hence, inoculation with low doses may enable EqHV to evade strong immune responses in the early phase and therefore promote robust, long-lasting infection.

cGAS/STING innate immunity antagonism by the hepatitis E virus open reading frame 1 protein

Robert LeDesma[1], Emily Mesev[1], Emma Guare[1], Celeste H. Claudio[1], Brigitte Heller[1], Saori Suzuki[1], Jiayu Zhang[1], Jared E. Toettcher, Alexander Ploss[1,*]

[1]Department of Molecular Biology, Lewis Thomas Laboratory, Princeton University, Princeton, NJ 08544, USA,
*Correspondence should be address to A.P. (aploss@princeton.edu)

Background and Aims: Hepatitis E virus (HEV) is a positive sense, single stranded RNA virus of the *Hepeviridae* family responsible for over 20 million infections causing approximately 70.000 deaths annually. HEV encodes three gene products, the open reading frame (ORF) 1 polyprotein encodes the viral replicase, ORF2 the capsid and ORF3 a phosphoprotein essential for viral egress. Viral protease-mediated innate immunity antagonism is well characterized across many other known (+) ssRNA viruses such as hepatitis C virus (HCV), though the existence of an HEV protease is unresolved and controversial. Prior bioinformatic analyses predicted a papain-like cysteine protease (PCP) domain within ORF1, with the highly conserved residue C483 as part of the putative catalytic dyad, though functional characterization of the PCP is still debated. Further, the ability of the ORF1 protein to antagonize and blunt the host innate immune response is not fully understood. Using comparative innate immunity profiling, our work highlights HEV ORF1 protein's ability to blunt host innate immunity via a viral protease *independent* mechanism, and explores mechanisms as to how ORF1 accomplishes this.

Methods: Human hepatoma cells stably expressing either the catalytically active or inactive protease domain from one of nine different viruses were independently subjected to innate immunity stimulation via agonists to the RIG-I like helicase/MAVS, Toll-like receptor 3/TRIF, or cGAS/STING signaling pathways. For HEV, a C483A mutation renders ORF1 non-functional. These protease domain-bearing cells respond to IFN-beta and IFN-lambda stimulation as evidenced by STAT1 translocation. Cellular RNA and total protein was harvested from viral protease expressing cells stimulated with innate immune agonists, were analyzed both by RT-qPCR -to measure transcript levels of IFN stimulated genes (ISGs), as well as IFN-beta and IFN-lambda, and immunoblotting for the quantification of host innate immunity signaling proteins.

Results: WT ORF1 expressed in human hepatic cells robustly blunts innate immune signaling across the cGAS/STING signaling pathway. Comparative innate immunity profiling likens HEV ORF1's profile of IFNs and ISGs to that of West Nile virus (WNV), though WNV accomplishes its own antagonism via its virally encoded NS2B3 protease. Further, ORF1's reduction of adapter protein expression is not accomplished via global transcription/translation repression and inhibition of the host proteasome alters the innate immunity antagonism profile exhibited by HEV ORF1, opening up a new and exciting avenue of investigation.

Conclusions: Our data demonstrate that the presence of WT HEV ORF1 creates a cellular environment conducive to viral replication by blunting the cell's ability to sense and respond to infection, though the mechanism is still undetermined.

The V141L variant in IFNAR1 alters the whole blood response to stimulation and is associated with resistance to hepatitis C virus infection in humans.

Jamie Sugrue[1], Céline Posseme[2], Ziyang Tan[3], Christian Pou[3], Bruno Charbit[4], Vincent Bondet[2], Nollaig M. Bourke[5], Petter Brodin[3], Darragh, Duffy[2,4], Cliona O'Farrelly[1,6].

1. School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland.
2. Translational Immunology Unit, Institut Pasteur, Paris, France.
3. Science for Life Laboratory, Department of Women's and Children Health, Karolinska Institutet, Stockholm 17165, Sweden.
4. Cytometry and Biomarkers UTechS, CRT, Institut Pasteur, Paris, France
5. Department of Medical Gerontology, School of Medicine, Trinity Translational Medicine Institute, Trinity College Dublin, Ireland.
6. School of Medicine, Trinity College Dublin, Ireland.

