STUDIES INVESTIGATING THE CLINICAL IMPACT AND IMMUNOLOGICAL HOST RESPONSE TO VIRAL ERADICATION IN HEPATITIS C INFECTION

Dr Swee Lin Ginette Chen Yi Mei

MBBS (Hons), FRACP

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

ORCID: 0000-0002-4764-0799

November 2019

Department of Medicine (St Vincent's Hospital)

The University of Melbourne

ABSTRACT

Australia now has improved access to HCV treatment for individuals with all stages of liver disease. There is limited data however, documenting any long-term clinical benefit of viral eradication in those with early stage liver disease. We report on the long-term outcomes of a well-characterised cohort of CHC subjects with predominantly early stage liver disease, whom paired liver fibrosis assessments were performed more than 10 years apart. We show in a real-world setting, that both early and curative HCV treatment halts fibrosis progression. Our data supports the early treatment of all people with CHC regardless of liver fibrosis stage, to prevent long-term liver sequelae.

In subjects with acute / subacute HCV infection, we identify predictors of innate immunological response in peripheral blood mononuclear cells, to differentiate spontaneous clearers to those who progress to chronic infection. Our data strongly implicates TLR4 signaling in the persistence of HCV infection. Those who developed chronic infection had higher TLR4 expression on peripheral monocytes and NK cells and increased IFN-γ response to TLR4 stimulation. We also observed increased TLR7 responsiveness in this group. We confirm the previously noted associations of IFNL4 genotype and plasma IP-10. Our data presents TLR4 as a potential biomarker for predicting clearance. This would clinically translate to individuals presenting with acute / subacute HCV infection, being able to defer drug therapy and its associated morbidity and cost.

In subjects with chronic HCV infection, we demonstrate a clear effect of direct antiviral agent (DAA) mediated viral suppression therapy on patterns of TLR signaling in subjects with chronic HCV-1. We show that peripheral monocytic TLR2, TLR4 and TLR7 signaling is down-regulated early on in treatment, with a strong trend to higher baseline TLR signaling being associated with viral clearance with the DAAs. We are the first study to demonstrate a relationship between TLR signaling activity and IFN-free therapy for HCV. We hypothesise that the HCV virus directly stimulates the TLR pathways to induce an antiviral effect, with higher TLR signaling evident among those

who respond to DAA therapy. This was accompanied by a reduction in PBMC ISG expression, NK activation markers and plasma levels of inflammatory cytokines / chemokines, with restoration of the innate immune response.

Despite excellent treatment options, the mechanisms responsible for viral eradication in HCV have remained poorly defined. Previous data suggest a link between innate immunity and HCV pathogenesis, as well as spontaneous viral clearance. The data suggested that antiviral therapy alone was not sufficient to clear virus and supported a key role for innate immunity contributing to viral clearance. Our results strongly implicate TLR signaling / expression in HCV viral clearance. We believe TLR agonists must be considered as a potential HCV vaccine candidate and further research in this area is required.

DECLARATION

This is to certify that this thesis:

i) contains no material that has been accepted for the award of any other degree or diploma in any university of other institution,

ii) comprises only my original work except where indicated in the Preface,

iii) is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices

Swee Lin G. Chen Yi Mei November 2019

PREFACE

The work presented in this thesis was undertaken under the supervision of Professor Alexander Thompson, Associate Professor Paul Desmond and Associate Professor Kumar Visvanathan, Department of Gastroenterology, St Vincent's Hospital and Department of Medicine, University of Melbourne.

Three original manuscripts are included in this thesis. They been published in international peer reviewed journals and put through a comprehensive peer review process prior to acceptance for publication. Multiple co-authors have been involved. All co-authors have completed and signed a co-author authorisation form.

The three included manuscripts are as follow:

1. S. G. Chen Yi Mei**,** P. V. Desmond, A. J. Thompson (2012) IL28B: Current and Future Use. **Current Hepatitis Reports** 11:136–145
2. Chen Yi Mei S, Thompson AJ, Christensen B, Cunningham G, McDonald L, Bell S, Iser D, Nguyen T, Desmond PV. Sustained virological response halts fibrosis progression: A long-term follow-up study of people with chronic hepatitis C infection. **PLoS ONE**. 2017;12(10): e0185609.
3. Chen Yi Mei S G, Burchell J, Skinner N, Millen R, Matthews G, Hellard M, Dore G, Desmond PV, Sundararajan V, Thompson AJ, Visvanathan, K, Sasadeusz J (2016) Toll-like Receptor Expression and Signaling in Peripheral Blood Mononuclear Cells Correlate With Clinical Outcomes in Acute Hepatitis C Virus Infection. **The Journal of infectious diseases**. 2016 Jun 9;214(5):739–47. PMID: 27284092.

In the first manuscript, co-author contributions included critical review of the manuscript. In the second manuscript, co-author contribution included data collection and critical review of the manuscript. In the third manuscript, co-author contributions included LOWESS plots, guidance and assistance with laboratory experiments and critical review of the manuscript. The steering committee from the ATACH study were included as co-authors of this manuscript, providing patient samples and critical review of the final manuscript.

The candidate's contribution included study concept proposals, ethics submissions, study design, data collection and statistical analyses (not including LOWESS curves), performing all laboratory experiments to include TLR expression and signaling on plasma and peripheral blood mononuclear cells (PBMCs), preparation of first drafts and editing of manuscripts following co-author reviews, online submission for each manuscript for publication, response to peer review process and final submission for publication.

ACKNOWLEDGEMENTS

Undertaking this PhD has been the one aspiration that has truly required a village to help me get there. It would not have been possible without both the professional and personal support I have received. I will be forever grateful for this.

I would firstly like to thank my primary supervisor, Professor Alexander Thompson. Thank you for your unwavering support, mentorship and guidance throughout the tenure of this thesis. Your commitment and patience have been truly appreciated. Your vision and immense knowledge have also been a continual inspiration, very much needed at times. To my supervisor, Associate Professor Paul Desmond, thank you for your mentorship, warmth, wisdom and 'big picture' perspective. I have had the privilege of working under your guidance since I was a junior doctor. You have supported me since this time, into my advanced training and throughout this thesis. I am very thankful for this. To my supervisor, Professor Kumar Visvanathan, thank you for your continual mentorship, guidance and expertise. It would not have been possible to undertake this research without your steady support, generosity and patience.

I must acknowledge Jackie Yu, Narelle Skinner, Mario Congiu and Rosie Millen and other fellow laboratory members, for generously taking me under your wings and teaching me the skills of the laboratory. I have treasured this opportunity to delve beyond clinical medicine and explore the science that underpins a lot of clinical practice. I greatly appreciate your time and generosity in showing me the way. A special thanks to Jackie Yu for truly going above and beyond, and for the friendship we have formed.

Thank you to Kate Mellor and Lucy McDonald, our beloved HCV nurses, who were a backbone of support particularly in the early stages – for identifying patients for my study, educating them on the visit schedule, looking after them with such care, and for also looking after me. I will always appreciate your warm smiles and our chats, as I found my way through the early years of this PhD. Thank you to the HCV trial nurses, led by Nicole Tresize, for your support and always generously helping with

blood collections when needed. To my fellow PhD students – we had a wonderful group, and I am thankful for the companionship, de-briefs and collegiality. A special thank you to Dr Jacinta Holmes, for your guidance, vision and support. You set the bar high, and have always been someone to look up to.

Thank you to my collaborators from the ATAHC and NETWORKS study, led by Joe Sasedeuz, for entrusting me with your clinical samples and data, and for your expertise and support. To Associate Professor Vijaya Sundararajan and Jodie Burchell for your expertise on data analysis. To Professor Golo Ahlenstiel for generously sharing your expertise on NK studies. Most importantly, thank you to the patients who participated in my studies. This would not have been possible, if it were not for your kindness and generosity, taking the time out of your lives to come in for those extra visits, particularly when you may have not felt the best due to treatment side effects - I am truly grateful for this. I would also like to acknowledge the Gastroenterology Society of Australia and the Department of Gastroenterology at St Vincent's Hospital, for generously providing funding support for this research.

To the Senior Female Gastroenterologists, who have quietly supported and encouraged me in the background. To Professor Sally Bell, thank you for your words of encouragement and belief in me. You have always been someone I can turn to, and look up to. I will forever cherish your support in my moment of need. Having just fallen pregnant with my third child, I did not know if I could get there, and was not sure where I fit in, in this professional world. Your belief in me, sharing your personal experience and your advice were beyond invaluable. To Professor Amanda Nicoll, your unyielding support has been greatly appreciated – holding a position for me, covering my clinics / endoscopy lists so that I could undertake my laboratory work and write up my thesis, is greatly appreciated. I am hopeful that in the future, I will also be able to support young women as you have both supported me.

To my nanny, 'big' Sophia, who is truly a part of the family and has supported me throughout this thesis. Thank you for taking such good care of my children, for caring for them like they are your own. For making them feel safe and loved, when I was in the lab late into the night and Domenic was at work, or I was only a few blocks away in the library analysing my data or writing my thesis. For always giving me a smile when I

would say ‘I am nearly finished’. Thank you for your nonjudgement, for your professionalism, for being a sounding board, and for all of your help running my house so that I could work on my thesis every evening after the kids were in bed. Domenic, Oliver, Hugh, baby Sophia and I are truly indebted to you, thank you for everything you have done for our family.

To Zanny and Ray, thank you for your support over the years – especially with the children, particularly in the early years when I was finding my way. Thank you Ray for your belief in me – you are always in our thoughts.

To my parents, who have been my biggest supporters. Mum, you always brought me up to believe in myself, thank you for this. Your unconditional love and support have helped me navigate the constant juggle of motherhood, family and having a career. Dad, you have and always will be my rock. I cherish, and thank you for all of our conversations, your wisdom and advice. I am very lucky to have you in my life. To my sisters, Mi-Lin and Wei-Lin, thank you for being there through the ups and downs. Our sisterly bond is so strong, this has helped me immensely. Thank you Wei-Lin for your amazing editing assistance and taking the time to read my thesis in such detail. Your feedback has been invaluable and greatly appreciated.

To my beautiful children – Oliver, Hugh and Sophia. Thank you for being on this journey with me, through the ups and the downs. The three of you know that it has not been easy at times. Thank you for your unconditional love and patience. I cherish you all, and hope that one day you will be proud of me as your Mother.

Finally, to my amazing husband Domenic. I will be and am forever indebted to you for your unconditional love and support. This PhD has been a labour of love, not just for me, but for you and our children. But through all of the ups and downs, I have never once doubted your commitment to me, us, and our family. Your loyalty, love and belief in me, has helped me to follow my dreams. Thank you for this. It is without a doubt, that I could not have done this without you.

Swee Lin Chen Yi Mei, November 2019

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1 Literature Review

1.1 Introduction

Up to 71 million people are chronically infected with the hepatitis C virus (HCV) worldwide.[1] Chronic hepatitis C (CHC) leads to the development of cirrhosis, hepatocellular carcinoma and liver failure and is a significant public health concern with HCV-related mortality surpassing HIV-related mortality in the United States, over a decade ago.[2]

The landscape of HCV treatment has changed remarkably over the last decade. HCV treatment was traditionally associated with significant morbidity and unacceptable cure rates. However, interferon-free combinations of direct acting antiviral (DAA) agents are now standard of care, with cure rates approaching 100% and excellent tolerability.

Despite these therapeutic advances, the exact mechanism of HCV eradication is yet to be unravelled. There is no doubt that there is a complex interplay between the virus and host response, that ultimately determines if an individual achieves spontaneous clearance in acute HCV infection, or a sustained virological response with HCV treatment. An understanding of the immunopathogenesis of HCV and treatment response remains very relevant to vaccine development and design, and is the next major challenge for HCV. As the antiviral drugs are costly, this remains a vital public health initiative for resource limited regions.

The first aim of this thesis was to investigate the clinical benefit of viral eradication. With improved access to HCV treatment for individuals with all stages of liver disease over the last half-decade, we felt that it was important to determine if there is any long-term clinical benefit to viral eradication. In a cohort of CHC subjects followed long term, we show that HCV eradication is associated with lower rates of liver fibrosis progression.

We also wished to explore the underlying immunopathogenesis of HCV by looking at the host response to viral eradication. This formed the basis of the second

and third aims, in which we wished to investigate the immunological host response to viral eradication in both acute and chronic hepatitis C infection. We firstly looked at a cohort of subjects with acute HCV infection, and identified predictors of innate immunological response in peripheral blood mononuclear cells (PBMCs) to differentiate spontaneous clearers to those who progressed to chronic infection. This led to the question of whether the innate immune response plays a critical role in HCV clearance and antiviral therapy in subjects with CHC. Similar to the acute HCV cohort, we were able to identify baseline predictors of treatment response to DAA therapy in the setting of CHC. During the course of this thesis, the treatment paradigm moved swiftly from IFN being the backbone of therapy, to IFN-free regimens consisting of a combination of DAAs, now the standard of care. We were able to capture this evolution and in doing so, identify key differences in innate immunological response to IFN-based therapy compared to DAAs alone.

1.2 Epidemiology

The prevalence of chronic HCV infection in Australia was estimated at 227,306 in 2014, with a reported 10,790 cases of new infection reported each year.[3,4] This data was prior to the introduction of the DAAs, with a reported 58,280 individuals treated for hepatitis C since March 2016.[5-7]

In Australia, approximately 80% of HCV infections occur through injecting drug use (IDU).[8,9] Prior to 1990, blood transfusions accounted for 5-10% of acute infections, but this was virtually eliminated after blood screening of HCV was implemented. Immigration from endemic regions is the other key risk factor in Australia, with approximately 11% of infected individuals being immigrants.[10,11] These include Asia, Africa and South America, where key modes of HCV transmission include inadequate sterilisation of medical equipment, unsterile vaccinations and transfusion of unscreened blood or blood products. Less frequent modes of transmission include vertical transmission with a 2-8% risk from mother to baby, and sexual transmission with a risk of <1% in heterosexual relationships. However, the incidence of per mucosally transmitted HCV in HIV-positive men who have unprotected sex with men,

has increased in Europe, the USA, Asia and Australia. This mini-epidemic has occurred since 2004, during the era of the new DAAs.[12-14]

1.3 HCV Virology

1.3.1 Classification

HCV belongs to the Hepacivirus genus, a member of the family *Flaviviridae*.

1.3.2 Viral Structure

HCV is a small (50nm) enveloped, single-stranded, positive-sense virus with an RNA genome of approximately 9.6kb in length. The RNA genome is surrounded by an icosahedral protective shell formed by the HCV core protein, known as the nucleocapsid. This is further encased by a host-derived lipid bilayer studded with complexes of two HCV envelope glycoproteins, E1 and E2.

1.3.3 Genome Organisation

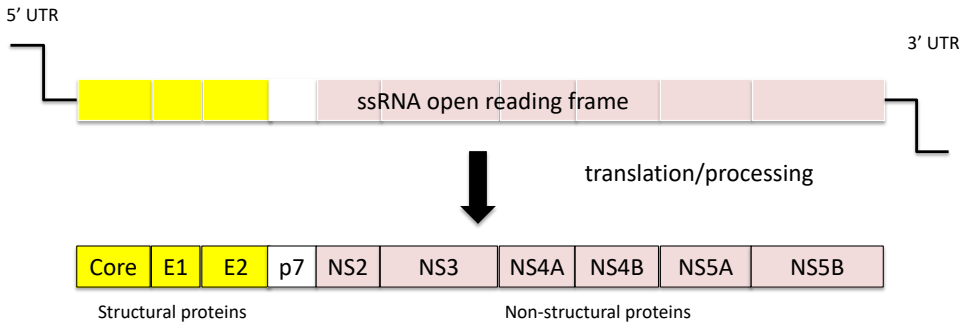


Figure 1.1 HCV genome.

The RNA genome consists of an open reading frame encoding a long viral polyprotein, consisting of 10 structural and non-structural proteins. Similarly, to other

viruses of the family Flaviviridae, co– and post– translational cleavage of the HCV polyprotein requires both host and viral proteases.

The N-terminal quarter of the genome encodes the core protein and two membrane-associated glycoproteins E1 and E2. The rest of the genome encodes the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. In addition, p7 is a polypeptide located at the junction between the structural region and the NS region, reported to be involved in ion channel activity.

The RNA genome is flanked by two short untranslated regions (UTR). A highly conserved 5' UTR of approximately 341 nucleotides, precedes the translation initiation codon, containing the ribosome binding site (IRES - internal ribosomal entry site). The 3' UTR is of variable length, consisting of a poorly conserved sequence of approximately 40 nucleotide, an internal poly (U)/polypyrimidine tract, followed by a highly conserved 98-nucleotide sequence.

1.3.4 Viral Replication Cycle

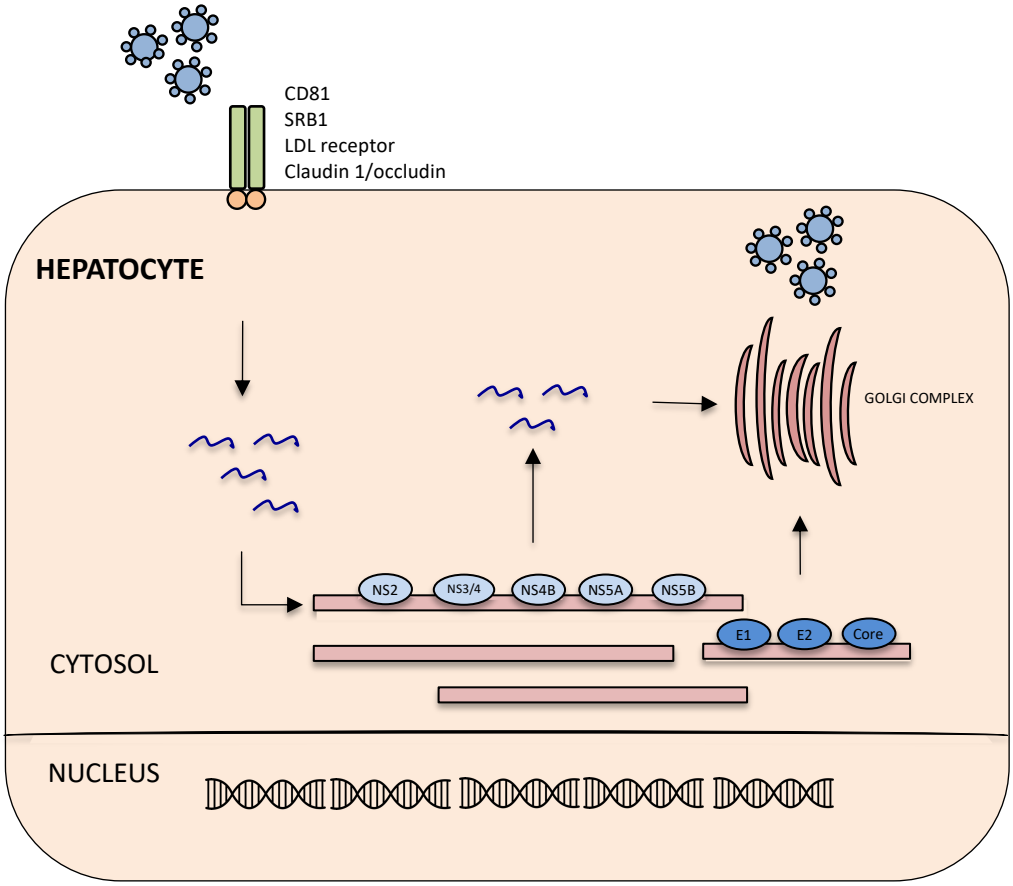


Figure 1.2 HCV viral replication cycle.

1.3.5 HCV Entry, Fusion and Uncoating

HCV enters its target cell, the hepatocyte, through clathrin-mediated endocytosis via a combination of four putative host co-receptors. These include two E2 binding proteins, the tetraspanin CD81[15-17] and scavenger receptor class B1 (SC-B1)[18-20], and the tight junction proteins claudin (CLDN1) and occluding.[21-25] Other putative receptors have been implicated in viral entry, such as the low density lipoprotein (LDL) receptor, glycosaminoglycans, CLDN6 and CLDN9 and two lectins, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and the related liver-specific intercellular adhesion molecule 3-grabbing nonintegrin (L-SIGN). Endocytosis of HCV is followed by Rab5-dependent transport to the early endosomal compartment, where a low pH environment leads to fusion of the endosomal membrane and viral envelope, releasing the viral genome into the cytosol.[26]

1.3.6 Viral Replication

Viral replication in the cytosol occurs on a modifed cytosolic membrane network in which the genome becomes amplified, to reach critical mass. The RNA genome is transcribed to form a complementary negative-sense RNA molecule, which then serves as a template for the synthesis of progeny positive-strand RNA molecules. The newly translated viral polyprotein is cleaved by a host-cell signalase as well as virus-specific non-structural proteins, NS2 and NS3. The NS3 protease, together with its cofactor, NS4A, cleaves the viral polyprotein at four sites, generating viral proteins essential for replication of the RNA genome. The NS3 protease also has helicase (unwindase) activity. The enzyme capable of performing both steps of RNA synthesis is the virally encoded RNA-dependent RNA polymerase NS5B. Alongside robust replication of HCV of approximately 1012 virons per day[18], the RNA polymerase lacks proof reading skills and these factors account for the high rate of mutation and presence of HCV quasispecies within a single host.[27,28]

1.3.7 Assembly and Release

New viral genomes are packaged into viral particles by the viral structural proteins. Virus is released from the hepatocyte in association with host lipoproteins, such that the blood HCV is present as a lipoprotein coated virus.

1.3.8 HCV Genotypes

HCV has been classified into at least 6 different genotypes (1-6), distinguished by differences in 30-35% of their nucleotide sequences. Numerous subtypes have been identified within the genotypes, bearing differences in 20-25% of their nucleotide sequences. The HCV genotypes vary according to geographical distribution, as well as the risk groups involved. The greatest variability occurs in the E1 and E2 glycoproteins. The core and non-structural proteins are highly conserved and the 5' UTR contains the least variability.

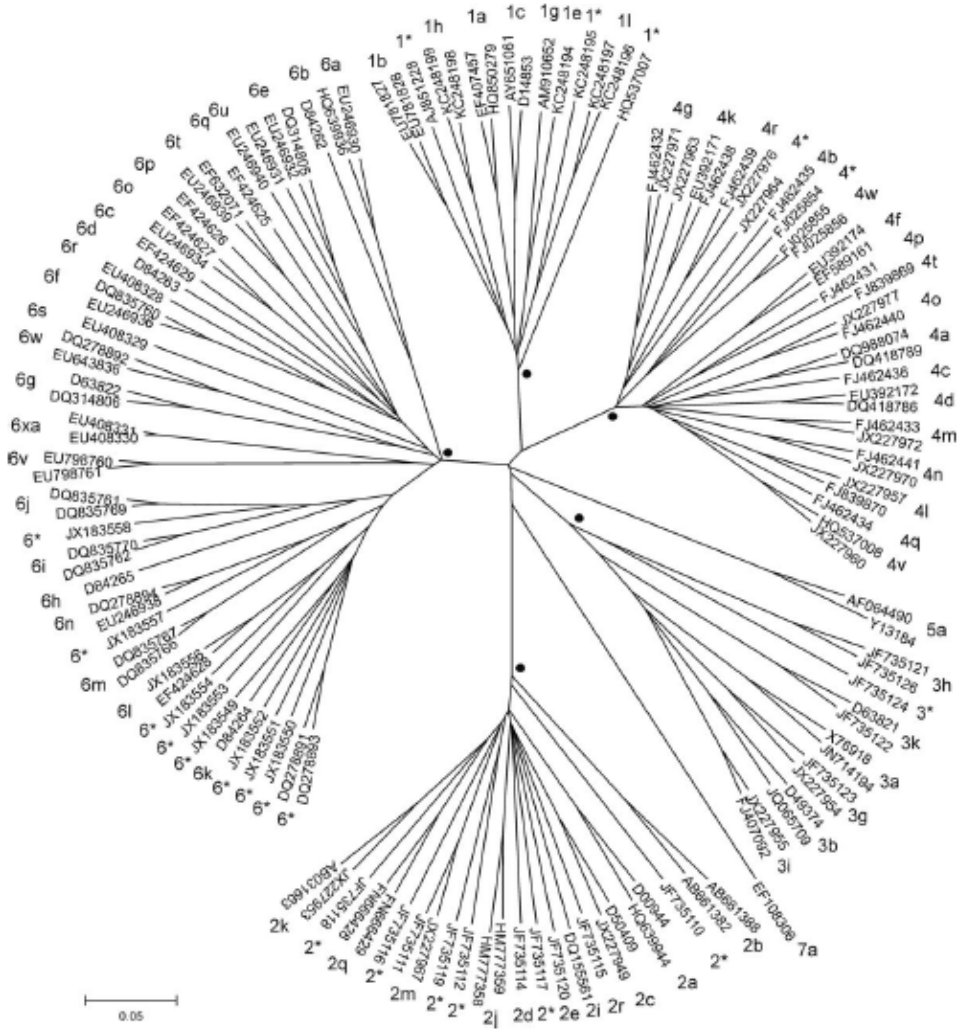


Fig. 1. Phylogenetic tree of 129 representative complete coding region sequences. Up to two representatives of each confirmed genotype/subtype were aligned (together with a third extreme variant of subtypes 4g and 6e) and a neighbor joining tree constructed using maximum composite likelihood nucleotide distances between coding regions using MEGA5.[83] Sequences were chosen to illustrate the maximum diversity within a subtype. Tips are labeled by accession number and subtype (*unassigned subtype). For genotypes 1, 2, 3, 4, and 6, the lowest common branch shared by all subtypes and supported by 100% of bootstrap replicates (n = 1,000) is indicated by •.

Figure 1.3 Phylogenetic tree of hepatitis C virus.[29]

1.3.8.1 Viral Epidemiology in Australia

HCV-1 is the most common genotype in Australia (55%), followed by HCV-3 (38%), and HCV-2 and HCV-4 in 7% and <1%, respectively.[30] Europe has similar prevalence rates to Australia.[31] Asia reports similar prevalence of HCV genotypes to Australia, but HCV-2 (18.6%) and HCV-6 (7.0%) are more common. In America, the prevalence of HCV-1 is higher (74.5%), with lower rates of HCV-3 (10.6%) and higher rates of HCV-2 (10.2%). In contrast, HCV-1 (26.3%), HCV-2 (23.7%) and HCV-4 (28.1%) are equally common in Africa, with HCV-3 (6.3%) the least common.

1.4 Diagnosis Of HCV

The screening blood test for hepatitis C is the anti-HCV antibody. If the anti-HCV antibody is positive, hepatitis C infection must be confirmed with a nucleic acid test for HCV RNA called the HCV PCR (polymerase chain reaction) assay. A HCV PCR detects the presence of the hepatitis C virus in the blood.

An individual with a positive anti-HCV antibody but a negative HCV PCR does not have hepatitis C infection. This would indicate that the individual has had previous exposure to hepatitis C, and has either spontaneously cleared the virus, or has achieved viral eradication with anti-viral therapy.

1.4.1 Acute Hepatitis C

Acute hepatitis C is defined as infection with HCV within the first six months of exposure. This can be identified with a positive HCV PCR and an undetectable anti-HCV antibody that within 12-weeks will become detectable.

1.4.2 Chronic Hepatitis C

Chronic HCV infection is defined as infection with HCV for greater than 6-months, confirmed by the detection of a positive HCV PCR for duration of greater than 6-months.

1.5 Natural history of HCV

15–50% of individuals with acute HCV infection spontaneously clear the virus.[2] The majority of individuals infected with HCV develop chronic infection, defined by

positive PCR for HCV RNA in serum / plasma for > 6 months. The majority of individuals with acute HCV infection are asymptomatic, but some may experience non-specific symptoms, such as fatigue, malaise, nausea, anorexia and mild to moderate abdominal pain. A small proportion develop clinical jaundice, and of those who are symptomatic, it may occur in up to 70%.[32] Clinical jaundice, elevated ALT levels, female gender, genotype 1, HBsAg positivity and IFNL4 polymorphisms all predict for spontaneous clearance.[33,34]

Chronic hepatitis C is characterized by hepatic inflammation, thought to be driven by the immune response to HCV viremia (the HCV is not directly cytopathic). It is typically a slowly progressive disease with studies suggesting that up to 10-20% will develop cirrhosis after 20-30 years of infection.[35] Once cirrhosis is established, individuals carry a 1.5% annual risk of developing hepato-cellular carcinoma and a 4% annual risk of hepatic decompensation.

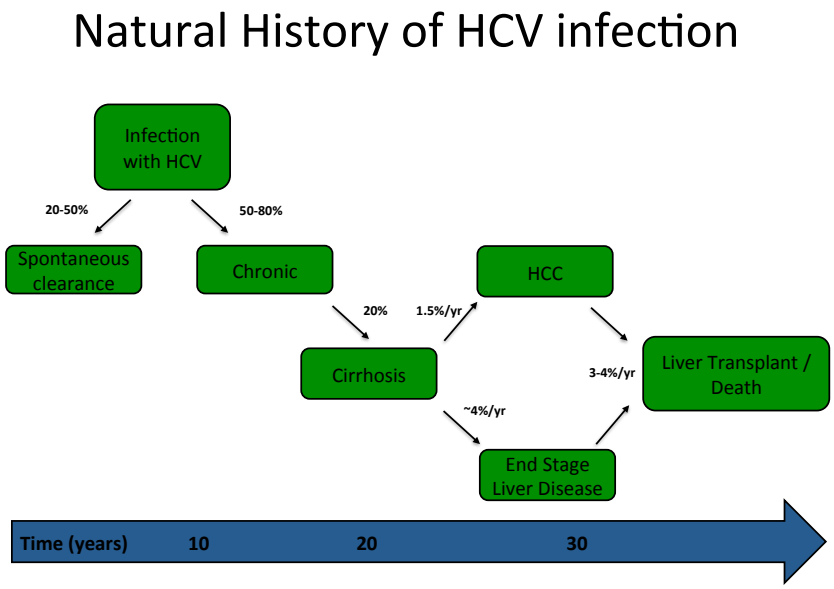


Figure 1.4 Natural History of HCV Infection.

1.5.1 Factors Associated with Fibrosis Progression and the Development of Cirrhosis

Factors that influence fibrosis progression are dependent on the stage of liver fibrosis of at initial assessment, either assessed via liver biopsy or non-invasive means, or by a myriad of host and viral factors.

Liver histology, informing the level inflammation and fibrosis stage, at initial assessment has been shown to predict disease progression.[36,37] A small cohort study showed that mild liver fibrosis at baseline conferred a 1.2% annual risk of cirrhosis progression, compared to almost all subjects with severe inflammation and bridging fibrosis developing cirrhosis at 10-years. Steatosis, particularly in those with genotype 3 HCV infection, has also been shown to be associated with fibrosis progression.[38-40]

Host related factors have been shown to influence fibrosis progression and include older age (>40 years), male gender, mode of transmission (blood transfusion), heavy alcohol use, co-infection with HIV or hepatitis B, diabetes mellitus or insulin resistance, obesity and vitamin D deficiency; data largely derived from cross-sectional analyses of large cohorts or clinical trials.[40-45] The only protective factor that has been identified to lower the rate of fibrosis progression is coffee consumption.[46,47]

Genetic factors may also influence fibrosis progression. In a study of 574 Caucasian subjects in the United States, seven SNPs were found to predict the development of advanced fibrosis / cirrhosis in CHC infection.[48] This data was validated in 938 subjects from the longitudinal HALT-C trial with an association between this genetic signature and fibrosis progression seen after 3.5-year follow up.[49] Subjects had previously failed pegIFN +/- RBV and were re-treated. Those with breakthrough or relapse at week 20 were randomised to a lower dose pegIFN maintenance therapy or no treatment for 3.5 years, with no benefit shown with maintenance treatment.[50,51] Fibrosis progression was assessed from liver biopsies obtained at baseline, 1.5 and 3.5 years.

There are data to suggest that SVR leads to fibrosis regression. Clinical trials based on paired liver biopsies have shown SVR to be associated with histological improvement in both grade and stage. However, these studies were limited by a short-term follow up of only 12–24 months.[41,42,52] Long-term data demonstrating any benefit of viral eradication on fibrosis progression is limited. Clinical trials evaluating antiviral therapy for HCV have typically used sustained virological response as a surrogate for clinical benefit. Data from Spain suggest that SVR is associated with significant reduction in the risk of progression to cirrhosis in a population of patients with median duration of follow-up of 10 years.[53] A similar retrospective study in Korea observed an

association between SVR and lower risk of cirrhosis over median follow-up of 4 years.[54] However, a recent Cochrane review concluded that there was no evidence that the new IFN-free DAA treatments reduced HCV-related morbidity or all-cause mortality.[55] This was based on 5-month follow up data. As such, more long-term studies are required to determine whether viral eradication does reduce long-term HCV-related morbidity and mortality.

1.6 Clinical Manifestations of HCV

1.6.1 Generalised Symptoms

In the context of compensated liver disease, the majority of individuals with chronic HCV infection are asymptomatic. However, some may report generalised symptoms such as fatigue, general malaise, sleep disturbance, anorexia, nausea, abdominal pain, weakness or diarrhea. It is unclear the degree to which chronic HCV infection contributes to these generalized symptoms. A recent study suggests that fatigue, pain and sleep disturbance are more strongly associated with socio-economic, demographic and psychiatric conditions as opposed to chronic HCV infection itself.[56]

1.6.2 Hepatic Manifestations

After the establishment of cirrhosis, individuals are at risk for hepatic decompensation. Symptoms related to decompensated liver disease and portal hypertension can include icterus (jaundice), reduced mental clarity (hepatic encephalopathy), increase in abdominal girth (ascites), ankle swelling (peripheral edema) or haematemesis and meleana.

1.6.3 Extra-hepatic Manifestations

Many extra-hepatic manifestations have been associated with chronic HCV infection. These occur as a result of the immune / inflammatory response to the virus. One study suggests that up to 40% of subjects with chronic HCV infection will present with at least one extra-hepatic manifestation. This data does not reflect our clinical experience, with the lymphoproliferative and dermatological conditions listed below encountered infrequently.[57] They are as follow:

1.6.3.1 Lymphoproliferative Disorders

Essential Mixed Cryoglobulinemia – This condition is characterised by deposition of immune-complexes into small and medium-sized blood vessels. More than 90% of individuals with this condition will have chronic HCV infection. Approximately half of individuals with chronic HCV infection will have detectable cryoglobulins.[58] Clinical manifestations include renal disease, arthralgias, neurological disease and a leukocytoclastic vasculitis. If individuals have chronic HCV infection, HCV eradication with anti-viral therapy is the mainstay of treatment. Additional therapies include plasmapheresis.

Lymphoma – Chronic HCV infection has been associated with the development of primary hepatic lymphoma, as well as B-cell non Hodgkin lymphoma, with a higher prevalence among those with lymphoma.[59,60] Furthermore, curative HCV therapy has been shown to risk the risk of lymphoma.[61]

1.6.3.2 Dermatological Conditions

Porphyria Cutanea Tarda – This condition presents as a chronic blistering, photo-sensitive skin rash, that results from accumulation of uroporphyrins in the liver, blood and urine due to reduced activity of the hepatic enzyme, uroporphyrinogen decarboxylase. It is also characterised by neurovisceral symptoms, such as abdominal pain and neuropsychiatric changes. The prevalence of HCV in such individual has been reported as up to 50%.[62] An improvement in symptoms may occur with HCV treatment.

Lichen Planus – This condition presents as hyperpigmented, flattened, violaceous skin papules, but may also involve the hair, nails and mucous membranes. The prevalence of HCV in individuals with lichen planus has been reported from 10 to 40%.[3]

1.6.3.3 Thyroid Disease

Thyroid disease has been found to be more common in individuals with chronic HCV infection. A prevalence of 2-13% of thyroid disease in CHC has been reported, being most common in older women.[5,7]

1.6.3.4 Diabetes Mellitus

A link between HCV and Type 2 diabetes mellitus has been suggested by several epidemiological studies. A meta-analysis reported an odds ratio of 1.7 of developing diabetes in the setting of HCV.[8] HCV has also been linked to the development of insulin resistance, but the underlying mechanism between the two conditions are still not known.

1.7 Treatment of HCV

1.7.1 Chronic HCV Infection

1.7.1.1 The Evolution of Antiviral Therapy for Chronic HCV Infection

The treatment for chronic HCV infection has changed dramatically over the past two decades. In the mid 1990s, treatment consisted of standard IFN at 3MU three times weekly for 12 months for treatment-naive chronic HCV patients, with a sustained virological response rate (SVR, defined as a negative HCV PCR 3-months post anti-viral therapy) of only 16%.[10] Ribavirin, a synthetic guanosine nucleoside analogue with antiviral therapy against DNA and RNA viruses, administered as monotherapy was found to improve serum transaminases for the duration of treatment but not at follow up, and had no effect on HCV RNA levels.[12,14] However, in combination with standard interferon proved to double rates of SVR of up to 33% with 24 weeks of therapy and up to 42% with 48 weeks of therapy.[15,17] This effect was attributed to lower relapse rates, more so than the higher rates of end of treatment response with the use of ribavirin.

Treatment response rates were still considered suboptimal and it was hypothesised that low rates of SVR could be partly related to inadequate serum concentrations of the interferon, in view of its short, 8-hour half-life. Viral kinetic studies of HCV indicated a high rate of turnover and an in-vivo half-life of only a few hours[18,20] suggesting that interferon was not providing enough immune pressure on the virus. The pharmacological properties of interferon were thus improved, with the covalent attachment of a branched 40-kd polyethylene glycol moiety to IFN-α-2a (pegylation), resulting in reduced clearance (10-fold) and a more sustained absorption (time to peak plasma concentration increased 7-fold), permitting once-weekly dosing. Pegylation also reduced immunogenicity of the interferon. Three pivotal registration

trials established pegylated interferon and ribavirin as standard of care for chronic hepatitis C infection for the next decade.[21,23,25] These studies delineated differences in response between HCV genotypes with SVR rates for Genotype 1 HCV of 42-46% with 48-weeks of therapy, in comparison to HCV-2/3 with rates of 76-82% requiring only 24 weeks of therapy and low dose daily ribavirin (800mg). Long-term studies confirmed that a SVR, defined as an undetectable serum HCV RNA 6-months post treatment, could safely be considered a cure.[52,63,64]

At the commencement of this thesis, we witnessed the advent of the first wave of direct acting antiviral agents (DAAs), in the form of the first generation NS3/4A serine protease inhibitors - Boceprevir and Telaprevir. Addition of these DAAs into the HCV treatment armamentarium revolutionised the treatment of HCV, with achievable SVR rates of up to 63-75% with Genotype 1 HCV (similar to that of HCV-2 and HCV-3), and the potential to reduce treatment duration with early virological response.[65,66] A lot of promise and hope was felt in the world of hepatitis C treatment, particularly with the discovery of IFNL4 polymorphisms and the ability to predict treatment response. Despite improved treatment response to the first generation NS3/4A protease inhibitors, the side effect profile of these agents were considerable and treatment remained a challenging process for patients. The second wave, first generation NS3/4A protease inhibitor, simeprevir saw improved tolerability however still required the use of pegylated interferon and ribavirin.[67,68] Biomarkers predicting treatment response during this era of DAAs in combination with PR remained clinically important. SVR rates were still suboptimal, particularly in the cirrhotics, and the decision as to whether to treat or await the newer DAAs due to the associated toxicity, was important in clinical practice.

Over the next few years, we bore witness to the rapid development of different classes of DAAs geared to targeting multiple steps in the HCV replication cycle. Combination therapy using the DAAs targeting different parts of HCV life cycle is now the standard of care, negating the requirement for pegylated interferon.

1.7.1.2 Current Treatment Regimens for Chronic HCV Infection

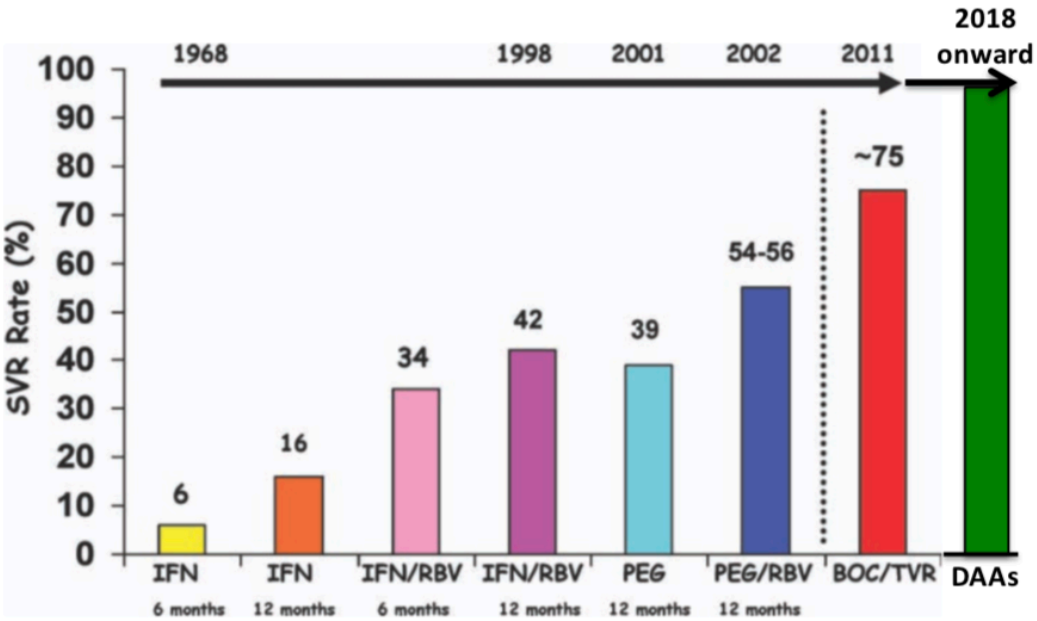


Figure 1.5 SVR rates according to differing treatment regimen and durations[69]

In March 2016, four of the newer direct antiviral agents were listed on the Australian Pharmaceutical Benefits Scheme (PBS) for the first time. All individuals with CHC, regardless of fibrosis stage (except Genotype 4/6) were now able to receive IFN-free treatment in the form of a combination of DAAs. Such regimens had been available in the United States and Europe from late 2014, with access in Australia limited to those participating in clinical trials, through compassionate access via the pharmaceutical company (typically reserved for advanced liver disease or IFN intolerability) or through the direct purchase of DAAs from the pharmaceutical company afforded by the patient themselves. The IFN-free regimens were initially limited to treatment of genotype 1 / 2 / 3, however the recent PBS listing of a pan-genotypic treatment option now allows for treatment of Genotype 4 and 6.

1.7.1.3 Direct Acting Antiviral Agents

The different subclasses of DAAs are discussed below.

1.7.1.3.1 NS3/4A Protease Inhibitors

The function of the HCV NS3/4A protease is to cleave the HCV polyprotein to generate mature non-structural proteins, essential for viral replication. The currently

approved third generation NS3/4a protease inhibitors in Australia include grazoprevir, paritaprevir (ritonavir-boosted), glecaprevir and voxilaprevir.

1.7.1.3.2 NS5A Inhibitors

The NS5A protein is essential for viral replication and assembly, however its complete structure and function is yet to be determined. The currently approved NS5A inhibitors available in Australia include velpatasvir, pibrentasvir ledipasvir, ombitasvir, daclatasvir and elbasvir.

1.7.1.3.3 NS5B Inhibitors

The NS5B protein is an RNA-dependent RNA polymerase that uses the HCV RNA virus as a template, to transcribe double-stranded messenger RNA. There are two subclasses of NS5B inhibitors – nucleoside/nucleotide analogue inhibitors (NIs) and non-nucleoside analogue inhibitors (NNIs). NIs target the catalytic site within the NS5B polymerase. As a false substrate, its incorporation into newly synthesised viral RNA leads termination of the transcription process. Through this mechanism of action, NIs act against all HCV viral genotypes and have a high barrier to resistance. In Australia, sofosbuvir is the first approved nucleotide inhibitor. NNIs also bind to the NS5B polymerase, however induces a conformational change, blocking the function of the polymerase. Dasabuvir is the only approved NNI inhibitor in Australia.

1.7.1.3.4 Ribavirin

Ribavirin is a guanosine analogue with antiviral activity against hepatitis C. Its exact mechanism is not known, however in combination with pegylated IFN-α, was essential in reducing the rates of relapse and breakthrough. In the era of the potent DAAs, ribavirin still has an important role, particularly in the more difficult to treat cirrhotic patients, offering shorter treatment duration as well as improved cure rates.

1.7.1.4 Approved IFN-free regimens for chronic HCV infection in Australia

1.7.1.4.1 Genotype 1a/1b HCV infection

Sofosbuvir plus Velptastavir

Treatment is for 12-weeks and is associated with cure rates of >=95%.[70]

Sofosbuvir plus ledipasvir

Treatment is for 12-weeks and is associated with cure rates of >= 95%.[71] The exception is for prior non-responders to pegIFN therapy who have cirrhosis require 24-weeks of treatment, with cure rates still at >=95%.[72] An abbreviated 8-weeks of treatment may be considered in individuals who are treatment naïve, non-cirrhotic and have a baseline HCV VL < 6 x 10^6 IU/mL.

Elbasvir plus grazoprevir

Treatment is 12-weeks for Genotype 1b HCV infection.[73,74] Among individuals with Genotype 1a HCV who are either treatment naïve, prior relapsers to pegIFN/RBV alone or pegIFN/RBV in combination with simeprevir, boceprevir or telaprevir, treatment is for 12-weeks. Among HCV-1a partial and non-responders to pegIFN/RBV alone or in combination with simeprevir, boceprevir or telaprevir, treatment is for 16-weeks. All regimens are associated with cure rates >=95%. As grazoprevir is a protease inhibitor, this regimen is contra-indicated in individuals with history of hepatic decompensation or decompensated cirrhosis.

Sofosbuvir plus daclatasvir

Treatment is for 12-weeks and associated with >=95% cure rates, with a few exceptions.[75] Individuals with cirrhosis should receive 24-weeks of treatment, or 12-weeks if administered with ribavirin. Secondly, 24-weeks of treatment is recommended in those who have failed triple therapy, in the form of pegIFN/RBV in combination with a protease inhibitor (simeprevir, boceprevir or telaprevir).

Paritaprevir–ritonavir, ombitasvir and dasabuvir ± ribavirin

This regimen is associated with cure rates of >=95%.[76-79] Treatment for HCV-1a is 12-weeks with the addition of ribavirin, except in those who have cirrhosis or history of null-response to pegIFN/RBV, and require 24-weeks. Treatment for HCV-1b infection is 12-weeks without ribavirin. This regimen contains the protease inhibitor, paritaprevir, and is therefore not recommended in those who have previously failed protease inhibitor therapy.

1.7.1.4.2 Genotype 2 HCV infection

Sofosbuvir plus Velpatasvir

Treatment is for 12-weeks and is associated with cure rates of >=95%.[70,80]

1.7.1.4.3 Genotype 3 HCV infection

Sofosbuvir plus Velptastavir

Treatment is for 12-weeks and is associated with cure rates of >=95%.[80] However, the addition of ribavirin should be considered in subjects who have failed pegIFN/RBV or have cirrhosis, with lower cure rates (89-93%) observed in clinical trials.[80]

Sofosbuvir plus daclatasvir

Treatment is for 12-weeks and associated with cure rates of 94-97% cure rates, with a few exceptions.[81] Individuals with cirrhosis have proven to be more difficult to cure, and should receive either 24-weeks of treatment or 12-weeks with the addition of ribavirin. These regimens are associated with a similar rate of cure at 86%. Secondly, 24-weeks of treatment is recommended in those who have previously triple therapy, in the form of pegIFN/RBV in combination with a protease inhibitor (simeprevir, boceprevir or telaprevir).

1.7.1.4.4 Genotype 4 HCV infection

Elbasvir plus grazoprevir

Treatment is for 12-weeks in individuals who are treatment naïve, and is associated with cure rates of 96%.[82] The addition of weight-based RBV and extending the treatment to 16-weeks is recommended for those who have previously failed pegIFN/RBV.

Sofosbuvir plus Velpatasvir

Treatment is for 12-weeks and associated with cure rates of 100%.(70)

Glecaprevir + Pibrentasvir

Treatment is for 8-weeks for subjects without cirrhosis, and 12-weeks for those with cirrhosis, and associated with cure rates of 93% and 100% (n=16), respectively.[83,84] This regimen is not recommended in subjects with Child Pugh B/C cirrhosis as this regimen includes an NS3/4a protease inhibitor.

1.7.1.4.5 Genotype 5 and 6 HCV infection

Sofosbuvir plus Velpatasvir

Treatment is for 12-weeks and associated with cure rates of 97% and 100% for genotype 5 and 6, respectively.[70]

Glecaprevir + Pibrentasvir

Treatment is for 8-weeks and associated with cure rates of 100% in patient without cirrhosis.[83,84] Treatment duration should be extended to 12-weeks in those with cirrhosis. This regimen is not recommended in subjects with Child Pugh B/C cirrhosis as this regimen includes an NS3/4a protease inhibitor.

DAA Regimen	Genotype	Duration	
		Non cirrhotic	Cirrhosis
Sofosbuvir plus Velptastavir	1a/b, 2, 3, 4, 5, 6	12-weeks	12-weeks
Sofosbuvir plus ledipasvir	1a/b	12-weeks	12-weeks
Elbasvir plus grazoprevir	1b 1a* 1a# 4 4**	12-weeks 12-weeks 16-weeks 12-weeks 16-weeks + ribavirin	12-weeks Contra-indicated in Child Pugh B/C cirrhosis
Sofosbuvir plus daclatasvir	1a/b, 3 1a/b, 3^	12-weeks 24-weeks	24-weeks, or 12-weeks + ribavirin
Paritaprevir–ritonavir, ombitasvir and dasabuvir	1a 1b	12 weeks + ribavirin 12 weeks	24-weeks + ribavirin 12-weeks
Glecaprevir + Pibrentasvir	4, 5, 6	8-weeks	12-weeks Contra-indicated in Child Pugh B/C cirrhosis

*relapsers to PR alone or PR + simeprevir, boceprevir or telaprevir
#partial or non responders to PR alone or PR + simeprevir, boceprevir or telaprevir
^failed triple therapy with PR + simeprevir, boceprevir or telaprevir
**failed PR

Table 1.1 Approved IFN-free regimens for chronic HCV infection in Australia

1.7.2 Acute HCV Infection

In the era of IFN-free DAA therapies, there is limited evidence with regards to the approach for treatment of acute HCV infection. In Australia, the IFN-free regimens require an individual to have 'chronic infection', defined as HCV seropositivity for 6-months with a detectable HCV viral load. Therefore, observation of individuals for acute HCV infection during the first 6-months should be considered and if spontaneous

clearance as not occurred at 6-months, treatment would be as for chronic hepatitis C infection.

1.8 Immunopathogenesis of HCV

The immune system relies on both an innate and adaptive response to combat invasion by microbial pathogens.

1.8.1 Innate immune response

1.8.1.1 Pattern Recognition Receptors are Thought to Initiate the Innate Immune Response to HCV

PRRs signaling plays a role in the pathogenesis of HCV. PRRS include the TLRs as well as RIG-I and PKR. Thirteen TLRs have been described so far, each responsible for recognition of different PAMPs, with ten functional TLRs identified in humans. A type 1 transmembrane protein, TLRs are characterised by a leucine-rich repeat (LRR) domain and a conserved region known as toll-interleukin (IL-1) receptor (TIR) domain. TLRs survey both the extracellular space (transmembrane TLR) as well as the cytoplasm (endosomal TLR). Bacterial components are detected by TLR2, 4 and 5 that reside on the cell surface. Specifically, TLR4 recognises lipopolysaccharide (LPS), an integral component of the outer membrane of gram-negative bacteria, TLR5 recognises flagellin, and TLR2 recognises lipoproteins and glycoproteins present in a variety of bacteria, yeasts and mycoplasma (Figure 1.6). TLR2 obtains its broad range of specificity through heterodimerisation with TLR1 and TLR6, recognising triacyl lipopeptides and diacyl lipopeptides, respectively. Various adapter and accessory molecules, such as MD-2 and CD14, also influence specificities of the TLRs.(85) Detection of bacterial and viral nucleic acids are mediated by TLR3, TLR7/8 and TLR9, all located within the cell in the endosomal/lysosomal compartment, although TLR3 can be detected on the cell surface in certain cell types.[86] It is postulated that the intracellular localisation of nucleic acid-specific TLRs minimises the potential reactivity with self-DNA and RNA.[87] TLR3 mediates the recognition of double stranded RNA, TLR7/8 recognises viral single-stranded RNA and TLR9 recognises unmethylated CpG DNA from bacteria and viruses.

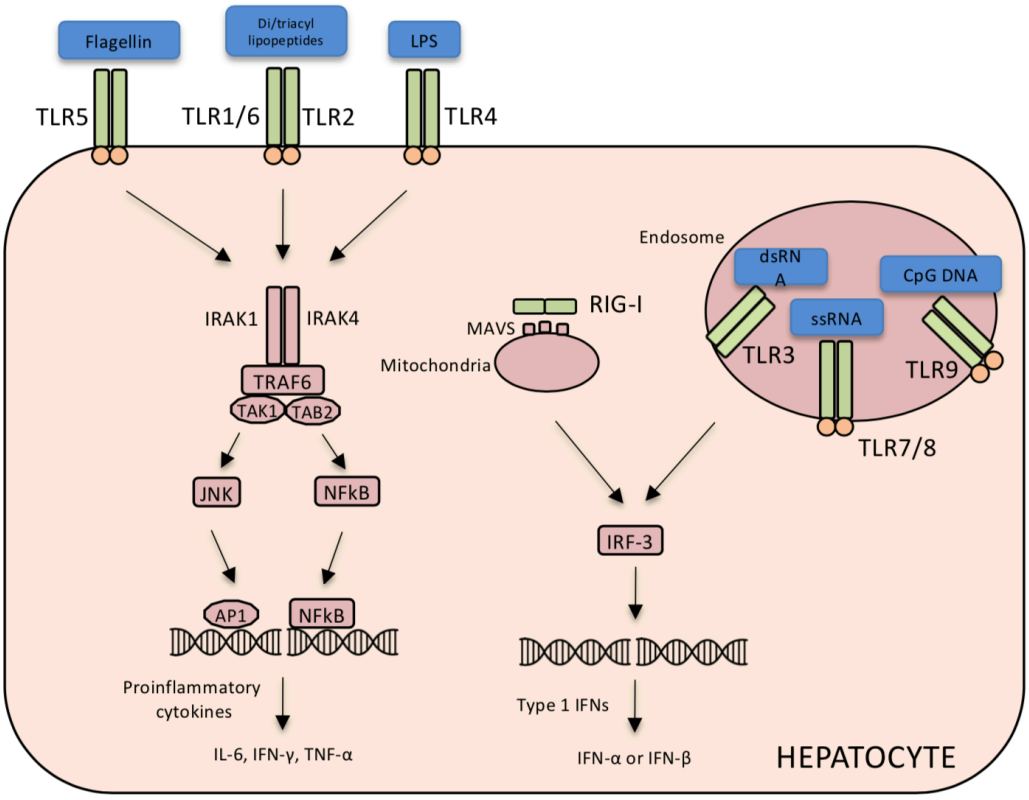


Figure 1.6 Innate immunity – the first line of defence

HCV is a non-cytolytic virus and viral replication within the hepatocyte drives the host immune response and underpins the pathogenesis of the virus. During viral replication within the hepatocyte, the host detects the presence of HCV, as 'non-self' nucleic acid via the cytosolic helicase retinoic acid induced gene-I (RIG-I) receptor or the endosomal toll-like 3 receptor (TLR3) (Figure 1.7). Recognition by RIG-I has been shown in vitro to occur within hours of infection, triggering downstream signaling before extensive viral protein synthesis has occurred.(88) RIG-I binds to the exposed 5' triphosphate and the 3' UTR region of the HCV RNA genome, rich in poly-U/UC nucleotides. In contrast, TLR3 senses HCV double-stranded RNA intermediates that accumulate late in HCV viral replication, shown to initiate downstream signaling in cultured hepatoma cells, 3-4 days after infection.[89] In vitro studies have identified specific components of the HCV polyprotein that interact with TLR2 and TLR4 receptors. TLR2 is expressed on the plasma membrane and has been shown to recognise the HCV core and NS3 protein[90,91] HCV NS5A protein interacts with TLR4.[92,93]

The endosomal TLR7/8 receptors detect single stranded RNA, and have been shown to sense the HCV genomic RNA mounting an inflammatory response.[94] The TLR9 receptors recognises genomic DNA, however there is no data yet to show a direct interaction with HCV.[94,95]

During HCV viral replication and activation of RIG-I or TLR3, a conformational change in the receptors leads to the recruitment of adaptor molecules mitochondrial antiviral signaling protein (MAVS) and toll-interleukin-receptor domain containing adaptor protein-inducing interferon beta (TRIF), respectively, that then prompt a downstream effect leading to the formation of transcription factors, IFN-regulatory factor 3 (IRF3) and IFN-regulatory factor 7 (IRF7). Dimerisation of IRF3 occurs, which enters the nucleus and targets the promoter for the type 1 IFN, IFN-β and other antiviral genes. IFN-β engages the IFN-α/beta receptor on the cell surface of hepatocytes in an autocrine and paracrine manner, and activates the JAK-STAT signaling pathway. Jak-1 phosphorylates STAT1 and STAT2, and together with IRF9 form ISG factor 3 (ISGF3) transcription factor complex, which binds to IFN-stimulated response elements (ISRE) within the promoters of ISGs to activate gene transcription.

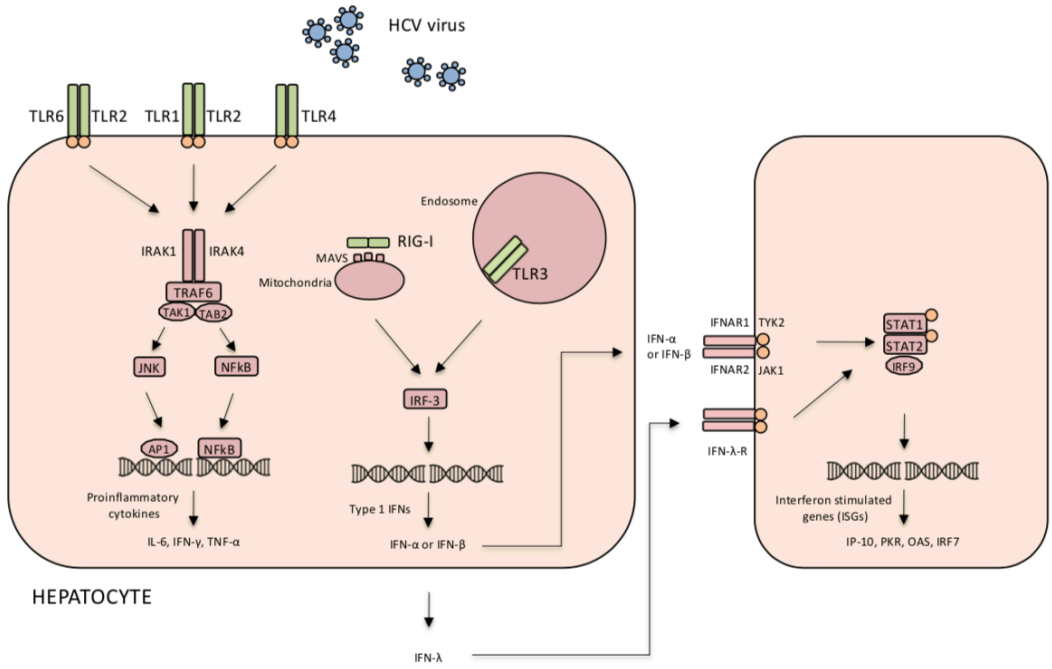


Figure 1.7 Innate immunity has been implicated in the host response to chronic HCV infection

A third pattern recognition receptor has also been identified, a cellular protein kinase PKR that senses the IRES of the HCV RNA genome. Activation of PKR triggers a kinase-independent signaling cascade in primary human hepatocytes, to drive the induction of ISGs and IFN-β, prior to RIG-I activation, using the adaptor proteins MAVS, tumour necrosis factor receptor-associated factor 3, IRF3 and NF-kB.[96]

Previous work in our laboratory has shown that TLR2/4 expression is up-regulated in CHC compared to healthy controls, on peripheral CD14+ monocytes and hepatic mRNA.[97,98] Increased peripheral monocytic TLR2 expression correlated with increased circulating TNF-α levels, and TLR2/4 hepatic mRNA expression correlated with hepatic necro-inflammatory activity. Other groups have produced data to support our findings.[92,99-102]

There is conflicting data however, regarding TLR signaling in the setting of CHC. TLR4 signaling has been shown to be compromised in 13 women with CHC with reduced IL-6 response, compared to healthy controls.[103] In another study that included 31 subjects with CHC, TLR 2 / 4 signaling was shown to be up-regulated with an increase in IL-6, IL-8, TNF-αlpha and IFN-β response.[99] There is no data yet to determine if there is correlation between TLR expression / signaling and IFNL3 / IFNL4 polymorphisms.

1.8.1.2 Interferon Signaling and Interferon Stimulated Genes

The type 1 IFN system in response to invading pathogens activates over 380 human ISGs. Among these, ISGs with specific anti-HCV activity have been identified through microarray RNA analyses of liver and peripheral blood mononuclear cells of chimpanzees and HCV infected patients and further characterised in in vitro studies.[104-107] Intra-hepatic up-regulation of 2',5'- oligoadenylate synthetase-like (OASL) gene correlates with a high HCV viral load, and in vitro inhibits HCV viral replication through degradation of RNA.[108] Activated PKR acts in parallel with the IRF3-activated gene P56, to both prevent initiation of viral protein translation, through the phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2α), and the binding of P56 to eIF3, respectively.[109,110] An RNA editing enzyme, adenosine deaminase that acts on dsRNA (ADAR1), catalyses the deamination of adenosines in dsRNA, leading to unstable RNA and a high propensity for mutations.[111] Finally, intra-hepatic viperin

expression is up-regulated in HCV patients[112] and has been shown to interact with the NS5A protein in vitro to limit viral replication.[113]

IRF7, IRF1, RIG-I, MDA5 and IRF2 are ISGs shown in a cell culture model to specifically inhibit HCV replication, impairing translation of the incoming genome.[114] This implies that ISGs target early in the life cycle of the virus. In microarray RNA analyses of peripheral blood mononuclear cells from HCV patients receiving treatment, Mx1, OASL, OAS2 and viperin were ISGs shown to be up-regulated, correlating with HCV viral load reduction.[107]

Type 3 IFNs are also produced in response to HCV infection or stimulation with the TLR3 ligand, polyinosinic:polycytidylic acid (poly I:C) – a synthetic analog of double-stranded RNA. They are produced at higher levels than type 1 IFNs, and ISG induction has been shown to correlate with type 3 but not type 1 IFN production.[115-117] This suggests that the type 3 IFN system may functionally be the predominant antiviral pathway induced in hepatocytes, rather than type 1 IFNs, and is largely responsible for the observed patterns of ISG induction. The importance of up-regulation of these antiviral genes by HCV is underscored by the fact that HCV-infected patients exhibiting high levels of intra-hepatic ISG expression are less responsive to pegIFN-α and ribavirin. This was evident in 16 patients, treated with PegIFN/RBV with liver biopsy/PBMCs obtained prior to treatment and 4-hours post PegIFN injection.[118]

The potential role of type 3 IFNs in the pathogenesis of HCV is intriguing, particularly in view of the discovery of the association between IFNL4 polymorphism and spontaneous clearance of HCV as well as IFN treatment response (see below). IFNL4 polymorphisms are associated with intra-hepatic ISG expression and may explain the relationship between hepatic ISG expression and HCV treatment outcome.[119,120] No association has been found however, between IFNL4 genotype and IFNL4 mRNA expression.[120-123] It may be that the poor response IFNL4 genotype leads to 'exhaustion' of the type 1 IFN response, incapable of further activation to IFN-based antiviral therapy. In the case of the good-response genotype however, signaling is quiet and therefore primed for a strong type 1 IFN response in the context of IFN-based therapy.

ISG expression has been shown to differ between different cell populations in the liver. In non-responders to IFN-based therapy, ISG up-regulation is more pronounced in hepatocytes, but more pronounced in Kupffer cells in responders.[124] Hence, it is possible that the IFNL4 polymorphisms have cell-specific effects on expression, not detected in the previous studies. This is an evolving area that still needs to be solved.

During the course of this PhD, down-regulation of ISG expression has been shown to occur in hepatocytes and peripherally, in the context of the new IFN-free DAA therapies regardless of treatment outcome.[125] Interestingly, this study showed that Type 1 IFN hepatic ISG expression at the end of treatment was higher among those who achieved SVR, as opposed to patients who later relapsed. The authors of this study hypothesised that innate immunity this may be in fact facilitating viral eradication in the context of the DAAs. A more recent small study showed that higher baseline hepatic ISG expression and an activated peripheral NK phenotype was also associated with SVR to DAA treatment.[126]

1.8.1.3 Cytokines and Chemokines

T helper type 1 (Th1) and T helper type 2 (Th2) cells are a vital part of the anti-viral response. Th1 cells drive the cell-mediated response through production of IL-2, TNF-α and IFN-γ, promoting activation of macrophages, cytotoxic CD8+T cells and NK cells. The humoral immune response is the domain of the Th2 cells, effected through cytokines such as IL-4 and IL-5 production that activate helper CD4+ T-cells promoting antibody production via B cells. IL-10, an immune-modulatory cytokine is also a product of Th2 cells that inhibits Th1 cytokine production and T cell proliferation, but is also produced by macrophages, dendritic cells and B cells. Chemokine response to viral infection is also an integral part of the innate immune response, leading to activation and migration of effector lymphocytes to the site of infection.

Subjects with CHC present with an activated intra-hepatic and peripheral cytokine / chemokine milieu. Numerous studies have shown CHC subjects to have elevated levels of circulating pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-6, IL-8 and IL-12.[92,97,127-132] Cytokine response to chronic HCV may play an important role in treatment response, as well as rates of liver fibrosis progression. In 47 CHC subjects,

low pre-treatment levels of serum TNF-α predicted a sustained virological response to IFN-based therapy.[127] This same group followed 56 untreated CHC subjects with paired liver biopsies at a 3-year interval and showed serum TNF-α levels to correlate with the degree of hepatic inflammation at baseline, and at follow-up.[128] Interestingly, the anti-inflammatory cytokine IL-10 is also elevated in CHC, with low levels predicting SVR in 79 CHC subjects who received IFN-based therapy.[133] A more recent study of 18 subjects with CHC found an association between high serum IL-10 levels and mild liver fibrosis, and low serum IL-10 and high levels of the pro-inflammatory cytokines IL-2, IFN-γ and TNF-α with severe fibrosis.[134]

Chemokines also play a role in the pathogenesis of HCV. IP-10 is a potent chemokine produced by hepatocytes and liver-infiltrating lymphocytes during CHC, and prompts the recruitment of T-cells and NK cells. Low pre-treatment levels of IP-10 have been shown to predict SVR in response to PR in CHC.[135] Riva et al looked at 16 patients with acute HCV, and found lower levels of IP-10, as well as a truncated biologically inactive form of IP-10, in those who spontaneously cleared the infection.[136] They postulated that IP-10, in its truncated form, acts as an antagonist to biologically active IP-10, as it retains its CXCR3 binding ability. This may explain the paradox of high IP-10 levels being associated poor treatment response in the context of CHC.

Mechanistic studies have looked further to elucidate the exact role of such cytokines / chemokines in the pathogenesis of HCV. Dolganiuc et al. elegantly showed increased monocytic production of TNF-α, as well as the anti-inflammatory cytokine IL-10, following TLR2-mediated stimulation with HCV core and NS3 protein.[90,91,137,138] HCV core protein was also shown in vitro, to reduce TLR-9 mediated IFNa production, implying impaired plasmacytoid dendritic cell (pDC) IFN-α production. pDCs being a critical antigen-presenting cell in viral infections producing large amounts Type 1 IFNs. This was posed as potential mechanism in which HCV evades the innate immune response. HCV RNA intermediates have been identified as the key pathogen associated molecular pattern, to trigger TLR3-mediated production of pro-inflammatory cytokines and chemokines.[89] Monocytic-derived and myeloid dendritic cells, but not pDCs, have been shown in PBMCs from CHC subjects, to produce TLR-3 mediated IL-12, IL-6, IL-10 and IFN-γ in response to cell culture produced hepatitis C.[139] NS5A protein has also

been shown in a cell culture model, to be a promoter for the TLR4 gene, leading to up-regulation of TLR4 expression and TLR4 mediated IFN-β and IL-6 production in hepatocytes and B-cells.[93] NS5A also induces IL-8 mRNA and protein expression in vitro, that partially inhibits IFN response.[140]

1.8.1.4 Natural Killer Cells

Natural killer (NK) cells form an integral part of the IFN-driven, innate immune response, constituting 5-15% of the PBMC population and 30% of the intrahepatic lymphocyte population. NK cells may be the key cell population driving the eradication of HCV during anti-viral therapy. Killer immunoglobulin-like receptor (KIR)-HLA genotypes have been associated with HCV outcome, with homozygosity for the KIR2DL3 haplotype predicting HCV clearance in the setting of acute infection.[141,142] The expression of activatory and inhibitory receptors on NK cells have been shown to differ between healthy subjects and those with chronic HCV infection, with an activated NK phenotype present in the setting of CHC.[143] One study compared 42 subjects with CHC to healthy controls, and found TRAIL expression to be up-regulated, with a corresponding increase in NK cell degranulation. IFN-γ response was poor however. It was hypothesised that these conflicting findings may represent NK cells contribution to liver disease progression, but with ineffectual viral clearance. NK phenotype and functional response to IFN-based therapy also differs between responders and non-responders to treatment, with responders demonstrating greater NK cytotoxicity.[144] Furthermore, higher pretreatment levels of NK inhibitory receptors is associated with the unfavourable IFNL3 genotype.[145] More recently, NK cell function was shown to be restored in the setting of IFN-free antiviral therapy.[146]

1.8.1.5 IFNL4 Genotype

Please refer to Chapter Appendices 1.10 for review on IFNL4 genotype. In late 2009 / early 2010, four independent groups simultaneously performed genome-wide association studies and identified linked single nucleotide polymorphisms (SNPs) in the region of the IFNL3 gene on chromosome 19 that predicted for viral eradication with pegylated IFN-α and RBV, for genotype 1 chronic HCV infection.[147-150] This landmark discovery had immediate clinical relevance for treatment decision-making, informing the timing of therapy prior to the availability of direct acting antiviral agents.

Individuals carrying the good response genotype were 2-3 fold more likely to be cured. IFNL4 polymorphism was subsequently associated with spontaneous clearance of acute HCV infection also.[150-152]

The top discovery SNPs were rs12979860 and/or rs8099917. These are both tag SNPs for a causal variant on a haplotype block in the region of the IFNL4 gene. The causal variant has not yet been identified. Rs12979860 and rs8099917 are in very strong linkage disequilibrium in most populations, and are similarly informative. The exception is patients of African ancestry where rs12979860 is more strongly associated with viral eradication. The frequency of the good response variant varies between patients of different ethnic background, being most common in Asian populations, and least common in patients with African ancestry.[147,151] This difference in genotype frequency between patients of different ethnic background explained much of the differences in pegIFN and RBV response rates historically observed according to ethnicity.[153-156]

The mechanism explaining the association between IFNL4 polymorphism and IFN treatment response is unclear. The IFNL4 gene is found on chromosome 19 and encodes for the antiviral cytokine, interferon lambda 3 (IFN-λ3).[157,158] This type 3 IFN, binds to a unique cell surface receptor, the IFN-lambda-receptor (IFNLR) that activates the Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathway and leads to the induction of interferon-stimulator genes (ISGs). The downstream signaling pathway is shared with the type 1 IFN receptor. A key point of distinction between the type 1 and type 3 IFN receptors is the distribution of expression, where type 1 IFN receptors are ubiquitous, but type 3 IFN receptors are restricted to epithelial cells, including hepatocytes, and plasmacytoid dendritic cells, but are not expressed by most haemopoietic cells.[159] IFN-λ inhibits HCV replication in cell culture models as well as in vivo.[160-162] Data suggests type 3 IFN may be the dominant IFN produced by the liver in response to HCV infection.[115,116]

As yet there is no convincing data to show a relationship between IFNL4 genotype and IFN-λ-3 expression in the liver or peripheral blood mononuclear cells.[120-123] This suggests that IFNL4 polymorphism may be associated with functional consequences for IFN-λ-3.[120,122,147] IFNL4 genotype has been associated with levels of

intrahepatic ISG expression, where patients with the good response variant display low level expression.[120-122,163] This is consistent with previous data linking liver ISG expression to IFN treatment response.[118] IFNL4 genotype has also been associated with NK cell activity.[145]

More recently, the IFNL4 gene was discovered through RNA sequencing performed in primary human hepatocytes from a liver donor uninfected with HCV, stimulated with the TLR3 ligand, poly I:C.[164] Found between IFNL3 and IFNL2, a dinucleotide polymorphism of IFNL4 (ss469415590, ΔG/TT) creates (ΔG) or disrupts (TT) an open reading frame, with transcription of either a functional or dysfunctional antiviral cytokine, IFN-λ4, respectively. Transcription of the unfavourable IFNL4 polymorphism results in transient production of IFN-λ4, activating STAT1 and STAT2 downstream of the JAK-STAT signaling pathway, leading to ISG induction. The favourable IFNL4 polymorphism leads to no IFN-λ4 production.[165] Similar to IFNL3 polymorphisms, IFNL4 polymorphims are associated with IFN-based treatment response and intra-hepatic ISG expression.[119] It is in complete linkage disequilibrium with the unfavourable genotype of the two top IFNL3 polymorphisms (rs8099917 and rs12979860) in individuals of Chinese and European ancestry, but in moderate linkage equilibrium in individuals of African ancestry. IFNL4 polymorphisms are a better predictor of response to IFN-based treatment in this group. Despite the recent discovery of IFNL4 polymorphisms, again the exact mechanism responsible for its strong association with treatment response is yet to be determined.

1.8.2 Adaptive Immune Response

The adaptive immune response to HCV ensues after the initial innate immune response. It is comprised of two components – a humoral response where B-cells recognise circulating pathogens leading to the production of antibodies, and a cell-mediated response involving T-cells that recognise processed viral pathogens within a major histocompatibility complex, presented on the surface of a cell.

1.8.2.1 Humoral Response

In stark contrast to the immediate innate immune response to acute HCV infection, HCV-specific antibodies take approximately 6-8 weeks to develop.[166] This is

despite the HCV virus being detectable in serum within days of infection. HCV-specific antibodies develop against epitopes on the HCV structural and non-structural viral proteins. The majority of these antibodies lack anti-viral activity. Spontaneous clearance has been demonstrated in subjects with hypogammaglobulinemia and primary antibody deficiency, suggesting that the humoral response is not essential in the control of HCV virus.[167]

A small proportion of the antibodies have been shown in vitro, to block HCV binding, entry and uncoating through targeting the hypervariable region-1 (HVR-1) of the HCV envelope glycoproteins, E1 or E2, but are isolate specific.[168,169] These are known as neutralising antibodies and have been associated with HCV clearance.[169,170] This was illustrated in a cohort of 49 women exposed to a single strain of Genotype 1b HCV (isolate AD78) when receiving contaminated anti-D immunoglobulin.[171] Spontaneous clearance was associated with early induction of neutralising antibodies (n=20), with late induction associated with persistent infection (n=19). Despite this association, neutralising antibodies demonstrate cross reactivity against differing HCV genotypes, making it difficult ascertain its exact role in viral clearance.[172-175] A more recent study addressed this issue by testing neutralising antibody response of 52 subjects with acute HCV infection, to a library of 19 HCV envelope glycoproteins derived from subjects with differing strains of genotype 1 HCV infection.[176] An early and broader anti-genotype 1 response was associated with spontaneous clearance, with a delayed response evident in those who developed persistent infection, regardless of HCV genotype. One CHC-infected subject followed for 26-years also demonstrated broadening of its neutralizing antibody response, with on-going escape of the HCV virus from antibody recognition.[177] In a study of 8 subjects with acute HCV infection, neutralising antibodies were shown to drive HCV envelope sequence evolution, with high antibody response in those who attained spontaneous clearance.[174] Further investigation as to the role of neutralising antibodies in controlling HCV viremia will be paramount in the development of a protective HCV vaccine.

1.8.2.2 Cell-mediated T-cell Response

The cell-mediated T-cell response is also evident 6-8 weeks after infection, but is thought to play a more critical role in HCV clearance.[178,179] A CD4+ T-cells response is essential in promoting B-cell mediated antibody production and priming CD8+ T-cells, through cytokine production produced by its respective, Th2 and Th1 cells. A strong and sustained, polyclonal CD4+ T-cell response, targeting numerous epitopes on the viral proteins, is associated with viral clearance.[179-183] This coincides with the presentation of clinical hepatitis and a rise in liver transaminases. A CD8+ T-cell response is also crucial, leading to lysis of virus-infected cells and cytokine production (predominantly TNF-α and IFN-γ) promoting viral clearance. In acute infection, HCV-specific CD8+ T-cell response is initially stunned with impaired IFN-γ production[184,185], however recovery is evident when the CD4+ T-cells come into effect.[186] In the setting of chronic infection, on-going antigenic stimulation leads to dysfunctional T-cell effector function.[187]

1.8.2.3 Viral Evasion Mechanisms

HCV has developed numerous mechanisms to evade the host innate immune response. In vitro studies have demonstrated its ability to block steps in the Type 1 IFN signaling pathway, as well as through direct antagonism of HCV specific anti-viral ISGs, accounting for its ability to establish persistent infection in the host.

Specifically, the HCV NS3/4A protease has been shown in cell culture models to cleave the adaptor molecules, Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) and mitochondrial antiviral signaling protein (MAVS), thereby blocking activation of interferon gene expression through the Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I) pathways.[188,189] The NS3 protein itself, has been shown in vitro to interfere with TLR3-mediated IFN-β production through binding to the protein kinase, TBK1, upstream of the transcription factor IRF3, inhibiting IRF3 activation.[190]

The HCV core protein, through its interference of the JAK/STAT signaling pathway, also attenuates the type 1 IFN response. It directly binds to the STAT1 protein preventing its phosphorylation and activation, as well as induces expression of the negative regulator, suppressor of cytokine signaling 3 (SOCS3).[191,192] In a cell

culture model, this pathway is also blocked through HCV polyprotein induced protein phosphatase 2A expression, causing decreased STAT1 activation and down-regulation of ISGF3-dependent ISG transcription.[193-197]

HCV also has direct effects against specific ISGs. The HCV NS5A protein inhibits the antiviral function of 2'-5' OAS and PKR through direct interaction, but also induces IL-8 expression leading to reduced ISG expression.[132,198-200] PKR is also inhibited by the HCV E2 protein.[201]

1.9 Hypothesis and Aims

1.9.1 Part A

Long-term data proving that viral eradication of chronic HCV reduces long-term HCV-related morbidity and mortality is limited. Clinical trials evaluating antiviral therapy for HCV have typically used sustained virological response as a surrogate for clinical benefit. Therefore, long-term clinical outcomes are not known. This is reflected in a recent Cochrane review that concluded that there was no evidence that the new IFN-free DAA treatments reduced HCV-related morbidity or all-cause mortality.[55] This was based on 5-month follow up data.

Hypothesis 1: Curative HCV treatment would halt fibrosis progression, and therefore improve long-term clinical outcomes.

Aim 1: To evaluate the impact of viral eradication on fibrosis progression over a decade.

Aim 2: To identify variables influencing liver fibrosis progression over a decade.

1.9.2 Part B

Mechanisms underlying viral eradication in the setting of both acute, and chronic HCV infection with anti-viral therapy are not clear. There is a wealth of literature that strongly implicates innate immunology as playing a key role. Spontaneous clearance of acute HCV infection is associated with IFNL4 polymorphisms and low serum chemokine IP-10 levels, but the immunopathogenesis of acute HCV remains poorly understood. The majority of individuals are asymptomatic, and those at highest risk of transmitting and acquiring HCV, are typically a difficult patient population to engage. As a result, there is limited data in this area.

In chronic HCV infection, innate immunology is thought to be a biomarker for IFN response, with poor response characterised by elevated pre-treatment intra-hepatic ISG expression, less pronounced on-treatment changes in PBMC ISG expression, reduced levels of NK cell degranulation, and elevated pre-treatment plasma chemokine IP-10 levels. IFNL4 polymorphisms are also associated with IFN response,

and although the underlying mechanism has not yet been identified, IFNL4 polymorphisms are associated with ISG and IP-10 expression.

When this thesis commenced, pegylated IFN was the backbone of HCV treatment in combination with a protease inhibitor. This was associated with significant toxicity and SVR rate were suboptimal. Therefore, it was very relevant to:

- identify biomarkers predicting SVR to aid clinical decision making;
- identify novel biological pathways that might lead to new therapeutic approaches.

With the introduction of DAA therapy, particularly early DAA therapy, SVR rates were 80-90% and stratification of treatment responses according to immunological markers was still an important goal. Even today, with highly effective DAA therapy, identification of biomarkers predicting high rates of SVR may identify groups suitable for shorter treatment duration.

We therefore wished to explore the innate immunological pathways involved in the spontaneous clearance of acute HCV infection, and viral eradication with HCV treatment in chronic HCV infection.

Hypotheses:

1. Peripheral TLR expression and signaling activity are associated with the clinical outcome of acute hepatitis C infection.

2. Peripheral TLR signaling and expression are associated with response to IFN-free antiviral therapy.

3. Peripheral markers of innate immunity (monocyte ISG expression / NK phenotype and function /cytokine, chemokine expression) are associated with the response to IFN-free antiviral therapy.

Aims:

1. To identify patterns of TLR signaling and expression in individuals who acquire acute HCV infection, comparing spontaneous clearance vs. persistent infection.

2. To investigate the innate immune effects of viral load decline with IFN-free DAAs by examining peripheral TLR expression and signaling, ISG expression, NK phenotype and function and cytokine / chemokine expression.

3. To compare this data to a parallel prospective cohort of patients treated with an IFN-containing regimen, allowing for a direct comparison of the innate immune responses to the different anti-viral therapies.

1.10 Appendix

Curr Hepatitis Rep (2012) 11:136–145
DOI 10.1007/s11901-012-0135-5

IL28B: Current and Future Use

S. G. Chen Yi Mei · P. V. Desmond · A. J. Thompson

Published online: 28 July 2012

Abstract Host genetic variability in the vicinity of the IL28B gene is strongly associated with the outcome of pegylated interferon-α and ribavirin treatment for genotype 1 hepatitis C infection. In the setting of protease inhibitor triple therapy, this association is attenuated. However, IL28B genotype continues to be useful for guiding duration of therapy, as well as selecting the most cost-effective treatment regimen. Recent data suggests that IL28B genotype may remain relevant to future interferon-free regimens. This review explores the clinical role of IL28B genotyping in the context of current and evolving therapies.

Keywords IL28B gene · Hepatitis C infection · Direct acting antiviral agents · NS3/4A protease · NS5B polymerase · NS5A protein

Introduction

Up to 170 million people are chronically infected with the hepatitis C virus (HCV) worldwide [1]. Chronic hepatitis C leads to the development of cirrhosis, hepatocellular carcinoma and liver failure and is a significant public health concern with HCV-related mortality recently surpassing HIV-related mortality in the United States [2]. Importantly, these complications can be prevented by viral eradication. HCV can be cured by interferon-based therapy. Prior to 2011, the standard of care treatment for genotype 1 HCV was pegylated interferon-α (pegIFN) and ribavirin (RBV) for 48 weeks. Unfortunately pegIFN and RBV therapy only cured at best 50 % of genotype 1 HCV patients, and was associated with considerable morbidity. In 2009, genome-wide association studies (GWAS) identified a strong association between a polymorphism in the region of the IL28B gene on chromosome 19, and treatment outcome with pegIFN and RBV [3••, 4••, 5••]. Patients carrying the good response genotype were 2–3 fold more likely to be cured. IL28B polymorphism was subsequently associated with spontaneous clearance of acute HCV infection also [6, 7••]. This discovery had immediate clinical relevance for treatment decision-making, informing the timing of therapy in the setting of the impending availability of direct acting antiviral agents (DAAs). Now that the 1st generation protease inhibitors telaprevir and boceprevir are widely approved, IL28B genotype remains relevant to clinical management, although the association with sustained virological response (SVR) is attenuated. In this article, we review the association between IL28B and IFN treatment outcomes, and discuss the utility of IL28B genotyping in the context of current and future treatment paradigms.

S. G. C. Y. Mei · P. V. Desmond · A. J. Thompson (✉)
St. Vincent's Hospital (Melbourne),
Melbourne, Victoria, Australia 3065
e-mail: alexander.thompson@svhm.org.au

S. G. C. Y. Mei
e-mail: sweelin.chenyimei@svhm.org.au

P. V. Desmond
e-mail: paul.desmond@svhm.org.au

A. J. Thompson
Victorian Infectious Diseases Reference Laboratory (VIDRL),
Melbourne, Victoria, Australia

A. J. Thompson
Department of Gastroenterology & Duke Clinical research
Institute, Duke University Medical Center,
Durham, NC, USA

IL28B Polymorphism Predicts for Response to Peg-Interferon and Ribavirin Treatment

The discovery that IL28B variation strongly predicts for the eradication of genotype 1 HCV infection in the setting of

pegIFN and RBV therapy was made simultaneously by four independent groups in late 2009/early 2010 [3••, 4••, 5••, 7••]. All four groups performed a genome-wide association study and identified linked single nucleotide polymorphisms (SNPs) in the region of the *IL28B* gene on chromosome 19 that predicted for viral eradication. The top discovery SNPs were rs12979860 and/or rs8099917. These are both tag SNPs for a causal variant on a haplotype block in the region of the IL28B gene. The causal variant has not yet been identified. Rs12979860 and rs8099917 are in very strong linkage disequilibrium in most populations, and are similarly informative. The exception is patients of African ancestry where rs12979860 is more strongly associated with viral eradication. The frequency of the good response variant varies between patients of different ethnic background, being most common in Asian populations, and least common in patients with African ancestry [3••, 6]. This difference in genotype frequency between patients of different ethnic background explains much of the differences in pegIFN and RBV response rates historically observed according to ethnicity [8–11]. IL28B genotype is also strongly associated with spontaneous clearance of acute HCV infection [6, 7••, 12].

The mechanism explaining the association between IL28B polymorphism and IFN treatment response remains unclear. The *IL28B* gene is found on chromosome 19 and encodes for the antiviral cytokine, interferon lambda 3 (IFN-λ3) [13, 14]. This type 3 IFN, binds to a unique cell surface receptor, the IFN-lambda-receptor (IFNLR) that activates the Janus kinase-signal transducers and activators of transcription (JAK-STAT) signalling pathway and leads to the induction of interferon-stimulator genes (ISGs). The downstream signalling pathway is shared with the type 1 IFN receptor. A key point of distinction between the type 1 and type 3 IFN receptors is the distribution of expression, where type 1 IFN receptors are ubiquitous, but type 3 IFN receptors are restricted to epithelial cells, including hepatocytes, and plasmacytoid dendritic cells, but are not expressed by most haemopoietic cells [15] IFN-λ inhibits HCV replication in cell culture models [16, 17] as well as in vivo [18]. Recent data suggest type 3 IFN may be the dominant IFN produced by the liver in response to HCV infection [19, 20].

The immunology associated with IL28B genotype in patients with genotype 1 HCV infection is just starting to be unravelled. As yet there are no convincing data to show a relationship between IL28B genotype and IFN-λ-3 expression in the liver or peripheral blood mononuclear cells. This suggests that IL28B polymorphism may be associated with functional consequences for IFN-λ-3 [3••, 21, 22]. IL28B genotype has been associated with levels of intrahepatic ISG expression, where patients with the good response variant display low level expression [21–24]. This is consistent with previous data linking liver ISG expression to IFN treatment response [25]. IL28B genotype has also been associated with NK cell activity [26]. There remains much to learn and this is a rapidly expanding area.

IL28B Genotyping in the Context of Pegifn and RBV Therapy

Protease inhibitor triple therapy is now standard of care in many parts of the Western world, however these drugs are not universally available and it remains relevant to discuss the clinical utility of *IL28B* genotyping for pegIFN and RBV therapy.

In the intent-to-treat analysis of the IDEAL study pharmacogenetics cohort, 69 % of Caucasian patients with the good response IL28B genotype achieved an SVR compared to 27 % to 33 % of patients with the poor response IL28B genotypes ($p<0.0001$) [27]. IL28B genotype was shown to be the strongest pre-treatment predictor of response to pegIFN and RBV therapy, after adjustment for other well-established baseline predictors such as ethnicity, hepatic fibrosis stage, plasma HCV RNA level and fasting glucose level. IL28B polymorphism was strongly associated with on-treatment viral kinetics. A greater proportion of individuals with the CC genotype achieved critical on-treatment virological milestones (Caucasians: rapid virological response (RVR): CC 28 % vs. CT/TT 5 %, $p<0.0001$; and partial early virological response (pEVR): CC 97 % vs CT/TT 72 %/68 %, $p<0.0001$). Recent data suggest that IL28B genotype is more strongly associated with phase 1 vs. phase 2 decline in the setting of IFN therapy [28]. IL28B genotype is also associated with treatment outcome in patients with genotype 4 HCV infection, and the effect size is similar to that seen in genotype 1 HCV [29]. The association between IL28B genotype and IFN response in genotype 2/3 HCV is less clinically relevant. Genotype 2/3 HCV are more sensitive to IFN. The literature linking IL28B genotype to treatment response is inconsistent, and most studies have not been powered to demonstrate small differences in SVR. Most studies have observed differences in early viral response according to IL28B genotype, but not all have identified an association with SVR [7••, 30–38]. IL28B genotype may be most relevant to genotype 3 HCV in the setting of other poor IFN response characteristics, at baseline (cirrhosis, insulin resistance), or on-treatment (non-RVR patients). Clearly there are important virological determinants of IFN sensitivity that differ between HCV-1 and HCV-2/3; these remain poorly defined.

There has been interest in how IL28B genotype compares to measurement of on-treatment viral kinetics as a predictor of pegIFN and RBV treatment outcome for genotype 1 HCV. In the clinic, IL28B genotype and on-treatment response can be considered to provide complementary information about IFN responsiveness. IL28B genotype is the critical pre-treatment predictor of SVR, and on-treatment virological decline provides a "real-time" measure of response, capturing the IL28B genotype effect, as well as the other clinically relevant

determinants of response, including fibrosis stage and insulin resistance. On-treatment virological response is particularly important for predicting eligibility for short duration pegIFN and RBV, and it has recently been shown that 24 weeks is sufficient only for the subset of patients who carry the good response IL28B genotype and have a low baseline HCV RNA level and achieve an RVR [39].

Prior to the availability of direct-acting antiviral (DAA) therapy, IL28B genotype was a useful piece of information for making decisions about a patient's the suitability for pegIFN and RBV therapy. Caucasian patients who carry the good response IL28B genotype achieve SVR rates ≥70 % with 48 weeks of pegIFN and RBV, and remain good candidates for therapy (Table 1). In the setting of the poor response IL28B genotypes SVR rates are disappointing (< 40 %), and in the absence of clinical urgency, deferral of therapy awaiting access to DAAs is reasonable.

Il28B Genotyping and Telaprevir/Boceprevir Triple Therapy

Telaprevir and boceprevir are now licensed as first-line treatment for genotype 1 HCV in many parts of the world. Both agents are inhibitors of the HCV NS3/4A protease, and must be used in combination with pegIFN and RBV to prevent the selection of HCV resistance-associated variants. The clinical utility of the *IL28B* genotype for predicting treatment response in the context of the new DAAs has been explored through retrospective analyses of the pivotal Phase 3 trials. The addition of an NS3/4A serine protease inhibitor to pegIFN and RBV therapy significantly improves the rate of SVR in both treatment-naïve and treatment-experienced patients, and therefore the association between IL28B genotype and likelihood of SVR is attenuated.

In the ADVANCE Trial, triple therapy with telaprevir, pegIFN and RBV was investigated in treatment naïve patients chronically infected with genotype 1 HCV [40••]. Telaprevir was given for either 8 (T8PR) or 12 weeks (T12PR) in combination with pegIFN and RBV, followed by pegIFN and RBV alone for an additional 12 or 36 weeks, according whether an extended RVR (eRVR) was achieved. In a retrospective analysis of the ADVANCE study cohort, 42 % of the participants, all Caucasian, were genotyped for the *IL28B* SNP, rs12799860 [41•]. Telaprevir therapy was associated with an increase in SVR across all *IL28B* genotypes, and the relationship between IL28B genotype and SVR appeared to be attenuated (T12PR: CC = 90 % vs. CT = 71 % vs. TT = 73 %). A greater increment in SVR was noted in carriers of the minor T variant compared to carriers of the CC genotype (Tables 2 and 3). CC patients were more likely achieve an eRVR and be eligible for short duration therapy (Tables 2 and 3).

The Phase 3 REALIZE study enrolled treatment-experienced patients. Patients were classified according to previous pegIFN and RBV treatment response (previous relapse, partial response and null response) [42••]. Patients were randomised to one of two groups receiving 12 weeks of telaprevir plus pegIFN and RBV for a total of 48 weeks (with or without a 4 week lead-in period of pegIFN and RBV) or a control group that received placebo plus pegIFN and RBV. A retrospective analysis of the association between the SNP rs12799860 and SVR was performed in 80 % of this cohort (n=527) [43•]. SVR rates were high across all *IL28B* genotypes in the telaprevir arms, remaining numerically higher in the CC patients in the overall analysis (Tables 2 and 3). However, once patients were stratified according to previous IFN response, IL28B genotype was no longer associated with the outcome of telaprevir therapy [43•].

Retrospective analyses of the boceprevir Phase 3 studies, SPRINT 2 and RESPOND 2, have also evaluated the association between *IL28B* genetic variability and SVR. The SPRINT 2 trial randomized treatment-naïve patients to one of two boceprevir containing arms (4 week lead-in phase of therapy with pegIFN and RBV, followed by response-guided boceprevir-based therapy for 28 to 48 weeks, or 4 week lead-in phase plus 44 weeks of boceprevir plus pegIFN and RBV), or the control arm (placebo plus pegIFN and RBV) [44••]. IL28B genotyping (rs12799860) was performed in 62 % (n= 653) of the SPRINT 2 cohort [45•]. High rates of SVR were observed in all individuals with the favourable CC genotype, with minimal increase in SVR with boceprevir therapy (Tables 2 and 3). It should be noted that the SVR rate in the pegIFN and RBV control arm was substantially higher than that seen in the ADVANCE trial. Although absolute SVR rates did not increase, 90 % of the CC patients in a combined analysis of SPRINT-2/RESPOND-2 were eligible for short duration therapy in the RGT arm [45•]. SVR rates in the non-CC patients were substantially higher with the addition of boceprevir (Tables 2 and 3). The lead-in phase of pegIFN and RBV has been shown to characterize IFN responsiveness and stratify patients for likelihood of subsequent SVR with boceprevir therapy, as well as risk of antiviral resistance. A 1 log10 IU/mL reduction in HCV RNA has been identified to be a critical threshold for predicting SVR/resistance. This has been proposed as a clinical decision point, where patients with poor IFN responsiveness might have treatment deferred for more effective future regimens (eg quadruple therapy or IFN-free therapy). The clinical utility of the lead-in for predicting IFN responsiveness varies according to IL28B genotype. In patients who carry the good response IL28B genotype, IFN responsiveness (1 log reduction) is almost universal [45•, 46]. In contrast, the lead-in has greater utility for identifying IFN responsiveness in patients with the poor response IL28B genotypes.

The RESPOND 2 trial enrolled treatment-experienced genotype 1 HCV patients, all of whom were previous

Table 1 IL28B genotyping is relevant to decision-making about 1st-line therapy for genotype 1 HCV IL28B genotype may be used to identify patients who benefit most from DAA therapy. Considerations include efficacy, duration of therapy, treatment-related toxicity, and cost effectiveness

IL28B genotype	Lead-in phase of peginterferon plus ribavirin (PR)	1st line therapy	Pros	Cons
Good response IL28B genotype	Not informative (1 $\log_{10}$ reduction is almost universal)	PR	SVR rate of ≥70 %[a]	48 weeks of therapy required for the majority
			May be more cost-effective than first-line treatment with triple therapy	Patients who relapse will require re-treatment
			HCV resistance to PR has not been described	
			Avoids DAA-related toxicity	
		Telaprevir/boceprevir triple therapy	High SVR rate>80 %[a]	SVR increment is small
			Majority eligible for short duration RGT	Increased treatment-related toxicity
			May be more effective for cirrhotic patients[b]	High pill burden
				Expensive, cost effectiveness not clear
Poor response IL28B genotype	≥ 1 $\log_{10}$ reduction in HCV RNA identifies patients who are IFN-responsive	PR	–	Low SVR rate compared to DAA triple therapy
		Telaprevir/boceprevir triple therapy	Large SVR benefit compared to PR (75–82 %)[a]	Increased treatment-related toxicity
			Cost effective	High pill burden
	< 1 $\log_{10}$ reduction in HCV RNA defines poor IFN-responsiveness	PR	–	Very low SVR rate
		Telaprevir/boceprevir triple therapy	Large SVR benefit compared to PR	Overall SVR rates remain suboptimal
				Risk of selection of resistance associated variants[c]
		Deferral for next generation agents	Future regimens will have:	Risk of disease progression, esp in patients with advanced liver fibrosis
			- Higher SVR rate	Risk of loss-to-follow-up
			- Reduced risk of resistance	May be more expensive (DAA failure may prevent enrolment in future clinical trials)
			- Reduced treatment-related toxicity	
			- Reduced pill burden	

[a] Caucasians enrolled in the IDEAL study

[b] The SVR rate in Caucasian patients with cirrhosis enrolled in the IDEAL study according to IL28B genotype was CC 41 % vs. CT 22 % vs. TT 11 %

[c] The clinical significance of resistance-associated variants remains unclear

relapsers or partial responders to prior pegIFN and RBV therapy [47••]. Null responders, defined by a failure to achieve a 2 log10 reduction in HCV RNA at week 12 of the previous IFN treatment, were excluded. Retrospective analysis of the relationship between IL28B genotype and treatment outcome was possible in 66 % (n=259) of the original cohort. SVR rates were substantially higher in individuals of all IL28B genotypes who received boceprevir therapy, vs. pegIFN and RBV alone [45•] (Tables 2 and 3). IL28B genotype was still associated with SVR, although the association was attenuated compared to that observed in the control arm. The major increment in SVR was observed in non-CC patients; the CC patients were more likely to be eligible for short duration therapy. Similar to the data from REALIZE, once IFN responsiveness was characterized in this study by measurement of the virological decline during the lead-in phase, IL28B genotype was no longer informative for predicting SVR [45•].

Table 2 Association of IL28B genotype and treatment response in telaprevir/boceprevir phase 3 studies. Rates of SVR according to IL28B genotype

Study	Treatment		CC	CT	TT
Advance [41•] (*N*=454/1088)	PR, *n*=161		64 %	25 %	23 %
	T8PR, *n*=153		84 %	57 %	59 %
	T12PR, *n*=140		90 %	71 %	73 %
Sprint-2 [45•] (*N*=653/1048)	PR, *n*=217		78 %	28 %	27 %
	BOC RGT, *n*=222		82 %	65 %	55 %
	BOC/PR48, *n*=214		80 %	71 %	59 %
Realize [43•] (*N*=527/622)	Relapsers	PR, *n*=52	33 %	20 %	30 %
		Pooled T12/PR48, *n*=209	88 %	85 %	85 %
	Partial Responders	PR, *n*=20	20 %	20 %	0 %
		Pooled T12/PR48, *n*=79	63 %	58 %	71 %
	Prior Null Responders	PR, *n*=33	(N/A)	6 %	7 %
		Pooled T12/PR48, *n*=134	40 %	29 %	31 %
Respond 2 [45•] (*N*=259/394)	Prior Relapsers	PR, *n*=32	50 %	33 %	57 %
		Pooled BOC arms, *n*=134	86 %	74 %	78 %
	Prior Partial Responders	PR, *n*=20	33 %	0 %	33 %
		Pooled BOC arms, *n*=73	60 %	55 %	45 %

The cost-effectiveness of drug therapy is an increasingly important issue in today's world. Telaprevir and boceprevir are both expensive, and there has been considerable interest in identifying patient populations in whom DAA may not be necessary as first-line treatment (Table 1). IL28B genotype is an attractive biomarker, as the good response genotype identifies a group of patient who achieve high rates of SVR with pegIFN and RBV alone. A number of groups have now modeled the cost effectiveness of different treatment strategies for boceprevir/telaprevir access. Telaprevir and boceprevir are most cost-effective for patients who carry the poor response IL28B genotypes [48, 49]. In patients who carry the good response IL28B genotype, cost effectiveness is less clear. First-line therapy with pegIFN and RBV RGT, followed by PI therapy for those patients who relapse, may be more cost-effective [48–50]. An important caveat is that there are little data evaluating the impact of PI therapy in patients with the good response genotype and advanced liver fibrosis. In the IDEAL study, the SVR rates in this group were <50 % with pegIFN and RBV alone [27]. It should also be noted that the results of cost effective analyses depend on the assumptions made, and will be strongly influenced by the set price for drug.

In summary, both telaprevir and boceprevir therapy are associated with a substantial increase in the rate of SVR compared to pegIFN and RBV alone. The association between IL28B genotype and treatment outcome is therefore attenuated. In treatment-naïve patients, IL28B genotype informs IFN responsiveness and provides clinically useful information. In patients who carry the good response IL28B genotype, PI therapy appears to be associated with only a small increment in SVR rate. As such, the cost effectiveness

Table 3 Association of IL28B genotype and treatment response in telaprevir/boceprevir phase 3 studies. Rates of key on-treatment virological milestones according to IL28B genotype

Study	Treatment	Outcome (%)	CC	Non-CC	
				CT	TT
Advance [41•]	T8PR, *n*=153	eRVR[a]	64 %	51 %	50 %
	T12PR, *n*=140	eRVR[a]	78 %	57 %	45 %
Sprint-2 [45•]	Pooled BOC arms, *n*=436	Week 8 Undetectability[a]	89 %	52 %	
Respond 2 [45•]	Pooled BOC arms, *n*=206	Week 8 Undetectability[a]	82 %	51 %	
Sprint 2 & Respond 2 (Combined) [45•]	Pooled BOC arms, *n*=633	Lead-in phase, HCV RNA reduction≥1 $\log_{10}$ IU/mL	97 %	75 %	56 %

[a] Criteria for short duration therapy according to RGT, response-guided therapy

of PI therapy for these patients is not clear. A major clinical benefit of PI therapy for these patients is to allow short duration therapy (>75 % of good response patients will be eligible for short duration therapy). Patients who carry the poor response IL28B genotypes clearly benefit from PI therapy, with large increments in SVR. An algorithm for the use of IL28B genotyping in the setting of telaprevir/boceprevir therapy is proposed in Table 1. In treatment-experienced patients, where previous IFN response has been well-characterized, IL28B genotyping is less useful.

Table 4 Association of IL28B genotype and treatment outcome of DAA regimens in development

Study	N	Treatment	Outcome (%)	CC	Non-CC
SILEN-C1 [59]	40	PR	SVR24	82 %	41 %
	70	BI201335 240 mg QD/PR (no lead in)		100 %	71 %
Pillar [60]	46	PR48	SVR24	100 %	55 %
	109	75 mg TMC 435/Pooled PR12 and PR24		84 %	73 %
	107	150 mg TMC 435/Pooled PR12 and PR24		97 %	78 %
Atlas [61]	24	PR24	SVR24	88 %	25 %
	51	Danoprevir 300 mg/PR		81 %	63 %
	57	Danoprevir 600 mg/PR		95 %	79 %
	38	Danoprevir 900 mg/PR		85 %	68 %
Essential [53]	73	PR48	SVR24	73 %	17 %
	72	Alisporivir[a]/PR 48 weeks		100 %	62 %
	71	Alisporivir/PR RGT		100 %	73 %
	72	Alisporivir/PR 24 weeks		71 %	33 %
Jump-C [62]	33	Mericitabine/PR	SVR12	80 %	72 %
Sound C2 [57]	59 (81)[a]	BI 201335/BI207127 (TDS)/ RBV, 16 wks	SVR12	67 %	57 % (HCV-1a, SVR12=32 %)
	61 (80)	BI 201335/BI207127 (TDS)/ RBV, 28 wks		71 %	57 % (HCV-1a, SVR12=38 %)
	68 (78)	BI 201335/BI207127 (BD)/ RBV, 28 weeks		79 % (HCV-1a, SVR12= 75 %) (HCV-1b, SVR12=82 %)	64 % (HCV-1a, SVR12=32 %) (HCV-1b, SVR12=84 %)
	39 (46)	BI 201335/BI207127 TDS/no RBV, 28 weeks		58 %	33 % (HCV-1a=0 %)
	56 (77)	BI 201335/BI207127 (TDS)/ RBV, 40 wks	SVR 4	68 %	52 % (HCV-1a=42 %)
Proton [63]	13	200 mg PSI 7977/PR "12+12"	SVR12	N/A	100 (TT)
Inform-SVR [64]	64	Mericitabine/Danoprevir-ritonavir/RBV	SVR12	HCV-1a 27 % (46/15)	HCV-1a 25 % (7/28)
				HCV-1b 50 % (2/4)	HCV-1b 76 % (13/17)
[65]	10	BMS-790052/BMS-650032 (null responder population)	SVR24	100 (2/2)	100 (8/8)
[66]	10	BMS-790052/BMS-650032/ PR (null responder population)	SVR24	100 (1/1)	100 (9/9)
	11	BMS-790052/BMS-650032 (null responder population)		100 (1/1)	40 (4/10)
Pilot [67]	11	ABT-450-ritonavir/ABT-072/ ABT-450/RBV	SVR24	91 % (10/11)	–
Co-Pilot [68]	19	ABT-450-ritonavir 250 mg/ ABT-333/RBV	SVR24	90 % (9/10)[b]–100 % PP	100 % (9/9)
	14	ABT-450-ritonavir 250 mg/ ABT-333/RBV	SVR24	80 % (4/5)[c]–100 % PP	100 % (9/9)

[a] The development of Alisporivir has recently been halted

[b] 1 patient withdrew week 2 due to asymptomatic ALT elevation

[c] 1 patient withdrew week 1 for personal reasons

IL28B Genotyping and Future DAA Therapies

The next wave of DAA therapy is on the horizon in the management of Genotype 1 HCV. Phase 2 data investigating second-generation NS3/4A protease inhibitors, cyclophilin inhibitors and NS5A inhibitors, all in combination with PegIFN and RBV, are emerging. Studies now stratify randomization according to IL28B genotype, and interim analyses of efficacy according to IL28B polymorphism are summarized in Table 4. In general, IL28B polymorphism has continued to be associated with treatment outcome in studies of triple therapy with a single DAA plus pegIFN and RBV, although the strength of association has been clearly attenuated compared to the pegIFN and RBV control arms. IL28B genotype was useful for discriminating those eligible for shortened therapy. The boundaries of short-duration therapy have not yet been fully explored in the setting of DAA therapy, and IL28B genotype may be important for defining the minimum necessary length of treatment. Recent interim data from the ATOMIC study suggests that IL28B genotype might be less relevant to outcomes of triple therapy with the very potent nucleotide analogue GS-7977, where SVR12 rates of 90 % where observed in 52 patients treated for only 12 weeks duration [51].

Quadruple therapy using a combination of two different classes of DAAs with pegIFN and RBV is also being evaluated, and shows particular promise for patients who are prior null responders to pegIFN and RBV alone. Very high rates of SVR have been observed in small cohorts of patients in early phase studies [52]. As yet there is little data evaluating the relationship between IL28B genotype and treatment response. Analyses will be limited by the small number of patients who carry the good response IL28B genotype in null responder cohorts, but the high SVR results obtained suggest that much of the IL28B effect may be overcome. Indeed, with SVR rates >90 % it will be difficult to identify any predictive biomarkers for treatment response.

Interferon-free therapy for HCV is greatly anticipated and preliminary results from Phase 2 clinical trials are promising [52–54]. Although the mechanism by which the *IL28B* genotype influences treatment outcome is yet to be elucidated, it is clearly a marker of interferon sensitivity. Therefore, the clinical utility in the context of an interferon-free regimen is not clear. Recent data suggest that IL28B genotype may still be associated with treatment response in the setting of IFN-free regimens, although the clinical relevance of this association is not yet known. The INFORM-1 study was the first study to demonstrate that IFN-free therapy could have a potent antiviral effect [55]. Patients were treated for 14 days with the NS5B nucleoside polymerase inhibitor, mericitabine, in combination with the protease inhibitor, danoprevir. Recent analysis of the on-treatment viral kinetics revealed a significant difference in second phase viral decline according to *IL28B* genotype [56], suggesting that IL28B genotype may influence the rate of clearance of infected hepatocytes. This might be consistent with the role of IL28B genotype in natural clearance of HCV. Interim results of the SOUND-C2 trial have demonstrated a clear difference in SVR12 according to *IL28B* genotype in HCV-1a patients (Table 4) [57]. The SOUND-C2 data suggest that IL28B genotype may remain particularly relevant to IFN-free therapy for HCV-1A, which is emerging as a "hard-to-cure" virus for DAA therapy. Patients who carry the good response IL28B genotype may continue to be an easy-to-cure population for IFN-free therapies. In fact, a number of IFN-free regimens are going forwards into populations limited to HCV-1b or the good response IL28B genotype [57, 58]. Whether more potent DAAs, or combinations of multiple DAAs, can overcome this association in HCV-1a patients remains to be seen. Patients who carry the poor response IL28B genotypes and have HCV-1a infection may remain hard-to-cure, and given the high response rates observed with quadruple therapy, may ironically remain the population who continue to require IFN therapy.

Conclusions

IL28B polymorphism predicts for IFN responsiveness and is a key determinant of the outcome of pegIFN and RBV therapy. In the setting of telaprevir/boceprevir treatment, IL28B genotype is associated with the likelihood of SVR, as well as eligibility for short duration therapy. IL28B genotype may also be relevant to cost-effectiveness strategies. The treatment for HCV is rapidly evolving, with the development of multiple classes of DAAs, quadruple therapy regimens, and IFN-free strategies. In the future it is likely that clinicians will have the option of using a number of different regimens of combination DAAs±pegIFN. All will be very effective, with SVR rates >90 % for most patient groups, and first-line treatment regimens will be IFN-free. With such high SVR rates, it is likely that IL28B genotype will no longer be associated with the overall rate of SVR. To remain clinically relevant, IL28B genotyping will need to be shown to allow individualization of treatment regimens. Patients with the good response IL28B genotype will need to be eligible for treatment that is cheaper, simpler, or shorter. Conversely, the poor response IL28B genotype may require more intensive, longer duration therapy.