Background & Aim: Resistance to viral infection is an oft overlooked and understudied outcome following exposure to viral infection. Previously, our group has recruited a cohort of women exposed the hepatitis C virus following receipt of contaminated anti-D immunoglobulin. Studies of the cohort, involving three infection outcomes – resistant (exposed seronegative; ESN), spontaneous resolvers (SRs) and those who were previously chronically infected but cleared infection following therapy (sustained virological responders; SVRs) uncovered a signature of viral resistance in the ESNs that implicated an enhanced polyIC induced type I interferon gene signature in whole blood.
Method: Here, using a gene candidate approach we sought to assess the genetics of viral resistance. The cohort was genotyped for tagSNPs in the TLR3-IFN-I pathway. Using this approach we found that a SNP in IFNAR1, rs2257167 (V141L), was associated with increased resistance to HCV infection in the dominant model (p=0.02; Odds ratio = 3.54; 95% confidence interval = 1.21 to 9.82).
Result: Analysis of transcriptomic data from two independent healthy female control cohorts revealed increased IFNAR1 mRNA expression in whole blood in those carrying at least one copy of the variant C allele compared to those homozygous for the wild-type G allele. Analysis of whole blood stimulation data in 500 females from the *Milieu Interieur* cohort showed increased IRG upregulation in response to stimulation with LPS, polyIC and the live influenza A virus (IAV) in those carrying the variant allele.
Conclusion: Conversely, GG individuals had an increased pro-inflammatory response to stimulation with LPS, polyIC and IAV. The increased pro-inflammatory response observed in GG donors may have contributed to resistance to HCV infection by ESNs in the anti-D cohort.

Modelling acute HCV infection kinetics in humanized mice

Zhenzhen Shi[1#], Yuji Ishida[2,3#], Nicholson T Collier[4], Michio Imamura[3,5], Chise Tateno-Mukaidani[2,3], Jonathan Ozik[4], Jordan J Feld[6], Harel Dahari[1*], Kazuaki Chayama[3,7,8*]

(1) The Program for Experimental & Theoretical Modeling, Division of Hepatology, Department of Medicine, Loyola University Medical Center, Maywood, IL, USA; (2) PhoenixBio Co., Ltd., Higashi-Hiroshima, Japan; (3) Research Center for Hepatology and Gastroenterology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan; (4) Consortium for Advanced Science and Engineering, University of Chicago, Chicago, IL; Decision and Infrastructure Sciences, Argonne National Laboratory, Argonne, IL, USA; (5)Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan; (6) Toronto Centre for Liver Disease, Toronto, ON, Canada; (7) Collaborative Research Laboratory of Medical Innovation, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan; (8) RIKEN Center for Integrative Medical Sciences, Yokohama, Japan #equally contributed; *corresponding authors.

Background and Aims:
uPA-SCID chimeric mice with humanized livers (SCID-MhL) are a useful tool for studying HCV infection in the absence of an adaptive immune response. Here we sought to analyze and model the HCV kinetics from inoculation to steady state in the uPA-SCID mouse model, using an agent-based modelling (ABM) approach.

Methods:

Ten male mice (5 PXB SCID-MhL with hepatocyte donor: JFC [1 year, male Caucasian] and human albumin > 9mg/mL, and 5 SCID mice without humanized livers, SCID-M) were 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.). HCV RNA was measured using quantitative real-time PCR (qRT-PCR) as previously reported (BBRC. 2006; 346(1):67-73). We developed an ABM that accounts for two types of agents: hepatocytes and virus in the blood, attempting to recapitulate the stochastic process of HCV infection of hepatocytes in mice. The ABM simulates a series of infection stages including initial infection of uninfected cells, infected cells in a non-productive viral eclipse phase, and infected cells in a productive infection phase releasing HCV virion, which then proceed to infect additional hepatocytes. Several model parameters (e.g., virion production cycle) were calculated based on the in vivo experimental design. Model parameter fitting was done using a Genetic Algorithm (GA) with the EMEWS framework on the Midway2 high-performance computing cluster at the University of Chicago.

Results: While in SCID-M HCV was rapidly cleared, a productive infection was established in SCID-MhL. After an initial viral decline, the virus resurged, followed by a transient decline (in 4 mice) that eventually stabilized at high steady state levels. To account for a transient decline, a decrease in viral production was assumed reminiscent of our previous observation of such transient HCV decline seen in chimpanzees (Gastroenterology.;128(4):1056-66). The ABM predicts that: (1) the viral eclipse phase lasts between 1-20 h; (2) Once the infected cell passes the eclipse phase, the viral production rate is not constant, but rather increases over time. Initially all mice started with a long production cycle of 1 virion per 9-15h but gradually reaching 1 virion per hour after 12 hr; (3) Within 5 days, the virion production reaches a steady state production rate of 1-3 virions per hour in each infected cell; and (4) a viral production drop of 83%-98% starting between 2-5 days p.i. in 4 mice.

Conclusions: The ABM provides novel insights into the HCV life cycle in vivo. The model suggests a partial block of virion production possibly due to an early stage of innate immune response.