Disclosure Dr. A. Thompson has worked as a consultant for Merck, Roche, and Janssen, has received grant support from Merck, Gilead Sciences, and Roche, as well as honoraria from Gilead Sciences, Roche, BMS, Merck, and Janssen. AT is also a co-inventor of a patent related to the IL28B discovery.

Dr. P. Desmond has worked as a consultant for Merck, Roche and Janssen.

References

Papers of particular interest, published recently, have been highlighted as:
- • Of importance
- •• Of major importance

1. Lavanchy D. The global burden of hepatitis C. Liver International: Official Journal of the International Association for the Study of the Liver. 2009;29 Suppl 1:74–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19207969. Accessed March 26, 2012.
2. Ly KN, Xing J, Klevens RM, Jiles RB, Ward JW. Original research the increasing burden of mortality from viral hepatitis in the United. Ann Intern Med. 2012;156(4):271–8.
3. •• Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461(7262):399–401. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19684573. Accessed July 25, 2011. *This was one of the landmark trials that led to the discovery of the IL28B gene as an important predictor of treatment outcome in Genotype 1 HCV patients receiving PegIFN and RBV.*
4. •• Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009;41(10):1105–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19749757. Accessed August 2, 2011. *This was one of the landmark trials that led to the discovery of the IL28B gene as an important predictor of treatment outcome in Genotype 1 HCV patients receiving PegIFN and RBV.*
5. •• Suppiah V, Moldovan M, Ahlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009;41(10):1100–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19749758. Accessed July 22, 2011. *This was one of the landmark trials that led to the discovery of the IL28B gene as an important predictor of treatment outcome in Genotype 1 HCV patients receiving PegIFN and RBV.*
6. Thomas DL, Thio CL, Martin MP, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 2009;461(7265):798–801. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19759533. Accessed July 10, 2011.
7. •• Rauch A, Kutalik Z, Descombes P, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology. 2010;138(4):1338–45. 1345.e1-7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20060832. Accessed June 27, 2011. *This was one of the landmark trials that led to the discovery of the IL28B gene as an important predictor of treatment outcome in Genotype 1 HCV patients receiving PegIFN and RBV.*
8. Conjeevaram HS, Fried MW, Jeffers LJ, et al. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. Gastroenterology. 2006;131(2):470–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16890601. Accessed March 21, 2012.
9. Muir AJ, Bornstein JD, Killenberg PG. Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. N Engl J Med. 2004;350(22):2265–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15163776.
10. McHutchison JG, Lawitz EJ, Shiffman ML, et al. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. N Engl J Med. 2009;361(6):580–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20133247.
11. Dev AT, McCaw R, Sundararajan V, Bowden S, Sievert W. Southeast Asian patients with chronic hepatitis C: the impact of novel genotypes and race on treatment outcome. Hepatology (Baltimore, Md). 2002;36(5):1259–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12395338. Accessed May 10, 2012.
12. Tillmann HL, Thompson AJ, Patel K, et al. A polymorphism near IL28B is associated with spontaneous clearance of acute hepatitis C virus and jaundice. Gastroenterology. 2010;139(5):1586–92. 1592.e1. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20637200. Accessed February 14, 2012.
13. Kotenko SV, Gallagher G, Baurin VV, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol. 2003;4(1):69–77. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12483210. Accessed June 14, 2011.
14. Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol. 2003;4(1):63–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12469119. Accessed August 19, 2011.
15. Witte K, Gruetz G, Volk H-D, et al. Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines. Genes Immun. 2009;10(8):702–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19798076. Accessed April 8, 2012.
16. Marcello T, Grakoui A, Barba-Spaeth G, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology. 2006;131(6):1887–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17087946. Accessed July 11, 2011.
17. Robek MD, Boyd BS, Chisari FV. Lambda interferon inhibits hepatitis B and C virus replication. Society. 2005;79(6):1–5.
18. Muir AJ, Shiffman ML, Zaman A, et al. Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. Hepatology (Baltimore, Md). 2010;52(3):822–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20564352. Accessed March 8, 2012.
19. Marukian S, Andrus L, Sheahan TP, et al. Hepatitis C virus induces interferon-λ and interferon-stimulated genes in primary liver cultures. Hepatology (Baltimore, Md). 2011;54(6):1913–23. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3219820&tool=pmcentrez&rendertype=abstract. Accessed February 29, 2012.
20. Thomas E, Gonzalez VD, Li Q, et al. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. Gastroenterology. 2012. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22248663. Accessed March 15, 2012.
21. Urban TJ, Thompson AJ, Bradrick SS, et al. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. Hepatology (Baltimore, Md). 2010;52(6):1888–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20931559. Accessed September 19, 2011.
22. Honda M, Sakai A, Yamashita T, et al. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology. 2010;139(2):499–509. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20434452. Accessed October 4, 2011.
23. Dill MT, Duong FHT, Vogt JE, et al. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. Gastroenterology. 2011;140(3):1021–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21111740. Accessed December 5, 2011.

24. McGilvray I, Feld JJ, Chen L, et al. Hepatic cell-specific gene expression better predicts HCV treatment outcome than IL28B genotype. Gastroenterology. 2012. Available at: http://linkinghub.elsevier.com/retrieve/pii/S001650851200145X. Accessed January 30, 2012.
25. Sarasin-Filipowicz M, Oakeley EJ, Duong FHT, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A. 2008;105(19):7034–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2383932&tool=pmcentrez&rendertype=abstract.
26. Golden-Mason L, Bambha KM, Cheng L, et al. Natural killer inhibitory receptor expression associated with treatment failure and interleukin-28B genotype in patients with chronic hepatitis C. Hepatology (Baltimore, Md). 2011;54(5):1559–69. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3206734&tool=pmcentrez&rendertype=abstract. Accessed April 9, 2012.
27. Thompson AJ, Muir AJ, Sulkowski MS, et al. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology. 2010;139(1):120.e18–9.e18. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20399780. Accessed July 24, 2011.
28. Bochud P-Y, Bibert S, Negro F, et al. IL28B polymorphisms predict reduction of HCV RNA from the first day of therapy in chronic hepatitis C. J Hepatol. 2011;55(5):980–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21354446. Accessed October 26, 2011.
29. Asselah T, De Muynck S, Broët P, et al. IL28B polymorphism is associated with treatment response in patients with genotype 4 chronic hepatitis C. J Hepatol. 2012;56(3):527–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21951981.
30. Mangia A, Thompson AJ, Santoro R, et al. An IL28B polymorphism determines treatment response of hepatitis C virus genotype 2 or 3 patients who do not achieve a rapid virologic response. Gastroenterology. 2010;139(3):821–7. 827.e1. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20621700. Accessed November 7, 2011.
31. Stättermayer AF, Stauber R, Hofer H, et al. Impact of IL28B genotype on the early and sustained virologic response in treatment-naïve patients with chronic hepatitis C. Clinical Gastroenterology and Hepatology: The Official Clinical Practice Journal of the American Gastroenterological Association. 2011;9(4):344.e2–50.e2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20728570. Accessed May 10, 2012.
32. Lindh M, Lagging M, Färkkilä M, et al. Interleukin 28B gene variation at rs12979860 determines early viral kinetics during treatment in patients carrying genotypes 2 or 3 of hepatitis C virus. J Infect Dis. 2011;203(12):1748–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21606533. Accessed March 16, 2012.
33. Chayama K, Hayes CN, Abe H, et al. IL28B but not ITPA polymorphism is predictive of response to pegylated interferon, ribavirin, and telaprevir triple therapy in patients with genotype 1 hepatitis C. J Infect Dis. 2011;204(1):84–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21628662. Accessed May 10, 2012.
34. Sinn DH, Kim YJ, Lee S-T, et al. Association of a single nucleotide polymorphism near the interleukin-28B gene with response to hepatitis C therapy in Asian patients. J Gastroenterol Hepatol. 2011;26(9):1374–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21501223. Accessed May 10, 2012.
35. Sarrazin C, Susser S, Doehring A, et al. Importance of IL28B gene polymorphisms in hepatitis C virus genotype 2 and 3 infected patients. J Hepatol. 2011;54(3):415–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21112657.
36. Moghaddam A, Melum E, Reinton N, et al. IL28B genetic variation and treatment response in patients with hepatitis C virus genotype 3 infection. Hepatology (Baltimore, Md). 2011;53(3):746–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21374656. Accessed September 6, 2011.
37. Yu M-L, Huang C-F, Huang J-F, et al. Role of interleukin-28B polymorphisms in the treatment of hepatitis C virus genotype 2 infection in Asian patients. Hepatology (Baltimore, Md). 2011;53(1):7–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21254157. Accessed May 10, 2012.
38. Scherzer T-M, Hofer H, Staettermayer AF, et al. Early virologic response and IL28B polymorphisms in patients with chronic hepatitis C genotype 3 treated with peginterferon alfa-2a and ribavirin. J Hepatol. 2011;54(5):866–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21145807.
39. Liu C-H, Liang C-C, Liu C-J, et al. Interleukin 28B genetic polymorphisms and viral factors help identify hepatitis C virus genotype 1 patients who benefit from 24 week of peginterferon plus ribavirin therapy. Hepatology (Baltimore, Md). 2011;54(S1):A414.
40. •• Jacobson IM, McHutchison JG, Dusheiko G, et al. Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med. 2011;364(25):2405–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21696307. *This was a pivotal Phase 3 trial that demonstrated a significant improvement in SVR with an NS3/4A protease inhibitor in combination with PegIFN and RBV in treatment naive Genotype 1 HCV patients vs. PegIFN and RBV alone.*
41. • Jacobson IM, Catlett I, Marcellin P, et al. Telaprevir substantially improved Svr rates across all Il28B genotypes in the advance trial. J Hepatol. 2011;54:S542–3. A1369. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827811613718. Accessed April 8, 2012. *This study analysed the effect of the IL28B gene on SVR in treatment naive Genotype 1 HCV patients treated with an NS3/4A protease inhibitor in combination with PegIFN and RBV.*
42. •• Zeuzem S, Andreone P, Pol S, et al. Telaprevir for retreatment of HCV infection. N Engl J Med. 2011;364(25):2417–28. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21696308. *This was a pivotal Phase 3 trial that demonstrated a significant improvement in SVR with an NS3/4A protease inhibitor in combination with PegIFN and RBV in treatment experienced Genotype 1 HCV patients vs. PegIFN and RBV alone.*
43. • Pol S, Aerssens J, Zeuzem S, et al. Similar Svr rates in Il28B Cc, Ct or Tt prior relapser partial- or null-responder patients treated with telaprevir/peginterferon/ribavirin: retrospective analysis of the realize study. J Hepatol. 2011;54:S6–7. A13. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827811600159. Accessed April 8, 2012. *This study analysed the effect of the IL28B gene on SVR in treatment experienced Genotype 1 HCV patients treated with an NS3/4A protease inhibitor in combination with PegIFN and RBV.*
44. •• Poordad F, McCone Jr J, Bacon B. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med. 2011;364(13):1195–206. Available at: http://www.nejm.org/doi/full/10.1056/NEJMoa1010494. Accessed March 14, 2012. *This was a pivotal Phase 3 trial that demonstrated a significant improvement in SVR with an NS3/4A protease inhibitor in combination with PegIFN and RBV in treatment naive Genotype 1 HCV patients vs. PegIFN and RBV alone.*
45. • Poordad F, Bronowicki J-P, Gordon SC, et al. IL28B polymorphism predicts virologic response in patients with hepatitis C genotype 1 treated with Boceprevir (Boc) combination therapy. J Hepatol. 2011;54(S6). A12. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827811600147. Accessed April 8, 2012. *This study performed a combined analysis on the effect of the IL28B gene on SVR in treatment naive and treatment experienced Genotype 1 HCV patients treated with an NS3/4A protease inhibitor in combination with PegIFN and RBV.*
46. Thompson AJ, Clark PJ, Tillmann HL, et al. IL28B C/C genotype is predictive of >1 log10 IU/mL reduction in plasma HCV RNA after 4

weeks of peginterferon and ribavirin therapy: implications for the use of the lead-in strategy for direct-acting antiviral-based treatment regimens. Hepatology (Baltimore, Md). 2011;54(S1):A157.

47. •• Bacon BR, Gordon SC, Lawitz E. Boceprevir for previously treated chronic hepatitis C virus genotype 1 infection. N Engl J Med. 2011;41(2):122–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21677916. *This was a pivotal Phase 3 trial that demonstrated a significant improvement in SVR with an NS3/4A protease inhibitor in combination with PegIFN and RBV in treatment experienced Genotype 1 HCV patients vs. PegIFN and RBV alone.*
48. Cammà C, Petta S, Enea M, et al. Cost-effectiveness of boceprevir or telaprevir for untreated patients with genotype 1 chronic hepatitis C. Hepatology (Baltimore, Md.). 2012:1–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22454336. Accessed April 9, 2012.
49. Liu S, Cipriano L, Holodniy M. New protease inhibitors for the treatment of chronic hepatitis C—a cost effective analysis. Ann Intern Med. 2012;156:279–90. Available at: http://www.annals.org/content/156/4/279.short. Accessed April 9, 2012.
50. Gellad ZF, Naggie S, Reed SD, et al. The cost-effectiveness of a telaprevir-inclusive regimen as initial therapy for genotype 1 hepatitis C infection in individuals with the CC IL-28B polymorphism. Hepatology (Baltimore, Md). 2011;54(S1):A118.
51. Kowdlev KV, Läwitz E, Crespo I, et al. Atomic: 97 % RVR for PSI-7977 + PEG/RBV × 12 week regimen in HCV GT1: an end to response-guided therapy? J Hepatol. 2012;56:abstract 1. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827812002437. Accessed 10 May 2012.
52. Lok A, Gardiner D, Lawitz E, et al. Preliminary study of two antiviral agents for hepatitis C genotype 1. N Engl J Med. 2012;366(3):216–24.
53. Gane EJ, Stedman C, Hyland RH, et al. Once daily PSI-977 plus ribavirin: pegylated interferon-alfa not required for complete rapid viral response in treatment-naive patients with HCV GT2 or GT3. *Hepatology (Baltimore, Md.)*. Hepatology (Baltimore, Md). 2011;54(Number 4 (Supp)):A34.
54. Chayama K, Takahashi S, Toyota J, et al. Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. Hepatology (Baltimore, Md). 2012;55(3):742–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21987462. Accessed March 14, 2012.
55. Gane EJ, Roberts SK, Stedman CAM, et al. Oral combination therapy with a nucleoside polymerase inhibitor (RG7128) and danoprevir for chronic hepatitis C genotype 1 infection (INFORM-1): a randomised, double-blind, placebo-controlled, dose-escalation trial. Lancet. 2010;376(9751):1467–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20951424. Accessed April 9, 2012.
56. Chu TW, Kulkarni R, Gane EJ, et al. Effect of IL28B genotype on early viral kinetics during interferon-free treatment of patients with chronic hepatitis C. Gastroenterology. 2012;142(4):790–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22248659. Accessed April 10, 2012.
57. Zeuzem S, Soriano V, Asselah T, et al. Svr4 and Svr12 with an interferon-free regimen of Bi201335 and Bi207127, +/− ribavirin, in treatment-naive patients with chronic genotype-1 Hcv infection: interim results of sound-C2. J Hepatol. 2012;56:S45-A101. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827812601159. Accessed May 10, 2012.
58. Lawitz E, Poordad F, DeJesus E, et al. Abt-450/Ritonavir (Abt-450/R) combined with pegylated interferon alpha-2a/ribavirin after 3-day monotherapy in genotype 1 (Gt1) Hcv-infected treatment-naive subjects: 12-week sustained virologic response (Svr12) and safety results. J Hepatol. 2012;56:S470-A1187. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827812611994. Accessed May 10, 2012.
59. Sulkowski MS, Asselah T, Ferenci P, et al. Treatment with the second generation HCV protease inhibitor BI201335 results in high and consistent SVR rates—results from SILEN-C1 in treatment-naive patients across different baseline factors. Hepatology (Baltimore, Md). 2011;54(4 (Supp))):A226.
60. Fried MW. TMC435 in combination with peginterferon and ribavirin in treatment-naïve HCV genotype 1 patients: final analysis of the PILLAR Phase IIB study. Hepatology (Baltimore, Md). 2011;54(4 (Supp)):LB-5.
61. Terrault NA, Cooper C, Balart LA, et al. High sustained virologic response (svr24) rates with response-guided danoprevir (dnv; rg7227) plus pegifn α-2a (40kd) and ribavirin (p/r) in treatment-naive hcv genotype 1 (g1) patients: results from the atlas study. Hepatology (Baltimore, Md). 2011;54(4 (Supp)):A79.
62. Pockros P, Jensen D, Tsai N, et al. First Svr data with the nucleoside analogue polymerase inhibitor mericitabine (Rg7128) combined with peginterferon/ribavirin in treatment-naive Hcv G1/4 patients: interim analysis from the jump-C trial. J Hepatol. 2011;54:S538. A1359. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827811613615. Accessed April 30, 2012.
63. Lawitz E. Once-daily PSI-7977 plus Peg/RBV in treatment-naïve patients with HCV GT1: robust end of treatment response rates are sustained post-treatment. Hepatology (Baltimore, Md). 2011;54 (S1):A225.
64. Gane EJ, Pockros P, Zeuzem S, et al. Interferon-free treatment with a combination of mericitabine and danoprevir/R with or without ribavirin in treatment-naive Hcv genotype 1-infected patients. J Hepatol. 2012;56:S470-A1412. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827812614238. Accessed May 10, 2012.
65. Chayama K, Takahashi S, Kawakami Y, Ikeda K. Dual oral combination therapy with the NS5A inhibitor BMS-790052 and the NS3 protease BMS-650032 achieved 90 % sustained virologic response (SVR12) in HCV genotype 1B-infected null responders. Hepatology (Baltimore, Md). 2011;54:A1428. LB-4.
66. Lok A, Gardiner D, Lawitz E, et al. Quadruple therapy with BMS-790052, BMS-650032 and Peg-IFN/Rbv for 24 weeks results in 100 % Svr12 in Hcv genotype 1 null responders. J Hepatol. 2011;54:S536. A1356. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827811613585. Accessed April 30, 2012.
67. Lawitz E, Poordad F, Kowdley KV, et al. A 12-week interferon-free regimen of Abt-450/R, Abt-072, and ribavirin was well tolerated and achieved sustained virologic response in 91 % treatment-naive Hcv Il28B-Cc genotype-1-infected subjects. J Hepatol. 2012;56:S7-A13. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0168827812600270. Accessed May 10, 2012.
68. Poordad F, Lawitz E, Kowdley KV, et al. 12-week interferon-free regimen of Abt-450/R +Abt-333 +Ribavirin achieved Svr12 in more than 90 % of treatment-naive Hcv genotype-1-infected subjects and 47 % of previous non-responders. J Hepatol. 2012;56:S549–50. A1399. Available at: http://linkinghub.elsevier.com/retrieve/pii/S016882781261410X. Accessed May 10, 2012.

2 Materials and Methods

The following materials and methods were used to perform for experiments in this thesis.

2.1 Clinical Methods

2.1.1 Liver Stiffness Measurement (LSM)

LSM was assessed using transient elastography according to the manufacturer's guidelines, and considered valid if a minimum of 10 measurements were obtained with at least a 60% success rate and an interquartile range < 0.3 of the median value.

2.2 Laboratory Methods

2.2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

40 mL of whole blood was collected from subjects via venepuncture in to four gel-free, lithium heparin tubes. The blood was centrifuged at 1000 rpm for 5 minutes, brake on, to separate plasma from the blood. Plasma was carefully collected from the surface using a pipette and stored in four, 1mL aliquots in plasma tubes at -80°C.

Blood in the tubes were poured in equal amounts, in to two falcon tubes and diluted 1:2 with sterile PBS. Equal amounts of diluted blood were carefully overlayed over 15mL of cold Ficoll Paque Plus solution (GE Healthcare, United Kingdom) using a sterile transfer pipette. Centrifugation at 2400 rpm for 25-minutes, brake off, was performed to allow gradient separation of the components of whole blood, as depicted in Figure 2.1.

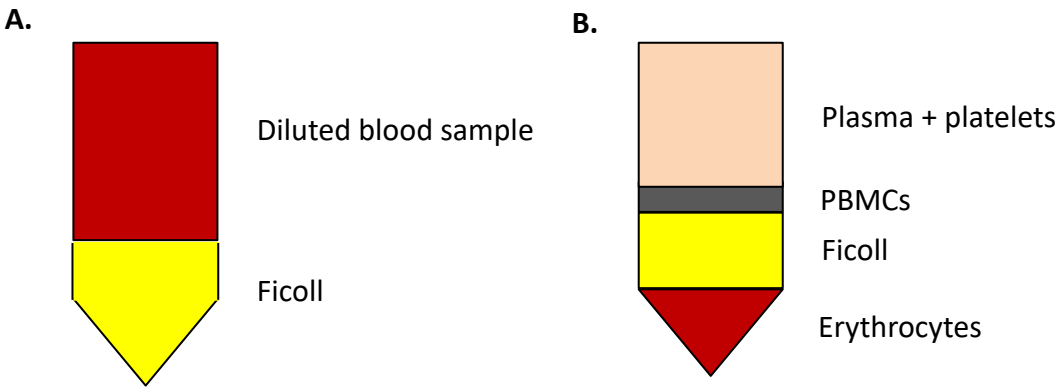


Figure 2.1 Isolation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation.
A. Layers before centrifugation. B. Layers after centrifugation.

The PBMCs were harvested using a transfer pipette, and placed in to falcon tubes at a maximum of 10mL. The PBMCs were diluted with PBS, up to 50mL in each falcon tube to wash the cells. Three washes were performed, with centrifugation performed at 1500 rpm for 10-minutes, brake on, in which the pelleted PBMCs would be re-diluted with 50mL of PBS each time. After the first wash, PBMCs pelleted from the same subject were combined to limit cell loss during the washing process. After the third wash, the PBS was carefully removed and the pelleted PBMCs were re-suspended in 1mL of media ((RPMI-1640 (Sigma Lifesciences, USA), 5% heat-inactivated foetal calf serum (FCS), penicillin, streptomycin, L-glutamine).

10µL of cells in media were added to 90 µL of Trypan blue stain for a cell viability exclusion test and counted with a haemocytometer. The PBMCs were re-suspended in media at a minimum concentration of 20 x 10^6 PBMCs/mL.

To prepare the PBMCs for long-term storage, 0.5mL of the re-suspended PBMCs in RPMI/5% FCS were added to 0.5mL of freezer mix (20% dimethylsulfoxide + 80% FCS) in cryo vials (Nunc, Copenhagen, Denmark), and gently mixed with the pipette. The cryo-vials were placed in a NalgeneTM Cryo 1-degree celcius freezing container (Nalgene, Thermo Fisher Scientific, USA) within 5-minutes (ensuring adequate isopropyl alcohol) and slowly cooled by being placed at -80°C for a minimum of 24-hours. The PBMCs were then placed in liquid nitrogen for long-term storage.

2.2.2 Flow Cytometry for Determination of TLR Intra-Cellular and Surface Expression on Peripheral CD14+ Blood Monocytes and Natural Killer Cells

1mL of frozen PBMCs from each subject, were thawed rapidly at 37°C in a warm water-bath. In a class II cabinet, PBMCs were added to 9mL of sterile PBS in a 10mL falcon tube, to remove any residual DMSO. The diluted PBMCs were mixed by inversion and pelleted through centrifugation at 1500rpm for 5-minutes. A transfer pipette was used to gently remove supernatant. The pelleted cells were resuspended in 1mL of supplemented RPMI (5% FCS-P/S (enriched with L-glutamax)). A cell count was performed using a haemocytometer and the trypan blue exclusion test was performed to ensure PBMC viability.

4 x 10^6 PBMCs were set aside for TLR functional assay and incubated at 37°C for a minimum of 1-hour, to reduce the background of cytokine production.

The remaining PBMCs were centrifuged at 1500 rpm for 5-minutes. The supernatant was removed and PBMCs were resuspended at 5 x 10^6 cells/mL (equivalent to 0.5 x 10^6 per 100uL).

400 uL of PBMCs (2 x 10^6 PBMCs in total) of each subject sample were aliquoted into an unsterile flow cytometry (FACS) tube and combined with 4 uL of FVS 510 to assess for PBMC viability. 3.6mL of PBS was added to each FACS tube and incubated for 15 minutes at room temperature in the dark to stain the PBMCs. The stained PBMCs were then centrifuged at 1500 rpm for 5 minutes, brake on, to pellet cells. The supernatant was removed and the stained PBMCs washed twice with 1mL of PBS/2% FCS. The cells were resuspended in 400 uL of PBS/2%FCS and gently vortexed.

FACS tubes were labelled in pairs, T1 representing the receptors of interest, and T2 representing the isotype controls. Four FACS tubes were labelled as TLRP1 T1, TLRP1 T2, TLRP2 T1 and TLRP2 T2.

Five FACS tubes were labelled as follows for single-stain compensation tubes: CD14 APC-Cy7, TLR2 AF647, TLR4 PE, CD3 Pac Bl and CD56 PE-Cy7.

Antibodies were aliquoted in to their respective FACS tubes as per Table 2.1.

FACS tube label	Marker	Fluorophore	Category number	Lot number	Recommended volume	Volume added
TLRP1 T1	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	CD14	APC-Cy7				1 µL
	TLR2	AF647	558319	5253914	0.125µg/20µL	2µL (0.0125µg)
	TLR4	PE	564215	5295895	0.25µg/5µL	1µL (0.05µg)
	CD86	APC				3 µL
TLRP1 T2	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	Mouse IgG1, κ	AF647	557732	4003600	0.03µg/5µL	2µL (0.0125µg)
	Mouse IgG1, κ	PE	554680		0.2µg/µL	0.25µL (0.05µg)
	IgG2	APC				1.5 µL
TLRP2 T1	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	CD14	APC-Cy7				1 µL
TLRP2 T2	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	CD14	APC-Cy7				1 µL
CD3 Pac Bl	CD3	Pacific blue				0.2 µL
CD56 PE Cy7	CD56	PE Cy7				1.5 µL
TLR4 PE	TLR4	PE	564215	5295895		0.5 µL
TLR2 AF647	TLR2	AF647	558319	5253914		1 µL
CD14 APC-Cy7	CD14	APC-Cy7				0.5 µL

Table 2.1 Antibodies for TLR surface expression

100µL of the stained PBMCs were transferred into each of the FACS tubes labelled TLRP1 (T1 & T2) and TLRP2 (T1 & T2) for surface and intra-cellular staining, respectively. These tubes were incubated for 20 minutes at room temperature in the dark. The stained PBMCs were then washed twice with 1mL PBS supplemented with

2% FCS and centrifuged at 1500rpm for 5 minutes. The supernatant was removed with a pipette.

100 µL of 2% formaldehyde was added to TLR P1 T1 and T2 for each subject. Centrifugation was performed at 1500 rpm for 5-minutes to pellet the stained, fixed PBMCs. The supernatant was carefully removed with a pipette, leaving approximately 75 µL of fluid. The FACS tubes were vortexed run on flow cytometry immediately.

Conversely, for the FACS tubes labelled TLR P2 T1 and T2, 250 µL of Cytofix/Cytoperm (BD Biosciences, CA, USA) was added using a chemical hood, and placed at 4°C overnight. This step permitted permeabilisation of cells for intracellular staining without disrupting the cell surface antibody staining.

The following day, each FACS tube was washed with 1mL of BD Perm/Wash (1:10 solution with distilled H_20) and spun at 2500 rpm for 5-minutes. This speed was employed due to account for the change in cell density after the Cytofix/Cytoperm step. Six FACS tubes were labelled as follows for single-stain compensation tubes - CD14 APC-Cy7, RIG-I AF647, TLR7 PE, CD3 Pac Bl and CD56 PE-Cy7. Antibodies were aliquoted in to their respective FACS tubes as per Table 2.2.

FACS tube label	Marker	Fluorophore	Category number	Lot number	Recommended volume	Volume added
TLRP2 T1	RIG-I	AF647	NBP2-27102		0.5µg/10µL	1µL (0.05µg)
	TLR7	PE	IC5875P	ABEV0312051	0.25µg/10µL	5µL (0.125µg)
TLRP2 T2	Mouse IgG1, κ	AF647	557732		0.03µg/5µL	8µL (0.05µg)
	Mouse IgG2a, κ	PE	349053		0.05µg/µL	2.5µL (0.125µg)
CD3 Pac Bl	CD3	Pacific blue				0.2 µL
CD56 PE Cy7	CD56	PE Cy7				1.5 µL
TLR7 PE	TLR4	TLR7 PE	IC5875P	ABEV0312051		1µL
RIG-I AF647	RIG-I	AF647	NBP2-27102			0.5 µL
CD14 APC-Cy7	CD14	APC-Cy7				0.5 µL

Table 2.2 Antibodies for TLR intra-cellular expression

These FACs tubes labelled TLRP2 T1 and T2 were incubated for 30 minutes at 4°C in the dark. The stained PBMCs were then washed twice with 1mL BD Perm/Wash and ready for flow cytometry. This was performed on the FACS Canto Flow Cytometer (Becton Dickinson, USA). Isotype matched non-binding control antibodies were used for comparison. A total of 10,000 CD14-positive monocytes and CD56-positive NK cells of each sample were analysed; dead cells were gated out based on their light scatter properties.

Data were analysed using FlowJo software (Tree Star Inc., Ashland, OR, USA). TLR2, TLR4, TLR7, RIG-I and CD86 values were expressed as a ratio of the geometric mean fluorescence of individual study patients to the geometric mean isotype control values for that patient.

2.2.3 NK Cell Phenotyping

1mL of frozen PBMCs were thawed rapidly at 37°C in a warm water-bath. In a class II cabinet, PBMCs were added to 9mL of sterile PBS in a 10mL falcon tube, to remove any residual DMSO. The diluted PBMCs were mixed by inversion and pelleted through centrifugation at 1500rpm for 5-minutes. A transfer pipette was used to gently remove supernatant. The pelleted cells were resuspended in 1mL of supplemented RPMI (5% FCS-P/S (enriched with L-glutamax)). A cell count was performed using a haemocytometer and the trypan blue exclusion test was performed to ensure PBMC viability. 2 x 10^6 PBMCs were set aside for NK functional assay and incubated at 37°C for a minimum of 1-hour, to reduce the background of cytokine production.

The remaining PBMCs were centrifuged at 1500 rpm for 5-minutes. The supernatant was removed and PBMCs were resuspended at 5 x 10^6 cells/mL (equivalent to 0.5 x 10^6 per 100uL).

Six unsterile flow cytometry (FACS) tubes were labelled as follows: NKP1 (NK panel 1) T1 and T2, NKP2 T1 and T2, and NKP3 T1 and T2.

Six unsterile FACS tubes were labelled as follows for single-stain compensation tubes: CD3 Pac Bl, CD56 PE-Cy7, NKp46 PE, TIM3 BB515, TRAIL APC, and HLA-DR FITC.

Antibodies were aliquoted in to their respective FACS tubes as per Table 2.3.

FACS tube label	Marker	Fluorophore	Category number	Lot number	Recommended volume	Volume added
NKP1 T1	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	NKp46	PE	557991	4255803	0.25µg/20µL	5µL (0.0625µg)
	NKG2D	APC	558071	5036864	1µg/20µL	4µL (0.2µg)
	TIM3	BB515	565568	5135962	0.5µg/5µL	2µL (0.2µg)
NKP1 T2	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	Mouse IgG1, κ	PE	554680		0.2µg/µL	0.3µL (0.0625µg)
	Mouse IgG1, κ	APC	554681		0.2µg/µL	1µL (0.2µg)
	Mouse IgG1, κ	BB515	564416		0.2µg/µL	1µL (0.2µg)
NKP2 T1	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	NKp30	PE	558407		0.2µg/µL	2µL (0.4µg)
	TRAIL	APC	563642		0.2µg/µL	2µL (0.4µg)
	HLA-DR	FITC	555811	4342790	0.5µg/20µL	2µL (0.05µg)
NKP2 T2	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	Mouse IgG1, κ	PE	554680		0.2µg/µL	2µL (0.4µg)
	Mouse IgG1, κ	APC	554681		0.2µg/µL	2µL (0.4µg)
	Mouse IgG2a, κ	FITC	555573	5104825	1µg/20µL	1µL (0.05µg)
NKP3 T1	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	CD85j	PE	551053	5156845	1µg/20µL	10µL (0.5µg)
	NKG2A	APC	130-098-812		10µL/test	2µL
	CD122	BB515	564688	4335880	0.5µg/5µL	5µL (0.5µg)

NKP3 T2	CD3	Pacific blue				1 µL
	CD56	PE Cy7				3 µL
	Mouse IgG2b, κ	PE	555743	5078858	1µg/20µL	10µL (0.5µg)
	REA Control (S)	APC	130-104-614			2µL
	Mouse IgG1, κ	BB515	564416		0.2µg/µL	2.5µL (0.5µg)
CD3 Pac Bl	CD3	Pacific blue				0.2 µL
CD56 PE Cy7	CD56	PE Cy7				1.5 µL
NKp46 PE	NKp46	PE	557991	4255803	1µL	1µL
TIM3 BB515	TIM3	BB515	565568	5135962		1 µL
TRAIL APC	TRAIL	APC	563642			0.5 µL
HLA-DR FITC	HLA-DR	FITC	555811	4342790		1µL

Table 2.3 Antibodies for NK receptor surface expression

100 µL of PBMCs were transferred into each of the FACS tubes labelled NKP1 (T1 & T2), NKP2 (T1 & T2) and NKP3 (T1 & T2) for surface staining. These tubes were incubated for 20 minutes at room temperature in the dark. The stained PBMCs were then washed twice with 1mL PBS supplemented with 2% FCS and centrifuged at 1500rpm for 5 minutes. The supernatant was removed with a pipette and 100 µL of 2% formaldehyde was added to fix the stained cells. Centrifugation was performed at 1500 rpm for 5-minutes to pellet the stained, fixed PBMCs. The supernatant was carefully removed with a pipette, leaving approximately 75 µL of fluid. The FACS tubes were vortexed and 1 uL of PI was added and mixed just prior to running on flow cytometry.

2.2.4 TLR Functional Assay

The thawed PBMCs in supplemented RPMI that were set aside (4 x 10^6 / subject) and rested at 37°C for a minimum of 1-hour were used to assess TLR function. The PBMCs were counted using the haemocytometer and viability re-assessed using the trypan blue exclusion test. The PBMCs were re-suspended in supplemented RPMI to obtain a concentration of 2 x 10^6 PBMC/mL.

To obtain a concentration of 2 x 10^6 PBMC/mL (0.5 x 10^6 PBMCs per TLR stimulation), the following formula was used:

V_{PBMCs} Patient 1 = 2 x 10^6/mL x V_{PBMCs}/[thawed PBMCs Patient 1]

$V_{PBMCs} = V_{PBMCs}$ Patient 1 + V_{RPMI}

$V_{RPMI} = V_{PBMCs} - V_{PBMCs}$ Patient 1 → to make total volume up to V_{PBMCs} = (n x 0.25mL) + 0.5mL

To determine the volume of PBMCs required, the following formula was used:

V_{PBMCs} = (n x 0.25mL) + 0.5mL

n = number of stimulation tubes

extra 0.5mL accounts for pipetting error

0.25mL of PBMCs per stimulation tube

To determine the volume of TLR ligand required, the following formula was used:

V_{ligand} = (n x 0.25mL) +0.5mL

n = number of patients

0.25mL per stimulation tube

*note - extra 0.5mL to account for pipetting error

To determine the volume of each TLR ligand required, the following formula was used:

V_{P3C} = 2 x [final] x V_{ligand}/[stock] → VP3C = 200ng/mL x V_{ligand}/10000

To make up the volume of TLR ligand, it was mixed with RPMI media with 1% L-glutamine, 1% penicillin and 5% fetal calf serum.

Table 2.4 details each TLR ligand with its corresponding stock concentration, the concentration used for TLR stimulation and an example of the volume of ligand and volume of RPMI media used.

TLR receptor	Ligand	Stock concentration	Stimulation concentration	2 x [final]	V_{ligand} (µL)	V_{media} (µL)
UnstimµLated						250µL
TLR2	P3C *(InVivoGen)*	10ug/mL	100ng/mL	200ng/mL (1:50)	30µL	1470µL
TLR3	Poly I: *(InVivoGen)*	5mg/mL	10ug/mL	20ug/mL (1:250)	6µL	1494µL
TLR4	LPS *(InVivoGen)*	10000ng/mL (10ug/mL)	100ng/mL	200ng/mL (1:50)	30µL	1470µL
TLR7/8	R848 *(InVivoGen)*	1000ug/mL	5ug/mL	10ug/mL (1:100)	15µL	1485µL
TLR9	CpGODN2006 *(InVivoGen)*	105.3uM (varies)	0.3uM	0.6uM	9µL	1580µL
RIG-I (positive)	5'ppp-dsRNA *(InVivoGen)*	100ug/mL (1ug/0.1mL)	0.5ug/mL	1ug/mL		
RIG-I (negative)	Control *(InVivoGen)*	100ug/mL (1ug/0.1mL)	0.5ug/mL	1ug/mL		

Table 2.4 Volume of TLR ligands used for TLR stimulation

Preparation of the RIG-I ligand (positive - 5'ppp-dsRNA, and negative - control) followed a different protocol. To prepare RIG-I stimulation the following steps were followed. The protocol recommends 10µL 5'ppp dsRNA to 100µL of lyovec. Therefore to make 5 samples, 50 µL 5'ppp dsRNA was added to 550µL of lyovec. This was combined with 700 µL of supplemented RPMI, resulting in a concentration of 5.0ug 5'ppp dsRNA in 1250µL. The same steps were followed for the RIG-I control.

250 µL of each TLR ligand was added to 250 µL of PBMCs into sterile FACS tubes. The sterile FACS tubes were capped only to the first stop, to allow gas exchange. The FACS tubes were placed in incubation at 37°C for 20 hours. The samples were centrifuged at 1500rpm for 5-minutes. The supernatants were aliquoted into three sterile Eppendorf's at 100 µL, 100 µL and 250 µL and stored at -80°C until batch analysis.

The concentrations of TLR ligand and duration of incubation period were chosen based on the results of optimization experiments, to maximize cytokine output.

2.2.5 Measurement of Cytokines by Enzyme-Linked Immunosorbent Assay (ELISA)

Commercial ELISA kits were used to measure the spontaneous secretion of cytokines in plasma. The cytokines measured are as follow:

a. IP-10 (BD Biosciences, CA, USA)

b. MCP-1 (BD Biosciences, CA, USA)

c. IL-6 (BD Biosciences, CA, USA)

d. IFN-γ (BD Biosciences, CA, USA)

e. CXCL9 (R&D systems, MN, USA)

If the cytokine measurements were found to be outside the standard curve, the samples were remeasured at a dilution of 1/10 or 1/100 in Assay Diluent (PBS + 10% FCS).

To perform ELISA for CXCL9, 1% Bovine Serum Albumin Millipore was used instead of Assay Diluent, as recommended in the manufacturer's guidelines (R&D systems, MN, USA)

One day prior to the experiment, a 94-well enhanced protein-binding ELISA plate was prepared with a Coating Buffer solution (0.1M Na2C03 pH 9.5). This consisted of 8.4g of NaHC03 and 3.56g Na2C03 to 50mL of distilled water. 100µL of coating buffer was required per well, therefore a 94 well plate required 9.4mL of coating buffer.

Capture antibody was diluted in Coating Buffer (1:250 dilution with 0.1M carbonate buffer, pH 9.5) and 100µL was added to each well. The plate was sealed in a humidified container and incubated overnight at 4°C.

Table 2.5 is an example of a template used to perform ELISA, with the standards in column 1 and 2. Standards were included in each 94-well ELISA plate, with a replicate of each sample included. If multiple time-points of the same subject were to be measured, these time-points were included on the same 94-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	500pg/ml	500pg/ml	T009-1	T009-1	T009-2	T009-2	T009-3	T009-3	T009-4	T009-4	T009-5	T009-5
B	250	250	T011-1	T011-1	T011-2	T011-2	T011-3	T011-3	T011-4	T011-4	T011-5	T011-5
C	125	125	B010-1	B010-1	B010-2	B010-2	B010-3	B010-3	B010-4	B010-4	B011-6	B011-6
D	62.5	62.5	B011-1	B011-1	B011-2	B011-2	B011-3	B011-3	B011-4	B011-4	B011-5	B011-5
E	31.3	31.3	T012-1	T012-1	T012-2	T012-2	T012-3	T012-3	T012-4	T012-4	T012-5	T012-5
F	15.6	15.6	B12-0	B12-0	B12-1	B12-1	B12-2	B12-2	B12-3	B12-3	B12-4	B12-4
G	7.8	7.8	B12-5	B12-5	T013-1	T013-1	T013-1	T013-1	T001-3	T001-3	T001-4	T001-4
H	0	0	T001-5	T001-5	B002-1	B002-1	B002-2	B002-2	B002-3	B002-3	B002-4	B002-4

Table 2.5 Template for ELISA plate

On the day of the experiment, the ELISA plate was removed from the fridge and brought to room temperature. The capture antibody solution was removed using a closed-circuit container and washed three times with 300µL of Wash Buffer (PBS/Tween® - 0.5 ml of Tween®-20 in 1 L PBS). The plate was blotted to remove any residual liquid.

250mL of Assay diluent was prepared (PBS + 10% FCS - 225mL PBS and 25mL FCS). 200 µl of Assay Diluent (10% FCS in PBS) was added to each well. The Assay Diluent was used as a blocking buffer to block non-specific binding. The plate was sealed in a humidified container and incubated at room temperature for 1 hour. The blocking buffer was removed and the plate was washed three times with 300µL of Wash Buffer. The plate was blotted to remove any residual liquid.

Stock standard solutions and their dilutions were prepared as per manufacturer's guidelines. One stock standard solution was made at a concentration of 500pg/mL. Six dilutions were derived from the stock standard solution, but combining 300µL of the stock standard solution with 300µL of assay diluent. This was vortexed and made a concentration of 250pg/mL. This step was repeated by combining 300 µL of the second standard (250pg/mL) with 300µL of assay diluent, to form a concentration of 125pg/mL. This step was repeated four more times to produce standards with

concentrations of 62.5pg/mL, 31.3 pg/mL, 15.6pg/mL, and 7.8pg/mL. Assay diluent was used for the final standard at a concentration of 0pg/mL. The standard concentrations were used to generate a standard curve to enable measurement of each cytokine.

1mL of frozen plasma for each subject was thawed to room temperature and spun at 13,500 rpm for 5 minutes. 100 µL of each sample and standard were aliquoted into the plate as per the template. The plate was sealed in a humidified container and incubated at room temperature for 2-hours. The standards and samples were removed and the plate was washed five times with PBS/Tween®.

100 µL of diluted detection antibody in Assay Diluent (detector antibody plus substrate enzyme Streptavidin-HRP, diluted 1:250 in Assay Diluent) was added to each well. The plate was sealed in a humidified container and incubated for 1 hour at room temperature. The detection antibody was removed and the plate was washed seven times with Wash Buffer, with residual fluid blotted from plate.

100µL of Substrate Solution (Sure Blue tetramethylbenzidine microwell peroxidase) was added to each well, and the plate was incubated at room temperature for 15-minutes (without a container).

50µL of Stop Solution (2M H2SO4) was then added to each well, at a similar rate to addition of the Substrate Solution. The plate was immediately placed into an ELISA microplate reader and the absorbance was measured at 450nm.

2.2.6 Measurement of Cytokines by Cytometric Bead Array Assay on TLR Stimulated PBMCs

The cytometric bead array (CBA) assay was performed on thawed tissue culture supernatants. The cytokines measured included:

a. Human IFN-γ (BD Biosciences, CA, USA)

b. Human IL-10 (BD Biosciences, CA, USA)

c. Human IFN-α (BD Biosciences, CA, USA)

d. Human TNF-α (BD Biosciences, CA, USA)

e. Human IL-1b (BD Biosciences, CA, USA)

f. Human IL-8 (BD Biosciences, CA, USA)

g. Human IL-6 (BD Biosciences, CA, USA)

h. Human IP-10 (BD Biosciences, CA, USA)

i. Human MCP-1 (BD Biosciences, CA, USA)

j. Human CXCL9 (BD Biosciences, CA, USA)

Human IFN-γ, IL-10 and IFN-α were measured at 1:1 concentration, with these analytes performed in the same experiment. The remaining cytokines were measured at dilutions of 1:1, 1:10 and 1:100, to accommodate high levels of cytokine, and performed in the same experiment.

Table 2.6 is an example of a template used to perform the CBA for one experiment. Each well was identified by the sample name, dilution factor and TLR stimulant. Wash buffer was allocated to one well per experiment to act as a control.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	wash buffer 1:1	A09-5 RIG-I (+) 1:1	A09-5 RIG-I cont 1:1	A10-1 US 1:1	A10-1 P3C 1:1	A10-1 PIC 1:1	A10-1 LPS 1:1	A10-1 R848 1:1	A10-1 CpG 1:1	A10-1 RIG-I (+) 1:1	A10-1 RIG-I cont 1:1	A10-2 US 1:1
B	A10-2 P3C 1:1	A10-2 PIC 1:1	A10-2 LPS 1:1	A10-2 R848 1:1	A10-2 CpG 1:1	A10-2 RIG-I (+) 1:1	A10-2 RIG-I cont 1:1	A10-4 US 1:1	A10-4 P3C 1:1	A10-4 PIC 1:1	A10-4 LPS 1:1	A10-4 R848 1:1
C	A10-4 CpG 1:1	A10-4 RIG-I (+) 1:1	A10-4 RIG-I cont 1:1	A10-5 US 1:1	A10-5 P3C 1:1	A10-5 PIC 1:1	A10-5 LPS 1:1	A10-5 R848 1:1	A10-5 CpG 1:1	A10-5 RIG-I (+) 1:1	A10-5 RIG-I cont 1:1	A11-1 US 1:1
D	A11-1 P3C 1:1	A11-1 PIC 1:1	A11-1 LPS 1:1	A11-1 R848 1:1	A11-1 CpG 1:1	A11-1 RIG-I (+) 1:1	A11-1 RIG-I cont 1:1	A11-2 US 1:1	A11-2 P3C 1:1	A11-2 PIC 1:1	A11-2 LPS 1:1	A11-2 R848 1:1
E	A11-2 CpG 1:1	A11-2 RIG-I (+) 1:1	A11-2 RIG-I cont 1:1	A11-4 US 1:1	A11-4 P3C 1:1	A11-4 PIC 1:1	A11-4 LPS 1:1	A11-4 R848 1:1	A11-4 CpG 1:1	A11-4 RIG-I (+) 1:1	A11-4 RIG-I cont 1:1	A11-5 US 1:1
F	A11-5 P3C 1:1	A11-5 PIC 1:1	A11-5 LPS 1:1	A11-5 R848 1:1	A11-5 CpG 1:1	A11-5 RIG-I (+) 1:1	A11-5 RIG-I cont 1:1					
G												
H												

Table 2.6 Template for CBA

Preparation of standards

1. One vial of lyophilised Human Inflammatory Cytokines Standard for each cytokine to be measured in the same multiplex assay, were pooled in a 10mL yellow top polypropylene tube. 2.0 mL of Assay Diluent was added and allowed to sit

for a minimum of 15-minutes, then gently mixed with a pipette. This tube was labelled as the Top Standard (5000 pg/mL).

2. 300 µL of Assay Diluent were pipetted into ten yellow top tubes and labelled as per the following:

a. 1:2 dilution – 2500 pg/mL

b. 1:4 dilution – 1250 pg/mL

c. 1:8 dilution – 625 pg/mL

d. 1:16 dilution – 312.5 pg/mL

e. 1:32 dilution – 156 pg/mL

f. 1:64 dilution – 80 pg/mL

g. 1:128 dilution – 40pg/mL

h. 1:256 dilution – 20pg/mL

i. Assay dilutent – 0pg/mL

3. Serial dilution was performed transferring 300 µL from the Top Standard into the tube labelled, 1:2 dilution. This was mixed thoroughly with gentle pipetting, and 300 µL was transferred into the 1:4 dilution, and so on, to the 1:256 tube.

Preparation of Mixed Human Inflammatory Cytokine Capture Beads

1. The number of tests to be performed were calculated, to include standards + controls + samples (including dilutions)

2. The total volume of capture beads and diluent was calculated as per the following:

= 25 µL x (number of samples + 10 standards + 4 controls)

The volume of each capture bead was calculated as per the following:

= 0.5 µL x (number of tests)

The volume of capture bead diluent was calculated as per the following:

= total volume – (volume of capture beads) x ‘number of analytes’

3. Each Capture Bead was vortexed aggressively into a suspension for a few seconds and the calcµLated volume was added to a single tube labelled “mixed Capture Beads” with the volume of Capture Bead Diluent.

4. The Bead mixture was vortexed thoroughly

Preparation of PE Detection Reagent

The same calculations apply as per the mixed capture beads.

Preparation of Cytometer Setup Beads

1. Add 15 µl of Cytometer Setup Beads to three cytometer setup tubes labeled A, B and C.

2. Add 15 µl of FITC Positive Control Detector to tube B.

3. Add 15 µl of PE Positive Control Detector to tube C.

4. Incubate tubes A, B, and C for 30 minutes at room temperature, in the dark.

5. Add 450 µl of Wash Buffer to tube A and 400 µl of Wash Buffer to tubes B and C.

Preparation of Test Samples

1. Frozen tissue culture supernatants were removed from the -80 C freezer and allowed to thaw to room temperature. The Eppendorf’s were spun for 5-minutes at 13,500 rpm.

2. Each test sample was diluted to a desired factor of 1:1, 1:10, 1:50 or 1:100 with Assay Diluent. An example is as follows:

1:10 dilution – 90 µL of Assay Diluent + 10 µL of NEAT sample

1:50 dilution – 245 Assay Diluent + 5 µL of NEAT sample

1:100 dilution – 90µL of Assay Diluent + 10µL of 1:10 diluted sample

3. The diluted test samples were vortexed to ensure thorough mixing.

Assay procedure

1. 15 µL of the mixed Capture Beads were added to all test tubes. This included samples, controls, tests and corresponding diluted tests. The mixed Capture Beads were vortexed after every 10 tubes, to ensure it remained thoroughly mixed.

2. 15 µL of each sample was aliquoted to its correspondingly labelled, non-sterile FACS tube, including the standards

3. A quick vortex was performed to mix with Capture Beads.

4. The tubes were incubated at room temperature for 1-hours, in the dark.

5. 1mL of Wash buffer was added to each tube and centrifuged at 200 x g for 5-minutes.

6. Supernatant was carefully removed with a pipette from each tube, leaving a residual 75µL of liquid in each tube.

7. 15 µL of PE Detection Regent was added into the residual fluid. A quick, gentle vortex was performed to ensure mixed.

8. The tubes were incubated at room temperature for 2-hours, in the dark.

9. 1mL of Wash buffer was added to each tube and centrifuged at 200 x g for 5-minutes.

10. Supernatant was carefully removed with a pipette from each tube, leaving a residual 75µL of liquid in each tube.

11. 150 µL of wash buffer was added to tube to resuspend the pellet, ready to analyse on a flow cytometer.

The intensity of fluorescence signal was measured on the FACS Canto Flow Cytometer (Becton Dickinson, USA) and data analysed using the FCAP Array Software (BD Biosciences).

2.2.7 Measurement of Cytokines in Plasma by Enhanced Cytometric Bead Array Assay

The enhanced sensitivity cytometric bead array (CBA) assay was used to detect the spontaneous secretion of the following panel of cytokines, to allow for the detection of levels as low as 0.274 pg/mL.

a. IFN-γ (BD Biosciences, CA, USA)

b. IL-6 (BD Biosciences, CA, USA)

c. IL-8 (BD Biosciences, CA, USA)

d. IL-10 (BD Biosciences, CA, USA), and

e. TNF-α (BD Biosciences, CA, USA).

All cytokines were measured at 1:1 concentration.

The protocol had variations to that used for the standard cytometric bead assay and is as follows.

Preparation of standards

1. One vial of lyophilised Human Inflammatory Cytokines Standard for each cytokine to be measured in the same multiplex assay, were pooled in a 10mL yellow top polypropylene tube. 4.0 mL of Assay Diluent was added and allowed to sit for a minimum of 15-minutes, then gently mixed with a pipette. This formed the reconstituted standards.

2. 460 µL of assay diluent was pipetted into a tube labelled as the Top Standard (200,000 fg/mL).

3. 400 µL of Assay Diluent were pipetted into yellow top tubes and labelled as per the following:

   a. 1:3 dilution – 66,667 fg/mL

   b. 1:9 dilution – 22,222 fg/mL

   c. 1:27 dilution – 7,407 fg/mL

d. 1:81 dilution – 2,469 fg/mL

e. 1:243 dilution – 823 fg/mL

f. 1:729 dilution – 274 fg/mL

g. Assay diluent – 0 fg/mL

4. Serial dilution was performed transferring 40 µL of the reconstituted standards into the Top Standard tube. This was mixed thoroughly with gentle pipetting, and 200 µL from the Top Standard was transferred into the 1:3 dilution, and so on, to the 1:729 tube. The final tube labelled 0 fg/mL had no standard added, acting as a negative control.

Preparation of Mixed Human Inflammatory Cytokine Capture Beads

1. The number of tests to be performed were calculated, to include standards + controls + samples

2. The total volume of capture beads and diluent was calculated as per the following:

= 15 µL x (number of samples + 10 standards + 4 controls)

The volume of each capture bead was calculated as per the following:

= 0.75 µL x (number of tests)

The volume of capture bead diluent was calculated as per the following:

= total volume – (volume of capture beads) x 'number of analytes'

3. Each Capture Bead was vortexed aggressively into a suspension for a few seconds and the calculated volume was added to a single tube labelled "mixed Capture Beads" with the volume of Capture Bead Diluent.

4. The Bead mixture was vortexed thoroughly

Preparation of Human Detection Reagent (Part A)

1. The number of tests were calculated as per for capture beads

2. The total volume of human detection reagent and diluent was calculated as per the following:

The total volume required is calculated as

= 15 µL x (number of tests)

The total volume of Human Detection Reagent (Part A)

= number of tests x 0.75 µL

The volume of Human Detection Reagent Diluent

= total volume – (volume of human detection reagent x 'number of analytes')

3. The Human Detection Reagent (Part A) was added to the Detection Reagent Diluent, labelled as 'Mixed Human Detection Reagents' and stored at 4-degrees, in the dark.

Preparation of Enhanced Sensitivity Detection Reagent (Part B)

1. One vial (per 50 tests) of lyophilised Enhanced Sensitivity Reagent was reconstituted in 550µL of Detection Reagent Diluent and incubated at 15 minutes at room temperature, in the dark. This was gently mixed with a pipette

2. 4.5mL of Detection Reagent Diluent was added into a 15mL conical polypropylene tube

3. 0.5mL of reconstituted Enhanced Sensitivity Reagent (Part B) was added to the conical tube and gently vortexed

Assay procedure

1. 15 µL of the mixed Capture Beads were added to all test tubes. This included standards, controls, and tests. The mixed Capture Beads were vortexed after every 10 tubes, to ensure it remained thoroughly mixed.

2. 15 µL of each sample was aliquoted to its correspondingly labelled, non-sterile FACS tube

3. 50µL of each standard dilution was added to its correspondingly labelled, non-sterile FACS tube

4. A quick vortex was performed to mix with Capture Beads.

5. The tubes were incubated at room temperature for 2-hours, in the dark.

6. 15 µL of Human Detection Regent (Part A) was added. A quick, gentle vortex was performed to ensure mixed.

7. The tubes were incubated at room temperature for 2-hours, in the dark.

8. 1mL of Wash buffer was added to each tube and centrifuged at 200 x g for 5-minutes.

9. Supernatant was carefully removed with a pipette from each tube, leaving a residual 75µL of liquid in each tube.

10. 100µL of Enhanced Sensitivity Detection Reagent (Part B) was added to each tube and gently mixed

11. The tubes were incubated at room temperature for 1-hour, in the dark

12. 1mL of Wash buffer was added to each tube and centrifuged at 200 x g for 5-minutes.

13. Supernatant was carefully removed with a pipette from each tube, leaving a residual 75µL of liquid in each tube

14. 150 µL of wash buffer was added to tube to resuspend the pellet, ready to analyse on a flow cytometer.

2.2.8 NK Functional Assay

The thawed PBMCs in supplemented RPMI that were set aside (4 x 10^6 / subject) and rested at 37°C for a minimum of 1-hour were used to assess TLR function. The PBMCs were counted using the haemocytometer and viability re-assessed using the trypan blue exclusion test. The PBMCs were re-suspended in supplemented RPMI to obtain a concentration of 2 x 10^6 PBMC/mL.

Table 2.7 details each ligand used to assess NK function, with its corresponding stock concentration, and the concentration used for stimulation.

Ligand	[stock]	[Stimulation]	2 x [final]
Supplemented RPMI (unstimulated)			
IL-12	10ug/mL	0.5ng/mL	1ng/mL
IL-15	10ug/mL	20ng/mL	40ng/mL

Table 2.7 NK ligands to assess NK function

To prepare the IL-12 ligand, the following steps were taken:

1. 10µL aliquot of IL-12 (10ug/mL) + 90µL media (1:10)

= 100µL at 1ug/mL or 100µL at 1000ng/mL

2. 100µL + 9900µL media (1:100)

= 10mL at 10ng/mL

To prepare the IL-15 ligand, the following steps were taken:

1. 10µL aliquot of IL-15 (10ug/mL) + 90µL media (1:10)

= 200µL at 1ug/mL or 200µL at 1000ng/mL

2. 100µL + 150µL media (1:2.5)

= 250µL at 400ng/mL

Two aliquots were prepared to make a total of 500µL.

To obtain 2 x the final concentration, 500µL of IL-12 ligand at 10ng/mL was added to 500µL of IL-15 at 400ng/mL and 4mL of supplemented RPMI, for 10 samples.

Using sterile, covered FACS tubes, 500µL of prepared media was added 2 separate FACS tubes labelled:

a. IL-12 (1ng/mL) + IL-15 (40ng/mL)

b. RPMI with 5% FCS only

500µL of resuspended PBMCs was then added to each tube with a final concentration of ($1x10^6$ cells/mL) to make:

a. IL-12 (0.5ng/mL) + IL-15 (20ng/mL)

b. RPMI with 5% FCS only

The FACS tubes were incubate for 14 hours at 37°C.

The following day, 1µL Brefeldin (Golgi Plug) was added to each FACS tube and incubated for 4 hours at 37°C. The FACS tubes were centrifuged at 1500rpm for 5 min and the supernatant removed. One wash was performed with 2 mL PBS (to remove media) and centrifuged 1500 rpm for 5 min. The supernatant was removed and 1mL PBS and 1.0 ML FVS 510 (as 1 x 10^6 cells, for 1:1000 dilution); a viability stain. The samples were incubated for 15 minutes at room temperature in the dark. To stop the staining process, the cells were washed twice with 1mL PBS 2% FCS and centrifuged at 1500rpm for 5-minutes. The supernatant was removed leaving the pellet in 100µL of PBS, ready for surface staining.

The following antibodies were added to each FACs tube for surface staining, as per Table 2.8.

FACS tube label	Marker	Fluorophore	Volume added
CD3 Pac Bl	CD3	Pacific blue	0.2 µL
CD56 PE Cy7	CD56	PE Cy7	1.5 µL

Table 2.8 Antibodies for TLR surface expression

Four unsterile FACS tubes were labelled as follows for single-stain compensation tubes - CD3 Pac Bl, CD56 PE-Cy7, IFN-γ PE and TNFa APC.

Antibodies were added to the labelled, single-stain compensation tubes as per Table 2.9.

FACS tube label	Marker	Fluorophore	Category number	Lot number	Volume added
CD3 Pac Bl	CD3	Pacific blue			0.2 µL
CD56 PE Cy7	CD56	PE Cy7			1.5 µL
IFNg PE	IFNg	PE	554701		0.5µL
TNFa APC	TNFa	APC	554514	4302508	0.3µL

Table 2.9 Antibodies for single stain compensation tubes

100µL PBMCs were added to each of the single stain compensation tubes.

These tubes were incubated for 20 minutes at room temperature in the dark. The stained PBMCs were then washed twice with 1mL PBS supplemented with 2% FCS and centrifuged at 1500rpm for 5 minutes. The supernatant was removed with a pipette and 100 µL of 2% formaldehyde was added to fix the stained cells. Centrifugation was performed at 1500 rpm for 5-minutes to pellet the stained, fixed PBMCs. The supernatant was removed.

In the chemical hood, 250 µl BD Cytofix/Cytoperm was added to in all FACS tubes, including compensation tubes, and incubated for 20 minutes at 4°C for intra-cellular staining. Two washes were performed with 1mL BD Perm/Wash (1:10 solution with distilled H_2O).

To make up 1x concentrated BD Perm/Wash at 1:10 dilution with dH_2O the following formula was used:

Total volume required = 4mL X # of FACS tubes (this includes extra as surface marker compensation tubes only need 2 washes).

The following antibodies were added to each FACs tube for intra-cellular staining, as per Table 2.10.

Antibody	Marker	Fluorophore	Category Number	Lot Number	Recommended volume	Volume added
IFNg PE	IFNg	PE	554701		0.2µg/µL	0.5µL (0.1µg)
TNFa APC	TNFa	APC	554514	4302508	0.2µg/µL	2µL (0.4µg)

Table 2.10 Antibodies for intra-cellular staining

The FACS tubes were incubated for 30 minutes at room temperature, in the dark. Two washes with 1 BD Perm/Wash were performed, with centrifugation at 2500rpm for 5min, and the supernatant removed from the pelleted cells, leaving a residual 100 µL, ready for flow cytometry.

Frequency of IFN-γ positive NK cells that were incubated in complete medium without cytokines was subtracted from the frequency of IFN-γ positive NK cells stimulated with cytokines.

2.2.9 RNA Extraction from Whole PBMCs

The RNAqueous®-4PCR Total RNA Isolation Kit (Ambion, Austin, TX, USA) was used for extraction of RNA. PBMCs from liquid nitrogen storage were thawed rapidly in a 37°C water-bath, then diluted with 9mL of sterile PBS in a 10mL falcon tube to remove any residual DMSO. The diluted PBMCs were mixed by inversion and pelleted by centrifugation at 1500rpm for 5-minutes. The supernatant was discarded and the pelleted cells resuspended in 1mL of supplemented RPMI Trypan blue exclusion was used to assess PBMC viability, which was greater than x%. PBMCs were then re-pelleted by centrifugation at 1500rpm for 5 minutes, and the cell pellet dissolved in 300µL of cell lysis buffer. The lysate was left to incubate at room temperature for a minimum of 10-minutes before continuing with the RNA extraction, or stored at -80°C long term.

2.2.9.1 RNA isolation

An equal volume (300µL) of 64% Ethanol was added to the lysate and mixed gently. The lysate/ethanol mixture (600µL total volume) was applied to a filter cartridge in a collection tube and centrifuged at 13,500 rpm for 1-minute. The flow-through was discarded and the cartridge was then washed with 650 µL of Wash solution#1 , twice with 450 µL Wash Solution 2/3 and finally dried by an additional centrifugation at 13,500 rpm for 2-minutes.

RNA was eluted into a fresh collection tube with 60 µL of Elution Solution (pre-heated to 80°C). After addition of the Elution Solution, the filter cartridge was allowed to incubate at room temperature for 1-minute to facilitate elution of RNA from the filter, then centrifuged at 13,500 rpm for 1-minute. Elution was repeated with an additional aliquot of 20 µL of elution solution and the eluates combined.

2.2.9.2 DNAseI Treatment and DNAseI Inactivation

Traces of DNA in the extracted RNA solution were removed by digestion with DNAseI. 1µL of DNAse 1 and 0.1 volume (8µL) of x10 DNAse Buffer were added to 80µL of the RNA sample, mixed gently and incubated on a 37°C heat block for 15 minutes. At the end of the incubation, DNAseI was inactivated by the addition of 0.1 volume (8µL) of DNAseI Inactivation Reagent. The collection tube was mixed gently to ensure dispersion of the DNAse Inactivation Reagent and allowed to sit at room

temperature for 2-minutes. After centrifugation at 13,500 rpm at 1-minute, the RNA was carefully transferred to a fresh tube and quantified on the NanoDrop™1000 Spectrophotometer v3.7 (ThermoScientific).

The RNA was stored in sterile RNAse/DNAse free tubes for long term storage at -80°C or used immediately for complimentary DNA (cDNA) preparation.

2.2.9.3 cDNA Preparation

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) was used to prepare cDNA. A 20 µL cocktail consisting of 4.0 µL of 10 x RT buffer, 1.6 µL of 25 x dNTP (100mM), 4.0µL x RT primers, 6.4 µL of sterile water, 2.0 µL of RNAseOut and 2.0 µL of Multiscribe RTAse, was added to 20 µL of RNA. The tube was mixed gently and allowed to incubate at room temperature for 10 minutes, then at 37°C for 120 minutes, followed by a 5-minute incubation at 85°C. To facilitate visualization of the RNA pellet, 2.0 µL of glycoblue (1ug/µL) was added to the sample. The mixture was then precipitated with 7.0 µL of 3M sodium acetate and 300 µL of 100% ethanol at -80°C overnight. The precipitated cDNA was then centrifuged at 13,500 rpm for 20-minutes, and the supernatant carefully removed with a pipette and discarded. The cDNA pellet was then washed with 400µL of 70% ETOH and centrifuged at 13,500 rpm for 10 minutes. The supernatant was again carefully removed and discarded. The pellet was allowed to air- dry for 5-minutes, then dissolved overnight in 300µL of TE (10mmol Tris buffer and 1mL EDTA) and used immediately or stored at -80°C long term.

2.2.9.4 cDNA Preparation

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) was used to prepare cDNA. A 20 µL cocktail consisting of 4.0 µL of 10 x RT buffer, 1.6 µL of 25 x dNTP (100mM), 4.0 µL x RT primers, 6.4 µL of sterile water, 2.0 µL of RNAseOut and 2.0 µL of Multiscribe RTAse, was added to 20 µL of RNA. The tube was mixed gently and allowed to incubate at room temperature for 10 minutes, then at 37°C for 120 minutes, followed by a 5-minute incubation at 85°C. To facilitate visualization of the RNA pellet, 2.0 µL of glycoblue (1ug/µL) was added to the sample. The mixture was then precipitated with 7.0 µL of 3M sodium acetate and 300 µL of

100% ethanol at -80°C overnight. The precipitated cDNA was then centrifuged at 13,500 rpm for 20-minutes, and the supernatant carefully removed with a pipette and discarded. The cDNA pellet was then washed with 400µL of 70% ETOH and centrifuged at 13,500 rpm for 10 minutes. The supernatant was again carefully removed and discarded. The pellet was allowed to air-dry for 5-minutes, then dissolved overnight in 300µL of TE (10mmol Tris buffer and 1mL EDTA) and used immediately or stored at -80°C long term.

2.2.9.5 PCR of cDNA

PCR was performed on an Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems, CA, USA). Forward and reverse primers were used a final concentration of 250nM (Table 2.11) in a 20 µL reaction containing 5 µL of cDNA, 1 µL each forward and reverse primers, 3 µL of sterile water, and 10 µL of SYBR Green Master Mix (Applied Biosystems) as listed in Table 2.11.

GENE	Forward Primer	Reverse Primer	Accession
OAS1	5'-CTTCGTACTGAGTTCGCTCCA	5'-TGCACTCCTCGATGAGCTTGA	NM_001032409
MX1	5'-AGATTGAGATTTCGGATGCTTCA	5'-GTCAGATCCGGGACATCTCG	NM_001144925
ISG15	5'-GCTGAGAGGCAGCGAACTCA	5'-CACGCCGATCTTCTGGGTGA	NM_005101
IP-10 (IP-10)	5'-GACTCTAAGTGGCATTCAAGGA	5'-GATTCAGACATCTCTTCTCACC	NM_001565
ACTBA (bActin)	5'-CATTGCYGACAGGATGCAGAA	5'-GCTGATCCACATCTGCTGGAA	NM_001101
GAPDH	5'-ATGGGTGTGAACCATGAGAAGT	5'-AGTCCTTCCACGATACCAAAGT	NM_002046
IL-10	5'-GAGATGCCTTCAGCAGAGTGA	5'-CTGGGTCTTGGTTCTCAGCTT	NM_000572
IL-6	5'-CAGACAGCCACTCACCTCTTC	5'-CAGGTTGTTTTCTGCCAGTGC	NM_000600
SOCS1	5'-TTCGCCCTTAGCGTGAAGATG	5'-CAGCTCGAAGAGGCAGTCGA	NM_003745
CXCL8 (IL-8)	5'-GCTCTGTGTGAAGGTGCAGTT	5'-TCCTTGGGGTCCAGACAGAG	NM_000584
CCL2 (MCP-1)	5'-GAAAGTCTCTGCCGCCCTTC	5'-GCTTCTTTGGGACACTTGCTG	NM_002982
CXCL9	5'-GAGAAAGGGTCGCTGTTCCTG	5'-CCTTCACATCTGCTGAATCTGG	NM_002416

Table 2.11 Forward and reverse mRNA PCR primer sequences

3 Sustained Virological Response Halts Fibrosis Progression: A Long-Term Follow-Up Study of People with Chronic Hepatitis C Infection

PLOS ONE

RESEARCH ARTICLE

Sustained virological response halts fibrosis progression: A long-term follow-up study of people with chronic hepatitis C infection

Swee Lin G. Chen Yi Mei[1,2]*, Alexander J. Thompson[1,2], Britt Christensen[1], Georgina Cunningham[1], Lucy McDonald[1], Sally Bell[1,2], David Iser[1], Tin Nguyen[1], Paul V. Desmond[1,2]

1 Department of Gastroenterology, St Vincent's Hospital, Melbourne, Australia, **2** Department of Medicine, University of Melbourne, Melbourne, Australia

* sweelin.chenyimei@svha.org.au

OPEN ACCESS

Citation: Chen Yi Mei SLG, Thompson AJ, Christensen B, Cunningham G, McDonald L, Bell S, et al. (2017) Sustained virological response halts fibrosis progression: A long-term follow-up study of people with chronic hepatitis C infection. PLoS ONE 12(10): e0185609. https://doi.org/10.1371/journal.pone.0185609

Editor: Chen-Hua Liu, National Taiwan University Hospital, TAIWAN

Received: July 11, 2017

Accepted: September 15, 2017

Published: October 24, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the Gastroenterological Society of Australia - postgraduate clinical research scholarship to Swee Lin G Chen Yi Mei. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background/Aims

Long-term follow-up studies validating the clinical benefit of sustained virological response (SVR) in people with chronic hepatitis C (CHC) infection are lacking. Our aim was to identify rates and predictors of liver fibrosis progression in a large, well characterized cohort of CHC patients in whom paired liver fibrosis assessments were performed more than 10 years apart.

Methods

CHC patients who had undergone a baseline liver biopsy pre-2004 and a follow up liver fibrosis assessment more than 10 years later (biopsy or liver stiffness measurement (LSM) using transient elastography [FibroScan]) were identified. Subjects who had undergone a baseline liver biopsy but had no follow up fibrosis assessment were recalled for LSM. Fibrosis was categorised as mild-moderate (METAVIR F0-2 / LSM result of $\leq$ 9.5 kPa) or advanced (METAVIR F3-4/ LSM >9.5 kPa). The primary objective was to assess the association between SVR and the rate of liver fibrosis progression over at least 10 years, defined as an increase from mild-moderate fibrosis at baseline liver biopsy (METAVIR F0-2) to advanced fibrosis at follow-up liver fibrosis assessment.

Results

131 subjects were included in this analysis: 69% male, 82% Caucasian, 60% G1 HCV, 25% G3 HCV. The median age at F/U fibrosis staging was 57 (IQR 54–62) years with median estimated duration of infection 33-years (IQR 29–38). At F/U, liver fibrosis assessment was performed by LSM in 86% and liver biopsy in 14%. The median period between fibrosis assessments was 14-years (IQR 12–17). 109 (83%) participants had received interferon-based antiviral therapy. 40% attained SVR. At F/U, there was a significant increase in the proportion of subjects with advanced liver fibrosis: 27% at baseline vs. 46% at F/U (p = 0.002). The prevalence of advanced fibrosis did not change among subjects who attained

Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: AJ Thompson: Research Grant – Gilead Sciences, Abbvie, BMS, Spring Bank Pharmaceuticals, Merck. Consultant – Gilead Sciences, Abbvie, BMS, Roche Diagnostics, Merck, Spring Bank Pharmaceuticals. Sponsored Lecture – Roche Diagnostics, Gilead Sciences, Abbvie, BMS. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

SVR, 30% at B/L vs 25% at F/U (p = 0.343). However, advanced fibrosis became more common at F/U among subjects with persistent viremia: 10% at B/L vs 31% at F/U (p = 0.0001). SVR was independently associated with protection from liver fibrosis progression after adjustment for other variables including baseline ALT (p = 0.011), duration of HCV infection and mode of acquisition.

Conclusion

HCV eradication is associated with lower rates of liver fibrosis progression. The data support early treatment to prevent long-term liver complications of HCV infection.

Introduction

Approximately 115 million people are chronically infected with hepatitis C (HCV) worldwide, including 230,000 Australians.[1,2] HCV is associated with complications including liver cirrhosis, liver failure and hepatocellular carcinoma. HCV is the most common indication for liver transplant in high-income countries including Australia. Antiviral therapy can eradicate HCV. It is assumed that eradicating HCV will prevent these complications.

The landscape of HCV treatment has changed dramatically over recent years. Interferon-free combinations of direct acting antiviral (DAA) agents are now standard of care, with cure rates approaching 100% and excellent tolerability. However the cost associated with the new DAAs is a major barrier to treatment access. Therefore, it is important to evaluate long-term clinical outcomes, to confirm that viral eradication is associated with predicted clinical benefit.

Clinical trials evaluating antiviral therapy for HCV use sustained virological response (SVR) as a surrogate for clinical benefit. Until recently, there has been little data available confirming the impact of SVR on long-term clinical outcomes of people with CHC. In particular, there are limited data concerning the long-term benefit of SVR on fibrosis progression in non-cirrhotic subjects.

In selected high-risk populations of subjects with advanced fibrosis or cirrhosis, SVR is associated with improved overall and liver-related survival, as well as reduced rates of hepatic de-compensation and hepatocellular carcinoma.[3–7] These results have been replicated in numerous, multi-centre studies of up to 500 subjects with a follow up of 3–8 years. However, data demonstrating a clinical benefit from SVR in people with less advanced disease are scarce.

There are data to suggest that SVR leads to fibrosis regression. Clinical trials based on paired liver biopsies have shown SVR to be associated with histological improvement in both grade and stage. However, these studies were limited by a short-term follow up of only 12–24 months.[8–10] Recent data from Spain suggest that SVR is associated with significant reduction in the risk of progression to cirrhosis in a population of patients with median duration of follow-up of 10 years. A similar retrospective study in Korea observed an association between SVR and lower risk of cirrhosis over median follow-up of 4 years. In both studies, the definition of cirrhosis at long-term F/U was based on radiological features for most patients. Transient elastography has greater sensitivity for the presence of advanced fibrosis / cirrhosis than ultrasound, meaning that these studies may have under-estimated the risk of fibrosis progression.

We describe a large, well-characterized cohort of CHC patients followed longitudinally in whom paired liver fibrosis assessments were performed more than 10 years apart. We used transient elastography and liver stiffness measurement to define the presence of advanced

fibrosis / cirrhosis at follow-up. We show that achievement of SVR halts liver fibrosis progression. The data supports early treatment of all people with CHC, regardless of liver fibrosis stage, to prevent long-term liver sequelae.

Methods

Data resource

The St Vincent's Hospital Hepatitis C electronic database (HCV database) is a hospital-based patient database that comprehensively records up-to-date patient characteristics (demographic and biochemical), treatment history, liver fibrosis stage and hospital clinic attendance.

Study population

This was a retrospective / prospective study. Subjects who had undergone a first time liver biopsy before 2004 were identified from the HCV database. For inclusion in the current study, subjects were required to have a second liver fibrosis assessment $\geq$ 10 years after the original assessment. 106 patients in the database had paired liver fibrosis assessments more than 10 years apart. If follow-up liver fibrosis staging had not been performed more than 10-years after baseline liver biopsy, subjects received an invitation by mail to present for liver stiffness measurement (LSM) and a blood test. 25 subjects were recalled by this method. We excluded subjects co-infected with hepatitis B or HIV. Demographic, clinical, histological and biochemical data were collected from the HCV database.

Timepoints

CHC was defined as persistent HCV RNA for at least 6-months after onset of acute infection or known exposure. The estimated duration of infection was calculated from the year of known exposure or episode of acute infection. A liver biopsy performed after diagnosis of chronic HCV infection formed the first liver fibrosis assessment. Second liver fibrosis measurement was defined as either a liver biopsy or LSM performed $\geq$ 10 years after first liver fibrosis assessment. HCV treatment was recorded as the completion date of antiviral therapy, with a sustained virological response (SVR) defined as an undetectable HCV RNA 24-weeks post antiviral therapy.

Blood test

Blood test included full blood count, electrolytes, liver function test, IL28B genotype, HCV viral load and genotype, and HBV serology.

Liver fibrosis evaluation and categorisation

Fibrosis was categorised as mild-moderate for a biopsy with a METAVIR stage of 0–2 or a LSM result of $\leq$ 9.5 kPa and as advanced for a biopsy with a METAVIR stage of $\geq$ 3 or a LSM result of $>$ 9.5 kPa.

Liver stiffness measurement by transient elastography

LSM was assessed using transient elastography according to the manufacturer's guidelines, and considered valid if a minimum of 10 measurements were obtained with at least a 60% success rate and an interquartile range $<$0.3 of the median value. Operators at our institution had performed a minimum of 100 procedures.

Liver histology

Liver histology was assessed by pathologists specialized in liver disease. Specimens were obtained by percutaneous liver biopsy, then fixed, paraffin-embedded, and stained with haematoxylin-eosin and Masson's trichrome. Histological fibrosis stage was scored according to the METAVIR classification, defined as F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.[11]

Outcomes analysis

The primary objective was to assess the effect of SVR on the rate of liver fibrosis progression over 10-years. Participants with advanced fibrosis (F3/F4), or cirrhosis (F4), at baseline were excluded in this analysis. SVR was defined as an undetectable HCV PCR 6-months post antiviral therapy. Fibrosis progression was defined as an increase from mild-moderate fibrosis at baseline liver biopsy (METAVIR F0-2) to advanced fibrosis at follow-up liver biopsy (METAVIR F3-4) or LSM (>9.5 kPa). The secondary objective was to identify variables influencing liver fibrosis progression, and included age, gender, hepatitis C genotype (genotype 1 vs. non-genotype 1), mode of HCV acquisition (blood transfusion vs. other), body mass index (BMI), baseline ALT, HCV viral load, IL28B genotype (rs12979860) (CC vs. non-CC), estimated duration of infection and SVR. Indirect fibrosis progression rate was defined as the ratio between the METAVIR fibrosis score at baseline liver biopsy and the estimated duration of infection. LSM measurements were converted to corresponding METAVIR fibrosis stage as previously validated.[12–18]

Statistical analysis

Participant characteristics were presented as median and inter-quartile ranges (IQR) for continuous variables and number (%) for dichotomous variables. McNemar's test was used to assess for liver fibrosis progression. In the analysis of variables that were associated with liver fibrosis progression, Fisher's exact test / Chi square test was used for dichotomous variables and Mann-Whitney for continuous variables. The probability level was set at $p \leq 0.05$. Variables that were associated with liver fibrosis progression with a p-value < 0.1 were included in multivariable logistic regression models. Statistical analysis was performed using PRISM v6.0.

The study protocol was approved by the Human Research Ethics Committee at St Vincent's Hospital and conducted according to the Declaration of Helsinki and ICH/GCP guidelines. Written informed consent was obtained for participants recalled for LSMs. Informed consent was not deemed necessary for subjects derived from the HCV database, given the retrospective nature of this study.

Results

Study population

131 subjects were included in this analysis. All subjects had had a liver biopsy at our institution prior to 2004. 18 subjects had paired liver histology available more than 10 years apart, 88 subjects had a liver biopsy with a follow-up LSM, and 25 subjects were actively recalled to present for a follow-up LSM and blood test. Of the 106 subjects who did not require recall, 7 did not have blood test results at the time of follow-up fibrosis staging.

Clinical characteristics of the cohort at baseline and follow up fibrosis assessment are summarized in Tables 1 and 2, respectively. 69% (91/131) were male, 82% were Caucasian (108/131) and the median age at follow-up assessment was 57 (IQR 54–62) years. As noted, liver fibrosis assessment at F/U was performed by transient elastography in 86% (113/131) and

Table 1. Participant characteristics at baseline.

Participant Characteristics at Baseline (n = 131)	Median (IQR) or percentage (n)
Age (years)	57 (IQR 54–62)
Male	69% (n = 91)
Ethnicity	82% Caucasian (108)
Age of HCV acquisition (years), n = 101	20 (IQR 17–24)
Estimated duration of infection at time of baseline liver biopsy (years)	17 (IQR 14–22)
Indirect Fibrosis Progression Rate (METAVIR Fibrosis stage/years of infection)	0.05 (IQR 0–0.16)
Patients treated for HCV	83% (n = 109)
Patients attaining SVR during study	40% (n = 44)
Initial ALT (U/L)	94 (IQR 60–152)
Baseline HCV viral load (log_{10} IU/mL)	6.10 (IQR 5.52–6.38)
Liver Fibrosis at baseline	
F0	41 (31%)
F1	43 (33%)
F2	20 (15%)
F3	10 (8%)
F4	17 (13%)
Mode of HCV acquisition	
IVDU	41 (31%)
Blood transfusion	22 (17%)
Tattoo	4 (3%)
Multiple risk factors	37 (28%)
No risk factors	21 (16%)
Unknown	3 (2%)
HCV genotype	
Genotype 1	79 (60%)
Genotype 2	7 (5%)
Genotype 3	33 (25%)
Genotype 4	2 (2%)
Genotype 6	1 (1%)
Unknown	9 (7%)

https://doi.org/10.1371/journal.pone.0185609.t001

14% (18/131) had a repeat liver biopsy. The median period between first and second liver fibrosis assessments was 14-years (IQR 12–17 years). The majority of our cohort (60%, n = 79) was infected with Genotype 1 HCV, followed by Genotype 3 HCV (25%, n = 33). At baseline, 79% (104/131) had mild-moderate liver fibrosis (F0 31% (41/131), F1 33% (43/131), F2 15% (20/131)), and 21% advanced fibrosis (F3 8% (10/131), F4 13% (17/131)). 109 (83%)

Table 2. Participant characteristics at follow-up fibrosis assessment.

Participant Characteristics at Follow-up Fibrosis Assessment (Liver Biopsy or LSM)	Median (IQR)
Estimated duration of HCV infection at follow-up fibrosis assessment (years), n = 101	33 (IQR 29–38)
Time between Liver biopsy and follow-up fibrosis assessment (years)	14 (IQR 12–17)
BMI	25.2 (IQR 23.5–28.3)
ALT (U/L)	48 (IQR 27–94)

https://doi.org/10.1371/journal.pone.0185609.t002

participants received antiviral therapy. 25% (27/109) were treated with standard interferon-α, 35% (38/109) with standard interferon-α and ribavirin, 33% (36/109) with pegylated interferon-α and ribavirin (PR), 6% (7/109) with a protease inhibitor in combination with (PR) and one participant with an interferon-free combination of DAAs. 40% (44/109) attained SVR. HCC developed in 3 participants during follow up. There were no reports of hepatic de-compensation or death at follow up.

Liver fibrosis progression secondary to hepatitis C infection

At baseline liver biopsy, mild-moderate liver fibrosis (F0-2) was detected in 79% (n = 104) and advanced fibrosis (F3-4) detected in 21% (n = 27). At follow-up, there was a statistically significant increase in the proportion of subjects with advanced liver fibrosis (35% (n = 46), p-value = 0.002, McNemar's test for comparison of baseline vs. 10 year F/U, (Fig 1A). 21% (n = 27) originally staged as mild-moderate fibrosis had progressed to advanced fibrosis after a median of 14 (IQR 12–17) years. 73% (n = 95) had no change in fibrosis stage, of which 59% (n = 77) and 15% (n = 19) had mild-moderate fibrosis and advanced fibrosis at baseline and follow up, respectively. Among patients with advanced fibrosis at baseline (n = 27), regression to mild-moderate liver fibrosis was observed in 6% (n = 8 (30%)). 7 had received curative HCV treatment. One participant demonstrated fibrosis regression (METAVIR F3 to LSM 5.8kPa after 17-years) despite relapsing to a course of pegylated interferon-a/ribavirin. Of note, they presented with a very low HCV viral load at baseline, with no hepatic inflammation at follow up (ALT 11 U/L).

114 subjects presented with METAVIR F0-F3 at baseline, of which 24% (n = 27) progressed to cirrhosis (F4 or LSM $\geq$ 13.5 kPa) over 17 years (IQR 12–16).

Predictors of liver fibrosis progression

Sustained virological response following HCV treatment. We considered the association between SVR and liver fibrosis progression. First, we separated patients into those who achieved SVR, and those who did not achieve SVR (Fig 1B and 1c). 83% (n = 109/131) of our cohort received HCV treatment and 40% (n = 44/109) achieved SVR. No significant change in liver fibrosis was observed over a median of 13.5 (IQR 11.5–16.9) years in subjects who were cured of HCV, p = 0.343 (Fig 1B), with fibrosis progression observed in only 3 subjects despite SVR. All had evidence of F2 fibrosis at baseline. One subject had biopsy proven F4 fibrosis at follow up, but required two courses of antiviral therapy (standard IFN and pegIFN/RBV) to achieve SVR. The remaining two subjects progressed to biopsy proven F3 fibrosis, both acquiring HCV secondary to IDU with previous exposure to HBV infection. Alcohol and diabetes were additional co-factors present in one subject. In contrast, 26% (n = 23/87) of subjects who did not receive antiviral therapy or achieve a sustained virological response to treatment, progressed from mild-moderate to advanced fibrosis over a median of 13.7 (IQR 12.3–16.0) years, p = 0.0001, McNemar's Test (Fig 1C).

A subset analysis was performed on participants who had baseline liver biopsy and follow up LSM (n = 114). This excluded those who had paired liver histology (n = 18). Similar to the findings of the overall cohort, there was a significant increase in the proportion of subjects (32%(n = 36) with advanced fibrosis at follow up, p = 0.038, McNemar's test for comparison of baseline vs. 10 year f/up (Panel A in S1 Fig). In those who attained SVR (n = 36), liver fibrosis regression occurred after 10-years, p = 0.013 (Panel B in S1 Fig), where as in those who did not attain SVR (n = 62), fibrosis progression was evident at follow-up, p = 0.0001 (Panel C in S1 Fig).

Fig 1A. Fibrosis staging of HCV cohort at baseline and follow-up

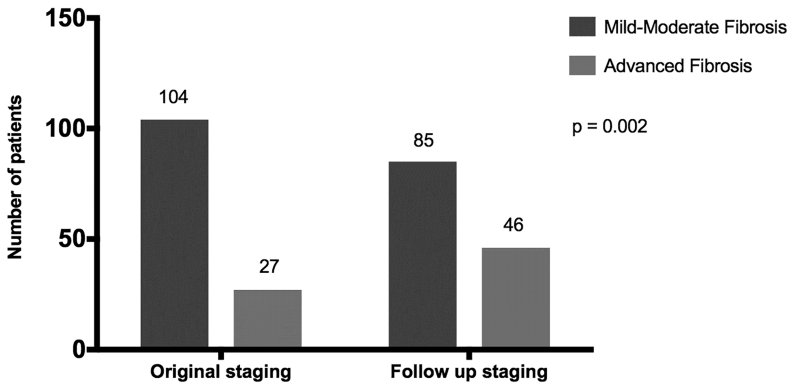


Fig 1B. Sustained virological response is associated with stable / improved liver fibrosis

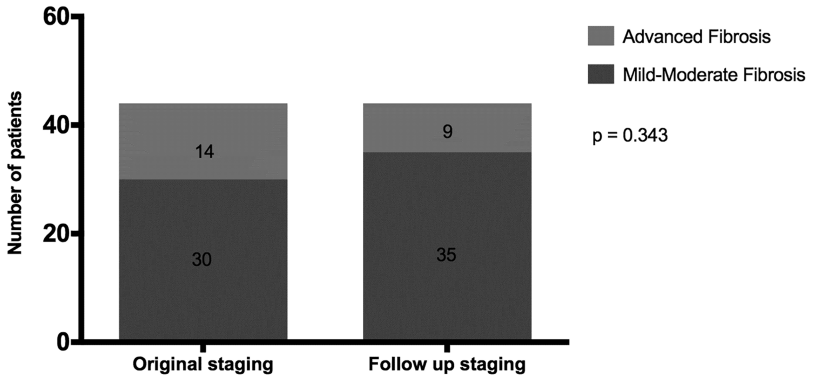


Fig 1C. Persistent HCV is associated with liver fibrosis progression

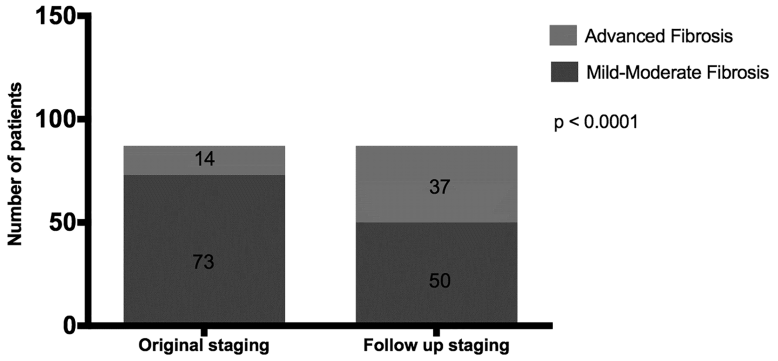


Fig 1. A. Fibrosis staging of HCV cohort at baseline and follow-up. B. Sustained virological response is associated with stable / improved liver fibrosis. C. Persistent HCV is associated with liver fibrosis progression.

https://doi.org/10.1371/journal.pone.0185609.g001

Failure to attain SVR and longer estimated duration of infection before HCV treatment initiation predict liver fibrosis progression. To adjust for potential confounding variables, we then considered the association between SVR and liver fibrosis progression using logistic regression analysis. The primary analysis included only those subjects with mild-moderate liver fibrosis at baseline. On univariable analysis, predictors of liver fibrosis progression among those

Table 3. Predictors of liver fibrosis progression—Failure to attain SVR and longer duration of infection prior to HCV treatment.

Co-variate	No Fibrosis progression	Fibrosis progression	*P*-value	Multivariable logistic regression (*P*-value)
n	76	28		
Age of patient (yrs)	56 (IQR 53–62)	60 (IQR 56–63)	0.188	
Gender	55 (72%)	18 (64%)	0.472	
HCV genotype (G1 vs. other)	47 (62%)	16 (57%)	0.351	
HCV acquisition (blood transfusion vs. other)	17 (22%)	16 (57%)	**0.002**	0.412
Estimated duration of HCV infection till liver biopsy (years)	16.5 (IQR 12.0–21.5)	19.0 (IQR 15.0–28.0)	**0.029**	0.318
Fibrosis rate to original liver biopsy (METAVIR stage/years of infection)	0.0387 (IQR 0–0.656)	0.0253 (IQR 0–0.0804)	0.984	
Age of acquisition	20 (IQR 17–22)	21 (IQR 15–25)	0.973	
Failure to attain SVR	47 (62%)	23 (82%)	**0.060**	**0.001**
Baseline ALT (U/L)	87 (IQR 55–121)	125 (IQR 76–175)	**0.011**	0.493
Baseline Viral Load (IU/mL)	891012 (IQR 344980–2405270)	1455260 (IQR 1148700–1744878)	0.521	
Baseline ferritin	226 (IQR 132–320)	273 (IQR 147–596)	0.170	
Baseline AFP	5 (IQR 5–6)	5 (IQR 1–7)	0.469	
Caucasian vs. other	62 (82%)	24 (86%)	0.774	
Estimated duration of infection before treatment initiation (years)	20.0 (IQR 15.0–28.0)	28.5(IQR 24.0–34.3)	**0.008**	**0.005**

https://doi.org/10.1371/journal.pone.0185609.t003

presenting with mild-moderate fibrosis at baseline (n = 104) were high baseline ALT (p = 0.011), longer estimated duration of HCV infection prior to liver biopsy (p = 0.029), longer estimated duration of infection before treatment initiation (p = 0.008) and mode of acquisition (p = 0.002) [Table 3]. On multivariable analysis, SVR was independently associated with protection from liver fibrosis progression (p = 0.001). Shorter duration of infection prior to HCV treatment initiation was also associated with protection from liver fibrosis progression (p = 0.005).

A separate analysis was performed to include subjects presenting with METAVIR F0 –F3 (n = 114) at baseline. Predictors of fibrosis progression on univariable analysis included longer estimated duration of infection prior to liver biopsy, failure to achieve SVR, high baseline ALT, high baseline ferritin and longer estimated duration before treatment initiation. Similar to the previous findings, attainment of SVR and shorter duration of HCV infection prior to treatment initiation were also associated with protection from liver fibrosis progression on multivariable analysis (see S1 Table).

Longer estimated duration of infection prior to HCV treatment initiation predicts liver fibrosis progression in HCV treatment failure. When we considered the population of participants who did not achieve SVR and had mild-moderate fibrosis at baseline (n = 57), the independent predictors of liver fibrosis progression were mode of acquisition, high baseline ferritin, and longer estimated duration of infection prior to HCV treatment initiation (see S2 Table). On multivariable analysis, shorter duration of infection prior to HCV treatment was associated with protection from liver fibrosis progression (p = 0.0002). This was also a significant predictor among participants who were untreated or did not attain SVR (p = 0.032) (see S3 Table).

Discussion

In this well-characterised cohort of patients followed longitudinally for more than 10 years, we have shown that SVR is associated with a reduction in the risk of liver fibrosis progression.

Fibrosis progression occurred in only 7% of those who achieved SVR in comparison to 30% of non-responders to antiviral therapy, over a decade. The three participants who did develop fibrosis progression despite SVR were noted to have additional co-factors such as alcohol and diabetes. This highlights the importance of early treatment initiation, as well as aggressive management of co-factors to avoid fibrosis progression.

Alongside SVR, early treatment initiation was found to be independently associated with protection against liver fibrosis progression. This was evident in both the overall cohort [20-yrs (IQR 15.0–28) vs. 29-yrs (IQR 24–35), p 0.005)], and among those who failed HCV treatment (see supplementary data). The importance of early curative therapy is underscored by the poor prognosis of individuals with advanced fibrosis/cirrhosis and failed HCV treatment. This particular cohort of patients is well described in the literature, most recently in a retrospective analysis of four hundred participants that observed disease progression and death to occur in 42% and 25% respectively, within a decade.[19]

Our results support the findings of two recent retrospective analyses with data to suggest that SVR significantly reduces the long-term risk of liver fibrosis progression. One study from Korea followed two hundred and eighty subjects for 4-years, of which 80% had received antiviral therapy. A lower incidence of cirrhosis was reported among those who attained SVR than without (0.6% vs. 33.9%, p = 0.001). A large study from Spain (n = 1289) also reported a lower incidence of cirrhosis with SVR after a 12-year follow-up period (2.2% vs. 28% of non-responders).[20] Of note, these studies included radiological findings, a less sensitive test than transient elastography, to define cirrhosis.

Fibrosis progression occurred in 21% of our cohort after a 14-year follow-up period, with the majority (n = 16, 59%) developing cirrhosis (Metavir F4 or LSM $\geq$ 13.5kPa). The cirrhosis rate at follow-up was 21%. This is consistent with previous estimates. A systematic review of 57 studies estimated progression to cirrhosis to be 21% at 20 years among liver clinic attendees.[21] More recently, a UK study found rates of cirrhosis of 23% at 20 years among individuals referred to a tertiary referral centre.[22] However, an Australian series of four hundred and fifty-five participants attending liver clinic reported a cirrhosis prevalence of 12% after 12 years (IQR 7-17yrs).[23] All of these studies formed estimates based on a single liver biopsy and an estimated date of infection, and may have underestimated the rate of HCV-related fibrosis progression in the liver-clinic setting.

Our results contribute to the growing body of evidence that curative HCV treatment prevents long-term fibrosis progression, and supports the notion that access to DAA therapy should be made widely available to all individuals regardless of fibrosis stage.

There are number of limitations to our study. Participants were identified from a HCV database, and although this database was inclusive of all HCV-infected attendees at our tertiary hospital, it is possible selection bias may have occurred. Furthermore, our patient population may be overrepresented by more severe illness and rapid disease progression, compared to a community-based cohort. We were not able to collect detailed data concerning [24–27] behavioural factors that have been associated with the natural history of CHC, including alcohol consumption, marijuana use and coffee consumption, were not included in our analysis.[28,29] Liver inflammation has been shown to alter LSMs and we potentially may have over estimated the degree of fibrosis in participants with high levels of ALT. However, fluctuating levels of inflammation / ALT are more characteristic of chronic hepatitis B infection and a study of one hundred and fifty HCV subjects who underwent a liver biopsy and LSM, showed no impact of ALT on LSM.[30] Despite these limitations, we believe the clinical message is strong, and the cohort is unique for the duration of long-term follow-up with paired liver fibrosis assessments.

In conclusion, we have shown in a well-characterised cohort of patients managed in a real-world setting, that curative HCV treatment halts fibrosis progression. The data support treatment for all people living with HCV, including people with mild-moderate liver fibrosis.

Supporting information

S1 Fig. A. Fibrosis staging of HCV cohort at baseline and follow-up (n = 114). B. Sustained virological response is associated with stable / improved liver fibrosis (n = 114). C. Persistent HCV is associated with liver fibrosis progression (n = 114).
(TIFF)

S1 Table. Predictors of fibrosis progression (F0-F3 vs. F4).
(DOCX)

S2 Table. Predictors of liver fibrosis progression in HCV treatment failure.
(DOCX)

S3 Table. Predictors of fibrosis progression—Analysis of non-SVR and untreated patients.
(DOCX)

Author Contributions

Conceptualization: Alexander J. Thompson, Paul V. Desmond.

Data curation: Swee Lin G. Chen Yi Mei, Britt Christensen, Georgina Cunningham, Lucy McDonald, David Iser, Tin Nguyen.

Formal analysis: Swee Lin G. Chen Yi Mei, Britt Christensen, Paul V. Desmond.

Investigation: Alexander J. Thompson.

Methodology: Swee Lin G. Chen Yi Mei, Alexander J. Thompson, Britt Christensen, Paul V. Desmond.

Supervision: Alexander J. Thompson, Sally Bell, Paul V. Desmond.

Writing – original draft: Swee Lin G. Chen Yi Mei.

Writing – review & editing: Alexander J. Thompson, Sally Bell, David Iser, Paul V. Desmond.

References

1. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. Journal of Hepatology. 2014 Nov; 61(1 Suppl):S45–57. https://doi.org/10.1016/j.jhep.2014.07.027 PMID: 25086286
2. Gidding HF, Topp L, Middleton M, Robinson K, Hellard M, McCaughan G, et al. The epidemiology of hepatitis C in Australia: Notifications, treatment uptake and liver transplantations, 1997–2006. Journal of Gastroenterology and Hepatology. 2009; 24(10):1648–54. PMID: 19798783
3. van der Meer AJ, Veldt BJ, Feld JJ, Wedemeyer H, Dufour J-F, Lammert F, et al. Association between sustained virological response and all-cause mortality among patients with chronic hepatitis C and advanced hepatic fibrosis. JAMA. 2012 Dec 25; 308(24):2584–93. https://doi.org/10.1001/jama.2012.144878 PMID: 23268517
4. Fernández-Rodríguez CM, Alonso S, Martinez SM, Forns X, Sánchez-Tapias JM, Rincón D, et al. Peginterferon plus ribavirin and sustained virological response in HCV-related cirrhosis: outcomes and factors predicting response. The American journal of gastroenterology. 2010 Sep 30; 105(10):2164–73. https://doi.org/10.1038/ajg.2010.294 PMID: 20700116
5. Veldt BJ, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, Zeuzem S, et al. Sustained virologic response and clinical outcomes in patients with chronic hepatitis C and advanced fibrosis. Annals of Internal Medicine. 2007 Nov 19; 147(10):677–84. PMID: 18025443

6. Morgan TR, Ghany MG, Kim H-Y, Snow KK, Shiffman ML, De Santo JL, et al. Outcome of sustained virological responders with histologically advanced chronic hepatitis C. Hepatology. 2010 Aug 31; 52 (3):833–44. https://doi.org/10.1002/hep.23744 PMID: 20564351
7. Cardoso AC, Moucari R, Figueiredo-Mendes C, Ripault M-P, Giuily N, Castelnau C, et al. Impact of peginterferon and ribavirin therapy on hepatocellular carcinoma: incidence and survival in hepatitis C patients with advanced fibrosis. Journal of Hepatology. 2010 May; 52(5):652–7. https://doi.org/10.1016/j.jhep.2009.12.028 PMID: 20346533
8. Poynard T, McHutchison J, Manns M, Trépo C, Lindsay K, Goodman Z, et al. Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. YGAST. 2002 May; 122 (5):1303–13.
9. Cammà C, Di Bona D, Schepis F, Heathcote EJ, Zeuzem S, Pockros PJ, et al. Effect of peginterferon alfa-2a on liver histology in chronic hepatitis C: a meta-analysis of individual patient data. Hepatology. 2004 Feb; 39(2):333–42. https://doi.org/10.1002/hep.20073 PMID: 14767986
10. Pearlman BLB, Traub NN. Sustained virologic response to antiviral therapy for chronic hepatitis C virus infection: a cure and so much more. Clin Infect Dis. 2011 Mar 31; 52(7):889–900. https://doi.org/10.1093/cid/cir076 PMID: 21427396
11. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology. 1996 Aug; 24(2):289–93. https://doi.org/10.1002/hep.510240201 PMID: 8690394
12. Cast e ra L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology. 2005; 128(2):343–50. PMID: 15685546
13. Sporea I, Sirli R, Deleanu A, Tudora A, Curescu M, Cornianu M, et al. Comparison of the liver stiffness measurement by transient elastography with the liver biopsy. World Journal of Gastroenterology. 2008 Nov 14; 14(42):6513–7. https://doi.org/10.3748/wjg.14.6513 PMID: 19030204
14. Sasso M, Tengher-Barna I, Ziol M, Miette V, Fournier C, Sandrin L, et al. Novel controlled attenuation parameter for noninvasive assessment of steatosis using Fibroscan(®): validation in chronic hepatitis C. J Viral Hepat. 2012 Mar 31; 19(4):244–53. https://doi.org/10.1111/j.1365-2893.2011.01534.x PMID: 22404722
15. Lupşor M, Badea R, Stefănescu H, Grigorescu M, Sparchez Z, Serban A, et al. Analysis of histopathological changes that influence liver stiffness in chronic hepatitis C. Results from a cohort of 324 patients. J Gastrointestin Liver Dis. 2008 May 31; 17(2):155–63. PMID: 18568136
16. Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal FEDER, Kazemi F, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. Hepatology (Baltimore, Md). 2005; 41(1):48–54.
17. Cross TJS, Calvaruso V, Maimone S, Carey I, Chang TP, Pleguezuelo M, et al. Prospective comparison of Fibroscan, King's score and liver biopsy for the assessment of cirrhosis in chronic hepatitis C infection. J Viral Hepat. 2010 Jul 31; 17(8):546–54. https://doi.org/10.1111/j.1365-2893.2009.01210.x PMID: 19874477
18. Wang J-H, Changchien C-S, Hung C-H, Eng H-L, Tung W-C, Kee K-M, et al. FibroScan and ultrasonography in the prediction of hepatic fibrosis in patients with chronic viral hepatitis. J Gastroenterol. 2008 Dec 31; 44(5):439–46.
19. van der Meer AJ, Hansen BE, Fattovich G, Feld JJ, Wedemeyer H, Dufour J-F, et al. Reliable prediction of clinical outcome in patients with chronic HCV infection and compensated advanced hepatic fibrosis: a validated model using objective and readily available clinical parameters. Gut. 2015 Jan 31; 64 (2):322–31. https://doi.org/10.1136/gutjnl-2013-305357 PMID: 24815676
20. Lens S, Torres F, Puigvehi M, Mariño Z, Londoño M-C, Martinez SM, et al. Predicting the development of liver cirrhosis by simple modelling in patients with chronic hepatitis C. Alimentary pharmacology \& therapeutics. 2016 Jan 31; 43(3):364–74.
21. Freeman AJ, Dore GJ, Law MG, Thorpe M, Overbeck Von J, Lloyd AR, et al. Estimating progression to cirrhosis in chronic hepatitis C virus infection. Hepatology (Baltimore, Md). W.B. Saunders; 2001; 34 (4):809–16.
22. Sweeting MJ, De Angelis D, Neal KR, Ramsay ME, Irving WL, Wright M, et al. Estimated progression rates in three United Kingdom hepatitis C cohorts differed according to method of recruitment. J Clin Epidemiol. 2006 Feb; 59(2):144–52. https://doi.org/10.1016/j.jclinepi.2005.06.008 PMID: 16426949
23. Khan MH, Farrell GC, Byth K, Lin R, Weltman M, George J, et al. Which patients with hepatitis C develop liver complications? Hepatology (Baltimore, Md). W.B. Saunders; 2000; 31(2):513–20.
24. Ostapowicz G, Watson KJ, Locarnini SA, Desmond PV. Role of alcohol in the progression of liver disease caused by hepatitis C virus infection. Hepatology (Baltimore, Md). W.B. Saunders; 1998; 27 (6):1730–5.

25. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet. 1997 Mar 22; 349 (9055):825–32. PMID: 9121257

26. Wiley TE, McCarthy M, Breidi L, McCarthy M, Layden TJ. Impact of alcohol on the histological and clinical progression of hepatitis C infection. Hepatology. 1998 Aug 31; 28(3):805–9. https://doi.org/10.1002/hep.510280330 PMID: 9731576

27. Monto A, Patel K, Bostrom A, Pianko S, Pockros P, McHutchison JG, et al. Risks of a range of alcohol intake on hepatitis C-related fibrosis. Hepatology (Baltimore, Md). Wiley Subscription Services, Inc., A Wiley Company; 2004; 39(3):826–34.

28. Hezode C, Roudot-Thoraval F, Nguyen S, Grenard P, Julien B, Zafrani E-S, et al. Daily cannabis smoking as a risk factor for progression of fibrosis in chronic hepatitis C. Hepatology. 2005 Jul; 42(1):63–71. https://doi.org/10.1002/hep.20733 PMID: 15892090

29. Modi AA, Feld JJ, Park Y, Kleiner DE, Everhart JE, Liang TJ, et al. Increased caffeine consumption is associated with reduced hepatic fibrosis. Hepatology (Baltimore, Md). 2010 Jan; 51(1):201–9.

30. Arena U, Vizzutti F, Abraldes JG, Corti G, Stasi C, Moscarella S, et al. Reliability of transient elastography for the diagnosis of advanced fibrosis in chronic hepatitis C. 2008.

4 Toll-like Receptor Expression and Signaling in Peripheral Blood Mononuclear Cells Correlate with Clinical Outcomes in Acute Hepatitis C Virus Infection

The Journal of Infectious Diseases

MAJOR ARTICLE

Toll-like Receptor Expression and Signaling in Peripheral Blood Mononuclear Cells Correlate With Clinical Outcomes in Acute Hepatitis C Virus Infection

Swee Lin G. Chen Yi Mei,[1,2] Jodie Burchell,[5] Narelle Skinner,[2] Rosie Millen,[2] Gail Matthews,[6] Margaret Hellard,[3,7] Gregory J. Dore,[6] Paul V. Desmond,[1] Vijaya Sundararajan,[5] Alexander J. Thompson,[1,2] Kumar Visvanathan,[1,2] and Joe Sasadeusz[4]

[1]Department of Gastroenterology, St Vincent's Hospital, [2]Immunology Research Centre, Department of Medicine, St Vincent's Hospital and the University of Melbourne, [3]Centre for Population Health, Burnet Institute, [4]Department of Infectious Diseases, Royal Melbourne Hospital, [5]Centre of Research Excellence, Department of Medicine, St Vincent's Hospital, Melbourne, [6]Kirby Institute, University of New South Wales, Sydney, and [7]Department of Infectious Diseases, the Alfred Hospital, Melbourne, Australia

Background. Mechanisms by which spontaneous clearance of acute hepatitis C occurs are unclear. A critical role for the innate immune system and IFNL4 polymorphisms has been proposed. This study investigates whether Toll-like receptor (TLR) expression and signaling during acute hepatitis C correlates with clinical outcomes.

Methods. Participants identified from the Australian Trial in Acute Hepatitis C and the Networks study were followed longitudinally from the time of diagnosis of acute hepatitis C. Peripheral blood mononuclear cells (PBMCs) and plasma were collected at and 2 time points after diagnosis. At each time point, TLR2, TLR4, and CD86 expression on peripheral blood monocytes, natural killer (NK) cells, and NK T cells was measured, as well as the response of PBMCs to stimulation with TLR ligands. Cytokine and chemokine levels were measured in stimulated PBMCs and plasma.

Results. We identified 20 participants with acute hepatitis C (10 with hepatitis C virus [HCV] monoinfection and 10 with HCV and human immunodeficiency virus coinfection). Eleven participants (55%) spontaneously cleared HCV. Acute hepatitis C and spontaneous clearance was associated with lower TLR4 expression on monocytes ($P = .009$) and NK cells ($P = .029$). Acute hepatitis C and spontaneous clearance was also associated with a reduced interferon γ response to TLR4 ($P = .038$) and TLR7/8 stimulation ($P = .035$), a reduced interleukin 6 response to TLR7/8 stimulation ($P = .037$), and reduced IFN-γ–inducible protein 10 (IP-10) response to TLR2 stimulation ($P = .042$). Lower plasma IP-10 levels were associated with spontaneous clearance ($P = .001$).

Conclusions. These findings implicate TLR4 signaling as playing a critical role in the outcome of acute hepatitis C.

Keywords. toll-like receptors; cytokines; hepatitis C.