A Picornavirus that retargets p90 ribosomal S6 kinases to phosphorylate new substrates

Belén Lizcano-Perret & Thomas Michiels

VIRO unit, de Duve Institute, Université Catholique de Louvain, Brussels, 1200, Belgium

Background and Aims: Proteins from highly unrelated pathogens, including viruses and bacteria, were shown to recruit and activate cellular kinases of the p90 ribosomal S6 kinases (RSK) family through a common linear motif (1). Interestingly, although these proteins evolved a convergent way to recruit RSKs, the outcome of RSK recruitment largely differs, ranging from inflammasome inhibition (Yersinia YopM) to nucleocytoplasmic trafficking perturbation (Cardiovirus leader protein). Data suggest the "model of the clamp" (Fig.1) whereby pathogens' proteins would recruit RSK through their conserved linear motif and recruit some targets through another domain of the protein. These targets would then serve as substrates for the hijacked RSK and the phosphorylated target protein would subsequently act as an effector to promote replication or escape host responses.

The leader (L) protein of Cardioviruses is a very small protein that interferes with innate immune responses. It notably triggers the diffusion of nuclear and cytosolic proteins across the nuclear pore complex and this activity correlates with L-mediated hyperphosphorylation of FG-nucleoporins (FG-NUP) (2).
The aim of this work was to assess the model of the clamp and to decipher the mechanism of nucleocytoplasmic perturbation by L.

Methods: A biotin ligase (BioID2) approach was used to identify L and RSK partners in infected cells. By fusing BioID2 to either L or RSK we identified the "proxeome" of the L-RSK complex. In order to confirm the direct phosphorylation by RSK of the L-RSK recruited targets, we used the analog-sensitive kinase system developed in the group of Kevan Shockat (UCSF). This strategy is based on kinase mutants bearing an enlarged ATP binding pocket, which can accommodate bulkier ATP-analogs that have a thiophosphate as donor.

Results: The BioID2 screens identified common partners for L and RSK, the most striking ones being FG-NUP. FG-NUP are hyperphosphorylated during Cardiovirus infection therefore we used analog-sensitive RSK mutants and showed that in cells infected with two different Cardioviruses, RSK directly phosphorylates at least two FG-NUP.

Conclusion: Our work illustrates a new virulence mechanism whereby pathogens' proteins redirect host kinases toward specific substrates and deciphers how cardioviruses trigger RSK-mediated FG-NUP hyperphosphorylation to perturb nucleocytoplasmic trafficking in the host cell.

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Cross-reactive antibodies facilitate innate sensing of dengue and Zika viruses

Kimberly E. Rousseau[1*], Laura K. Aisenberg[1*], Katherine Cascino[1], Guido Massaccesi[1], William H. Aisenberg[1], Wensheng Luo[2], Kar Muthumani[3], David B. Weiner[3], Stephen S. Whitehead[4], Michael A. Chattergoon[1], Anna P. Durbin[2], Andrea L. Cox[1]

[1]Johns Hopkins University School of Medicine, Department of Medicine, Baltimore, MD, USA
[2]Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA
[3]The Wistar Institute, Vaccine & Immunotherapy Center, Philadelphia, PA, USA
[4]National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA
*Authors contributed equally to the manuscript.

Background and Aims: Individuals who live in areas endemic with the *Aedes aegypti* mosquito are at risk of infection with both dengue (DENV) and Zika (ZIKV) viruses. These individuals are also at risk for repeated DENV infection. Outcomes of secondary DENV infection range from mild to life-threatening, largely dependent on the presence of anti-DENV antibodies. However, the full role of cross-reactive DENV antibodies on the course of DENV and ZIKV infections remains unclear.

Methods: We employed an *in-vitro* co-culture model with primary human plasmacytoid dendritic cells (pDCs) and DENV- or ZIKV- infected hepatoma cells to assess the ability of heterotypic cross-reactive DENV monoclonal antibodies (or polyclonal immunoglobulin isolated after DENV vaccination) to upregulate type I interferon (IFN) production by pDCs.

Results: We found a range in the ability of antibodies to increase pDC IFN production and a positive correlation between IFN production and the ability of an antibody to bind to the infected cell surface. Engagement of Fc receptor on the pDC and Fab binding of an epitope on an infected cell was required to mediate increased IFN production by providing specificity to and promoting pDC sensing of DENV or ZIKV.

Conclusions: This represents a mechanism independent of neutralization by which pre-existing cross-reactive DENV antibodies could protect a subset of individuals from severe outcomes during secondary heterotypic DENV or ZIKV infection.