Mechanisms by which spontaneous clearance of acute hepatitis C occurs are unclear. Only 15%–50% of individuals with acute hepatitis C virus (HCV) infection spontaneously clear the virus [1]. Individuals with acute hepatitis C are particularly difficult to identify, in part because the majority are asymptomatic at the time of infection. As a result, limited data exist correlating host immune response with clinical outcomes of acute hepatitis C, in either HCV monoinfection or in HCV and human immunodeficiency virus (HIV) coinfection. Despite the recent development of very effective direct antiviral therapy for HCV, these drugs are expensive, and the development of a preventive HCV vaccine remains a public health priority. Therefore, understanding the pathogenesis of early HCV infection and the immunological determinants of clinical outcome remain important.

Received 1 February 2016; accepted 23 May 2016; published online 9 June 2016.

Correspondence: S. G. Chen Yi Mei, Department of Gastroenterology, St Vincent's Hospital, Level 4 Daly Wing, 35 Victoria Parade, Fitzroy, Victoria 3065, Australia (sweelin.chenyimei@svha.org.au).

The Journal of Infectious Diseases® 2016;214:739–47

DOI: 10.1093/infdis/jiw235

The innate immune system is understood to play a critical role in the pathogenesis of HCV infection. The major cytoplasmic sensor for HCV is thought to be retinoic acid–inducible gene 1 (RIG-I), which recognizes double-stranded RNA intermediates produced during the viral lifecycle and leads to stimulation of the interferon (IFN) signaling pathway. Toll-like receptor 3 (TLR3) also signals through this pathway, but it detects double-stranded RNA intermediates within endosomes. Both RIG-I and TLR3 signaling have been shown in vitro to have antiviral responses to HCV [2–4]. More recently, variants in the region of the *IL28B* gene that encodes the type 3 IFN, IFN-λ4, have been associated with spontaneous clearance of acute hepatitis C [5]. This association may occur via IFNL4-mediated induction of IFN-stimulated genes (ISGs). IFNL4 polymorphism is highly predictive of spontaneous and IFN-α–induced HCV clearance in HCV monoinfection and HCV and HIV coinfection [6–10]. Other components of HCV are known to engage with the TLR4 and TLR2 receptors, and signaling through these TLRs leads to the production of proinflammatory cytokines, as well as stimulating type 1/3 IFN through IFN regulatory factor 3 [11, 12].

In chronic hepatitis C, TLR expression is increased in peripheral blood mononuclear cells (PBMCs) in comparison to expression in

healthy controls [11, 13–15]. Additionally, patients with chronic hepatitis C demonstrate higher cytokine production following ex vivo TLR stimulation. Increased TLR expression is also seen in HIV monoinfection [16, 17]. Our laboratory has previously shown that individuals with chronic hepatitis C, with either HCV monoinfection, HCV and HIV coinfection, or HIV monoinfection upregulate peripheral monocyte TLR2 and TLR4 protein expression, in comparison to healthy controls [13, 18]. Of note, no differences in TLR expression were identified between the virally infected groups. These findings suggest that TLR expression and function are intimately involved in the pathogenesis of chronic hepatitis C.

There are no data yet looking at TLR2 or TLR4 expression in the setting of acute hepatitis C. It is also not known whether HCV and HIV coinfection alters TLR expression and thus influences different rates of spontaneous clearance. Overall, spontaneous viral clearance occurs less frequently in HCV and HIV coinfection, suggesting that HIV infection may attenuate the immune mechanism responsible for clearance [19]. Interferon responsiveness is also reduced in HIV and HCV coinfection as compared to HCV monoinfection [20–23].

In collaboration with the Australian Trial in Acute Hepatitis C (ATAHC-I) and the Networks study, we performed a detailed evaluation of the role of innate immune signaling as a determinant of clinical outcomes in a unique cohort of individuals with acute hepatitis C [24, 25]. This is the first study to investigate TLR expression and functional cytokine response in the setting of acute hepatitis C, in both HCV monoinfection and HCV and HIV coinfection.

METHODS

Subjects

This was a longitudinal study of 10 HCV and HIV–coinfected and 10 HCV-monoinfected participants with stored PBMCs and plasma identified from the ATAHC-I and Networks studies of acute hepatitis C, as previously described [24, 25]. In the ATAHC-I, patients were followed longitudinally from time of diagnosis of acute/early HCV infection, defined as first detection of anti-HCV antibody within 6 months and clinical hepatitis C (jaundice or an alanine aminotransferase [ALT] level of >10 times the upper limit of normal) within the past 12 months or documented anti-HCV seroconversion within the past 24 months. Estimated date of infection in ATAHC-I participants was calculated either as 6 weeks prior to the onset of seroconversion, if present, or before the first ALT level of >400 IU/mL or as the midpoint between last negative anti-HCV antibody test and the first positive anti–HCV antibody test result, if participants were asymptomatic. In the Networks study, people who inject drugs were followed longitudinally for up to 5 years, with blood samples collected up to 3 times monthly. Estimated date of infection in Networks participants was calculated as the midpoint between last HCV RNA–negative test and the subsequent HCV RNA–positive test. Spontaneous clearance of acute hepatitis C was defined as 2 tests >4 weeks apart in which HCV RNA was undetectable (lower limit of detection, 10 IU/mL). PBMCs were collected at the time of diagnosis of acute/early HCV infection and at 2 different time points after diagnosis. This varied between each participant, based on participant follow up and availability of study samples for analysis. HCV RNA was detected in ATAHC-I participants with the transcription-mediated amplification assay for qualitative and quantitative HCV RNA (Versant, Bayer, Australia [lower limit of detection, 10 IU/mL]; and Versant HCV RNA 3.0, Bayer, Australia [lower limit of detection, 615 IU/mL], respectively), and HCV genotype was determined by the Versant LiPa2 assay (Bayer, Australia). Qualitative HCV RNA testing was performed for Networks participants by using the Cobas Amplicor HCV Test, version 2.0 (Roche Diagnostics, Branchberg, NJ). IFNL4 genotype (rs12979860) was determined by Sanger sequencing. The ATAHC-I protocol was approved by the Human Research Ethics Committee at St Vincent's Hospital and conducted according to the Declaration of Helsinki, the International Council on Harmonization of Technical Requirements for Pharmaceuticals for Human Use, and good clinical practice. The Human Research Ethics Committee at the Alfred Hospital approved the Networks study protocol.

Flow Cytometry for Determination of TLR Expression on Peripheral $CD14^+$ Blood Monocytes and Natural Killer (NK) Cells

Detailed methods were previously described elsewhere [13]. In brief, cell surface staining was performed on rapidly thawed PBMCs, using the following fluorochrome-conjugated anti-human monoclonal antibodies: anti-TLR2- fluorescein isothiocyanate, anti-TLR4-phycoerythrin (eBioscience), anti-CD86-allophycocyanin, anti-CD14-allophycocyanin Cy7, anti-CD3-Pacific Blue, and anti-CD56-phycoerythrin Cy7 (eBioscience). Isotype-matched nonbinding control antibodies were used for comparison. A total of 10 000 $CD14^+$ monocytes and $CD56^+$ NK cells of each sample were analyzed; dead cells were gated out, based on their light scatter properties on the FACS Canto Flow Cytometer (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). TLR2, TLR4, and CD86 values were expressed as a ratio of the geometric mean fluorescence of individual study patients to the geometric mean isotype control values for that patient.

PBMC Stimulation

A total of 1×10^6 thawed PBMCs from subjects were cultured at 37°C for 20 hours in Roswell Park Memorial Institute medium with 1% L-glutamine, 1% penicillin, and 5% fetal calf serum (FCS) and TLR ligands. PBMCs were stimulated with RPMI 5% FCS (unstimulated control), 100 ng/mL Pam-3-Cys (a TLR2 ligand), 10 μg/mL polyinosinic:polycytidylic acid (a TLR3 ligand), 100 ng/mL lipopolysaccharide (LPS; a TLR4 ligand), 5 μg/mL R848 (a TLR7/8 ligand), and 0.3 μM CpG ODN 2006 (a TLR9 ligand). All TLR ligands were from InVivoGen. Supernatants were harvested after culture and stored frozen at −70°C until

batch analysis. The concentrations of TLR ligand and the duration of the incubation period were chosen on the basis of results of optimization experiments, to maximize cytokine output.

Measurement of Cytokine Levels by the Cytometric Bead Array Assay and Enzyme-Linked Immunosorbent Assay (ELISA)

The cytometric bead array assay was performed on thawed tissue culture supernatants at dilutions of 1:1, 1:10, and 1:100, to accommodate high levels of cytokine. IFN-γ–inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), interleukin 8 (IL-8), interleukin 10 (IL-10), IFN-γ, tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) levels were measured simultaneously, according to the instructions provided by the manufacturer (BD Biosciences, San Jose CA). PBMCs cultured in medium alone were used as controls. A series of 10 dilutions using cytokine standards were run in each assay, for generation of standard curves. The intensity of the fluorescence signal was measured on the FACS Canto Flow Cytometer (Becton Dickinson), and data were analyzed using FCAP Array Software (BD Biosciences). Frozen plasma from subjects permitted measurement of spontaneous secretion of cytokines. The enhanced-sensitivity cytometric bead array assay was performed to measure IFN-γ, IL-6, IL-8, IL-10, and TNF-α levels. Commercial ELISA kits were used to measure IP-10 and MCP-1 levels, according to the manufacturer's guidelines.

Table 1. Baseline Characteristics of the Study Population

Characteristic	Spontaneous Clearance	Chronic Infection	*P* Value
Subjects	11 (55)	9 (45)	
Age, y	34.4 (26.2–39.4)	31.2 (29.3–46.2)	.700
Male sex	10 (91)	9 (100)	1.000
Estimated duration of infection, wk			
At screening	25 (9–33)	20 (8–38)	1.000
At first visit	46 (32–59)	43 (22–58)	.669
At second visit	56 (43–76)	66 (45–90)	.718
HCV aviremic			
At screening	6 (55)	0 (0)	
At first visit	9 (82)	0 (0)	
At second visit	11 (100)	0 (0)	
White	11 (100)	9 (100)	1.000
HIV coinfection	6	4	1.000
$CD4^+$ T-cell count, $\times 10^6$ cells/L	686 (613–806)	766 (534–882)	.873
Receiving ART			
Yes	4	2	.429
No	0	1	
Unknown	2	1	
HIV load, copies/mL	73.5 (43.8–7338)	48.5 (41.8–2338)	.471
HCV genotype			
1	5	3	.447
3	4	3	
4	0	1	
Unknown	2	0	
Favorable IFNL4 genotype	8 (73)	1 (11)	.010
Jaundice	3 (27)	2 (22)	1.000
Peak ALT level, U/L	589 (102–2030)	225 (124–502)	.226
BMI	22.3 (20.4–28.3)	23.9 (20.4–27.1)	.835

Data are no. of subjects, no. (%) of subjects, or median value (interquartile range).

Abbreviations: ALT, alanine aminotransferase; ART, antiretroviral therapy; BMI, body mass index; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

Statistical Analysis

Statistical analysis was performed using Stata, version 13.0. Participant characteristics and laboratory measurements are presented as median values and interquartile ranges. The Fisher exact test was used to test for significant group differences in dichotomous variables, and the independent samples *t* test was used for continuous variables. As time points of blood collection for each participant varied during the course of infection, group differences (acute hepatitis C and spontaneous clearance vs persistence, HIV positive vs negative, and IFNL4 favorable vs unfavorable) for each of the immunological outcomes were visualized using locally weighted scatterplot smoothing (LOWESS) curves. Group differences between the group with acute hepatitis C with spontaneous clearance and the group with acute hepatitis C without clearance that appeared marked on the LOWESS graphs were further investigated using random-intercepts multilevel modeling, with time and clearance status entered as the level 1 variables and the patient identifier as the level 2 variable. Probability level was set at a P value of $\leq .05$. No corrections for multiple comparisons were used, owing to the exploratory nature of the analyses [26]. Subgroup analyses for group differences by HIV and IFNL4 status were not performed because of sample size limitations.

RESULTS

Characteristics of the Study Population

Twenty participants were included in this analysis (10 with HCV monoinfection and 10 with HCV and HIV coinfection). Baseline characteristics of the cohort are summarized in Table 1. Eleven patients achieved spontaneous HCV clearance (5 with HCV monoinfection and 6 with HCV and HIV coinfection), and 9 developed chronic HCV infection (5 with HCV monoinfection and 4 with HCV and HIV coinfection). Estimated duration of infection at screening was similar in both groups (25 vs 20 weeks). All HIV-coinfected patients had $CD4^+$ T-cell counts of $>350 \times 10^6$ cells/L at the time of HCV infection, and 6 were receiving antiretroviral therapy. PBMCs and plasma were available for analysis at 3 time points for 15 patients and at 2 time points for 5 patients. Five with spontaneous clearance were HCV viremic at the first time point (2 participants were also viremic at the second time point) before clearing the virus. The remaining 6 with spontaneous clearance were aviremic at all time points.

A favorable IFNL4 genotype predicted spontaneous clearance ($P = .010$). Participants who achieved spontaneous clearance were otherwise comparable to those who developed chronic HCV infection in terms of age, sex, race, HCV genotype, body mass index, and jaundice.

TLR2, TLR4, and CD86 Expression on Peripheral Blood Monocytes, NK Cells (Dim and Bright), and NK T Cells

Low TLR4 expression was associated with the likelihood of spontaneous clearance. TLR4 expression on $CD14^+$ peripheral monocytes was significantly lower in participants who achieved spontaneous clearance ($P = .009$; Figure 1). TLR4 expression was also reduced on peripheral NK cells, on bright NK cells, and on dim NK cells among individuals with spontaneous clearance ($P = .029$, $P = .038$, and $P = .050$, respectively). The subjects with spontaneous clearance were separated into those who were initially viremic and those who were aviremic at the first time point, and TLR4 expression was found to be low and at a similar level in both groups (Figure 2). The HCV load of participants with persistent infection did not differ significantly from that of the subjects with spontaneous clearance who were viremic at the first time point ($P = .171$). No difference in TLR4 expression was seen on NK T cells ($P = .096$). HIV coinfection did not alter TLR4 expression when visualized on LOWESS curves (data not shown). LOWESS curve analysis of TLR2 and CD86 expression on peripheral $CD14^+$ monocytes, NK cells, and their subtypes showed no differences according to clinical outcome of acute hepatitis C. IFNL4 polymorphisms were associated with spontaneous HCV clearance when visualized on LOWESS curves. There was a strong but nonsignificant trend for the unfavorable IFNL4 genotype to be associated with higher expression of TLR4 ($P = .127$) and TLR2 ($P = .291$) on $CD14^+$ monocytes (Supplementary Data). Differences were not seen on NK cells or their subtypes.

Proinflammatory Cytokine Secretion

Plasma Levels

Plasma IP-10 levels were lower in participants who achieved spontaneous clearance as compared to those with persistent infection ($P < .001$; Figure 3). No significant differences were seen in the plasma levels of IFN-γ, IL-6, IL-8, IL-10, TNF-α, or MCP-1.

Levels of plasma IP-10 remained high on the LOWESS curve among individuals carrying the unfavorable IFNL4 genotype, but the difference did not reach statistical significance.

Cytokine Responses to TLR Stimulation of PBMCs

Reduced cytokine response to TLR4 (LPS) stimulation was associated with the likelihood of spontaneous clearance. TLR4

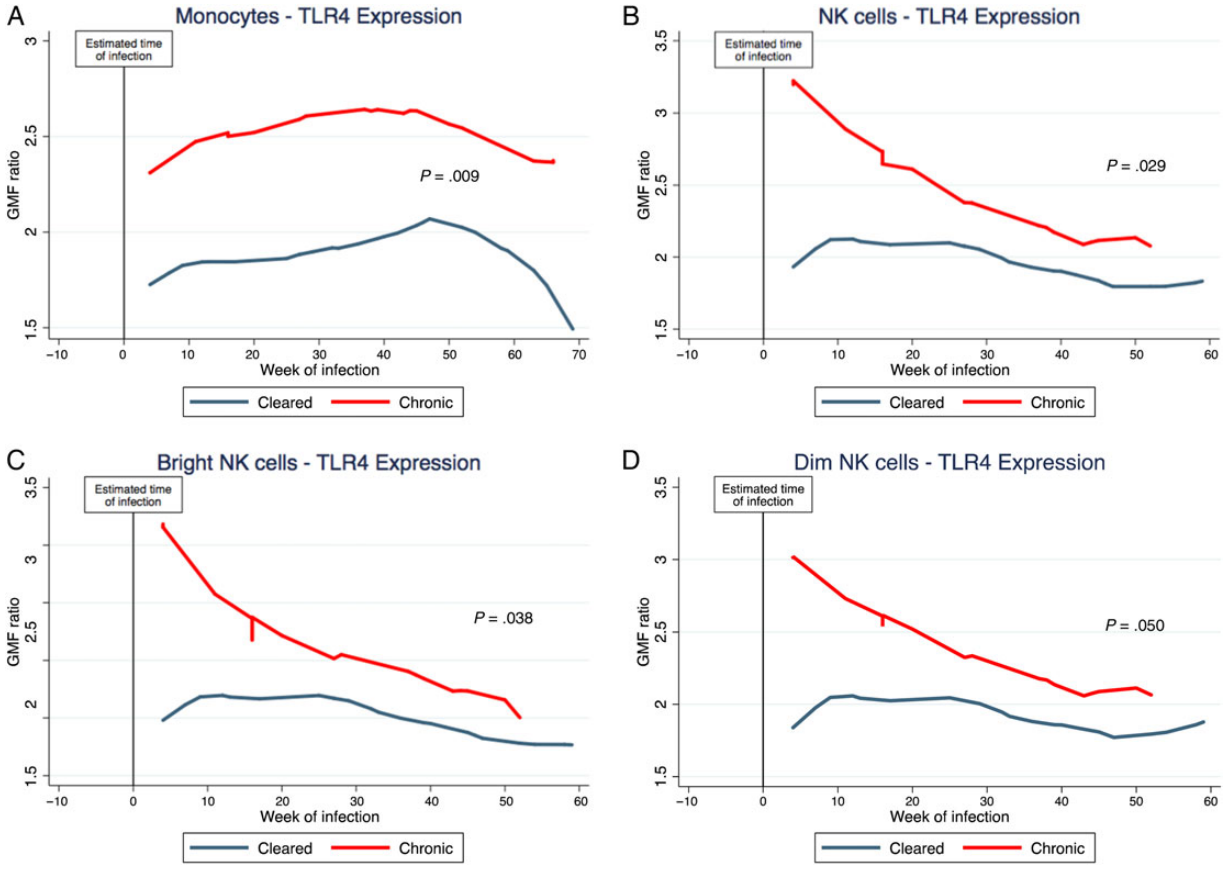


Figure 1. Locally weighted scatterplot smoothing curves illustrating Toll-like receptor 4 (TLR4) expression during acute hepatitis C with or without spontaneous clearance. Random-intercepts multilevel modeling found that TLR4 expression was significantly reduced on peripheral monocytes (*A*), natural killer (NK) cells (*B*), and bright (*C*) and dim (*D*) NK cells during acute hepatitis C with spontaneous clearance (n = 9; blue/dark gray, cleared) than during acute hepatitis C without spontaneous clearance (n = 11; red/light gray, chronic). The geometric mean fluorescence (GMF) ratio is calculated as the ratio of the geometric mean fluorescence of individual study patients to the geometric mean isotype control values for that patient. This figure is available in black and white in print and in color online.

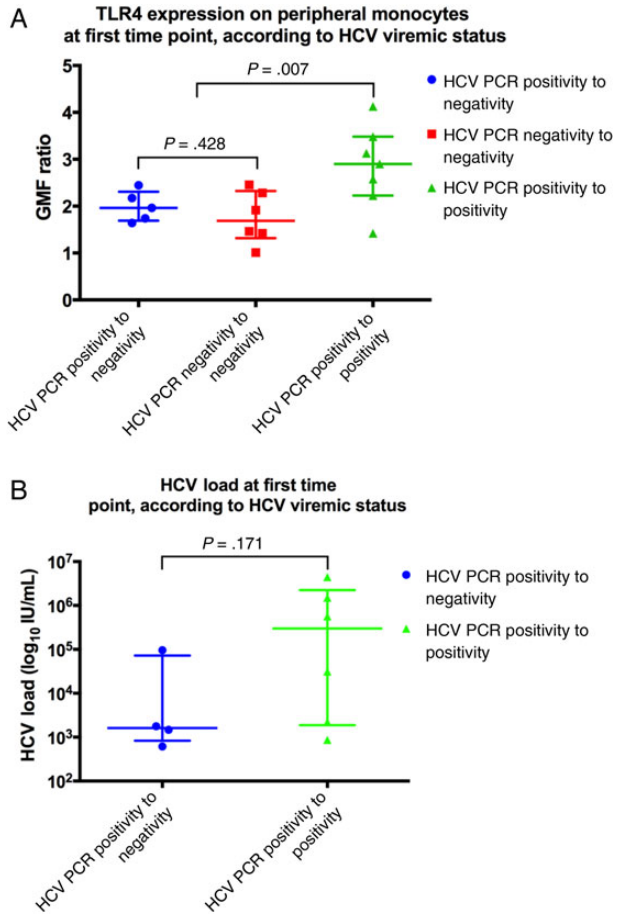


Figure 2. Hepatitis C virus (HCV) viremic status at the first time point. *A*, Toll-like receptor 4 (TLR4) expression on peripheral monocytes at the first time point, with participants grouped according to HCV viremic status. *B*, HCV load at the first time point, with participants grouped according to HCV viremic status. Abbreviations: GMF, geometric mean fluorescence; PCR, polymerase chain reaction.

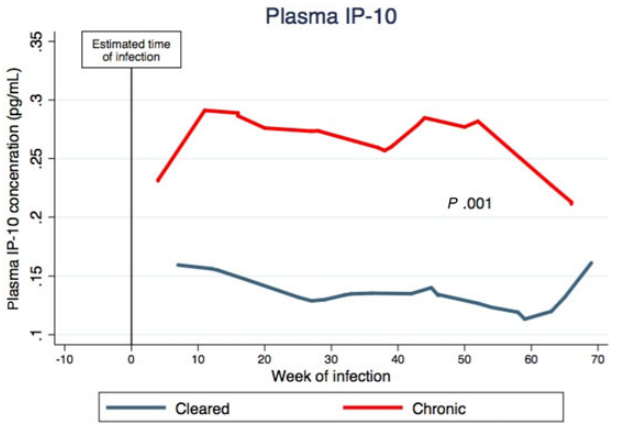


Figure 3. Locally weighted scatterplot smoothing (LOWESS) curve illustrating plasma inducible protein 10 (IP-10) levels in acute hepatitis C (AHC) with and without spontaneous clearance. Unstimulated plasma IP-10 levels were significantly lower in AHC and spontaneous clearance, n = 11, (blue/dark grey, Cleared - LOWESS) than AHC without spontaneous clearance, n = 9 (red/light grey, Chronic - LOWESS). This figure is available in black and white in print and in color online.

stimulation resulted in significantly reduced levels of IFN-γ among subjects with spontaneous clearance ($P = .038$) and a nonsignificant trend toward lower IL-6 production ($P = .078$; Figure 4). Similarly, TLR7/8 (R848) stimulation resulted in significantly lower IFN-γ ($P = .029$) and IL-6 ($P = .037$) production in the setting of spontaneous clearance. Finally, TLR2 (P3C) stimulation elicited lower levels of IP-10 among participants who spontaneously cleared the virus as compared to those who did not ($P = .042$). No differences in cytokine response were seen with TLR3 or TLR9 stimulation. There was no association between HIV status and any innate immune marker in this study.

IFNL4 polymorphisms predicted the response to TLR4 (LPS) stimulation. Individuals with the favorable IFNL4 genotype demonstrated significantly lower IFN-γ production following TLR4 stimulation ($P = .016$), in comparison to those with the unfavorable IFNL4 genotype. Similarly, significantly lower IFN-γ levels following TLR7/8 (R848) stimulation were found in this group ($P = .029$).

DISCUSSION

We have performed a prospective, longitudinal analysis of the innate immune response in a cohort of participants with acute hepatitis C, half of whom were coinfected with HIV. Despite the small number of participants in this study, key differences in TLR expression, spontaneous cytokine secretion, and functional cytokine response were observed between those with acute hepatitis C who had spontaneous clearance and those without spontaneous clearance. No clear effect of HIV was identified, but our numbers were small.

Individuals with spontaneous clearance of acute hepatitis C was associated with significantly lower levels of TLR4 expression on peripheral blood monocytes, NK cells, and their subtypes (dim and bright NK cells) as compared to those without spontaneous clearance. These findings are consistent with previous work in our laboratory that has shown an upregulation of TLR2 and TLR4 expression on peripheral monocytes in subjects with chronic hepatitis C, with or without HIV coinfection, compared to healthy controls [13]. Further evidence implicating TLR4 receptors as a critical player in the innate immune response to acute hepatitis C were the reduced levels of IFN-γ in response to TLR4 (LPS) stimulation in subjects with spontaneous clearance. TLR7/8 (R848) stimulation also elicited differences between the 2 groups, with reduced IFN-γ and IL-6 responses in the setting of spontaneous clearance. Of note, no changes in plasma levels of IFN-γ or IL-6 were observed in those with and those without spontaneous clearance.

Our results are the first to show differences in TLR protein expression and signaling in the setting of acute hepatitis C in relation to clinical outcome. We hypothesize that upregulation of TLR4 expression and an increase in the TLR4 signaling pathway is promoting viral persistence. The exact mechanism underlying

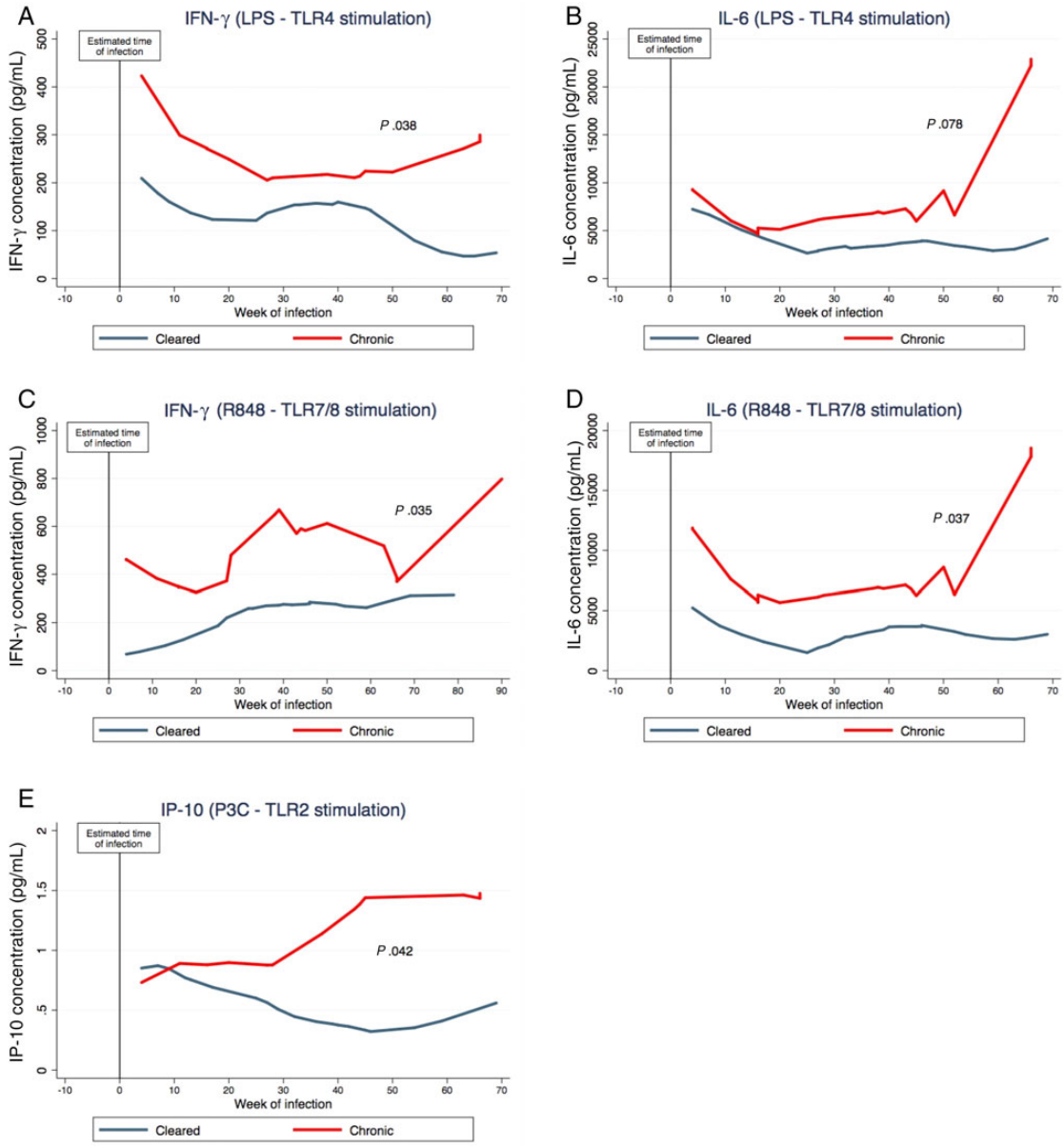


Figure 4. Locally weighted scatterplot smoothing (LOWESS) curves illustrating levels of interferon γ (IFN-γ), interleukin 6 (IL-6) and inducible protein 10 (IP-10) produced by stimulated peripheral blood mononuclear cells in acute hepatitis C (AHC) with or without spontaneous clearance. Toll-like receptor 4 (TLR4) stimulation resulted in significantly reduced levels of IFN-γ (*A*), and a non-significant trend toward lower IL-6 production (*B*) in AHC and spontaneous clearance, n = 11, (blue/dark grey, Cleared - LOWESS) than AHC without spontaneous clearance, n = 9, (red/light grey, Chronic - LOWESS). TLR7/8 stimulation resulted in significantly lower IFN-γ (*C*) and IL-6 production (*D*) in AHC and spontaneous clearance. IP-10 levels were significantly lower in AHC and spontaneous clearance following TLR2 stimulation (*E*). This figure is available in black and white in print and in color online.

this is not clear, however, and further studies are required to investigate this. It is possible that HCV may be driving this increase in TLR4 expression in those with persistent infection. However, when we separated the subjects with spontaneous clearance into those who were initially viremic and those who were aviremic at the first time point, TLR4 expression was low and at a similar level in both groups. Furthermore, no difference in HCV load was found between subjects with spontaneous clearance who were initially viremic and participants with persistent infection, indicating that TLR expression may be independent of HCV load.

Impairment of TLR4 signaling in HCV-infected dendritic cells has been demonstrated in vitro [27]. In a murine replicon model, TLR4 stimulation has been shown to suppress HCV viral replication [28]. These findings, however, were in the setting of chronic infection. TLR4 gene polymorphisms have been shown to prognosticate liver fibrosis risk in a white population [29]. In a Saudi Arabian population, an association between TLR4 gene polymorphisms and chronic HCV infection was identified but not with fibrosis [30]. Larger genome-wide association studies are needed to validate these findings. The TLR4

pathway has also been implicated as proviral in other infectious diseases. Respiratory syncytial virus requires TLR4 signaling to facilitate its clearance, whereas engagement of mouse mammary tumor virus with TLR4 promotes its viral persistence [31, 32]. The data suggest that TLR4 expression and signaling may be involved in the pathogenesis and persistence of HCV, and further mechanistic studies are required.

IFNL4 polymorphisms may at least in part explain the association of acute hepatitis C and spontaneous clearance with lower TLR expression and functional cytokine response to ex vivo TLR stimulation. IFNL4 polymorphisms predict spontaneous clearance of acute hepatitis C. As expected, the majority of subjects (8 of 11) with spontaneous clearance carried the favorable IFNL4 genotype, and, correspondingly, the unfavorable IFNL4 genotype was prevalent in those (8 of 9) who developed persistent infection. The type III IFN IFN-λ4 is encoded by the *IL28B* gene and upon binding to its corresponding receptor leads to the induction of ISGs. Low pretreatment intrahepatic ISG expression is strongly associated with the favorable IFNL4 genotype (rs12979860; rs8099917) and is a positive predictor of cure by IFN-based therapy in chronic hepatitis C [33–37]. Interestingly, our laboratory also found an increase in intrahepatic TLR2/TLR4 and TNF-α messenger RNA expression in subjects with chronic hepatitis C, with or without HIV coinfection, compared with healthy controls [18]. The paradigm seen in chronic hepatitis C, whereby IFN nonresponse is characterized by a preactivated innate immune state, is potentially relevant in acute hepatitis C. In the setting of chronic hepatitis C, Sarasin-Filipoicz et al showed that slow responders to pegylated interferon-alfa/ribavirin exhibited high pretreatment intrahepatic ISG expression, with no change in ISG expression following commencement of treatment. This was in contrast to rapid responders, who exhibited low pretreatment intrahepatic ISG expression with strong ISG induction following treatment. Similarly, Taylor et al showed greater changes during treatment in ISG expression in PBMCs in rapid responders versus slow responders. IFNL4 genotype may determine these differences. No comparable intrahepatic data exists for acute hepatitis C. Higher TLR expression and functional cytokine response in those who develop persistent infection may reflect an innate immune system incapable of mounting a response great enough to achieve viral clearance, similar to that seen in chronic hepatitis C. Our findings extend those of Villacres et al, who showed significantly higher basal IL-6 secretion levels but reduced levels of IL-6 secretion following TLR4 (LPS) stimulation in peripheral blood of 13 chronic HCV infected subjects, compared with healthy controls ($P < .05$).

Intrahepatic and peripheral blood levels of IP-10 are high in chronic hepatitis C. Additionally, low pretreatment serum IP-10 level is a positive predictor of sustained virological response to IFN-based therapy, with or without HIV coinfection [38–44]. Grebely et al and Beinhardt et al found, in large cohorts of patients with acute hepatitis C (n = 187 and n = 62, respectively), lower IP-10 levels in screening blood samples from subjects with spontaneous clearance (mean level [±SD], 248 ± 32 pg/mL vs 142 ± 22 pg/mL [$P = .008$]; and median level, 764 pg/mL vs 1481 pg/mL [$P = .006$]) [6]. Approximately half of the subjects in the group studied by Beinhardt et al had multiple measurements available on follow-up, in which IP-10 concentrations remained low in subjects with spontaneous clearance. Similarly, our study found that lower levels of plasma IP-10 were present in individuals with acute hepatitis C achieving spontaneous clearance, compared with those with persistent infection. This difference persisted following TLR2 (P3C) stimulation, with significantly reduced levels of IP-10 associated with spontaneous clearance. We hypothesize that a high level of IP-10, alongside an increase in IP-10 response to TLR2 signaling, promotes viral persistence. Our data fit with the growing body of evidence of the role IP-10 plays in the clearance of acute hepatitis C. A recent longitudinal study by Riva et al in 16 patients with acute hepatitis C similarly found significantly lower levels of IP-10, as well as a truncated biologically inactive form of IP-10, in those who spontaneously resolved the infection over the course of 12 months. They postulated that IP-10, in its truncated form, acts as an antagonist to biologically active IP-10, as it retains its CXCR3 binding ability. This may explain the paradox of high IP-10 levels being associated with the development of persistent HCV infection in acute hepatitis C and a poor treatment response in the context of chronic HCV infection.

There are a number of limitations to our study. Identifying the exact cell populations producing cytokines following ex vivo TLR stimulation would provide further depth to our analysis, alongside comparative intrahepatic TLR expression/signaling data. As liver biopsy is rarely clinically relevant in the setting of acute hepatitis C, this will prove a difficult area to study. A caveat to our findings was the inability to discern whether TLR4 upregulation in the setting of persistent infection was attributable to HCV or ongoing hepatic inflammation. Finally, owing to low numbers, we were not able to assess whether HIV-infected patients who had low $CD4^+$ T-cell counts or were not receiving antiretroviral therapy had impaired clearance.

In conclusion, our study is the first to illustrate differences in TLR4 expression on peripheral monocytes and NK cells (and their subtypes) and differing functional TLR responses, according to clinical outcomes in acute hepatitis C. Although new IFN-free therapies for chronic hepatitis C approach cure rates of nearly 100%, acute hepatitis C will continue to remain an ongoing public health issue, and unraveling the immunology behind viral clearance will be important in guiding management for these patients, in addition to development of a HCV vaccine. Last, owing to the high cost of the new HCV drugs, the prediction of spontaneous clearance by analysis of biomarkers will allow identification of patients who are likely to spontaneously clear infection, so that they can defer therapy and thereby avoiding unnecessary cost and toxicity.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Acknowledgments. We thank the Australian Trial in Acute Hepatitis C (ATAHC), Immunovirology Research Network, and Networks groups for sample provision and support in conducting this research.

S. G. C. performed and designed experiments, drafted and revised the manuscript, and performed statistical analysis. J. B. designed models for statistical analysis, performed statistical analysis, and critically revised the manuscript. N. S. and R. M. performed and designed experiments and revised the manuscript. G. M., M. H., and G. J. D. acquired data and critically revised the manuscript. V. S. designed models for statistical analysis, performed statistical analysis, and critically revised the manuscript. P. D., A. T., K. V., and J. S. conceived and designed experiments, performed statistical analysis, drafted and critically revised the manuscript, and supervised the study. J. S. conceived and designed the study and obtained funding.

Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health (NIH). The views expressed in this publication do not necessarily represent the position of the Australian or Victorian governments.

Financial support. This work was supported by the Gastroenterological Society of Australia (postgraduate clinical research scholarship); the National Institute on Drug Abuse, NIH (award R01DA015999 to the ATAHC); the Australian Department of Health and Ageing (funding to the Kirby Institute); the National Health and Medical Research Council (project grant 331212 to the Networks study); and the Victorian government (operational infrastructure support to the Burnet Institute).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J Viral Hepat **2006**; 13:34–41.
2. Loo Y-M, Gale M. Immune signaling by RIG-I-like receptors. Immunity **2011**; 34:680–92.
3. Wang N, Liang Y, Devaraj S, et al. Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. J Virol **2009**; 83:9824–34.
4. Li K, Li NL, Wei D, et al. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. Hepatology **2012**; 55:666–75.
5. Prokunina-olsson L, Muchmore B, Tang W, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet **2013**; 45:164–71.
6. Beinhardt S, Aberle JH, Strasser M, et al. Serum level of IP-10 increases predictive value of IL28B polymorphisms for spontaneous clearance of acute HCV infection. Gastroenterology **2012**; 142:78–85.e2.
7. Thomas DL, Thio CL, Martin MP, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature **2009**; 461:798–801.
8. Grebely J, Feld JJ, Applegate T, et al. Plasma interferon-gamma-inducible protein-10 (IP-10) levels during acute hepatitis C virus infection. Hepatology **2013**; 57:2124–34.
9. Tillmann HL, Thompson AJ, Patel K, et al. A polymorphism near IL28B is associated with spontaneous clearance of acute hepatitis C virus and jaundice. Gastroenterology **2010**; 139:1586–92,
10. Rauch A, Kutalik ZAN, Descombes P, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology **2010**; 138:1338–45,
11. Sato K, Ishikawa T, Okumura A, et al. Expression of Toll-like receptors in chronic hepatitis C virus infection. J Gastroenterol Hepatol **2007**; 22:1627–32.
12. Machida K, Cheng KTH, Sung VMH, et al. Hepatitis C virus induces Toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6. J Virol **2005**; 80:866–74.
13. Riordan SM, Skinner NA, Kurtovic J, et al. Toll-like receptor expression in chronic hepatitis C: correlation with pro-inflammatory cytokine levels and liver injury. Inflamm Res **2006**; 55:279–85.
14. Dolganiuc A, Garcia C, Kodys K, et al. Distinct Toll-like receptor expression in monocytes and T cells in chronic HCV infection. World J Gastroenterol **2006**; 12:1198–204.
15. Shehata MAH, El-Enein AA, El-Sharnouby GA. Significance of toll-like receptors 2 and 4 mRNA expression in chronic hepatitis C virus infection. Egypt J Immunol **2005**; 13:141–52.
16. Sanders CM, Cruse JM, Lewis RE. Toll-like receptors, cytokines and HIV-1. Exp Mol Pathol **2007**; 84:31–6.
17. Lester RT, Yao X-D, Ball TB, et al. Toll-like receptor expression and responsiveness are increased in viraemic HIV-1 infection. AIDS **2008**; 22:685–94.
18. Berzsenyi MD, Roberts SK, Preiss S, et al. Hepatic TLR2 & TLR4 expression correlates with hepatic inflammation and TNF-α in HCV & HCV/HIV infection. J Viral Hepat **2011**; 18:852–60.
19. Vogel M, Boesecke C, Rockstroh JURK. Acute hepatitis C infection in HIV-positive patients. Curr Opin Infect Dis **2011**; 24:1–6.
20. Côté P, Baril J-G, Hébert M-N, et al. Management and treatment of hepatitis C virus in patients with HIV and hepatitis C virus coinfection: A practical guide for health care professionals. Can J Infect Dis Med Microbiol **2007**; 18:293–303.
21. Laguno M, Murillas J, Blanco JL, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for treatment of HIV/HCV co-infected patients. AIDS **2004**; 18:F27–36.
22. Carrat F, Bani-Sadr F, Pol S, et al. Pegylated interferon alfa-2b vs standard interferon alfa-2b, plus ribavirin, for chronic hepatitis C in HIV-infected patients: a randomized controlled trial. JAMA **2004**; 292:2839–48.
23. Chung RT, Andersen J, Volberding P, et al. Peginterferon Alfa-2a plus ribavirin versus interferon alfa-2a plus ribavirin for chronic hepatitis C in HIV-coinfected persons. N Engl J Med **2004**; 351:451–9.
24. Dore GJ, Hellard M, Matthews GV, et al. Effective treatment of injecting drug users with recently acquired hepatitis C virus infection. Gastroenterology **2010**; 138:123–35.e1–2.
25. Aitken CK, McCaw RF, Bowden DS, et al. Molecular epidemiology of hepatitis C virus in a social network of injection drug users. J Infect Dis **2004**; 190:1586–95.
26. Freemantle N. Interpreting the results of secondary end points and subgroup analyses in clinical trials: should we lock the crazy aunt in the attic? BMJ **2001**; 322:989–91.
27. Agaugué S, Perrin-Cocon L, André P, et al. Hepatitis C lipo-viro-particle from chronically infected patients interferes with TLR4 signaling in dendritic cell. PLoS One **2007**; 2:e330.
28. Broering R, Wu J, Meng Z, et al. Toll-like receptor-stimulated non-parenchymal liver cells can regulate hepatitis C virus replication. J Hepatol **2008**; 48:914–22.
29. Li Y, Chang M, Abar O, et al. Multiple variants in toll-like receptor 4 gene modulate risk of liver fibrosis in Caucasians with chronic hepatitis C infection. J Hepatol **2009**; 51:750–7.
30. Al-Qahtani AA, Al-Anazi MR, Al-Zoghaibi F, et al. The association of toll-like receptor 4 polymorphism with hepatitis C virus infection in Saudi Arabian patients. Biomed Res Int **2014**; 2014:357062.
31. Haynes LM, Moore DD, Kurt-Jones EA, et al. Involvement of Toll-like receptor 4 in innate immunity to respiratory syncytial virus. J Virol **2001**; 75:10730–7.
32. Jude BA, Pobezinskaya Y, Bishop J, et al. Subversion of the innate immune system by a retrovirus. Nat Immunol **2003**; 4:573–8.
33. Urban TJ, Thompson AJ, Bradrick SS, et al. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. Hepatology (Baltimore, Md) **2010**; 52:1888–96.
34. Honda M, Sakai A, Yamashita T, et al. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology **2010**; 139:499–509.
35. Dill MT, Duong FHT, Vogt JE, et al. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. Gastroenterology **2011**; 140:1021–31.
36. McGilvray I, Feld JJ, Chen L, et al. Hepatic cell-specific gene expression better predicts HCV treatment outcome than IL28B genotype. Gastroenterology **2012**; 142:1122–31.
37. Sarasin-Filipowicz M, Krol J, Markiewicz I, et al. Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. Nat Med **2009**; 15:31–3.
38. Diago M, Castellano G, García-Samaniego J, et al. Association of pretreatment serum interferon gamma inducible protein 10 levels with sustained virological re-

sponse to peginterferon plus ribavirin therapy in genotype 1 infected patients with chronic hepatitis C. Gut **2006**; 55:374–9.
39. Romero AI, Lagging M, Westin J, et al. Interferon (IFN)-gamma-inducible protein-10: association with histological results, viral kinetics, and outcome during treatment with pegylated IFN-alpha 2a and ribavirin for chronic hepatitis C virus infection. J Infect Dis **2006**; 194:895–903.
40. Butera D, Marukian S, Iwamaye AE, et al. Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C. Blood **2005**; 106:1175–82.
41. Zeremski M, Markatou M, Brown QB, et al. Interferon gamma-inducible protein 10: a predictive marker of successful treatment response in hepatitis C virus/HIV-coinfected patients. J Acquir Immune Defic Syndr **2007**; 45:262–8.
42. Askarieh G, Alsio A, Pugnale P, et al. Systemic and intrahepatic interferon-gamma-inducible protein 10 kDa predicts the first-phase decline in hepatitis c virus RNA and overall viral response to therapy in chronic hepatitis C. Hepatology **2010**; 51:1523–30.
43. Darling JM, Aerssens J, Fanning G. Quantitation of pretreatment serum interferon-gamma–inducible protein-10 improves the predictive value of an IL28B gene polymorphism for hepatitis C treatment response. Hepatology **2011**; 53:14–21.
44. Vijgen L, Talloen W, Scholliers A, et al. 1167 Pre-treatment IP-10 levels and IL28B genotype in prediction of SVR in prior treatment-experienced genotype 1 HCV patients treated with telaprevir/peginterferon/ribavirin in the realize study. J Hepatol **2012**; 56:S462.

5 Peripheral Monocyte TLR Signaling and its Association with the Outcome of DAA Therapy in Patients with Genotype 1 HCV Infection

5.1 Introduction

Toll-like receptors (TLR) are a well-characterised family of pattern recognition receptors that reside predominantly on cells of the innate immune system and detect the presence of invading microbes, such as viruses, bacteria, fungi and parasites. Such invading microbes contain highly conserved structural motifs, known as pattern-associated molecular patterns (PAMPs), and through recognition by pattern recognition receptors (PRRs), trigger a diverse number of signaling pathways, including TLR signaling, to mount an immune inflammatory response.

There is a body of evidence to highlight the importance of TLR signaling as a key determinant of viral disease pathogenesis including hepatitis B, HIV, cytomegalovirus, respiratory syncytial virus and herpes simplex virus.[202] Toll-like receptor signaling has also been previously implicated in the pathogenesis of acute and chronic HCV infection. Previous work in our laboratory has shown that patients with chronic hepatitis C display higher levels of TLR2/4 protein expression on peripheral blood monocytes, as well as higher TLR2/4 gene expression in liver compared to healthy controls.[97,98] Monocyte TLR2 expression correlated with increased levels of circulating TNF-α and serum ALT, and TLR2/4 mRNA expression in the liver correlated with hepatic necro-inflammatory activity. Similar results have been observed in other studies.[92,99-103] In Chapter 4, we demonstrated that persistence of HCV infection was associated with high level reactivity of TLR4 and TLR7/8 signaling pathways in response to toll ligand-specific stimulation, as well as with high levels of TLR4 expression on monocytes and NK cells.[203]

Experimental models also suggest an important link between TLR signaling and HCV pathogenesis. In vitro studies have identified specific components of the HCV

polyprotein that interact with the TLRs leading to induction of a pro-inflammatory response. In peripheral blood mononuclear cells, the HCV core and NS3 protein have been shown to stimulate TLR2 signaling.[92] In a cell culture model the HCV NS5A protein enhanced TLR4 gene transcription and IL-6 / IFN-β production.[92,93] HCV is also sensed by cytoplasmic RIG-I and endosomal TLR3 during viral replication leading to stimulation of the interferon (IFN) signaling pathway. This interferon stimulated gene (ISG) pathway has been recognised as important in the HCV host-virus interaction and to be a determinant of sensitivity to exogenous IFN.[118,204] Poor response to exogenous IFN-α has been associated with elevated pre-treatment intra-hepatic ISG expression, as well as changes in peripheral ISG expression. It has also been associated with IFNL4 polymorphisms, where the "good response" genotype is associated with lower levels of hepatic ISG expression and good clinical response to exogenous IFN-based therapy.[120,122]

This link with TLR signaling has been clinically translated into the development of TLR agonists as potential anti-viral agents for HCV. In a phase 1b study, a small-molecule TLR7 agonist was shown in humans to have an IFN-dependent anti-viral effect against HCV.[205] Despite good tolerability, clinical development of this agent was discontinued due to the development of more effective direct acting antivirals (DAAs).

In summary, these data suggest a potential role for TLR signaling in the pathogenesis of chronic hepatitis C. To date there have been no studies of host-virus interactions in the setting of potent DAA therapy for HCV. In particular, it is not known how TLR expression / signaling responds to the inhibition of HCV replication, nor whether endogenous TLR signaling pathways are associated with the outcome of antiviral therapy. We hypothesized that the high level TLR2/4±7 expression and signaling observed in the setting of persistent HCV infection (Chapter 3), although insufficient to clear the virus, may contribute to antiviral efficacy in the setting of potent interferon-free DAA therapy.

5.2 Aims

The aims of this study were to investigate the early effect of potent viral suppression using DAA therapy on TLR expression and signaling on peripheral

monocytes in subjects with genotype 1 chronic HCV infection, and to determine whether baseline TLR expression/signaling or early changes in TLR expression/signaling during therapy were predictive of treatment outcome.

5.3 Methods

5.3.1 Description of Patient Cohort

Patients with Genotype 1 CHC who were referred for antiviral therapy between 2012 and 2014 were enrolled prospectively. Patients were treated with the direct acting antiviral regimen of ombitsavir (12.5mg), ritonavir (50mg) boosted paritaprevir (75mg), dasabuvir (250mg) +/- weight-based ribavirin. Plasma and PBMCs were collected from subjects at baseline, day-1, day-14 and day-28 of treatment.

HCV RNA levels were measured using the COBAS® TaqMan HCV test (Roche Molecular Diagnostics). The HCV genotype was tested using the COBAS® TaqMan HBV test (Roche Molecular Diagnostics). IL28B genotype (rs12979860) was typed using the TaqMan allelic discrimination kit (Applied Biosciences, Foster City, CA, USA).(206)

The study protocol was approved by the Human Research Ethics Committee at St Vincent's Hospital and conducted according to the Declaration of Helsinki and ICH/GCP guidelines. Written informed consent was obtained for participants.

5.3.2 Functional Evaluation of the Activity of TLR Signaling Pathway via PBMC Stimulation

The activity of TLR signaling pathways was tested by evaluating cytokine production from PBMCs in response to stimulation with TLR-specific ligands. Detailed methods were presented in Chapter 2.2.4. In brief, a total of 1×10^6 thawed PBMCs from subjects were cultured at 37°C for 20 hours in Roswell Park Memorial Institute (RPMI) medium with 1% L-glutamine, 1% penicillin, and 5% fetal calf serum (FCS) and TLR ligands. PBMCs were stimulated with RPMI 5% FCS (unstimulated control), 100 ng/mL Pam-3-Cys (a TLR2 ligand), 10 μg/mL polyinosinic:polycytidylic acid (a TLR3 ligand), lipopolysaccharide (LPS; a TLR4 ligand), 5 μg/mL R848 (a TLR7/8 ligand), and 0.3 μM CpG ODN 2006 (a TLR9 ligand). All TLR ligands were supplied by InVivoGen. Supernatants were harvested after culture and stored frozen at −70°C until batch analysis. The concentrations of TLR ligand and the duration of the incubation period

were chosen on the basis of results of optimization experiments, to maximize cytokine output.

5.3.3 Measurement of Cytokine Levels by the Cytometric Bead Array Assay and Enzyme-Linked Immunosorbent Assay (ELISA)

The cytometric bead array assay was performed on thawed tissue culture supernatants (post stimulation) at dilutions of 1:1, 1:10, and 1:100, to accommodate high levels of cytokine. IFN-γ–inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), interleukin 8 (IL-8), interleukin 10 (IL-10), interferon-gamma (IFN-γ), tumour necrosis factor α (TNF-α), chemokine (C-X-C motif) ligand 9 (CXCL9), interleukin-1b (IL-1b) and interleukin 6 (IL-6) levels were measured simultaneously, according to the instructions provided by the manufacturer (BD Biosciences, San Jose CA). Supernatants from PBMCs cultured in medium alone were used as controls. A series of 10 dilutions using cytokine standards were run in each assay, for generation of standard curves. The intensity of the fluorescence signal was measured on the FACS Canto Flow Cytometer (Becton Dickinson), and data were analysed using FCAP Array Software (BD Biosciences). To assess differences in TLR functional response on-treatment, each time-point was presented as a ratio in comparison to the baseline (day-0) time-point.

5.3.4 Flow Cytometry for Determination of TLR Expression on Peripheral CD14+ Blood Monocytes and Natural Killer Cells

Detailed methods have been previously described.(97) In brief, cell surface staining was performed on rapidly thawed PBMCs, using the following fluorochrome-conjugated anti-human monoclonal antibodies: anti-TLR2- fluorescein isothiocyanate, anti-TLR4-phycoerythrin (eBioscience), anti-TLR7-phycoerythrin (R&D Systems), anti-CD14-allophycocyanin Cy7, anti-CD3-Pacific Blue, and anti-CD56-phycoerythrin Cy7 (eBioscience). Isotype-matched nonbinding control antibodies were used for comparison. A total of 10 000 CD14+ monocytes and CD56+ NK cells of each sample were analysed; dead cells were gated out, based on their light scatter properties on the FACS Canto Flow Cytometer (Becton Dickinson). Data were analysed using FlowJo software (Tree Star, Ashland, OR). TLR2, TLR4, and TLR7 values were expressed as a ratio of the geometric mean fluorescence of individual study patients to the geometric

mean isotype control values for that patient. To assess differences in TLR receptor expression / signaling on-treatment, each time-point was presented as a ratio in comparison to the baseline (day-0) time-point.

5.3.5 Statistical Analysis

Participant characteristics and laboratory measurements are presented as median values and interquartile ranges. The Fisher exact test was used to test for significant group differences in dichotomous variables, and the independent samples t-test was used for continuous variables. The Mann-Whitney-U test was used to determine if differences existed between baseline TLR receptor expression / signaling, in comparison to TLR expression / signaling at each time-point. This was performed using Prism (v7). Graphs were presented as Tukey box plots, with the middle line representing the median, and the box extending from the 25th to 75th percentile.

5.4 Results

5.4.1 Characteristics of the Study Population

16 subjects with HCV-1 infection were prospectively enrolled and treated with a combination of IFN-free antiviral therapy, comprised of ombitsavir, ritonavir boosted paritaprevir, dasabuvir +/- ribavirin. The baseline characteristics and demographics of this group are detailed in Table 5.1. In brief, 8/16 (50%) were male, 15/16 (94%) were Caucasian and 14/16 (86%) had advanced liver fibrosis (METAVIR F3-4). 7/16 (44%) were prior non-responders to treatment with peginterferon-α plus ribavirin. 4/16 (25%) carried the CC genotype (rs12979860) which has been associated with good response to peginterferon-based treatment for HCV.

Participant characteristics	
n (%)	16 (48%)
Age, years (median, IQR)	55 (51 – 61)
Male (n,%)	8 (50%)
Caucasian (n,%)	15 (94%)
Treatment naive	9 (56%)
Baseline ALT, U/L (median, IQR)	128 (149 – 154)
HCV genotype 1a/1b	11 (69%) / 5 (31%)
Baseline HCV RNA level, IU/mL (median, IQR)	1 910 000 (55400 – 2 655 000)
Advanced fibrosis (≥F3)	14 (86%)
Favourable IFNL4 Gt (n,%) (CC, rs 12979860) • Treatment naïve	4 (25%) • 2 (50%)
Prior IFN experience	7 (44%)
Ribavirin (n,%)	11 (69%)
SVR (%)	14 (86%)

Table 5.1 Participant Characteristics and Demographics at Baseline

All subjects experienced a rapid decrease in serum HCV RNA levels during DAA therapy. Significant reductions in serum HCV RNA level were observed as early as day-1 of treatment (median reduction at day-1 = 1,908,623 IU/mL) (Figure 5.1). 10/16 (63%) subjects had an undetectable serum HCV RNA level at day-28. Serum ALT levels also fell rapidly with IFN-free therapy and median ALT fell significantly by day-7 of treatment. 14/16 (88%) subjects had ALT < ULN at day-28. One subject was lost to follow up. 14/16 (86%) subjects went on to achieve a sustained virological response at week 12 post-treatment (SVR12). 2/16 (19%) achieved an EOT response but then relapsed post-treatment. Both had HCV-1a infection, with one subject treatment experienced, cirrhotic and with the poor response IFNL4 genotype, and the other was treatment naïve with F3 fibrosis, carrying the good response IFNL4 genotype.

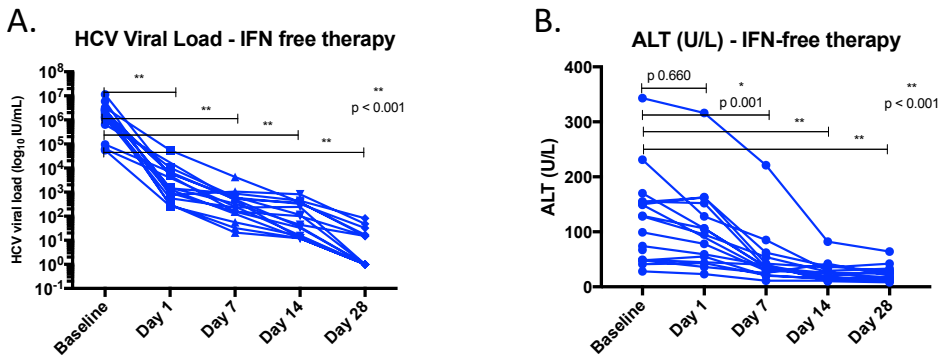


Figure 5.1 Serum HCV RNA and ALT response to IFN-free therapy.
Serum HCV RNA and ALT levels fell with IFN-free therapy. (A) Serum HCV RNA; (B) ALT.

5.4.2 IFN-free DAA Treatment was Associated with Reduction in PBMC TLR Signaling Activity Over Time

5.4.2.1 Analysis of On-treatment TLR Signaling Activity

We first tested whether DAA therapy and rapid viral suppression was associated with any effect on monocyte TLR signaling activity. The first analysis considered the entire cohort who received IFN-free DAA therapy. PBMCs were collected at days 0, 1, 14 and 28 of DAA therapy. As previously described, we measured cytokine responses to stimulation of PBMCs with the specific TLR ligands Pam-3-Cys (TLR2), Poly I:C (TLR3), LPS (TLR4), R848 (TLR7/8) and CpG ODN 2006 (TLR9).

DAA therapy was associated with a significant reduction of TLR signaling activity over time (Figure 5.2). Reduced responses to TLR2 stimulation (IL-10, IL-8), TLR7 stimulation (IL-10, MCP-1 and IL-8) and TLR9 stimulation (MCP-1) were detectable as early as day-1 after starting DAA therapy. Reduced cytokine response to stimulation with TLR2, TLR4 and TLR7/8 ligands was detectable at day-14 and day-28 (Figure 5.2), and there was a trend to progressive decline over time for all cytokine responses. Reduced IL-10 response was most marked and most common for TLR2, TLR4 and TLR7 ligands at all timepoints. Pro-inflammatory cytokine responses were also lower (Figure 5.2). No detectable cytokine response was observed following TLR3 ligand stimulation (as seen with our acute HCV cohort, data not shown).

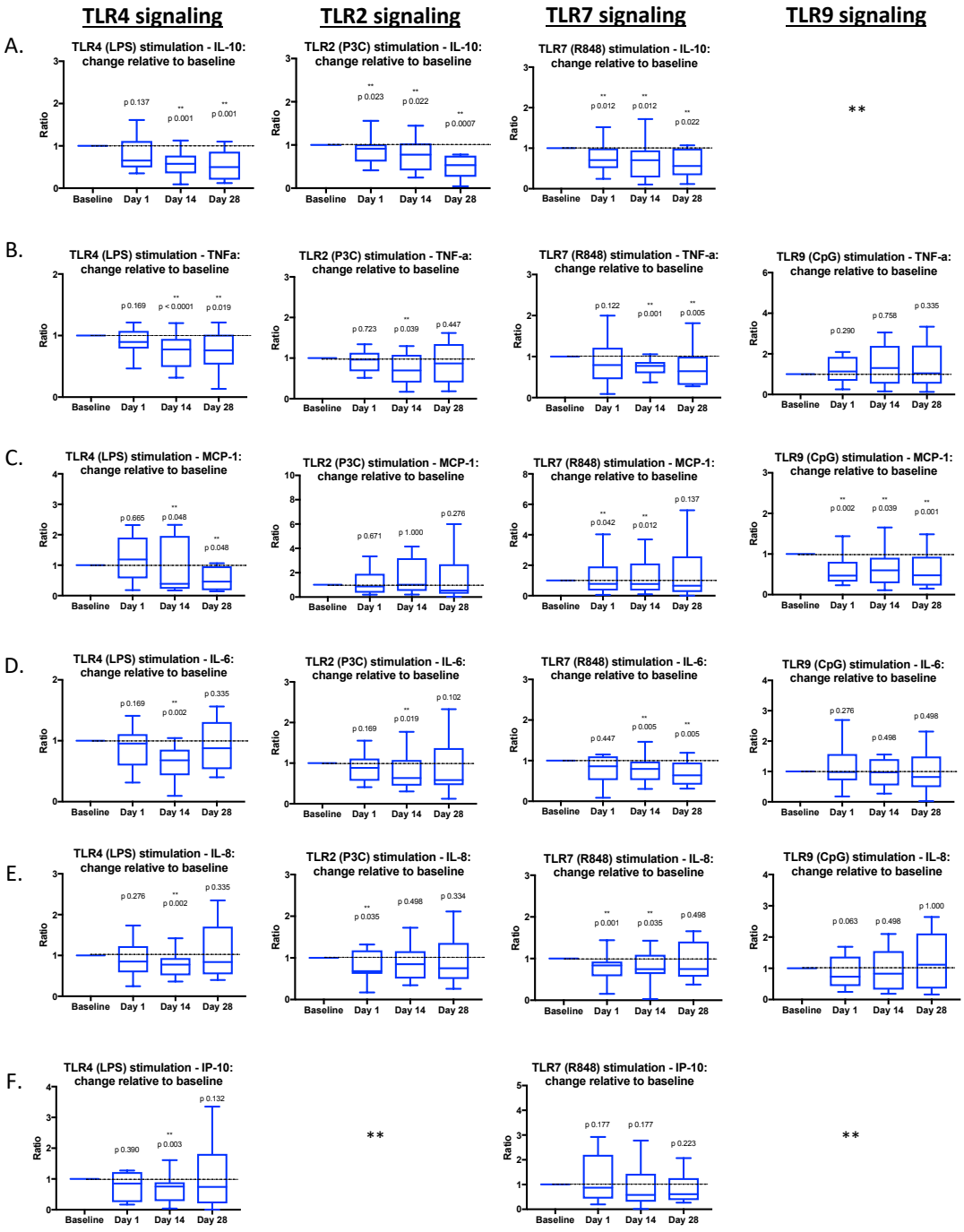


Figure 5.2 Reduced TLR 4 / 2 / 7 / 9 signaling with DAA therapy.

A) IL-10; B) TNF-α; C) MCP-1; D) IL-6; E) IL-8; F) IP-10.

**undetectable levels of measured cytokine to TLR stimulation.

Refer to Appendices (Figure 5.8) for cytokines: IFN-γ, IL-1b, CXCL9.

No detectable cytokine response was observed following TLR3 ligand stimulation.

5.4.3 Baseline TLR Signaling Activity and the Outcome of DAA Therapy for Genotype 1 HCV

We next tested for an association between TLR signaling activity at baseline and response to DAA therapy. We measured cytokine responses to stimulation of PBMCs with the specific TLR ligands Pam-3-Cys (TLR2), Poly-IC (TLR3), LPS (TLR4 ligand), R848 (TLR7/8) and CpG ODN 2006 (TLR9) as described above. Cell culture supernatants were tested post-stimulation for a cytokine panel that included IL-10, TNF-α, MCP-1, IL-6, IFN-γ, IL-8, CXCL9, IP-10 and IL-1b.

Analysis of pre-treatment samples demonstrated a strong trend for markers of PBMC TLR2, TLR4 and TLR7 signaling to be elevated in patients who went on to achieve an SVR following DAA treatment (Figure 5.3). A similar pattern was observed for higher on-treatment levels of TNF-α, MCP-1, IL-6, IL-8, IL-1b, IFN-γ and CXCL9 secretion following TLR ligand stimulation among subjects who achieved an SVR (see Chapter Appendices 5.8 - Figure 5.9). No differences according to treatment response were observed following TLR9 ligand stimulation.

We were also interested in whether TLR signaling activity differed according to IFNL4 genotype or liver fibrosis stage. No significant difference in baseline TLR reactivity was observed between patients with different IFNL4 genotype or different stage of liver fibrosis (see Chapter Appendices 5.8 - Figure 5.10).

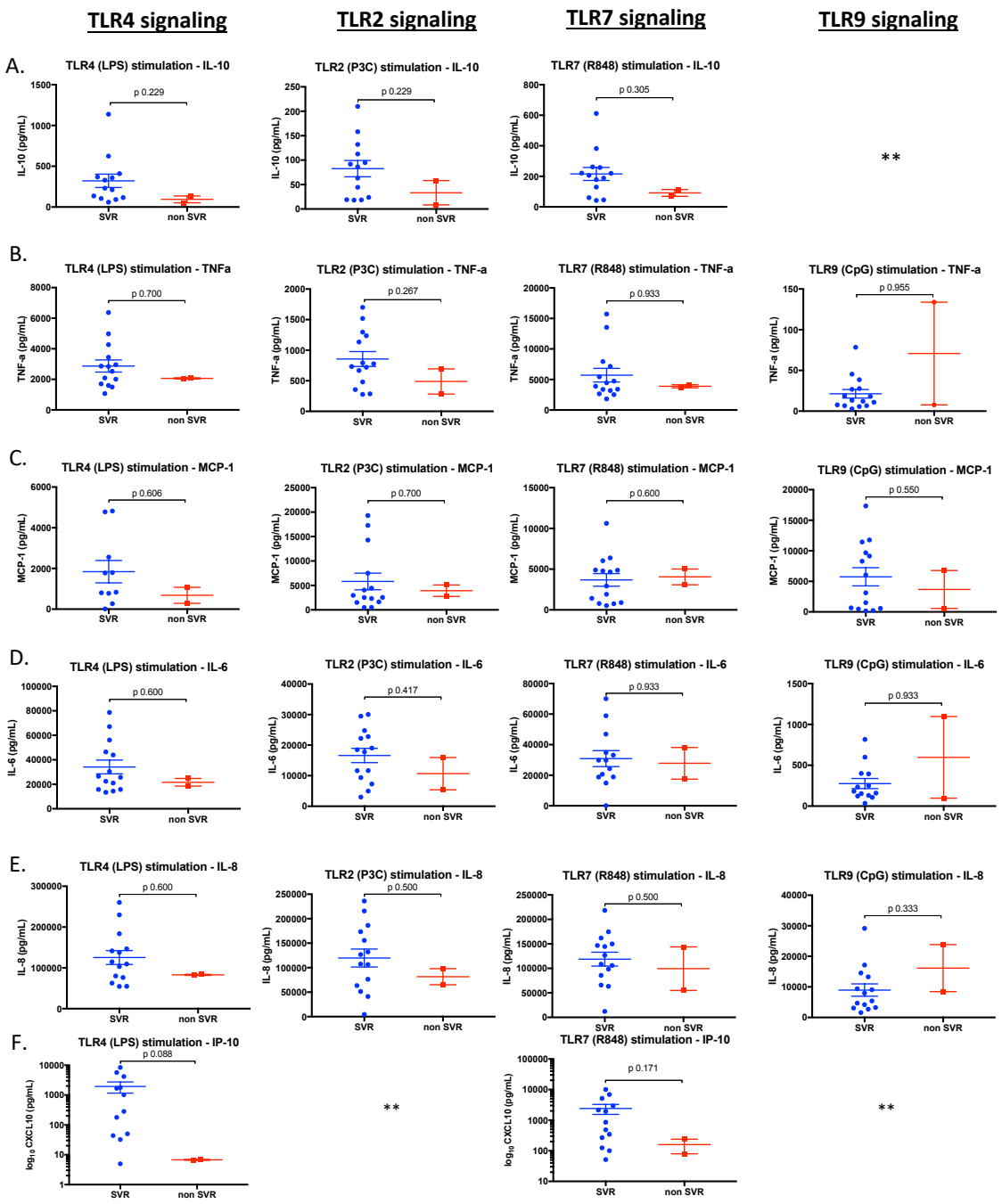


Figure 5.3 TLR signaling activity in PBMCs at baseline according to SVR vs. non SVR. A trend for markers of PBMC TLR2, TLR4 and TLR7 signaling to be elevated in patients who went on to achieve an SVR following DAA treatment was observed. A) IL-10; B) TNF-α; C) MCP-1; D) IL-6; E) IL-8; F) IP-10.

**undetectable levels of measured cytokine to TLR stimulation

Refer to appendices (Figure 5.9) for cytokines: IFN-γ, IL-1b, CXCL9.

No detectable cytokine response was observed following TLR3 ligand stimulation.

5.4.3.1 Analysis of PBMC TLR Signaling Activity Over Time According to Treatment Outcome (SVR12 vs non-SVR12)

We then analysed patterns of TLR signaling over time according to treatment outcomes. Although there were no statistically significant differences observed, there

was a trend to reduced TLR signaling 'on-treatment' among those who achieved SVR12. (Figure 5.4). No difference was seen according to IFNL4 genotype (C/C n = 4 vs. non-C/C n = 12) (see Chapter Appendices 5.8 - Figure 5.11).

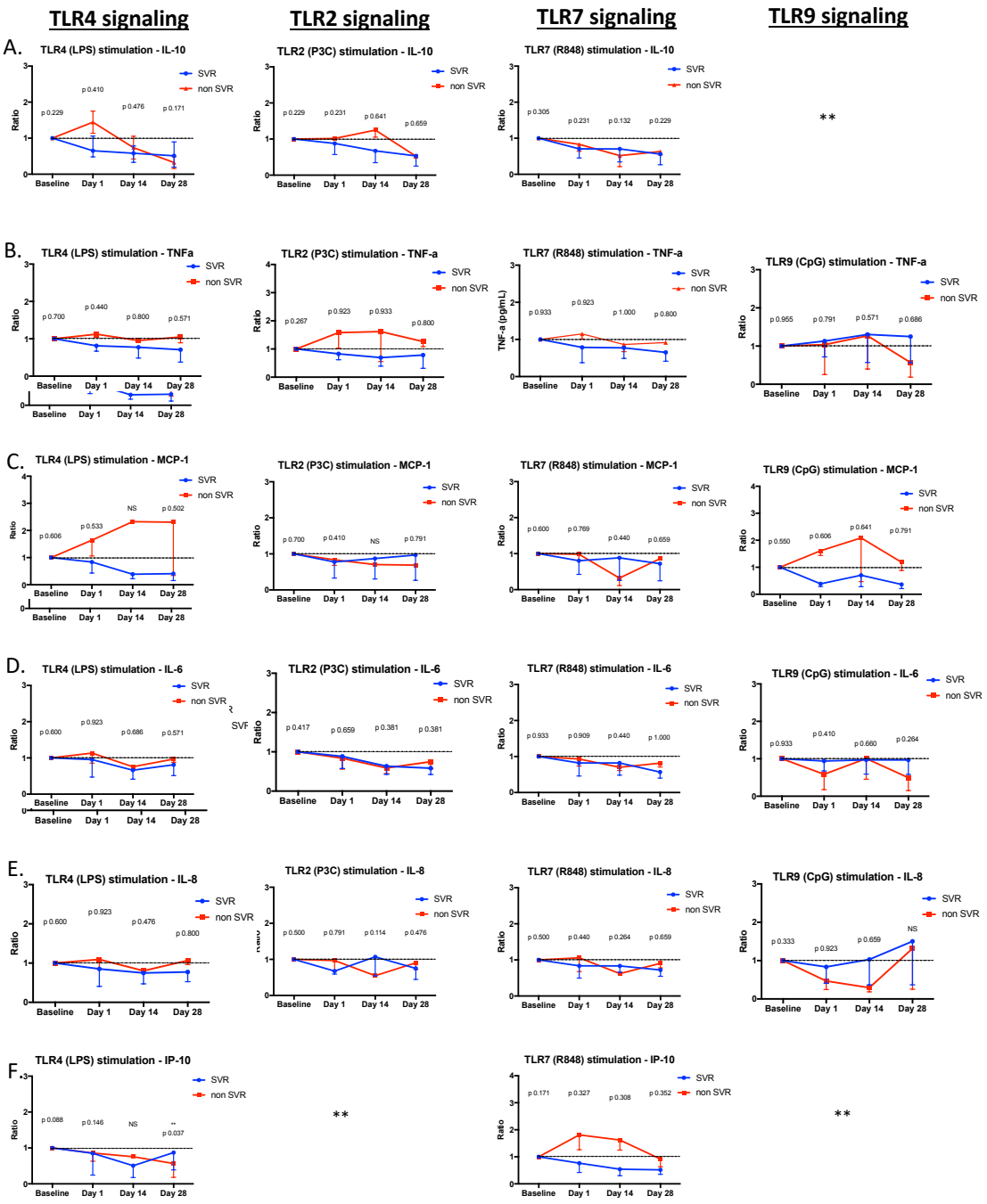


Figure 5.4 TLR 4/ 2 / 7 / 9 reactivity with DAA treatment according to SVR vs. non SVR.

A trend to reduced TLR signaling on-treatment was observed among those who achieved SVR. A) IL-10; B) TNF-α; C) MCP-1; D) IL-6; E) IL-8; F) IP-10.

**undetectable levels of measured cytokine to TLR stimulation

No detectable cytokine response was observed following TLR3 ligand stimulation.

5.4.3.2 PBMC TLR Signaling Activity was not Associated with Baseline Serum ALT Levels

After observing the trend for higher baseline levels of TLR signaling activity amongst subjects who then went on to achieve an SVR, we then compared baseline ALT levels between groups, as a simple test of whether the difference observed might be a non-specific reflection of underlying hepatic inflammation. However, there was a trend to higher ALT levels at baseline and day-1 in subjects who did not attain SVR12 (Figure 5.5). Subjects who did not achieve SVR12 also had higher baseline HCV RNA levels than those who did not attain SVR12.

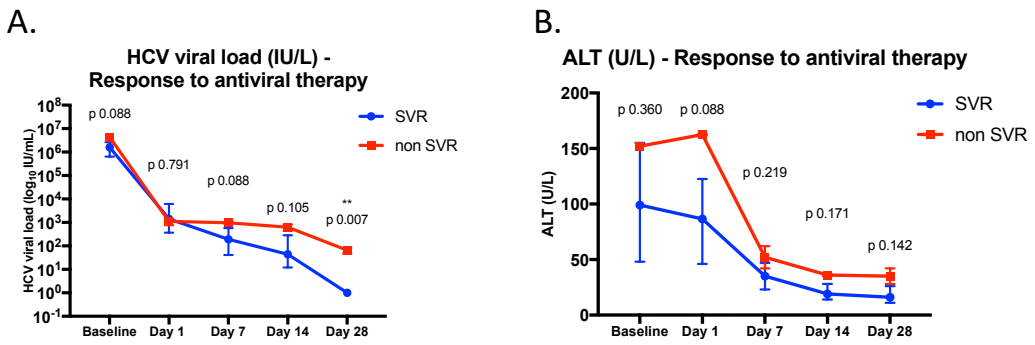


Figure 5.5 Serum HCV RNA and ALT response to DAA therapy according to SVR vs. non SVR.
A trend to higher ALT levels at baseline and day-1 was observed in subjects who did not attain SVR. (A) Serum HCV RNA; (B) ALT.

5.4.4 TLR2, TLR4, and TLR7 Expression on Peripheral Blood Monocytes and NK Cells (Dim and Bright) with DAA therapy

After observing that TLR signaling activity was down-regulated during antiviral therapy, and that there was a potential signal for baseline levels of TLR signaling activity to be associated with the outcome of DAA therapy, it was important to test whether this reflected difference in TLR expression. We tested expression of TLR2/4/7 on PBMC populations, including CD14+ monocytes, NK cells and NK bright/dim subsets, at baseline and longitudinally during treatment. However, there was no difference in CD14+ monocyte expression of TLR2, TLR4 or TLR7 evident over time (Figure 5.6). There was also no difference in levels of monocytic TLR expression comparing subjects who achieved SVR12 vs. those who did not achieve SVR12 at baseline (see Chapter Appendices 5.8 - Figure 5.13) or during treatment (Figure 5.7).

Small increases in the NK cell expression of TLR2, TLR4 and TLR7 were observed at day-14 / day-28 (Figure 5.6). These changes were most apparent on the NK dim cell subset. SVR12 was associated with higher day-1 / day-14 TLR4 expression on NK cells and NK bright/dim subsets, with a trend to higher TLR2/7 expression (Figure 5.7). No differences were seen according to IFNL4 genotype (see Chapter Appendices 5.8 - Figure 5.12).

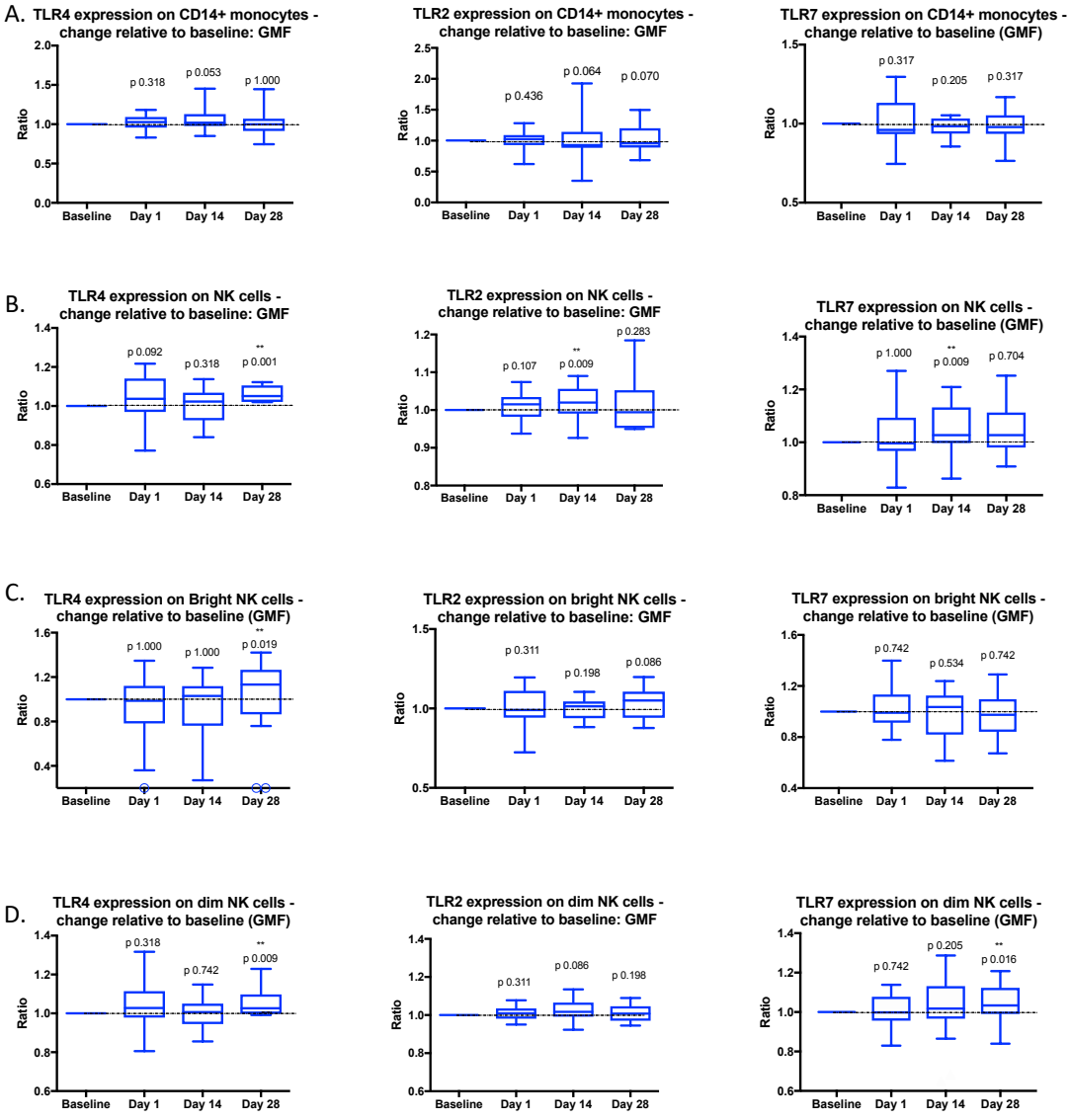


Figure 5.6 TLR 4 / 2 / 7 expression with DAA treatment.
No change in CD14+ monocyte expression of TLR 4 / 2 / 7 or TLR7 was observed with DAA treatment. Small increases in NK cell expression of TLR 4/ 2 / 7 was observed at day-14 and day-28. (A) CD14+ monocytes; (B) NK cells; (C) Bright NK cells; (D) Dim NK cells.

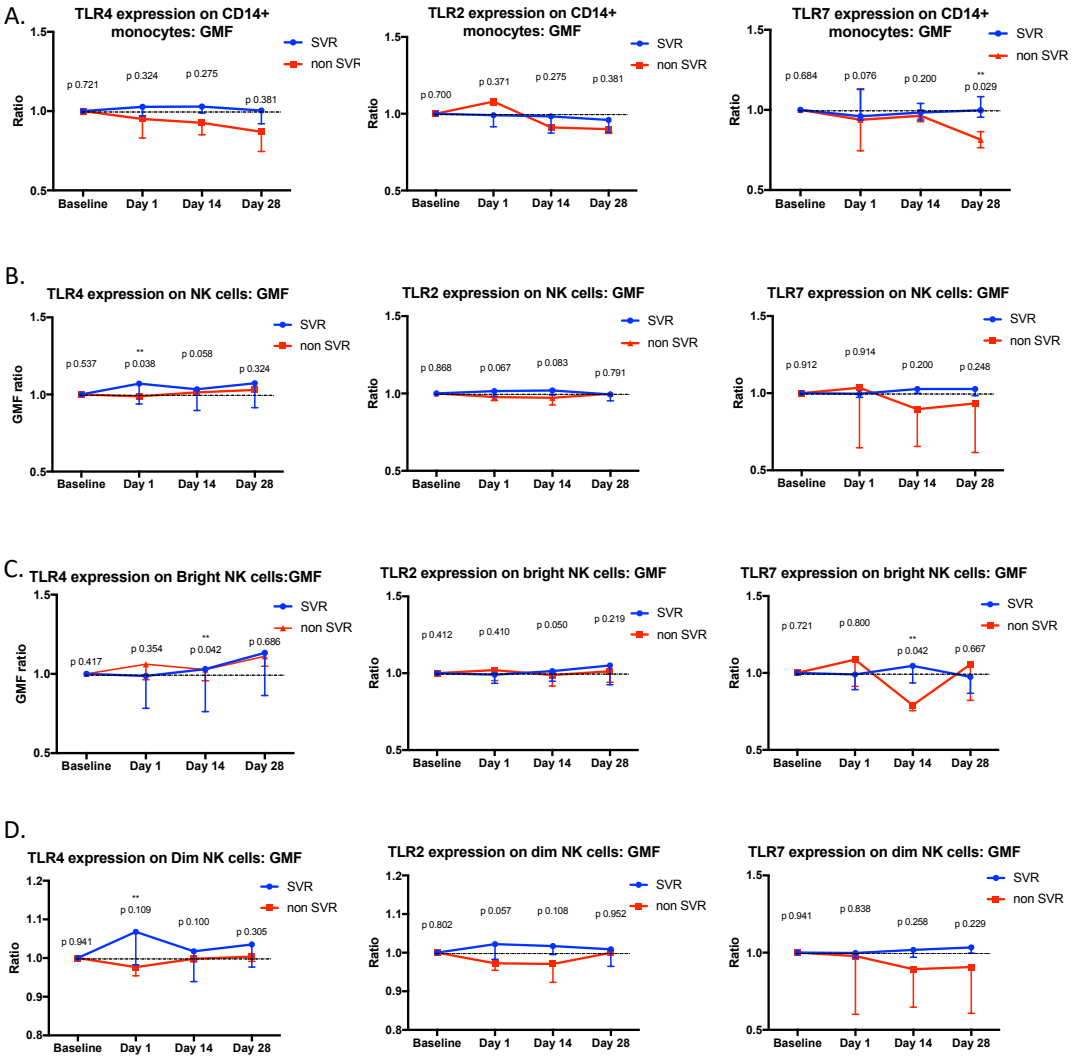


Figure 5.7 TLR 4 / 2 / 7 expression with DAA treatment, according to SVR vs. non SVR. No difference in levels of monocytic TLR expression was observed according to treatment response. (A) CD14+ monocytes; (B) NK cells; (C) Bright NK cells; (D) Dim NK cells.

5.5 Discussion

This is the first study to demonstrate a relationship between TLR signaling activity and IFN-free therapy for HCV. DAA-mediated viral suppression was associated with down-regulation of peripheral monocyte TLR4, TLR2 and TLR7 and TLR9 signaling. This occurred as early as day-1 with the TLR 2/7/9 pathways. We also observed a strong trend for higher baseline TLR 2/4/7 signaling activity in subjects who achieved viral clearance compared to those who subsequently relapsed.

Our data suggests an important role for TLR signaling in the anti-viral response to HCV. In the context of chronic HCV infection, active viral replication sensed through the host's extra and intra-cellular PRRs in response to the numerous viral PAMPs, leads to an increase in TLR expression / signaling, with ineffectual viral clearance.[97,99,103] Data from our acute HCV cohort demonstrated an association between increased TLR4 signaling and the development of persistent infection. In response to a potent set of DAAs, we propose a model whereby the early drop in TLR signaling is a direct reflection of the reduced levels of circulating extra and intra-cellular viral PAMPs, potentially moving toward the restoration of a HCV-induced dysregulated immune state. Although this drop in TLR signaling was observed in all treated subjects, the trend to increased pre-treatment TLR signaling in those who achieved viral clearance, suggests that the TLR pathway may be play a role in viral eradication in the context of DAAs. Increased TLR signaling reflecting a host-virus interaction geared toward a greater pro-inflammatory and type-1 endogenous IFN response, accelerated viral kinetics, and the prevention of HCV-related resistance associated variants. A trend to higher early on-treatment IL-10 / IP-10 response to TLR 4/2/7 signaling was also observed in those who achieved viral clearance, also supports this model.

No difference in TLR signaling or expression was seen according to IFNL4 genotype. Further studies are required to determine if IFNL4 is linked to TLR signaling and the response to DAAs. Our cohort was underpowered to determine if IFNL4 genotype is relevant to viral clearance. As the majority had advanced fibrosis, and nearly half had prior IFN non-response, this may also have confounded our results.

Although TLR signaling was down-regulated on treatment, no change in TLR expression was evident with the DAAs when tested on all PBMC populations. Previous data has shown that levels of TLR expression do not necessarily correlate with TLR pathway activity.[90] This suggests that the TLR-mediated anti-viral activity against HCV lies within the intrinsic property of the TLR pathways. TLR4 is known to induce both a pro-inflammatory and endogenous type 1 IFN response, via its MyD88 dependent and independent pathways, respectively. In a cell culture model, the HCV NS5A protein enhanced TLR4 gene transcription with increased IL-6 and IFN-β production.[92,93] In recognising viral single stranded HCV RNA, the endosomal pattern recognition receptor

TLR7 has similarly been shown to mediate both a pro-inflammatory cytokine / chemokines and type 1 IFN response.[94] Other components of the HCV polyprotein, the HCV core and NS3/4a, have been shown in vitro to stimulate TLR2 leading to activation of the NFKB pathway inducing a pro-inflammatory state.[90] As such, our data suggests that the baseline up-regulation of these TLR signaling pathways is in direct response to HCV replication.

We considered whether the increase in TLR signaling may reflect a non-specific pro-inflammatory state due to chronic HCV infection. However in our DAA therapy cohort there was a trend to lower ALT levels among those who achieved viral clearance, suggesting this is a true reflection of the host-virus interaction. Our data is consistent with the literature that has shown a strong association between TLR signaling and viral disease pathogenesis. In chronic HIV infection, an increase in TLR2/4 expression and signaling was found to correlate with HIV viral load, potentially promoting disease progression.[207] In contrast, cell culture and animal models investigating chronic HBV infection have shown increased TLR signaling leads to suppression of HBV viral replication.[208] This has clinically translated into the development of TLR agonists in the management of HBV, with TLR7/8 agonists currently in phase 2 development.[209,210]

TLR agonists have been trialled in HCV, but halted due to a toxicity signal.[205] Our data support a potential role for TLR agonists in refractory patients with chronic HCV, such as DAA failures or Genotype 3 HCV cirrhotics. In view of the highly effective first and second-line DAA regimens licensed in the years since this study was commenced, this is unlikely to be pursued.

5.6 Limitations

Although we were able to demonstrate clear patterns of innate immunological response to antiviral therapy, our cohort is small. Validation of our findings in a larger cohort of CHC patients, would allow for a more detailed analysis, including stratification according to treatment response and IFNL4 genotype would strengthen our results. Future studies should consider analysis of intra-hepatic TLR signaling; the data from this project support more invasive testing. We acknowledge that data from

our refractory cohort cannot be translated to treatment naïve HCV individuals with early fibrosis, or subjects with non-genotype 1 HCV. Further studies are required in such subjects. Furthermore, the direct acting antiviral regimen used in this study is no longer widely used.

5.7 Conclusion

Our data demonstrate a clear effect of DAA mediated viral suppression therapy on patterns of TLR signaling in subjects with G1 CHC. We have shown that peripheral monocytic TLR2, TLR4 and TLR7 signaling is down-regulated early on in treatment, with a strong trend to higher baseline TLR signaling being associated with viral clearance with the DAAs. No on-treatment changes in monocytic TLR expression were evident, suggesting that this observation reflects downstream activity of the signaling pathway. Future studies should evaluate the role of TLR signaling in other hard-to-cure populations including subjects with Gt 3 HCV and DAA non-responders. There may be a role for TLR-active agents in such hard-to-cure populations, or as adjuvant treatment to shorten overall treatment duration.

5.8 Appendices

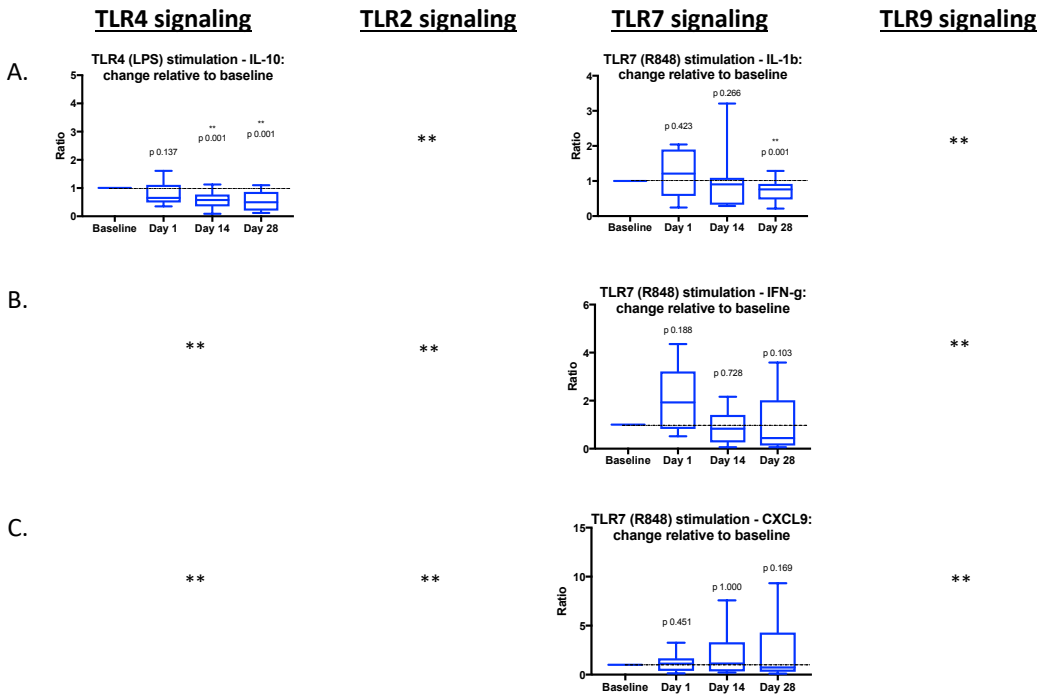


Figure 5.8 Reduced TLR 4 / 2 / 7 / 9 signaling with DAA therapy.

A) IL-1b; B) IFN-γ; C) CXCL9

**undetectable levels of measured cytokine to TLR stimulation.

No detectable cytokine response was observed following TLR3 ligand stimulation.

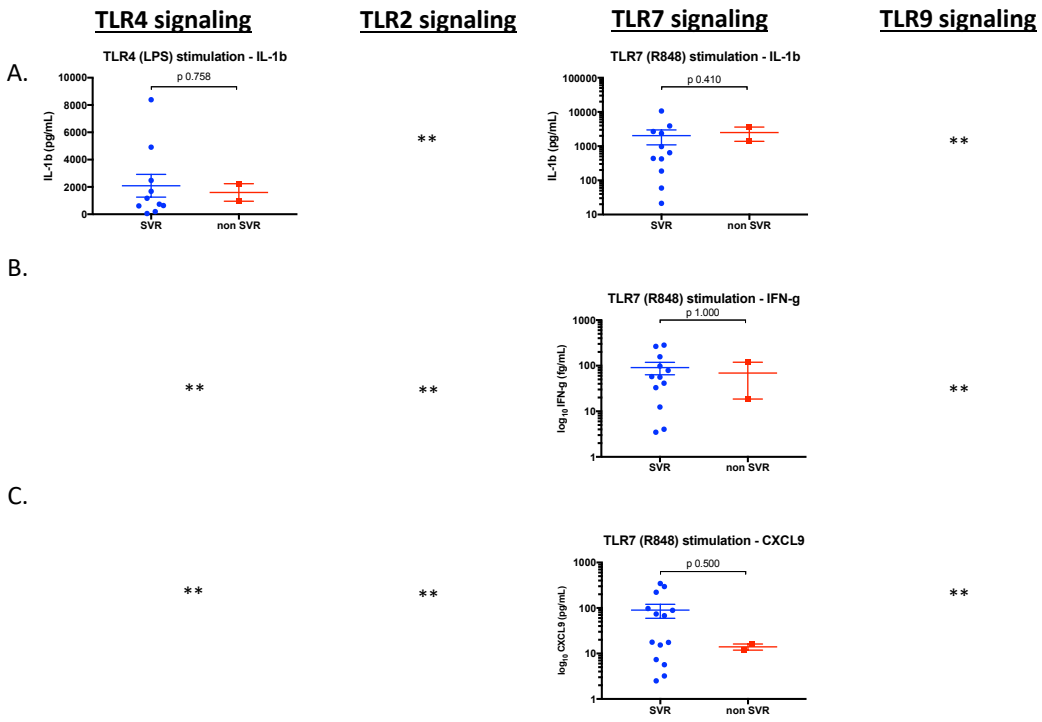


Figure 5.9 TLR signaling activity in PBMCs at baseline according to SVR vs. non SVR.

A trend for markers of PBMC TLR2, TLR4 and TLR7 signaling to be elevated in patients who went on to achieve an SVR following DAA treatment was observed. (A) IL-1b; (B) IFN-γ; (C) CXCL9

**undetectable levels of measured cytokine to TLR stimulation.

No detectable cytokine response was observed following TLR3 ligand stimulation.

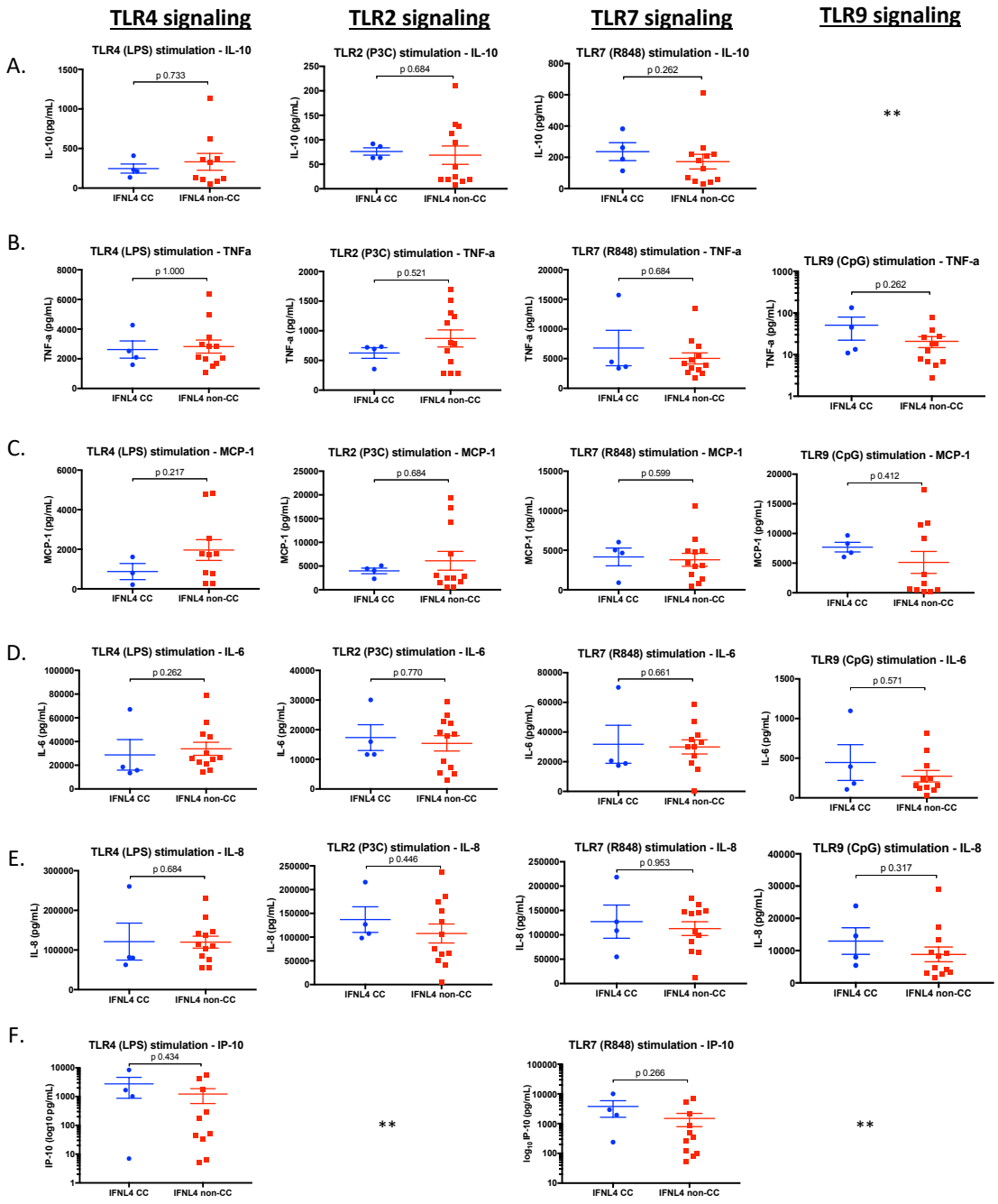


Figure 5.10 TLR signaling activity in PBMCs at baseline according to IFNL4 genotype.

No significant difference in baseline TLR reactivity was observed according to IFNL4 genotype. (A) IL-10; (B) TNF-α; (C) MCP-1; (D) IL-6; (E) IL-8; (F) IP-10.

**undetectable levels of measured cytokine to TLR stimulation.

No detectable cytokine response was observed following TLR3 ligand stimulation.

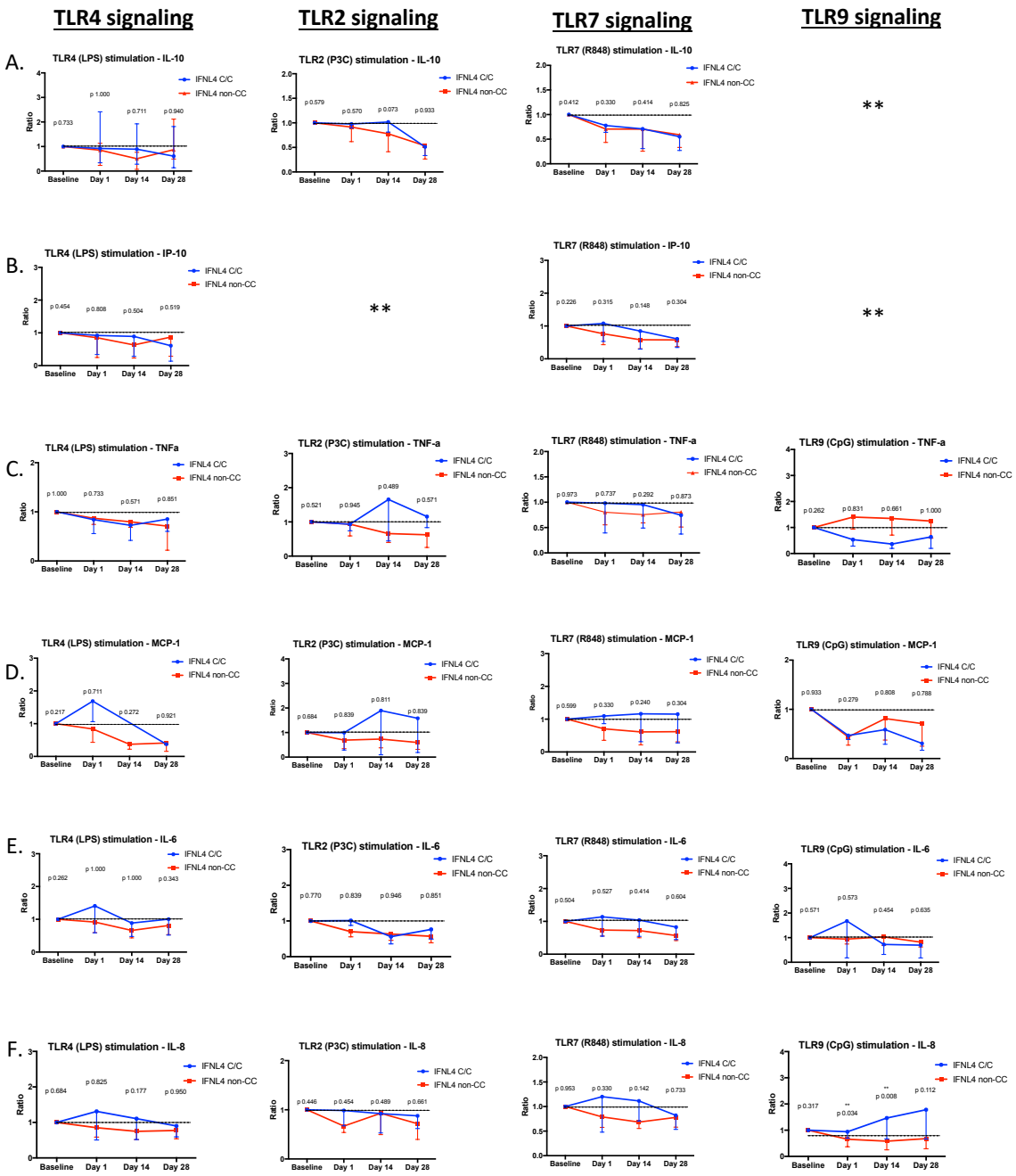


Figure 5.11 TLR 4 /2 /7/9 reactivity with DAA treatment according to IFNL4 genotype.

No change in on-treatment TLR signaling was observed according to IFNL4 genotype. (A) IL-10; (B) IP-10; (C) TNF-α; (D) MCP-1; (E) IL-6; (F) IL-8

**undetectable levels of measured cytokine to TLR stimulation

No detectable cytokine response was observed following TLR3 ligand stimulation.

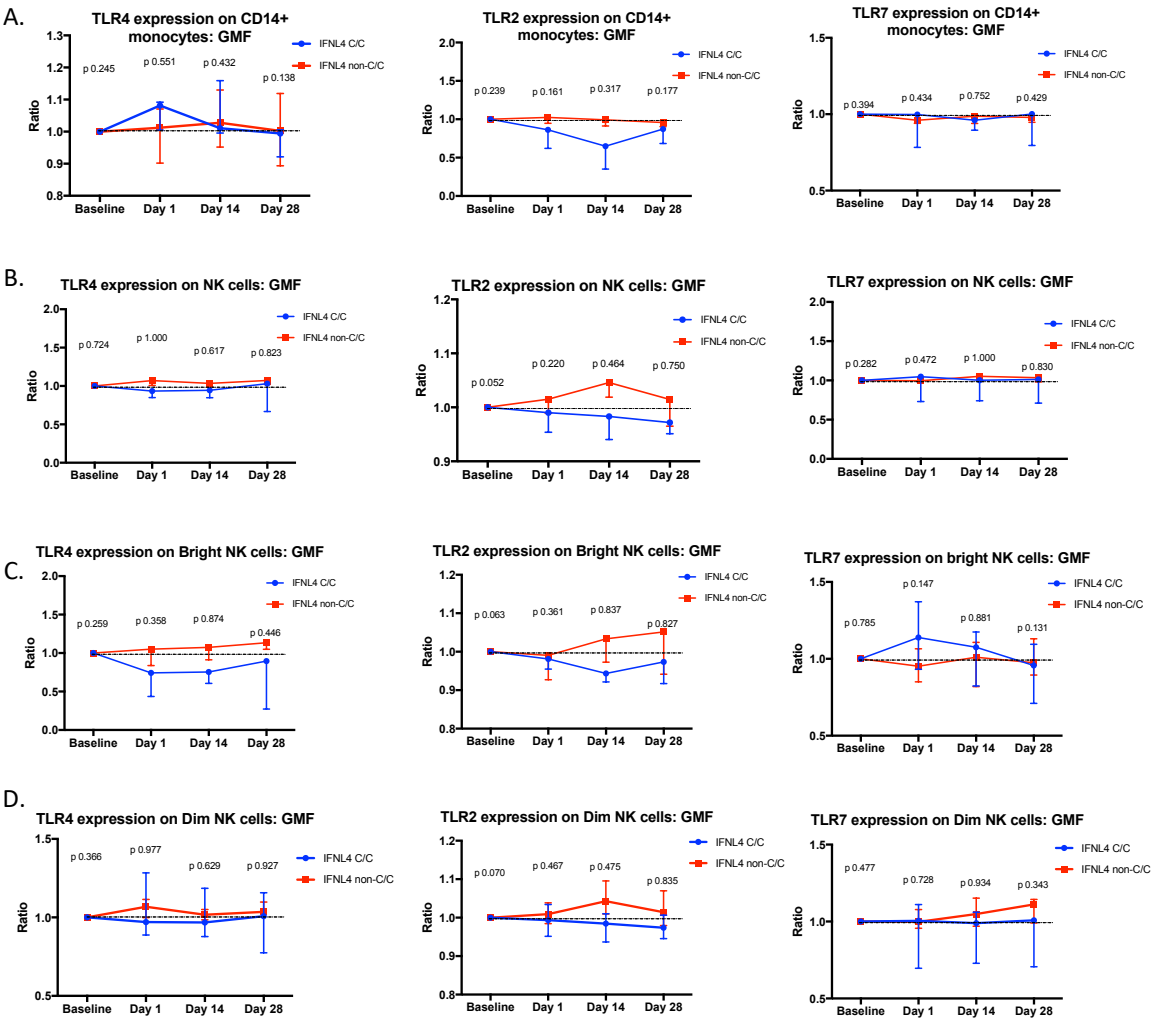


Figure 5.12 TLR 4/ 2 / 7 expression with DAA treatment, according to IFNL4 genotype.

No difference in levels of monocytic TLR expression was observed according to IFNL4 genotype. (A) CD14+ monocytes; (B) NK cells; (C) Bright NK cells; (D) Dim NK cells.

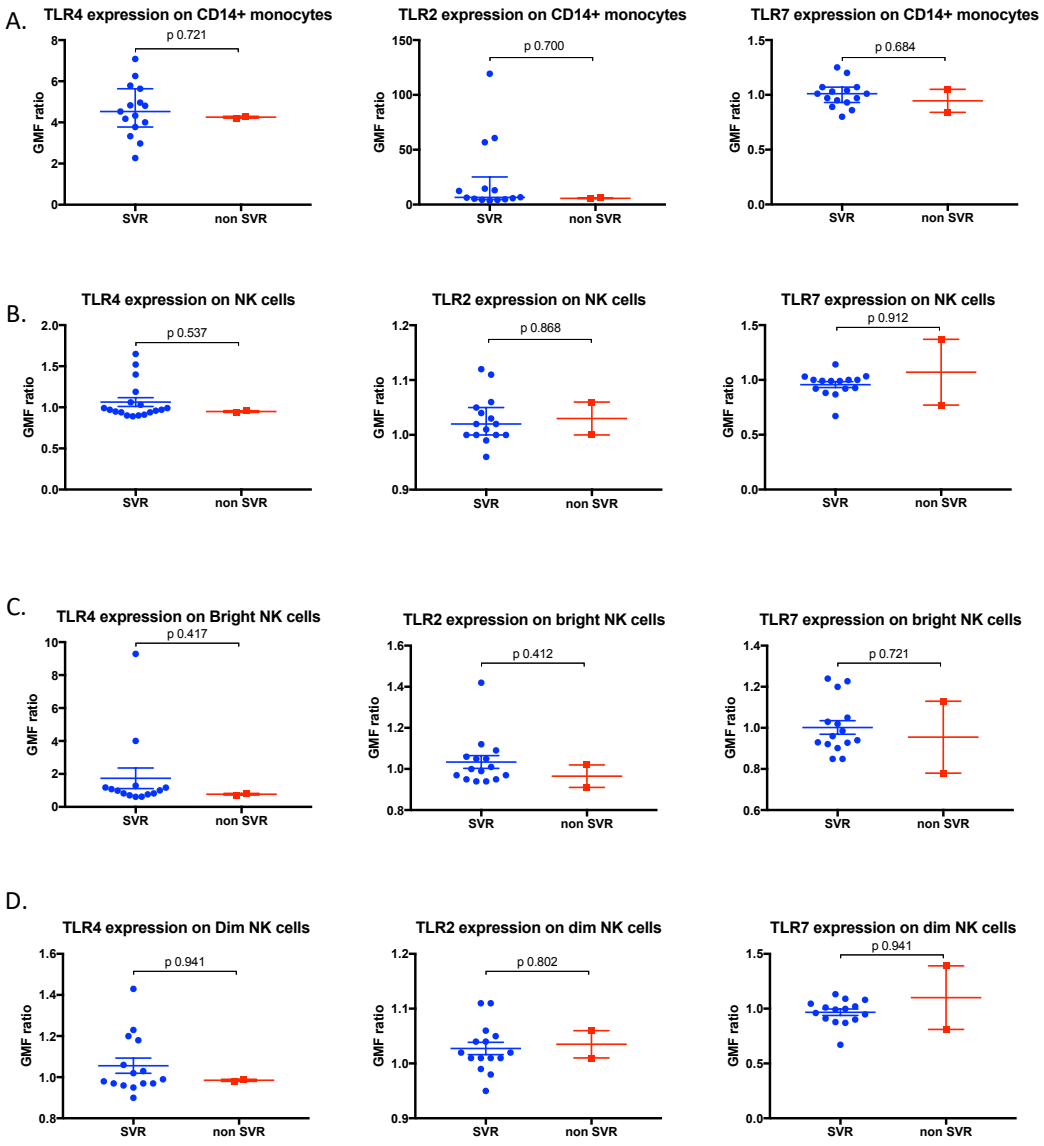


Figure 5.13 Baseline TLR 4 / 2 / 7 expression on with DAA treatment, according to SVR vs. non SVR.

No difference in levels of monocytic or NK TLR expression was observed according to treatment response. (A) CD14+ monocytes; (B) NK cells; (C) Bright NK cells; (D) Dim NK cells.

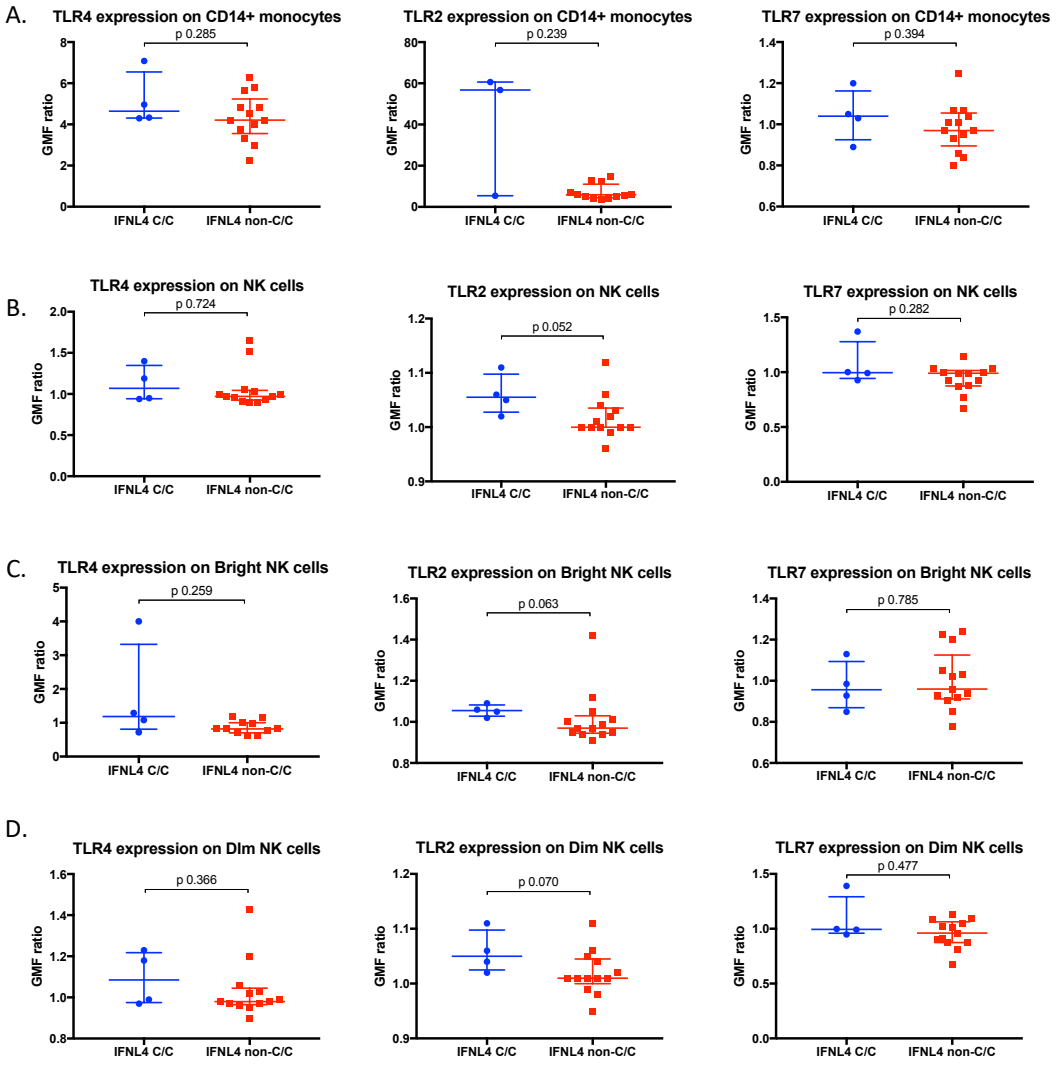


Figure 5.14 Baseline TLR 4 / 2 / 7 expression with DAA treatment, according to IFNL4 genotype.

No difference in levels of monocytic or NK TLR expression was observed according to IFNL4 genotype. (A) CD14+ monocytes; (B) NK cells; (C) Bright NK cells; (D) Dim NK cells.

6 Direct Acting Antiviral Therapy for HCV is Associated with Rapid Reduction in Markers of Innate Immunity

6.1 Introduction

The innate immune system is strongly associated with the pathogenesis of HCV infection, as well as interferon (IFN) treatment response. Interferon treatment response is strongly associated with patterns of intrahepatic interferon-stimulated gene (ISG) expression[118,204], thought to reflect host IFNL4 genotype.[120,122] Interferon treatment response is also associated with levels of circulating chemokines and cytokines, in particular IP-10[135,211], and natural killer cell function.[144,145]

An important translational question is whether innate immunity is associated with the outcome of IFN-free DAA therapy. To date there have been few studies of host-virus interactions in the setting of potent DAA therapy for HCV. In Chapter 5, we performed a detailed analysis of the association between monocyte TLR expression and signaling during antiviral therapy for genotype 1 HCV. We showed that viral suppression was associated with down-regulation of TLR2/4/7 signaling activity. There was a trend for treatment response to be associated with increased baseline TLR signaling activity in response to ligand stimulation. TLR signaling leads to induction of pro-inflammatory cytokines, chemokines and antimicrobial peptides, as well as endogenous type 1 IFNs which effect the innate immune response and are also important in initiating adaptive immune responses. Given the results observed in Chapter 5, as well as the well-described association between ISG expression, host IFNL4 genotype, chemokine levels, NK cell activity and IFN-treatment response, we were interested to test for an association between these downstream markers of innate immunity and HCV suppression in the context of IFN-free DAA therapy.

There are still limited data evaluating the reversal of impaired immune responses in the context of DAA therapy, and in particular how the immune system responds to the inhibition of replication without concomitant exogenous interferon administration.

Very recent data from a number of small studies suggests that viral clearance during DAA therapy is associated with downregulation of intrahepatic and peripheral ISG expression. DAA therapy has also been associated with reductions in circulating levels of chemokines, and NK cell activity[126,146,212-215]. It remains unclear whether ISG levels at baseline or their decline during treatment are correlated with virological breakthrough or relapse. Data suggesting a role for the endogenous intrahepatic interferon response in viral clearance during DAA therapy are conflicting. A gene signature based on pre-treatment liver and/or peripheral ISG expression predicting treatment outcome has been observed in some studies[126] but not others. Similarly, an association between IFNL4 genotype and the outcome of DAA therapy has not been consistently observed. A number of studies have observed an association between EOT liver ISG expression[125], or post-treatment peripheral ISG or serum IFN-α expression and SVR.[216] Limitations of previous data include small sample sizes, use of early DAA regimens that are no longer used because of suboptimal efficacy, failure to collect very early time-points limiting analysis of the kinetics of dynamic change in innate immune markers, and in one recent manuscript, analysis of acute rather than chronic HCV in a HIV-coinfected subjects[216] limiting extrapolation to HCV-mono-infected individuals with chronic infection.

We hypothesized that:

i) Viral replication drives the up-regulation of markers of innate immunity that are observed in the context of chronic HCV infection, and that rapid viral suppression with DAA therapy will be associated with rapid down-regulation of innate immune marker in the periphery.

ii) Expression levels of innate immune markers in the periphery will be a biomarker predicting response to treatment with potent DAA therapy in people with hard-to-cure characteristics.

6.2 Aims

The primary aim of the current study was to investigate the early effects of viral suppression on peripheral ISG expression, circulating cytokines/chemokine and NK cell phenotype during therapy with an interferon-free, DAA-only regimen, in subjects with

genotype 1 chronic HCV infection and hard-to-cure characteristics. Data were compared a parallel cohort treated with IFN-based therapy. The secondary aim was to determine whether baseline expression levels, or early changes, in innate immune markers were predictive of treatment outcome.

6.3 Methods

6.3.1 Description of Patient Cohort

Patients with Genotype 1 CHC who were referred for antiviral therapy between 2012 and 2015 were enrolled prospectively. Patients were treated with one of three regimens:

2012 – 2013

- A combination of telaprevir (750mg orally three times a day), pegylated-IFN-α-2a (180mcg subcutaneously, once a week) and weight-based ribavirin (Telaprevir / PR)

- A combination of boceprevir (800mg orally, three times a day), which was commenced after a 4-week lead in with pegylated-IFN-α-2a and weight-based ribavirin (PR)

2014

- The direct acting antiviral regimen of ombitsavir (12.5mg), ritonavir (50mg) boosted paritaprevir (75mg), dasabuvir (250mg) +/- weight-based ribavirin (PrOD +/- RBV)

The differing treatment regimens reflect the evolving treatment for CHC over the last half-decade.

Our laboratory also had data from a healthy control reference group. This data was included in some analyses.

Plasma and PBMCs were collected at baseline, day-1, day-14 and day-28 of treatment from subjects who received PrOD +/- RBV and Telaprevir / PR. Subjects who were treated with boceprevir / PR had plasma / PBMCs collected at baseline and

day-28. This allowed for a PR control for the early innate immunological studies, but not as a comparator for treatment outcome.

Notably, all subjects presented with at least one 'hard-to-cure' characteristic, to include either previous IFN non response, advanced fibrosis, high HCV viral load, IFNL4 non C/C genotype or HCV-1a infection (vs. HCV-1b).

6.3.2 Interferon Signaling Gene Expression

Please refer to Chapter 2 for a detailed description of the methods involved. We determined the gene expression profiles of a panel of biologically relevant ISGs (Table 6.1) previously reported to be important in HCV[118,120,217-219] in PBMCs at baseline, day-1, day-14 and day-28. In brief, PBMCs from each time-point were thawed and cell viability was assessed using the trypan blue exclusion test. RNA was extracted using the RNAqueous®-4PCR Total RNA Isolation Kit (Ambion, Austin, TX, USA). cDNA was then prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). PCR was performed on the Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems, CA, USA), to attain mRNA levels of Mx1, OAS1, SOCS1 and ISG15, using their respective forward and reverse primers. To assess the differences in ISG mRNA expression on-treatment, each time-point was presented as a ratio in comparison to the baseline (day-0) time-point.

ISG		Function	Chronic HCV
Mx1	Myxovirus (influenza virus) resistance 1	A strictly type I IFN-specific response marker	↑
OAS1		Degrades viral and cellular RNA. Encodes OAS2,5 proteins are expressed at low levels, then considerably induced by type I IFNs that are subsequently activated by viral RNA	↑
SOCS1	Suppressor of cytokine signaling 1	A negative regulator of the IFN signaling pathway	↑
ISG15		Free ISG15 stabilizes the USP18 protein by preventing its ubiquitination, leading to an	↑

		attenuated response to IFN-α	

Table 6.1 Panel of Interferon Signaling Genes[118,120,217-219]

6.3.3 Measurement of Plasma Cytokine /Chemokine Levels by Cytometric Bead Array Assay and Enzyme-Linked Immunosorbent Assay (ELISA)

Please refer to Chapter 2.2.5 for a detailed description of the methods involved.

In brief, frozen plasma from each time-point was thawed and enhanced cytometric bead array was performed to measure IL-1b, TNF-α, IL-8 and IL-10 levels simultaneously. ELISA was used to measure the chemokine IP-10, MCP-1, IL-6, CXCL9 and IFN-γ levels, according to the manufacturer's guidelines. The different treatment time points for each subject were performed in the same experiment.

The intensity of the fluorescence signal was measured on the FACS Canto Flow Cytometer (Becton Dickinson), and data were analysed using FCAP Array Software (BD Biosciences). To assess the differences in cytokine /chemokine response on-treatment, each time-point was presented as a ratio in comparison to the baseline (day-0) time-point.

6.3.4 Measurement of mRNA Levels of Cytokine / Chemokine in PBMCs by RNA Extraction and Real-Time PCR

Please refer to Chapter 2.2.9 for a detailed description of the methods involved. In brief, PBMCs from each time-point were thawed and cell viability was assessed using the trypan blue exclusion test. RNA was extracted using the RNAqueous®-4PCR Total RNA Isolation Kit (Ambion, Austin, TX, USA). cDNA was then prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). PCR was performed on the Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems, CA, USA), to attain mRNA levels of the measured cytokines /chemokines, using the respective forward and reverse primers. To assess the differences in chemokine /cytokine mRNA expression on-treatment, each time-point was presented as a ratio in comparison to the baseline (day-0) time-point.

6.3.5 NK Phenotype

There is data to suggest that a dysregulated peripheral NK cell phenotype / function in CHC also contributes to ineffectual viral clearance.[143] Certain NK phenotypic features have been associated with IFN response[144], and more recently to first-generation DAAs.[146,215,220]

We wished to investigate for NK phenotypic changes in response to second generation DAAs, and if associated with treatment outcome. A combination of biologically relevant activating and inhibitory NK receptor were measured on cryopreserved PBMCs at each time point. These are listed in Table 6.2.

NK receptor		Receptor Function
NKp46 / NKp30	+	Natural cytotoxicity receptors
HLA-DR	+	Activation marker
NKG2D	+	Activating receptor
TRAIL	+	Activation marker
CD122	+	IL-2 receptor beta chain
TIM3	+/-	Inhibitory receptor
NKG2A	-	Inhibitory receptor

Table 6.2 Panel of NK Receptors

Please refer to Chapter 2.2.3 for a detailed description of the methods involved. In brief, PBMCs from each time-point were thawed and cell viability was assessed using the trypan blue exclusion test. Surface staining was performed with CD markers and panel of NK receptors. These samples were analysed using flow cytometry and FlowJo (v10.1). The level of expression of the NK receptors are presented as (a) a percentage of the total cell population; and (b) geometric mean fluorescence. To assess the

differences in NK receptor expression on-treatment, each time-point was presented as a ratio in comparison to the baseline (day-0) time-point.

6.3.6 NK Cell Function

The activity of NK cell function was tested by evaluation of IFN-γ / TNF-α response to stimulation with IL-12 and IL-15 ligands. Detailed methods were presented in Chapter 2.2.8. In brief, a total of 1×10^6 thawed PBMCs from subjects were cultured at 37°C for 14 hours in Roswell Park Memorial Institute (RPMI) medium with 1% L-glutamine, 1% penicillin, and 5% fetal calf serum (FCS) and IL-12 or IL-15 ligands. 1uL Brefeldin was then added and incubated at 37°C for 4 hours. PBMCs were stimulated with RPMI 5% FCS (unstimulated control), 10ug/mL IL-12, or 10ug/mL IL-15. IL-12 / IL-15 ligands were supplied by InVivoGen. Surface staining was then performed with CD markers. Cells were fixed and permeabilized with the Cytofix/Cytoperm Kit and stained with anti-IFN-γ-PE and anti–tumour necrosis factor (TNF) α –APC (InVivoGen). A total of 10,000 CD56+ NK cells of each sample were analysed; dead cells were gated out, based on their light scatter properties on the FACS Canto Flow Cytometer (Becton Dickinson). Data were analysed using FlowJo software (Tree Star, Ashland, OR). Frequency of IFN-γ-positive NK cells that were incubated in complete medium without cytokines was subtracted from the frequency of IFN-γ-positive NK cells stimulated with cytokines.

6.3.7 Statistical Analysis

Participant characteristics and laboratory measurements are presented as median values and interquartile ranges. The Fisher exact test was used to test for significant group differences in dichotomous variables, and the independent samples t-test was used for continuous variables. The Mann-Whitney-U test was used to determine if differences existed between baseline ISG expression, in comparison to ISG expression at each time-point. This was performed using Prism (v7). Graphs were presented as Tukey box plots, with the middle line representing the median, and the box extending from the 25th to 75th percentile.

6.4 Results

6.4.1 Description of Patient Cohort

16 subjects with HCV-1 infection were prospectively enrolled and treated with a combination of IFN-free antiviral therapy, comprised of ombitsavir, ritonavir boosted paritaprevir, dasabuvir +/- ribavirin (PRoD +/- RBV). The baseline characteristics and demographics of this group are detailed in Table 6.3. In brief, 8/16 (50%) were male, 15/16 (94%) were Caucasian and 14/16 (86%) had advanced liver fibrosis (METAVIR F3-4). 7/16 (44%) were prior non-responders to treatment with peginterferon-α plus ribavirin. 4/16 (25%) carried the CC genotype (rs12979860) which has been associated with good response to peginterferon-based treatment for HCV.

As a parallel comparator, 17 subjects with HCV-1 infection were prospectively enrolled and treated with IFN-based therapy. 12 subjects were treated with telaprevir, pegylated-IFN-α-2a and weight-based ribavirin (Telaprevir / PR), and 5 subjects were treated with pegylated-IFN-α-2a and weight-based ribavirin (PR), with the addition of boceprevir at week 5. Their baseline characteristics and demographics are detailed in Table 6.3. In brief, of the subjects who received Telaprevir/PR, 9/12 (75%) were male or Caucasian, and 7/12 (58%) had advanced liver fibrosis (METAVIR F3-4). 7/16 (44%) were prior non-responders to treatment with peginterferon-α plus ribavirin. 4/16 (25%) carried the CC genotype (rs12979860) which has been associated with good response to peginterferon-based treatment for HCV. Of the subjects who received PR, 1/5 (20%) were male, 5/5 (100%) were Caucasian and 3/5 (60%) had advanced fibrosis. 3/5 (60%) were prior non-responders to treatment with peginterferon-α plus ribavirin and 0/5 carried the favourable IFNL4 CC genotype.

Participant characteristics	IFN-free therapy	IFN-based therapy	
	PRoD +/- RBV	Telaprevir/PR	PR
n (%)	16 (48%)	12 (36%)	5 (15%)
Age, years (median, IQR)	55 (51 – 61)	51 (57 – 62)	64 (57 – 65)
Male (n,%)	8 (50%)	9 (75%)	1 (20%)
Caucasian (n,%)	15 (94%)	9 (75%)	5 (100%)
Treatment naïve (n,%)	9 (56%)	7 (58%)	2 (40%)
Baseline ALT, U/L (median, IQR)	128 (149 – 154)	90 (54 – 135)	56 (48 – 86)
HCV genotype 1a/1b	11 (69%) / 5 (31%)	8 (67%) / 4 (33%)	1 (20%) / 4 (80%)
Baseline HCV RNA level, IU/mL (median, IQR)	1 910 000 (55400 – 2 655 000)	2 056 000 (471500 – 3777500)	4 930 000 (375000 – 6250000)
Advanced fibrosis (≥F3) (n,%)	14 (86%)	7 (58%)	3 (60%)
Favourable IFNL4 Gt (n,%) (CC, rs 12979860) Treatment naïve (n,%)	4 (25%) 2 (50%)	3 (25%) 2 (67%)	0 (0%) 0 (0%)
Prior IFN experience	7 (44%)	5 (42%)	3 (60%)
Ribavirin (n,%)	11 (69%)	12 (100%)	5 (100%)
SVR (n,%)	14 (86%)	6 (55%)	1 (20 %)

Table 6.3 Participant Characteristics and Demographics at Baseline

Subjects who were treated with PRoD +/- RBV, experienced a rapid decrease in serum HCV RNA levels during DAA therapy. Significant reductions in serum HCV RNA level were observed as early as day-1 of treatment (median reduction at day-1 = 1,908,623 IU/mL) (Figure 6.1). 10/16 (63%) subjects had an undetectable serum HCV RNA level at day-28. Serum ALT levels also fell rapidly with IFN-free therapy and median ALT fell significantly by day-7 of treatment. 14/16 (88%) subjects had ALT< ULN at day-28. 15/16 (94%) subjects achieved an end-of-treatment response. One subject was lost to follow up. 14/16 (86%) subjects went on to achieve a sustained virological response at week 12 post-treatment (SVR12). 2/16 (19%) achieved an EOT response but then relapsed post-treatment. Both had HCV-1a infection, with one subject treatment experienced, cirrhotic and with the poor response IFNL4 genotype, and the other was treatment naïve with F3 fibrosis, carrying the good response IFNL4 genotype.

Those who received T/PR had a significant reduction in HCV RNA levels as early as day-1 (median reduction at day-1 = 2,046,830 IU/mL, IQR 295,696 – 4,694,750) (Figure 6.1). 8/12 (67%) subjects had an undetectable serum HCV RNA level at day-28. Treatment with PR also saw a significant reduction in serum HCV RNA at week 4 (median reduction at day-28 = 4,834,800 IU/mL), but all had detectable virus. Serum ALT levels dropped by day-7 with T/PR with a trend to lower levels with PR, highlighting the differing potencies of the antiviral regimens. There was no difference in the mean levels of HCV RNA at baseline or day-28, for each of the treatment regimens.

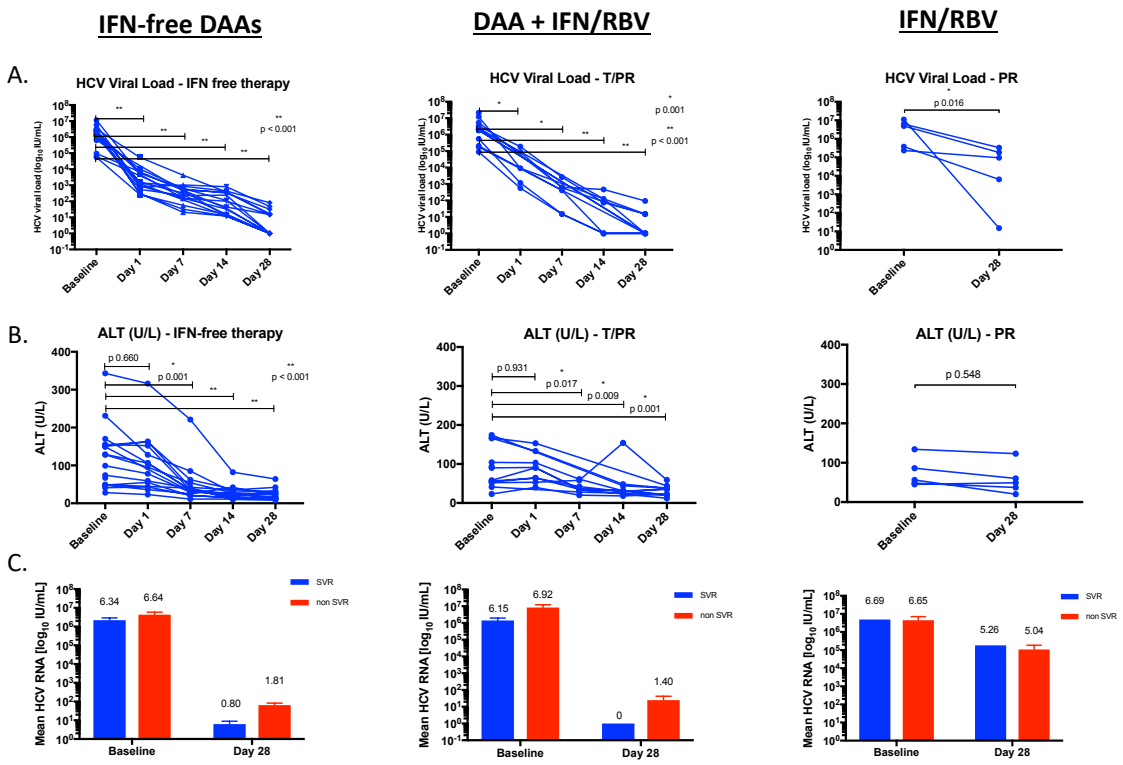


Figure 6.1 Viral kinetics and biochemical (ALT) response to antiviral therapy.
On-treatment viral load (A) and ALT (B) response to antiviral therapy. (C) Average viral load at baseline and day-28 according to treatment response.

6.4.2 Analysis of ISG mRNA Expression in PBMCs During Anti-Viral Therapy

The well-recognised link between intra-hepatic/peripheral ISG expression and virological response to exogenous IFN-α, as not yet been fully defined with the newer IFN-free DAAs. We first determined the gene expression profiles of a panel of biologically relevant ISGs (Mx1, OAS1, ISG15 and SOCS1, previously reported to be important in HCV[118,120,217-219] in PBMCs at baseline, day-1, day-14 and day-28. During treatment with IFN-free DAAs, the expression of all ISGs was significantly reduced over time with viral suppression. Reduced expression of SOCS1 was detectable at day-1, and non-significant trends were also noted for reduced levels of OAS and MX1 (Figure 6.2). Statistically significant reduction of all ISGs was detectable by day-14, plateauing there (Figure 6.2). In contrast, patients treated with telaprevir plus PR therapy experiencing rapid and marked induction of Mx1, OAS1 and ISG15 mRNA that peaked at day-1, before plateauing (Figure 6.2). SOCS1 mRNA expression was significantly down-

regulated at day-1, before returning to baseline. This was similar to the expression pattern observed at day-28 in patients treated with PR alone (Figure 6.2).

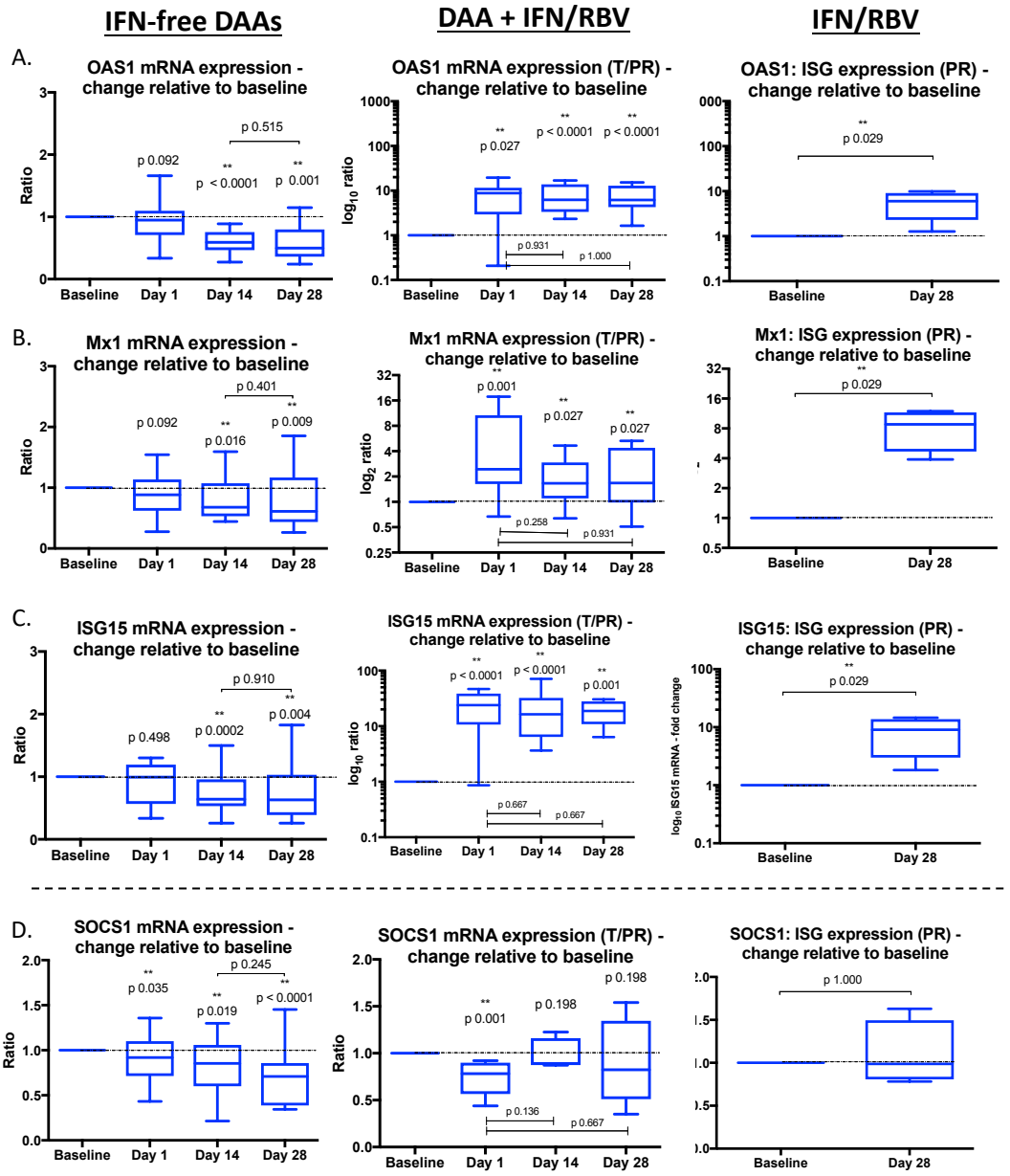


Figure 6.2 On-treatment viral suppression with DAA treatment is associated with reduced PBMC ISG expression.
(A) OAS; (B) Mx1; (C) ISG15; (D) SOCS1.

6.4.3 Pre-treatment ISG mRNA Expression in PBMCs was not Associated with Viral Clearance during IFN-Free DAA Therapy

We then tested for an association between baseline PBMC ISG mRNA expression and response to DAA therapy and T/PR + PR therapy, combined (Figure 6.3). In our

small cohorts, no significant differences were seen. Similarly, pre-treatment expression was not associated with IFNL4 genotype (Figure 6.4).

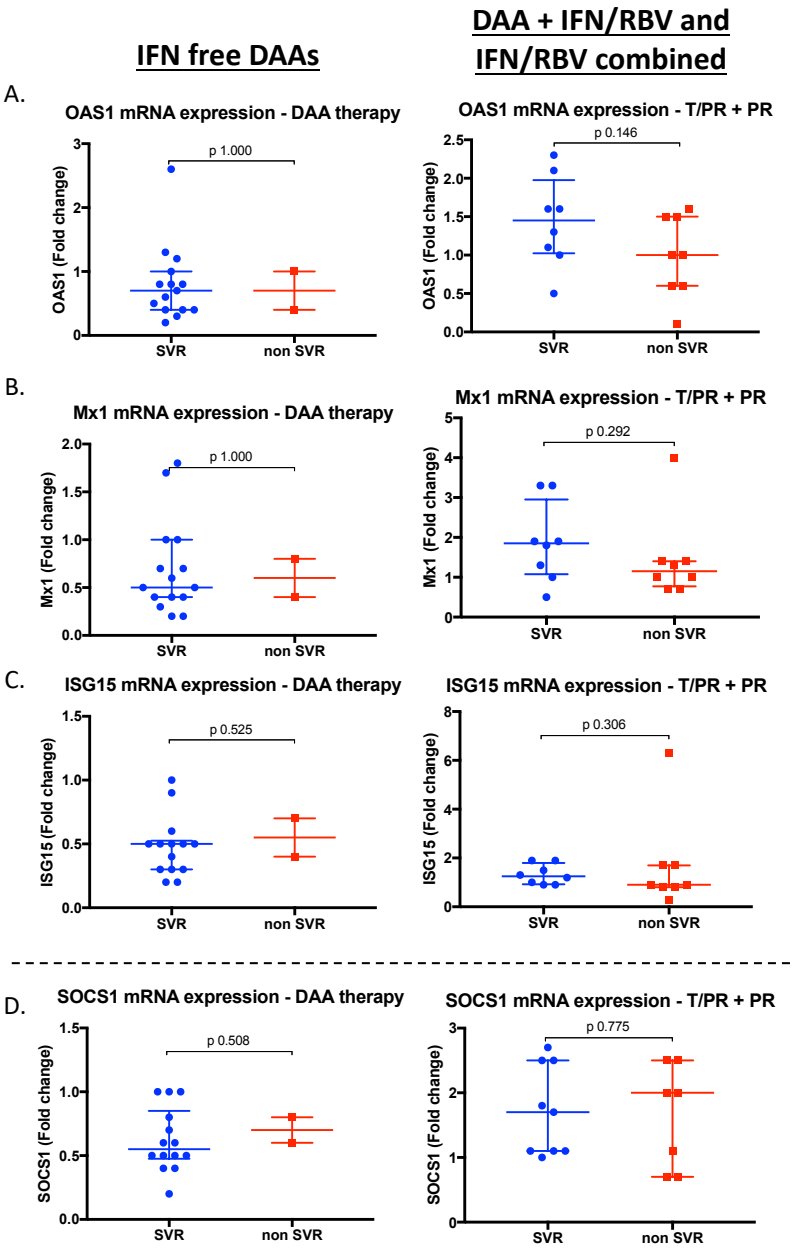


Figure 6.3 Pre-treatment ISG expression according to treatment response. No difference in pre-treatment ISG expression was observed according to treatment response to anti-viral therapy. (A) OAS; (B) Mx1; (C) ISG15; (D) SOCS1.

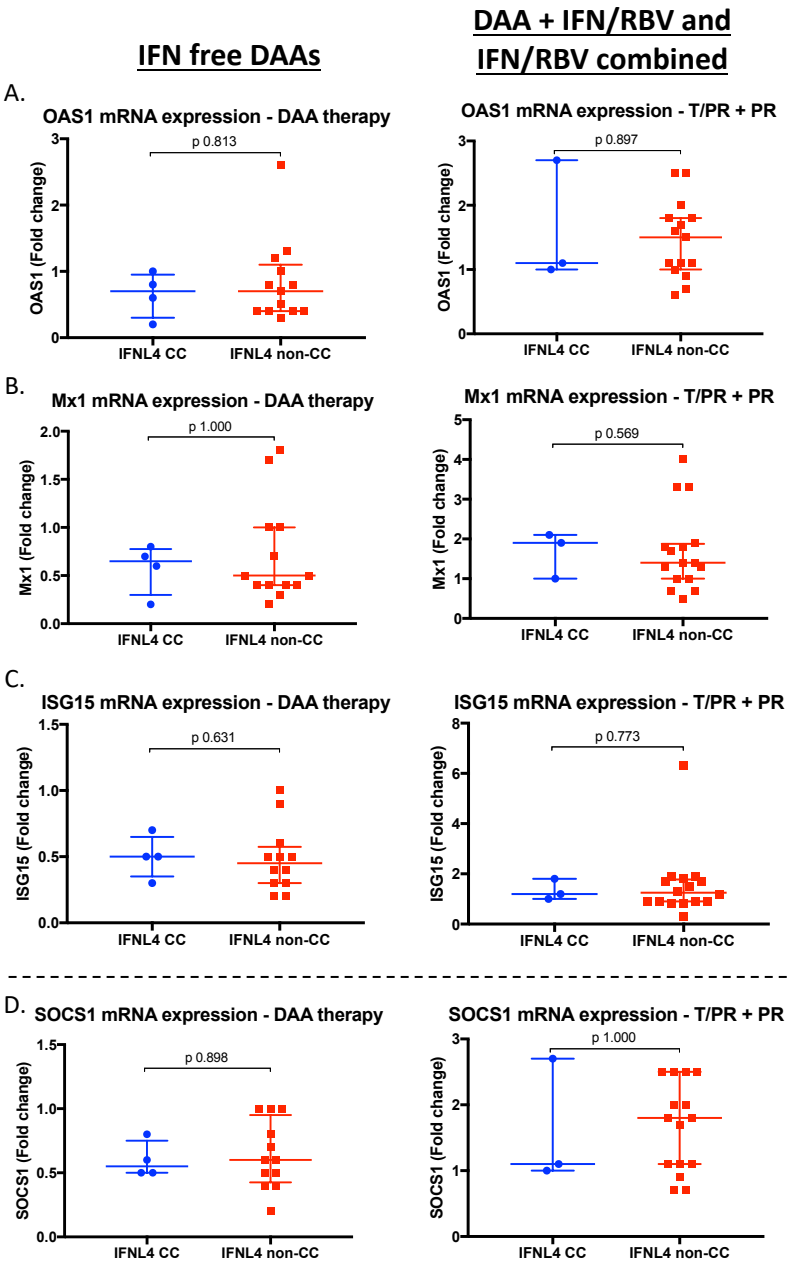


Figure 6.4 Pre-treatment ISG expression according to IFNL4 genotype.
No difference in pre-treatment ISG expression was observed according to IFNL4 genotype. (A) OAS; (B) Mx1; (C) ISG15; (D) SOCS1.

6.4.4 Analysis of On-Treatment Peripheral ISG Expression According to Treatment Outcome (SVR12 vs non-SVR12) and IFNL4 Genotype

No effect was seen when on-treatment changes in ISG expression were analysed according to treatment outcome (Figure 6.5). Given previous interest in IFNL4, we also analysed the data according to IFNL4 genotype, but did not see an IFNL4 effect (Figure

6.6). However, only 4 of 16 subjects received IFN-free DAA therapy, and 3 of 12 who received IFN-based therapy carried the favourable IFNL4 genotype.

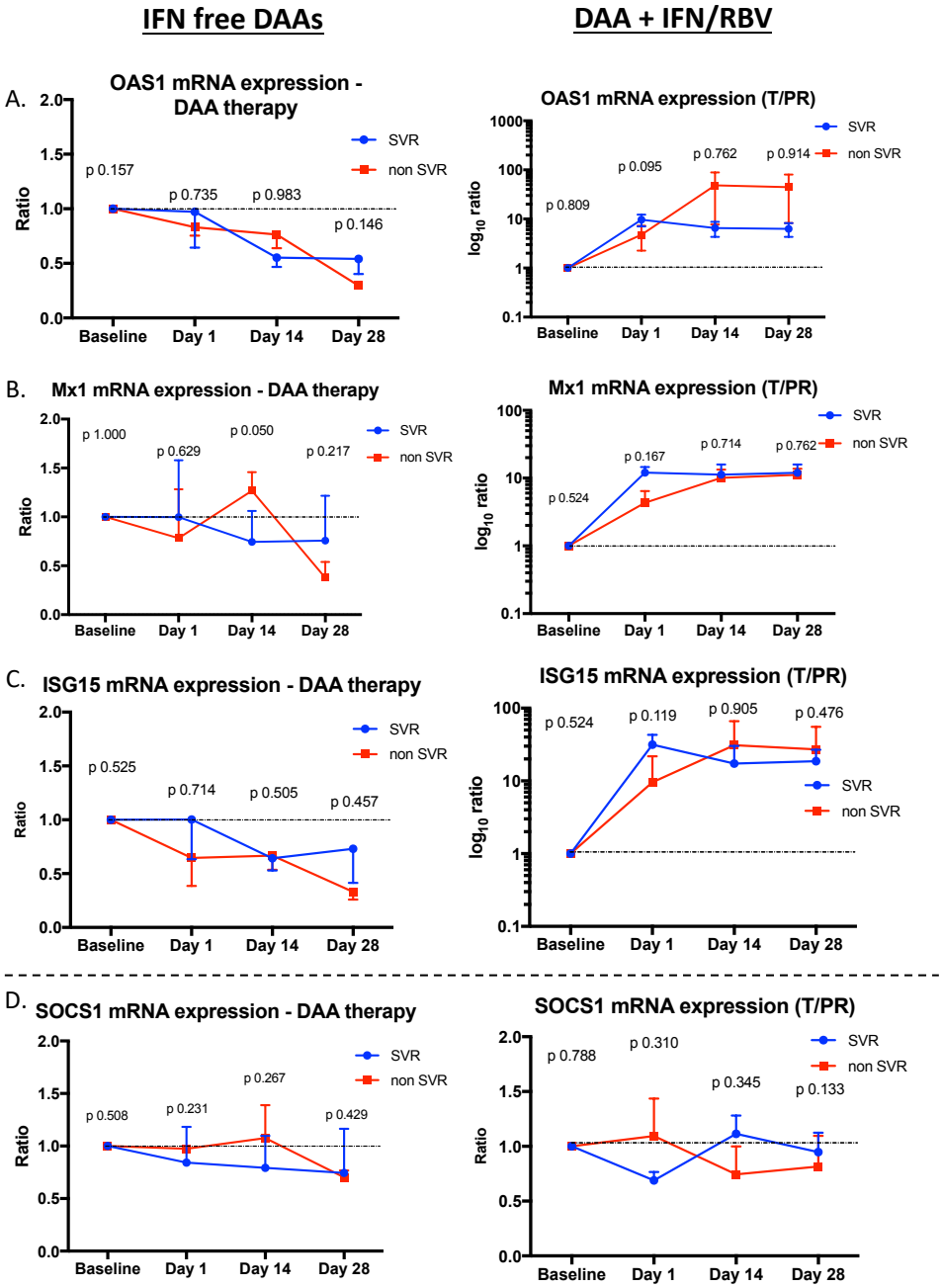


Figure 6.5 On-treatment changes in ISG expression are not associated with treatment outcome.
(A) OAS; (B) Mx1; (C) ISG15; (D) SOCS1.

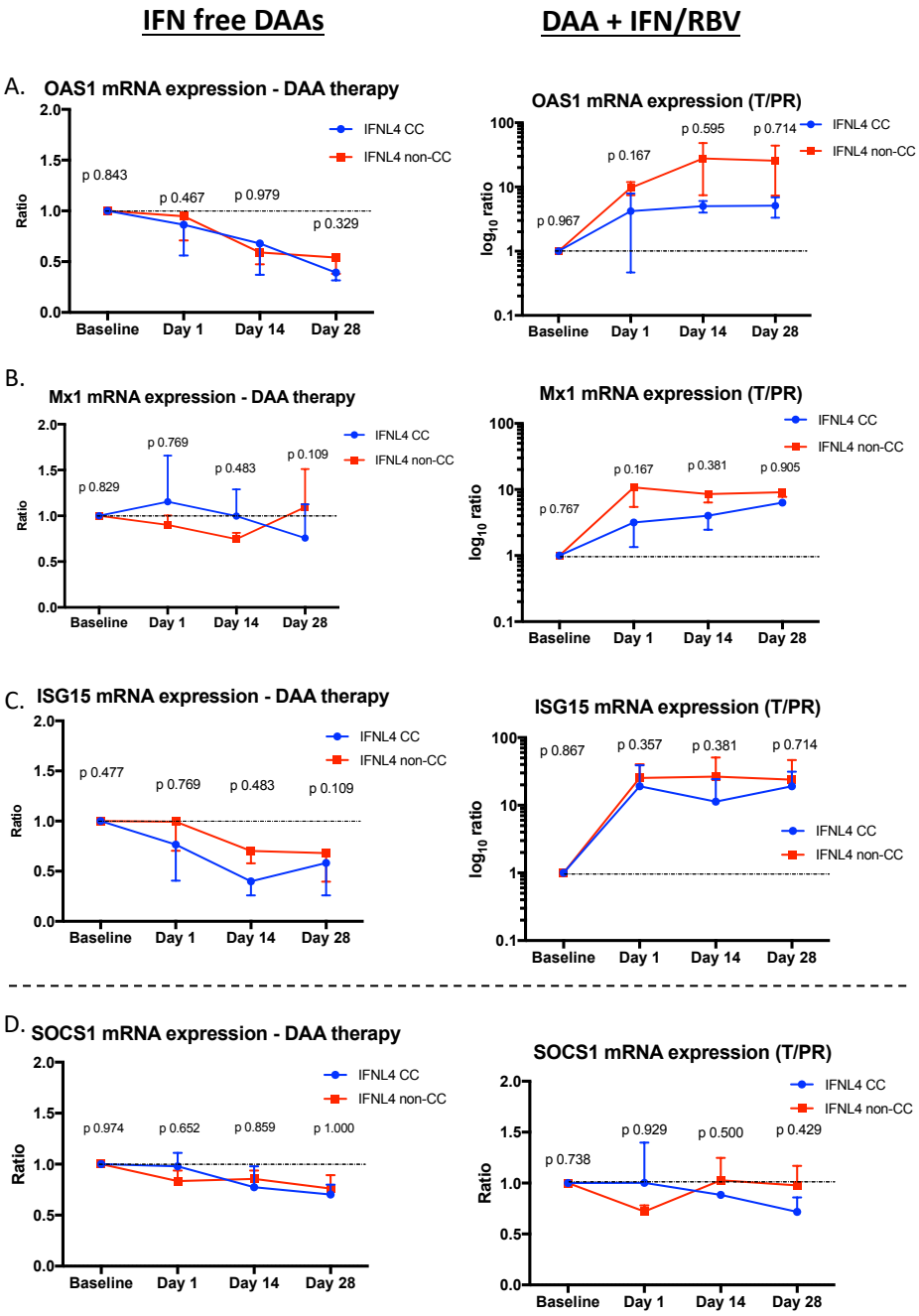


Figure 6.6 On-treatment changes in ISG expression are not associated with IFNL4 genotype.
(A) OAS; (B) Mx1; (C) ISG15; (D) SOCS1.

6.4.5 Levels of Plasma Chemokine / Cytokine Expression Fall On-Treatment with IFN-Free DAA Therapy

We determined the expression profiles for a panel of biologically relevant chemokines(IP-10, IL-8, MCP-1 and CXCL9), previously reported to be important in HCV[127,128,133-135] by testing both serum protein levels and PBMC mRNA expression.

Again, direct comparison was made between patients treated with IFN-free DAA therapy and IFN-based therapy. Levels of plasma IP-10, IL-8, MCP-1 and CXCL9 decreased rapidly within the first month of IFN-free DAA therapy. Reductions in levels of IP-10 were detectable at day-1 post treatment; reductions of all chemokines / cytokines was present at day-14 and 28 (Figure 6.7). Analysis of mRNA levels in PBMCs demonstrated a similar pattern, with rapid and sustained down-regulation of IP-10, MCP-1, and CXCL9 (Figure 6.8). No change was seen in IL-8 mRNA levels. Reduction in plasma levels and PBMC mRNA expression of the immuno-modulatory cytokine IL-10 were detectable by day-14 and day-28, respectively. We also tested for the pro-inflammatory cytokines IL-6, TNF-α and IL-1b; all were undetectable in plasma, despite the use of an enhanced sensitivity cytometric bead array. We proceeded to measure IL-6 mRNA levels in PBMCs, as a representative of the pro-inflammatory cytokines, known to act in concert with other pro-inflammatory cytokines, such as TNF-α. IL-6 mRNA levels in PBMCs significantly decreased in the first month of IFN free therapy, as early as day-1.

In contrast, patients treated with telaprevir plus PR therapy experienced a rapid and marked induction in plasma levels of chemokines IP-10, MCP-1, CXCL9 and immunomodulatory cytokine IL-10. A similar pattern was observed with PBMC mRNA expression of IP-10, MCP-1, IL-10 and IL-6. No change was seen in levels of plasma IL-8 or mRNA expression. There was no consistent pattern observed in the levels of chemokines / cytokines in both plasma and mRNA during the remaining month of treatment, likely reflecting the complexity of the innate immune response to exogenous IFN-α. mRNA levels did not always reflect the patterns observed in plasma. MCP-1 was the only chemokine to remain persistently elevated in both plasma and mRNA. After induction, plasma IP-10 levels dropped below baseline after day-14, but mRNA expression remained high. Plasma IL-10 levels also dropped to baseline by day-14, however IL-10 mRNA expression remained high. IL-6 mRNA expression remained elevated after induction. Plasma CXCL9 levels returned to baseline by day-14, whereas no day-1 change in CXCL9 mRNA expression was seen, with a reduction from day-14. Expression patterns observed at day-28 in patients treated with PR alone were similar.

On-treatment chemokine / cytokine expression was analysed according to treatment response and IFNL4 genotype, with no effect seen, although our numbers were small (IFN free, C/C n=4, IFN-based C/C n=3) limiting this analysis (see Chapter Appendices 6.8 - Figure 6.16; Figure 6.17).

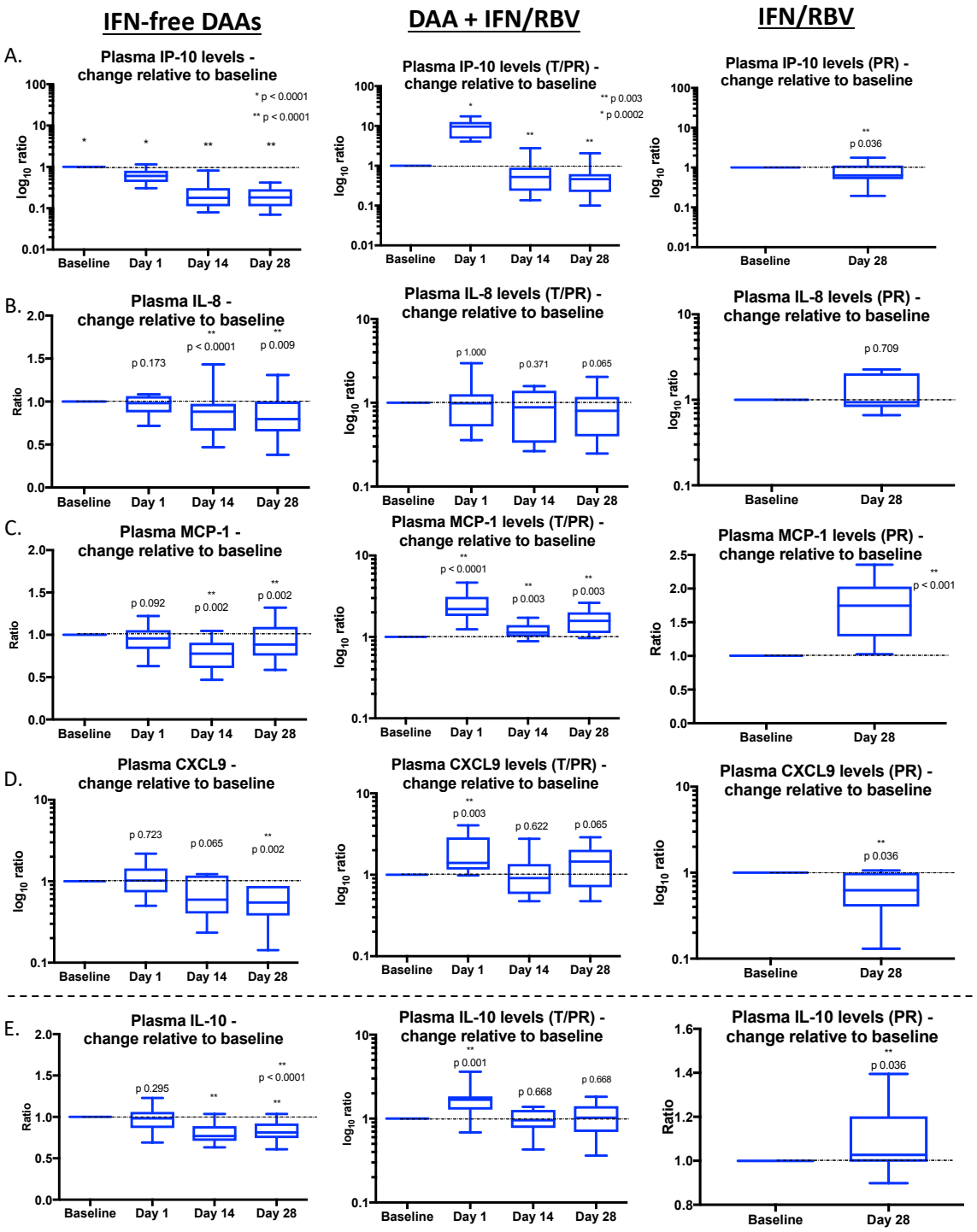


Figure 6.7 On-treatment viral suppression during IFN-free DAA therapy is associated with down-regulation of circulating plasma chemokine / cytokine levels – changes relative to baseline.

(A) IP-10; (B) IL-8; (C) MCP-1; (D) CXCL9; (E) IL-10.

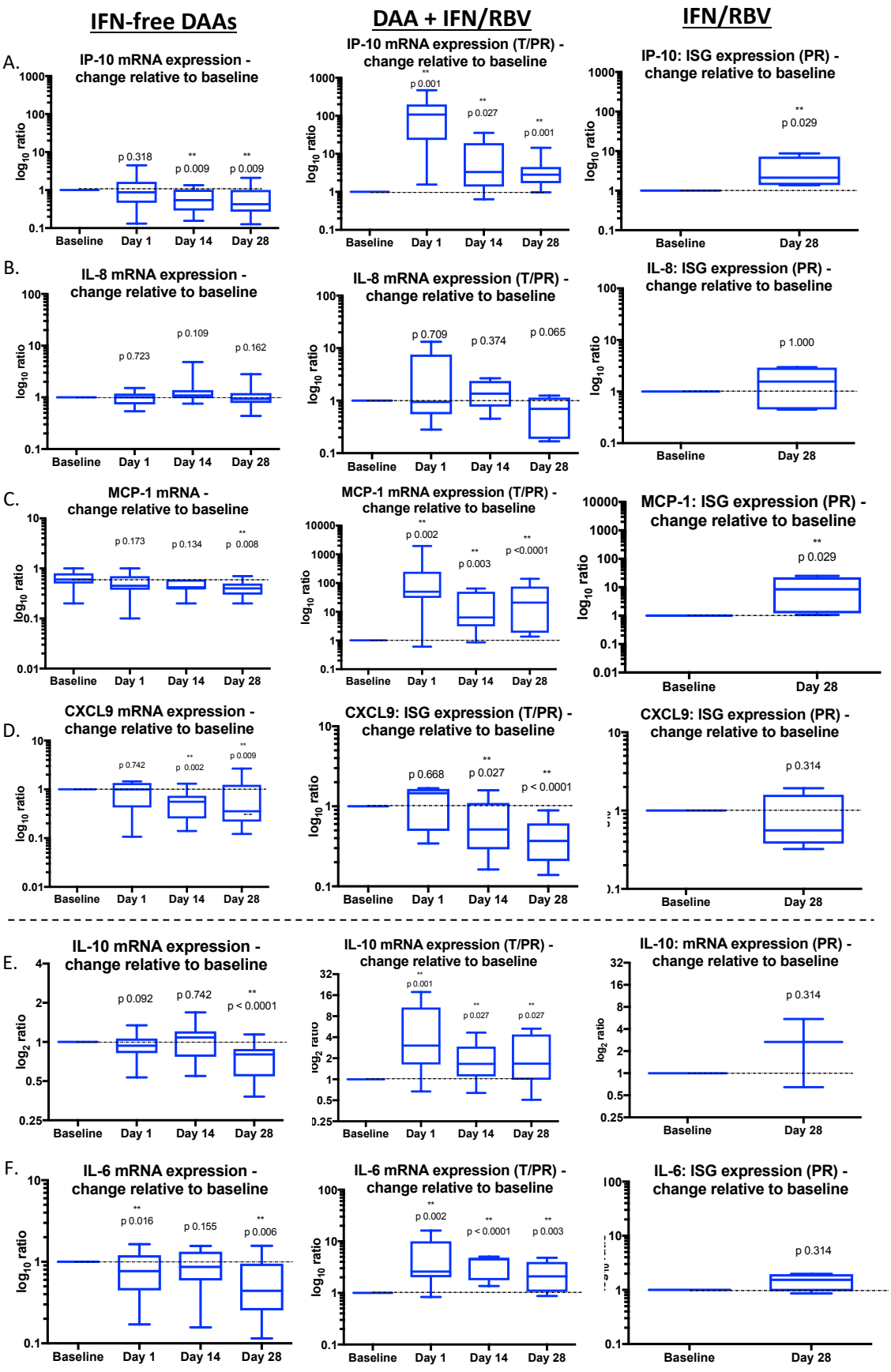


Figure 6.8 On-treatment viral suppression during DAA therapy is associated with down-regulation of chemokine/cytokine mRNA expression in PBMCs - changes relative to baseline.

(A) IP-10; (B) IL-8; (C) MCP-1; (D) CXCL9; (E) IL-10; (F) IL-6.

6.4.6 Baseline Plasma Cytokine / Chemokine Levels did not Predict SVR12 with Antiviral Therapy for Genotype 1 Chronic HCV infection

Pre-treatment levels of cytokines / chemokines in plasma were then analysed according to treatment response. No differences were seen within each treatment regimen (Figure 6.9), or when the entire CHC cohort was combined (Figure 6.9). A trend to lower pre-treatment IP-10 and IL-10 levels was seen in those who attained SVR12. Pre-treatment levels of IL-8, MCP-1 or CXCL9 did not differ according to treatment response. The type-1 inflammatory cytokines IL-6, IL-1b, TNF-α, and IFN-γ were not detectable in plasma.

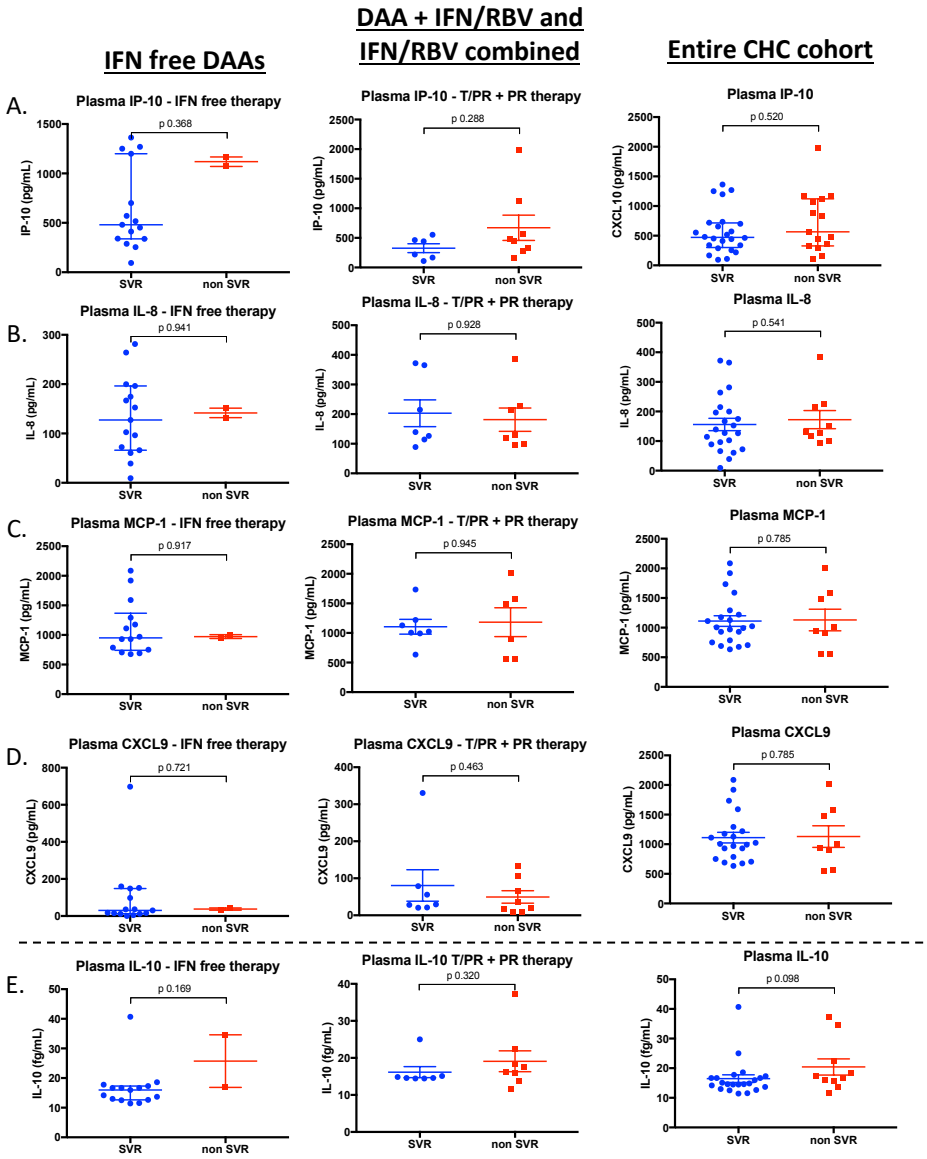


Figure 6.9 Pre-treatment levels of chemokines / cytokines in plasma according to treatment response.

A trend to lower pre-treatment IP-10 and IL-10 levels was seen in those who attained SVR. (A) IP-10; (B) IL-8; (C) MCP-1; (D) CXCL9; (E) IL-10.

IL-6 mRNA expression was detectable in PBMCs, with lower IL-6 mRNA expression associated with SVR12 with IFN-based therapy (Figure 6.10). When the entire cohort was combined, lower pre-treatment IL-6 and higher pre-treatment CXCL9 mRNA expression was associated with SVR12. No differences were seen when measuring mRNA expression of IP-10, IL-8, MCP-1 or IL-10 in PBMCs.

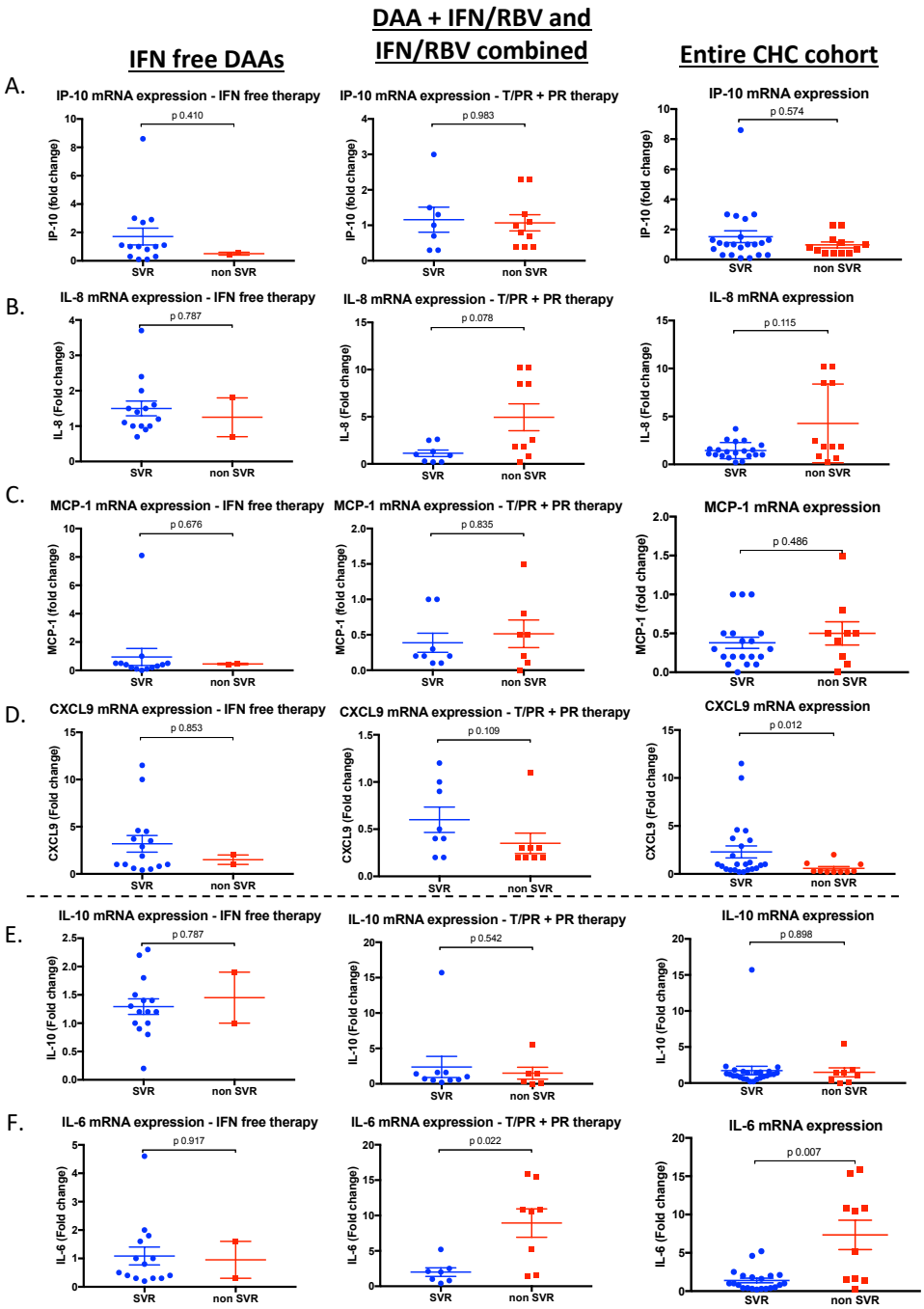


Figure 6.10 Pre-treatment mRNA levels of chemokines / cytokines according to treatment response.

Lower pre-treatment IL-6 mRNA expression was associated with SVR with IFN-based therapy. Lower pre-treatment IL-6 and higher pre-treatment CXCL9 mRNA expression was associated with SVR when analysed in the entire CHC cohort. (A) IP-10; (B) IL-8; (C) MCP-1; (D) CXCL9; (E) IL-10; (F) IL-6.

A trend to lower pre-treatment IP-10 plasma levels and IP-10 mRNA expression was seen with the favourable IFNL4 CC genotype (see Chapter Appendices 6.8 - Figure 6.18, Figure 6.19). Higher pre-treatment CXCL9 levels was associated with the IFNL4 CC genotype in those who received IFN-based therapy. A trend to higher pre-treatment MCP-1 mRNA expression was seen when the entire cohort was combined. No other differences in plasma levels of cytokines / chemokines or mRNA in PBMCS, were seen within each treatment group or as a whole cohort, when analysed according to IFNL4 genotype.

6.4.7 IFN-free DAA Treatment was Associated with a Rapid Reduction in Expression of TRAIL and CD122 on NK Cells During Treatment

In order to more comprehensively examine the role of innate immunity in DAA-mediated clearance, we analysed peripheral NK cells, which represent the primary IFN-sensitive innate immune effector cell type, as biomarkers of peripheral IFN responses. Finally, we also wished to assess if IFN-free DAA therapy was associated with modulation of NK cell activation. We tested for NK cell expression levels of the activation markers TRAIL and CD-122 using flow cytometry. In the first month of treatment, we saw that the viral clearance induced by the DAAs led to a lowering of the activation state of peripheral blood NK cells, as reflected by reduced levels of TRAIL and CD122. Differences were seen with IFN-based therapy. Treatment with T/PR and PR saw an increase in NK cell expression of TRAIL, most pronounced on the dim NK cell subset, with no change seen on the bright NK cell subset. CD122 expression momentarily dropped at day-14 with T/PR on NK cells and its dim NK cell subset. A reduction also occurred within the dim NK subset with PR. No differences were seen according to treatment response (Figure 6.12) or IFNL4 genotype (data not shown).

There was no change in the NK expression of activation markers NKp30, NKp46, HLA-DR or NKG2D in response to IFN-free therapy (see Chapter Appendices 6.8 - Figure 6.20). Furthermore, only a momentary drop in expression of inhibitory receptors, NKG2A and TIM3 was seen at day-14. Differences were seen with IFN-based therapy, in which a clear IFN-effect was mostly notable on day-1 of treatment. Data on the inhibitory receptor CD85j was incomplete due to issues with the reagent at the time of the experiment.

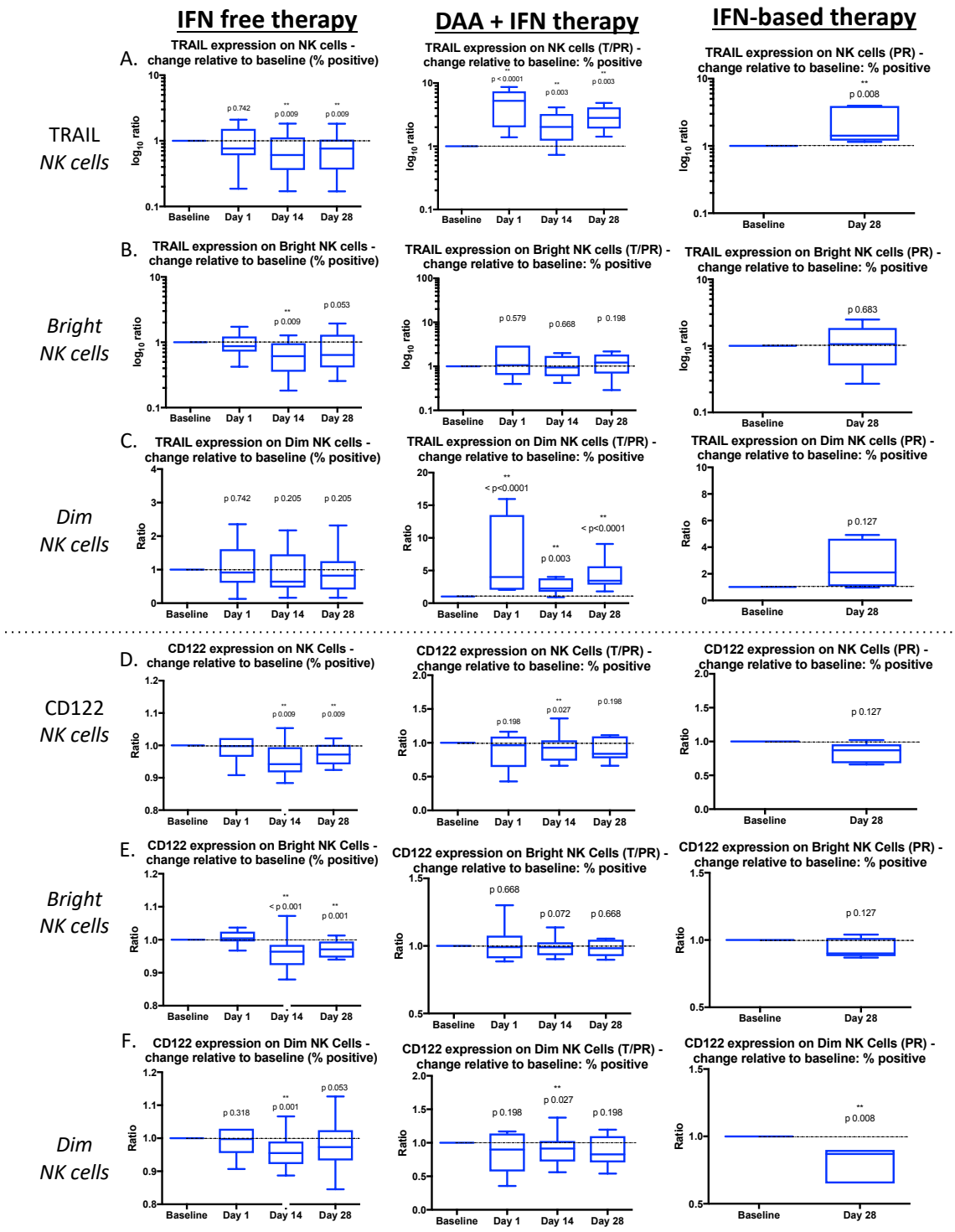


Figure 6.11 Reduced NK TRAIL and CD122 expression with DAA treatment. An increase in NK TRAIL expression was observed with IFN-based therapy. A drop in NK CD122 expression was observed at day 14. TRAIL expression on (A) NK cells; (B) Bright NK cells; and (C) Dim NK cells. CD122 expression on (C) NK cells; (D) Bright NK cells; and (E) Dim NK cells.

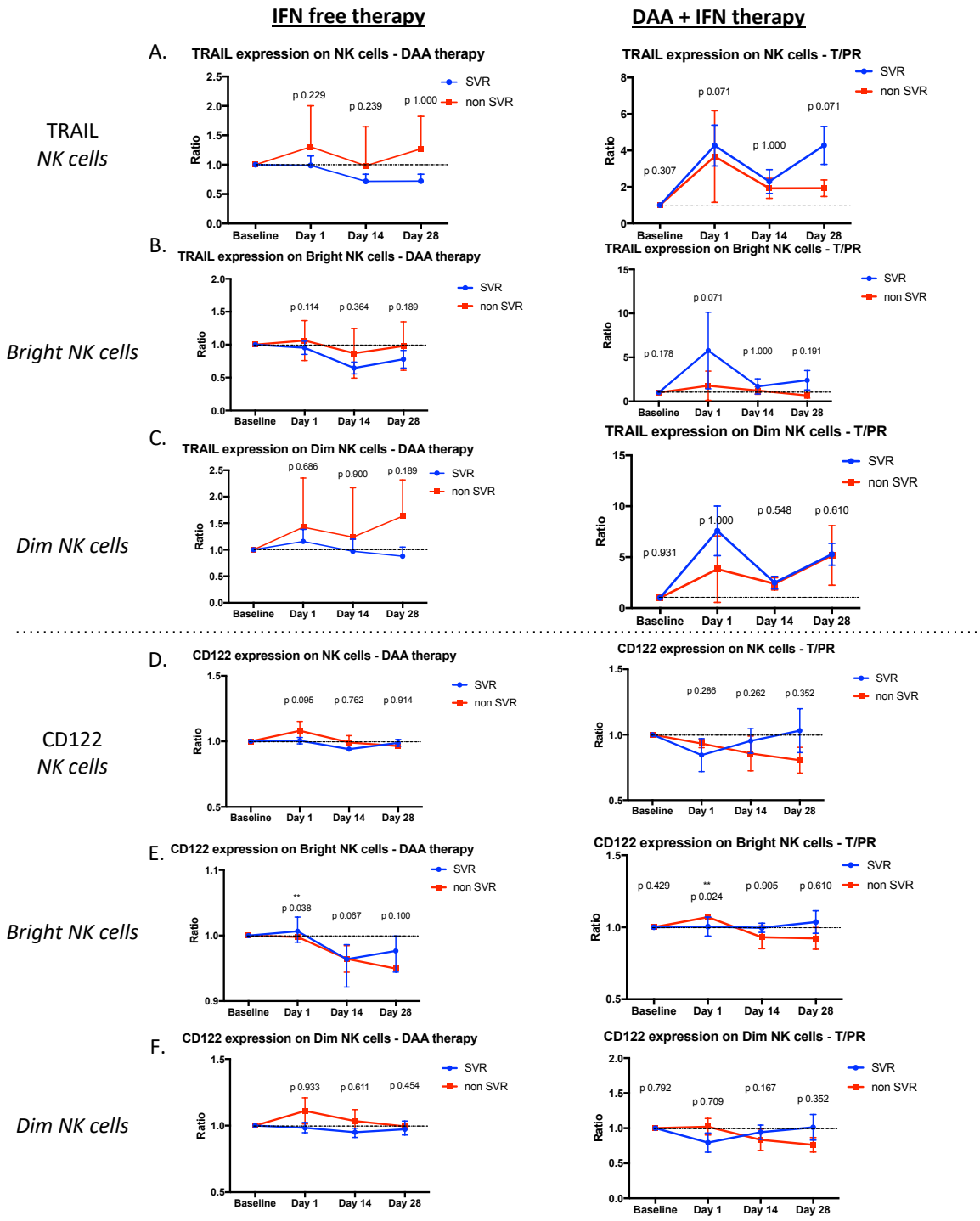


Figure 6.12 On-treatment NK TRAIL and CD122 expression according to SVR vs. non SVR.

No differences in NK TRAIL and CD122 expression were observed according to treatment response. NK TRAIL expression on (A) NK cells; (B) Bright NK cells; and (C) Dim NK cells. CD122 expression on (C) NK cells; (D) Bright NK cells; and (E) Dim NK cells.

6.4.8 Pre-Treatment NK Phenotype was not Associated with Treatment Outcome in Genotype 1 Chronic HCV infection

Next we tested for an association between pre-treatment NK phenotype and treatment response. No difference in pre-treatment NK receptor expression and treatment response was found (Figure 6.13). Similarly, no differences according to IFNL4 genotype were evident.

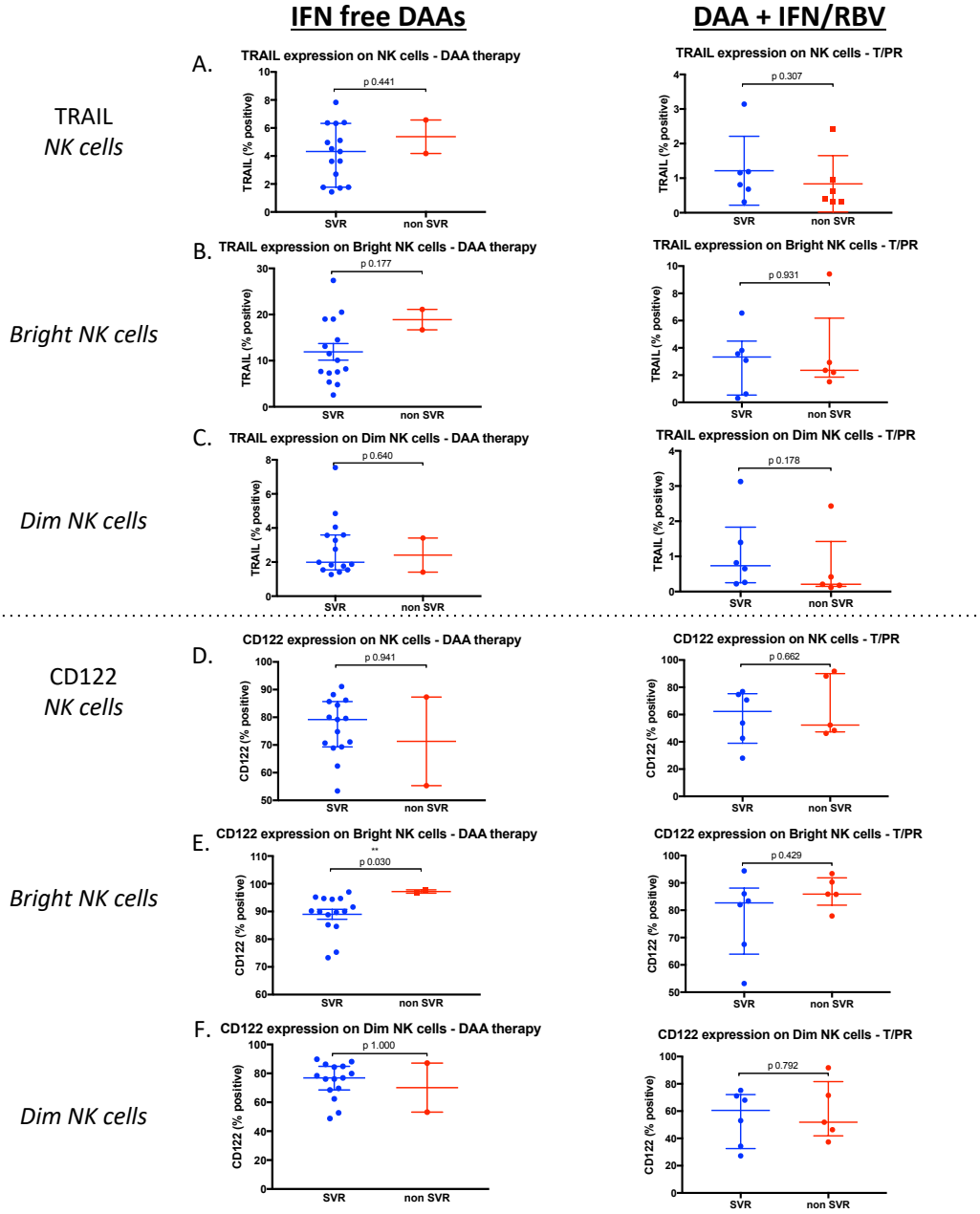


Figure 6.13 Pre-treatment NK TRAIL and CD122 expression was not associated with treatment response.
NK TRAIL expression on (A) NK cells; (B) Bright NK cells; and (C) Dim NK cells. CD122 expression on (C) NK cells; (D) Bright NK cells; and (E) Dim NK cells.

6.4.9 IFN-Free Therapy does not alter the Frequency of NK Cells and its Subsets During the First Month of Antiviral Therapy

We then assessed if the frequency of NK cells and its subtypes were modulated with IFN-free therapy, which may account for the reduction in TRAIL / CD122 expression. However, no change was observed with IFN-free DAA therapy (Figure 6.14). With T/PR there appeared to be a trend to an increase in frequency of the bright NK subset, with a corresponding decrease in the dim NK subset. Subjects receiving PR appeared to follow this pattern, although the numbers are small.

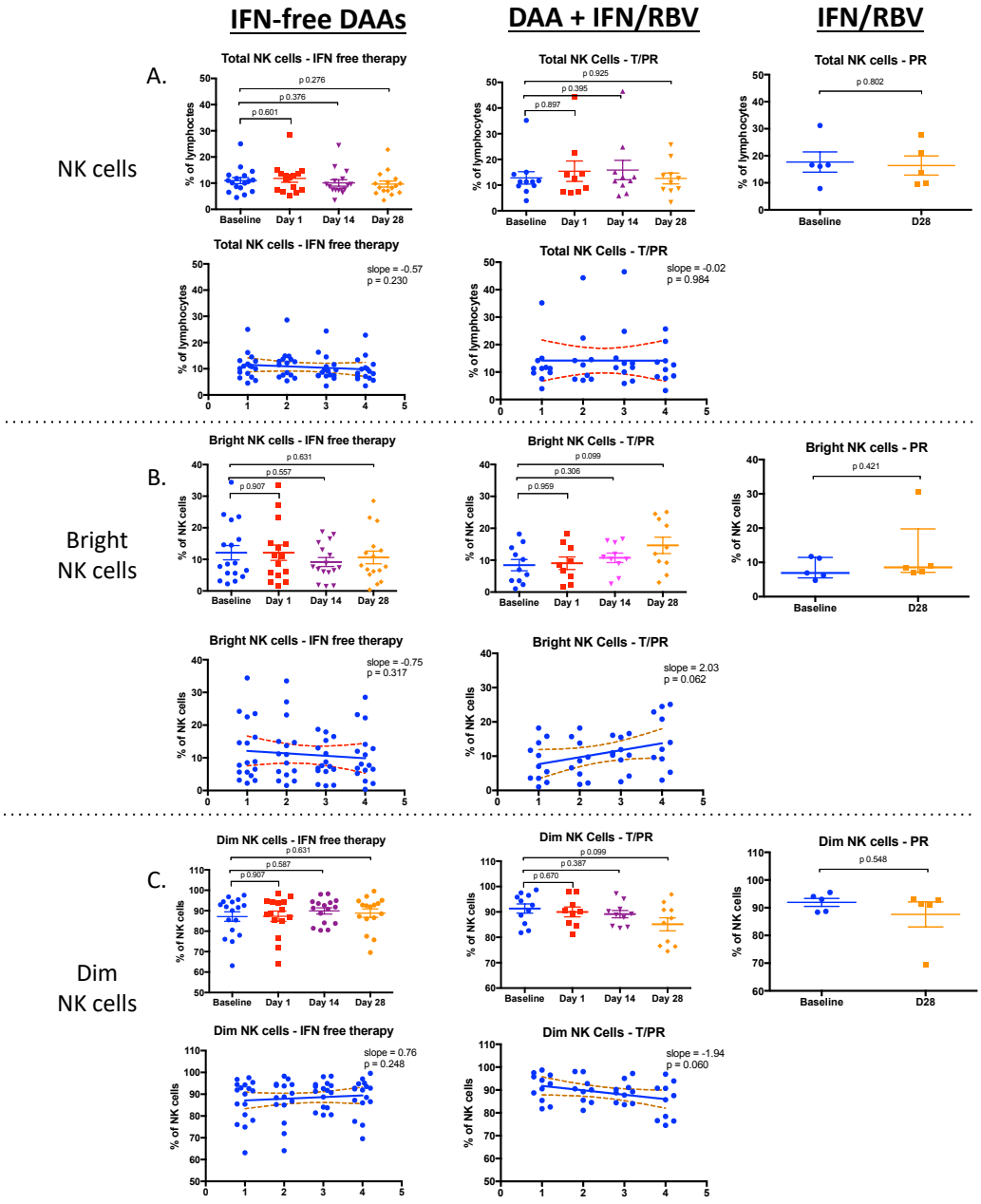


Figure 6.14 Anti-viral therapy does not alter the frequency of NK cells.
(A) NK cells; (B) Bright NK cells; and (C) Dim NK cells.

6.4.10 Early NK cell Cytokine Production is not altered with IFN-Free Therapy During the First Month of Treatment

To assess whether NK cell cytokine production contributes to viral clearance, this was assessed longitudinally during the first month of treatment. NK cytokine production (IFN-γ and TNF-α) production is impaired in CHC, but during the first month of IFN-free DAA therapy there was no corresponding improvement in IFN-γ or TNF-α production (Figure 6.15).

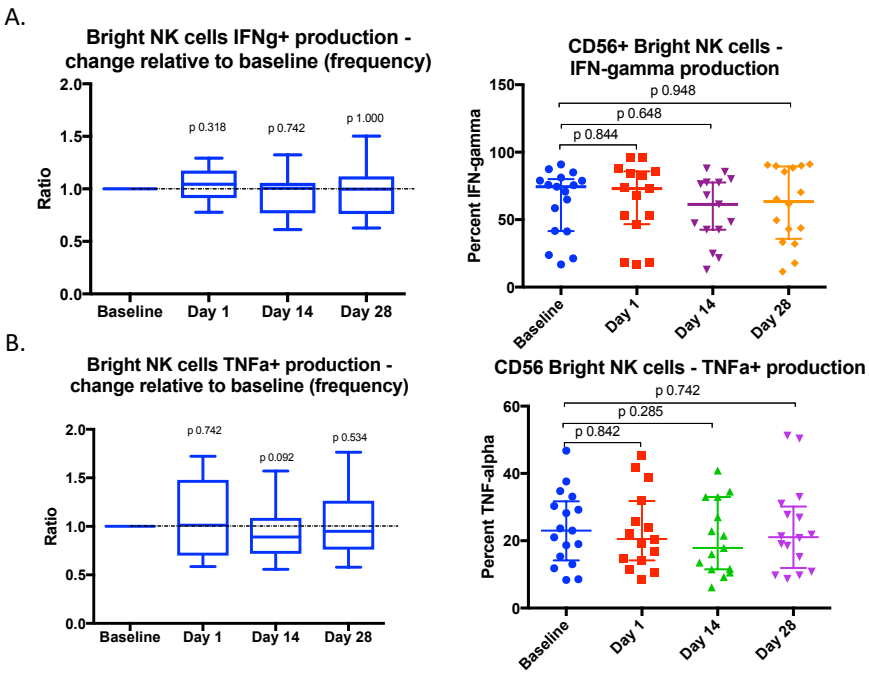


Figure 6.15 Early NK Cell cytokine production is not altered with IFN free therapy. Bright NK cell (A) IFN-γ production; and (B) TNF-α production.

6.5 Discussion

The innate immune system has been shown to play an important role in the host response to HCV, as well as the response to interferon-based treatment for HCV infection.[118,120,135,221,222] Previous studies have demonstrated an association between baseline and on-treatment expression of intrahepatic ISG, circulating chemokines / cytokines and as well as NK cell activity.[118,120,122,135,147,222] Intrahepatic and peripheral ISG expression, as well as circulating IP-10 levels and IFNL4 genotype have also been shown to be associated with the outcome of IFN-based treatment.[118,120,122,135,147,222] The role of the innate immune system in the setting of DAA therapy has not been well defined. Data in Chapter 5 suggested that TLR signaling is important in the host

response to HCV infection, and that baseline TLR signaling may be associated with the response to DAA therapy. In this series of experiments we studied baseline and on-treatment expression of intrahepatic ISG, circulating chemokines / cytokines and as well as NK cell activity, comparing a well characterized cohort of DAA treated patient to a control cohort treated with interferon-based triple therapy.

We confirmed previous descriptions that IFN-based treatment is associated with rapid induction of monocyte ISG expression, monocyte chemokine expression as well as circulating plasma chemokine levels, and NK cell activity (TRAIL/CD122+ NK cells) (Table 6.4). This phenomenon was clearly detectable at 24 hours following the first injection of IFN. Indeed, these changes were most prominent at 24 hours, but remained higher than baseline on-treatment to day-28. At day-28, there was no difference between patients treated with a protease inhibitor plus IFN/RBV compared to patients treated with IFN/RBV alone, suggesting that this effect is due to IFN/RBV. In this cohort, there was no difference in the on-treatment expression of these innate immune markers according to IFNL4 genotype or whether patients went on to achieve SVR. Pre-treatment levels of cytokines / chemokine in plasma were not associated with IFNL4 genotype or SVR. But in PBMCs, lower pre-treatment IL-6 mRNA expression predicted SVR, and higher pre-treatment CXCL9 mRNA expression was associated with IFNL4 CC genotype, consistent with recent data. In vitro studies have previously shown that the HCV NS3/4a protease inhibits RIG-I signaling via cleavage of IPS-1, blocking downstream IRF3 activation and IFN-β production.[88] In a cell culture model, protease inhibitors have been shown to restore IRF3 signaling, but at much higher concentrations than can be achieved in vivo.[223] Our data showed no difference in the induction of ISGs when comparing T/PR with PR.

In contrast, induction of these immune markers was not observed in the setting of DAA therapy. DAA therapy was associated with a rapid and progressive reduction in monocyte ISG expression, monocyte chemokine expression as well as circulating plasma chemokine levels, and NK cell activity (TRAIL/CD122+ NK cells) (Table 6.4). Again, these changes were detectable as early as 24-hours after DAA therapy was started. This was seen in all individuals and correlated with virological suppression. No difference in expression of these innate immune markers according to IFNL4 genotype

or whether patients went on to achieve SVR, either at baseline or on-treatment. The data suggest strongly that these pathways are involved in the host response to HCV replication, but that signaling activity reverts to baseline with potent HCV inhibition in the setting of DAA therapy.

On-treatment	DAA	IFN-based treatment
Monocyte ISG expression* (OAS1, MX1, ISG15, SOCS15)	↓	↑
Plasma chemokine* (IP-10, IL-8, MCP-1, CXCL9, IL-10)	↓	↑
Monocyte chemokine expression (IP-10, IL-8, MCP-1, CXCL9, IL-10, IL-6)	↓	↑
NK activity* (TRAIL/CD122 expression)	↓	↑
* No difference in baseline or on-treatment expression according to SVR or IFNL4 genotype		

Table 6.4 Summary of markers of innate immune response to antiviral therapy in chronic HCV infection

A number of recent studies have evaluated ISG expression and related chemokines / cytokines in patients being treated with DAA therapy.[125,126] Similar to our findings, they demonstrate on-treatment reduction in ISG / chemokine expression with DAA therapy. One study did identify higher end of treatment Type 1 IFN hepatic ISG expression was associated with SVR in 12 subjects treated with sofosbuvir / ribavirin, (SVR, n=7 vs. non SVR, n=5).[125] The other study observed higher pre-treatment hepatic ISG expression correlated with SVR in 13 subjects treated with asunaprevir / daclatasvir (SVR, n=9 vs. non SVR, n=4).[126] Notably, these studies utilised DAA regimens associated with suboptimal SVR rates, suggesting a possible role for innate immunity in the context of less potent DAA regimens. Our cohort was not powered to detect such differences with PrOD associated with cure rates > 95%. Although PrOD is no longer used first-line, this is due to poor tolerability associated with ribavirin, required as a

backbone for HCV-1a infection, as well as the fact that it is a genotype specific regimen. Elevated post-treatment peripheral ISG expression and lower serum IFN-α expression has recently been associated with virological relapse, but in subjects with acute HCV and HIV co-infection.[216] We did not collect samples post-treatment in this study. In contrast to the suboptimal DAA regimens used in the previous studies, we are the first to investigate peripheral ISG changes with a highly effective DAA regimen.

We were also interested to look at the effect of IFN-free DAA therapy on NK phenotype / function and whether HCV-induced NK dysfunction would be reversed. On-treatment reduction in the activation state of peripheral blood NK cells was observed, evidenced by reduced NK TRAIL and CD122 expression. However, this did not correlate with treatment response or IFNL4 genotype. Similarly, no differences in pre-treatment NK TRAIL / CD122 expression were seen according to these variables. Our results are consistent with recent data. Two small studies (n=13, n=12) similarly showed a reduction in peripheral and intra-hepatic NK TRAIL-expression with daclatasvir/asunaprevir, but with no stratification according to treatment response.[146,215] In contrast, a rapid induction of NK TRAIL / CD122 expression was observed in our telaprevir / PR cohort, consistent with previous literature.[144]

Despite our data suggesting there is lowering of peripheral NK activation state with DAA therapy, we observed no change in NK cell cytokine production. We hypothesised that that this may improve with the DAAs, and somewhat contrasts with previous descriptions in the literature. Serti et al. showed restored IFN-γ production after 8-weeks of treatment. We cannot exclude late changes in NK cytokine production in our cohort, with data up to 4-weeks, but our results suggest that NK cell activity does not play a critical role in viral clearance during potent DAA therapy, even in patients with hard to cure characteristics.

DAA-mediated viral suppression was associated with a lowering in the circulating levels of IP-10, IL-8, MCP-1, CXCL9, IL-10 and IL-6 in plasma, as well as PBMC mRNA expression, consistent with recent data.[125,146,215] Again, no differences were seen according to treatment response or IFNL4 genotype. A comparative analysis in our telaprevir / PR patients saw the early induction of IP-10, CXCL9, IL-10, MCP-1 and IL-6,

even in the presence of telaprevir, a potent DAA, illustrating clear differences in the innate immunological response between antiviral therapies.

6.6 Limitations

The main limitation to this analysis was the small cohort size, particularly when stratifying according to treatment response and IFNL4 genotype. The very high virological response rate further limited analyses of biomarkers for treatment response. Our data was based on the analysis of the peripheral innate immune response and whether this reflects the intra-hepatic innate immune response, should ideally be confirmed with intra-hepatic data. In an era where liver biopsy is not routinely requested, this could be investigated further in a representative animal or cell-culture model.

6.7 Conclusion

In a group of CHC subjects with 'hard-to-cure' characteristics, our data suggest that disappearance of the HCV virus correlates with changes in ISG expression, levels of chemokines, and phenotypic changes in the NK cell population. This is a very rapid process, occurring as early as day-1 post commencement of a potent set of DAAs. In particular, the rapid kinetics are consistent with previous suggestions that HCV replication is directly responsible for the high levels of ISG expression, high levels of chemokines/cytokines, and NK cell dysfunction reported in patients with chronic HCV. Our data supports a model whereby HCV stimulates the innate immune system, and with removal of the virus, the system reverts to baseline. In Chapter 5, a trend to higher TLR signaling was observed in those who achieved SVR. In the panel of innate immune markers investigated in this chapter, no baseline or on-treatment biomarkers predicted SVR, but with such a potent DAA regimen, our cohort was underpowered to detect this. Treatment with telaprevir / PR led to early induction of peripheral innate immune markers. Here we have shown clear on-treatment differences according to mode of antiviral therapy, broadening our current understanding of the role of peripheral ISG expression, NK cells and cytokines / chemokines in the pathogenesis of CHC.

6.8 Appendices

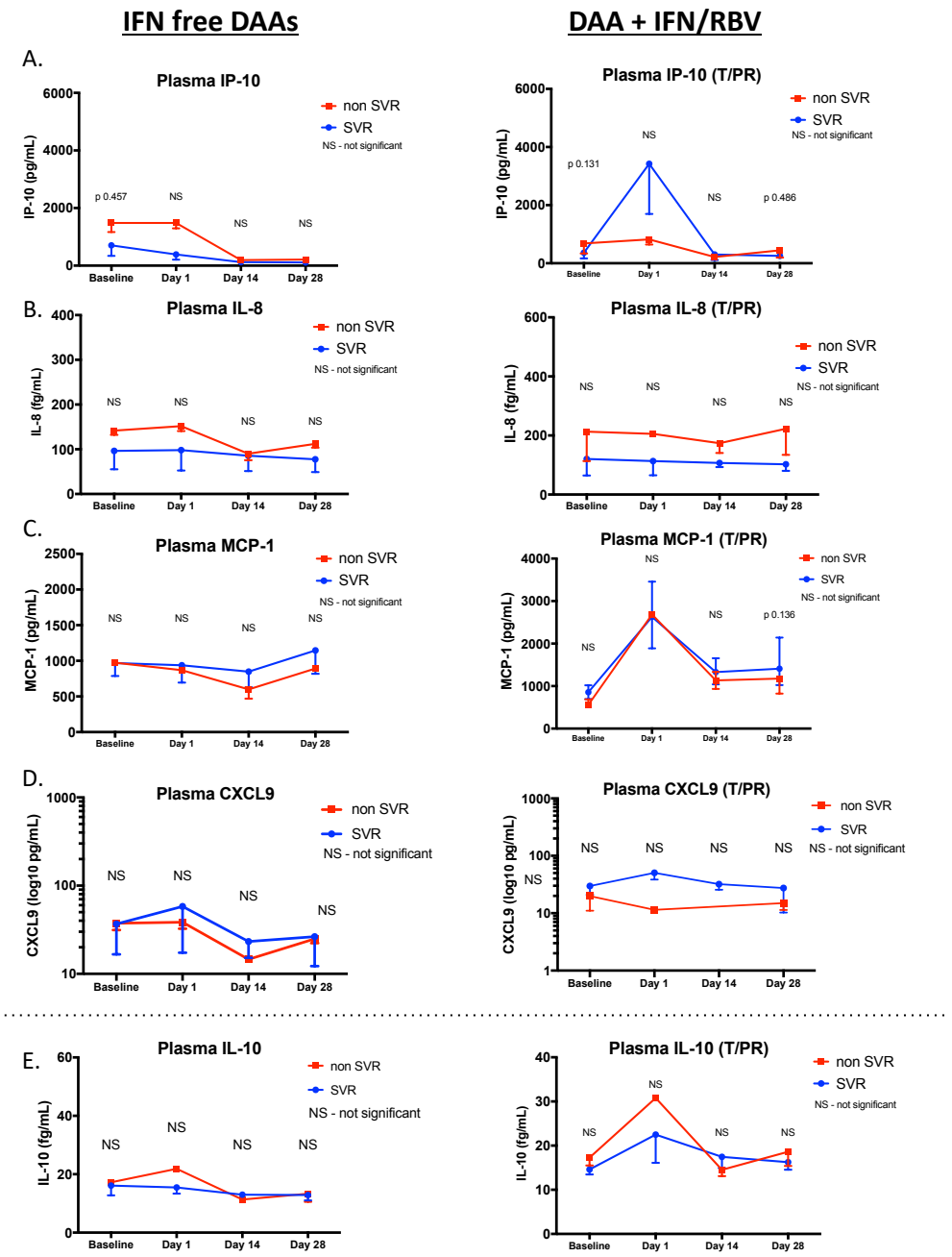


Figure 6.16 No on-treatment differences in circulating plasma chemokine / cytokine levels according to SVR vs. non-SVR.
(A) IP-10; (B) IL-8; (C) MCP-1; (D) CXCL9; (E) IL-10.

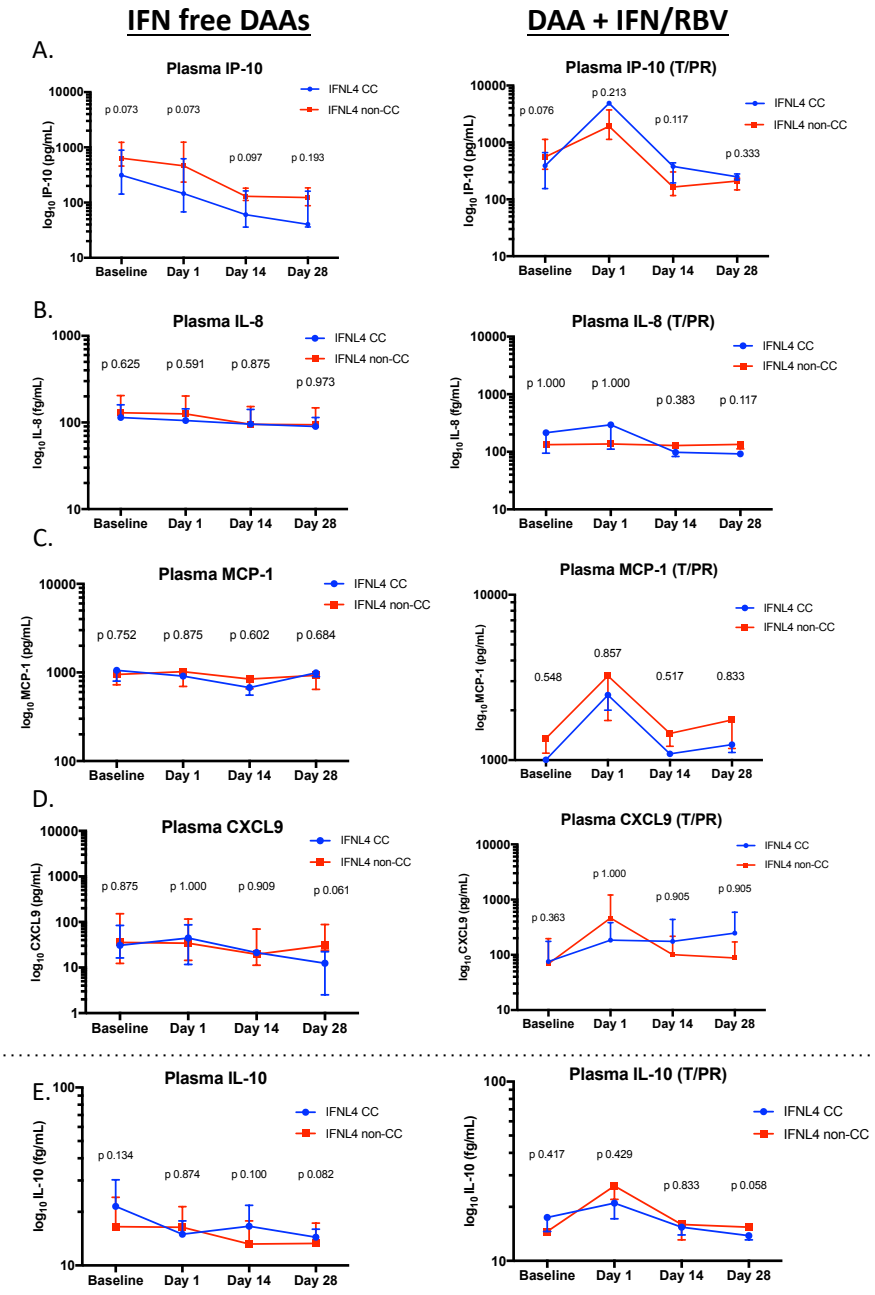


Figure 6.17 No on-treatment differences in circulating plasma chemokine / cytokine levels according to IFNL4 genotype.
(A) IP-10; (B) IL-8; (C) MCP-1; (D) CXCL9; (E) IL-10.

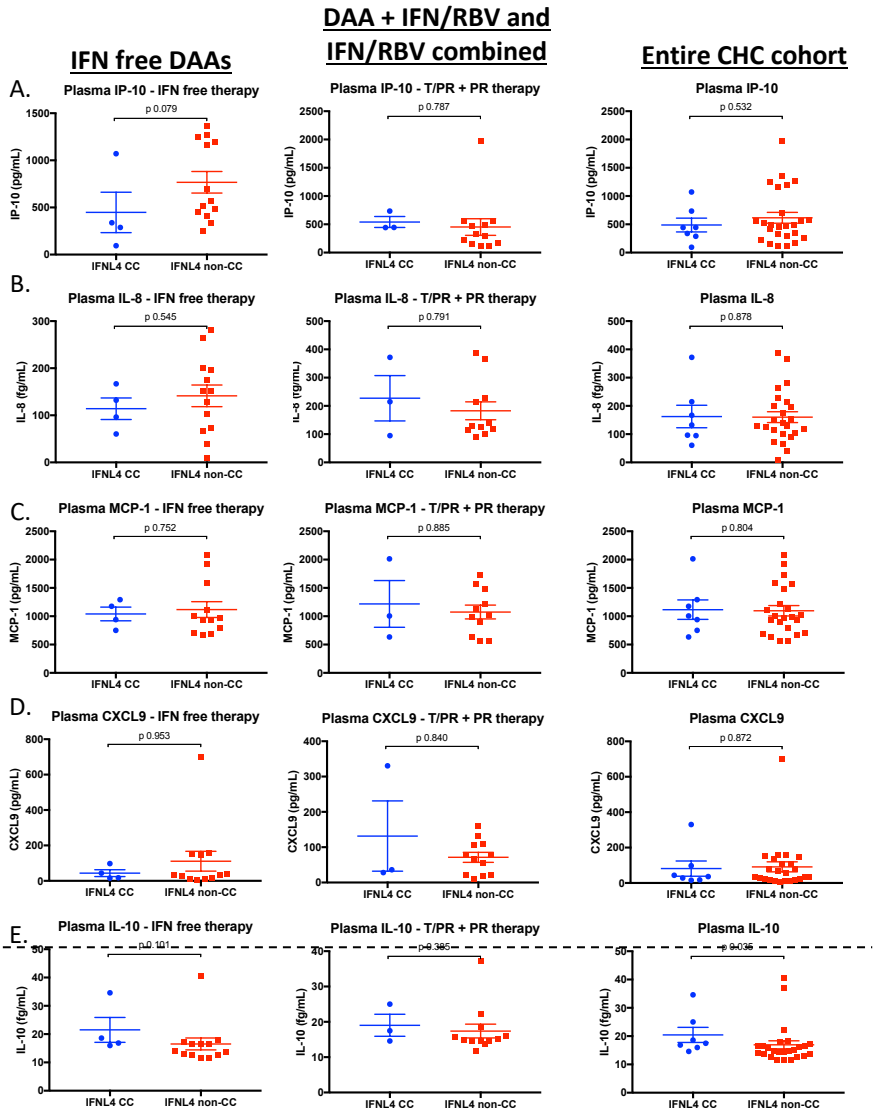


Figure 6.18 Pre-treatment levels of chemokines / cytokines in plasma according to IFNL4 genotype.

A trend to lower pre-treatment IP-10 plasma levels was seen with the favourable IFNL4 CC genotype with DAA therapy. No other differences in plasma levels of cytokines / chemokines were seen within each treatment group or as a whole cohort, according to IFNL4 genotype. (A) IP-10; (B) IL-8; (C) MCP-1; (D) CXCL9; (E) IL-10.

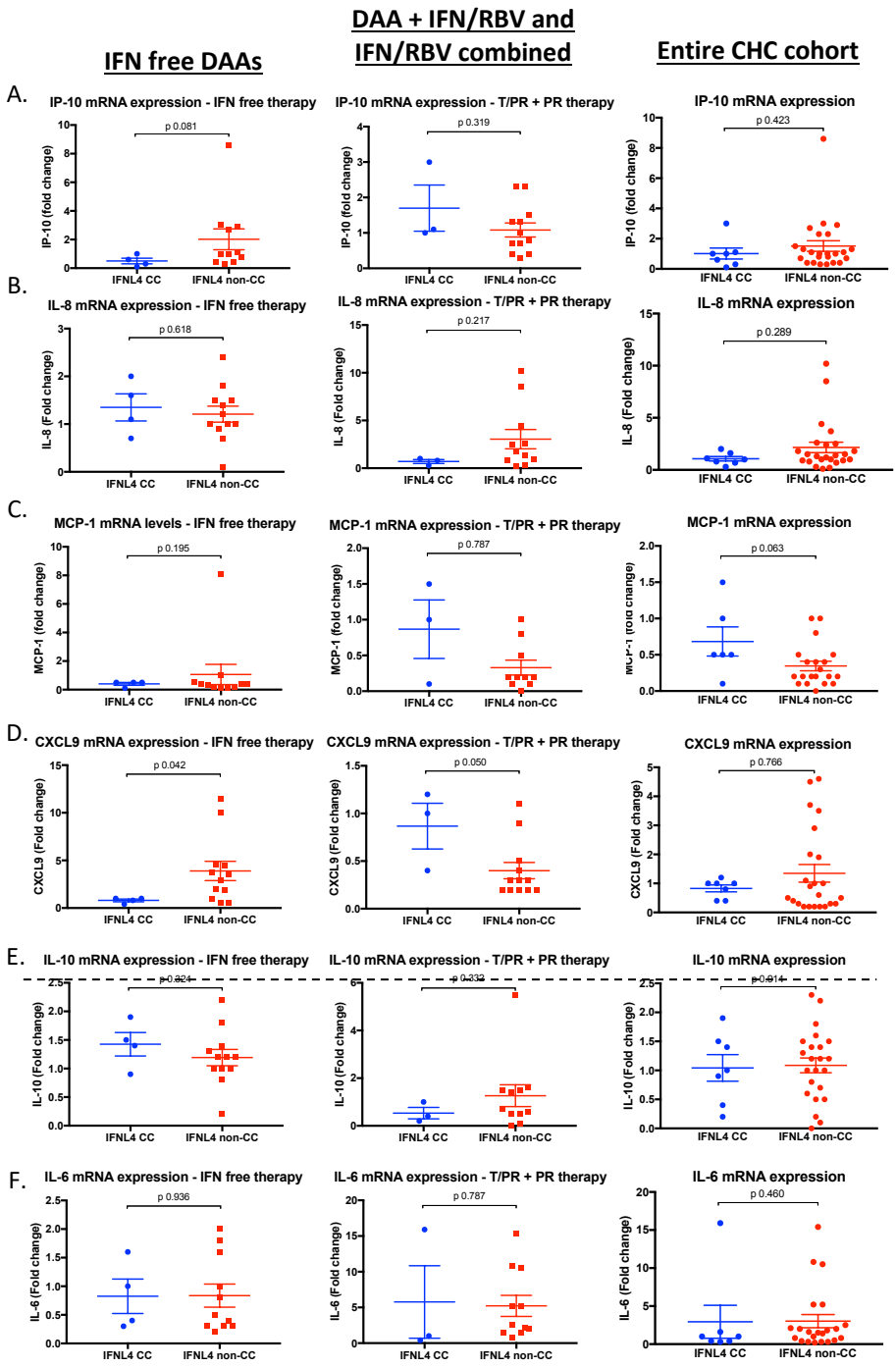
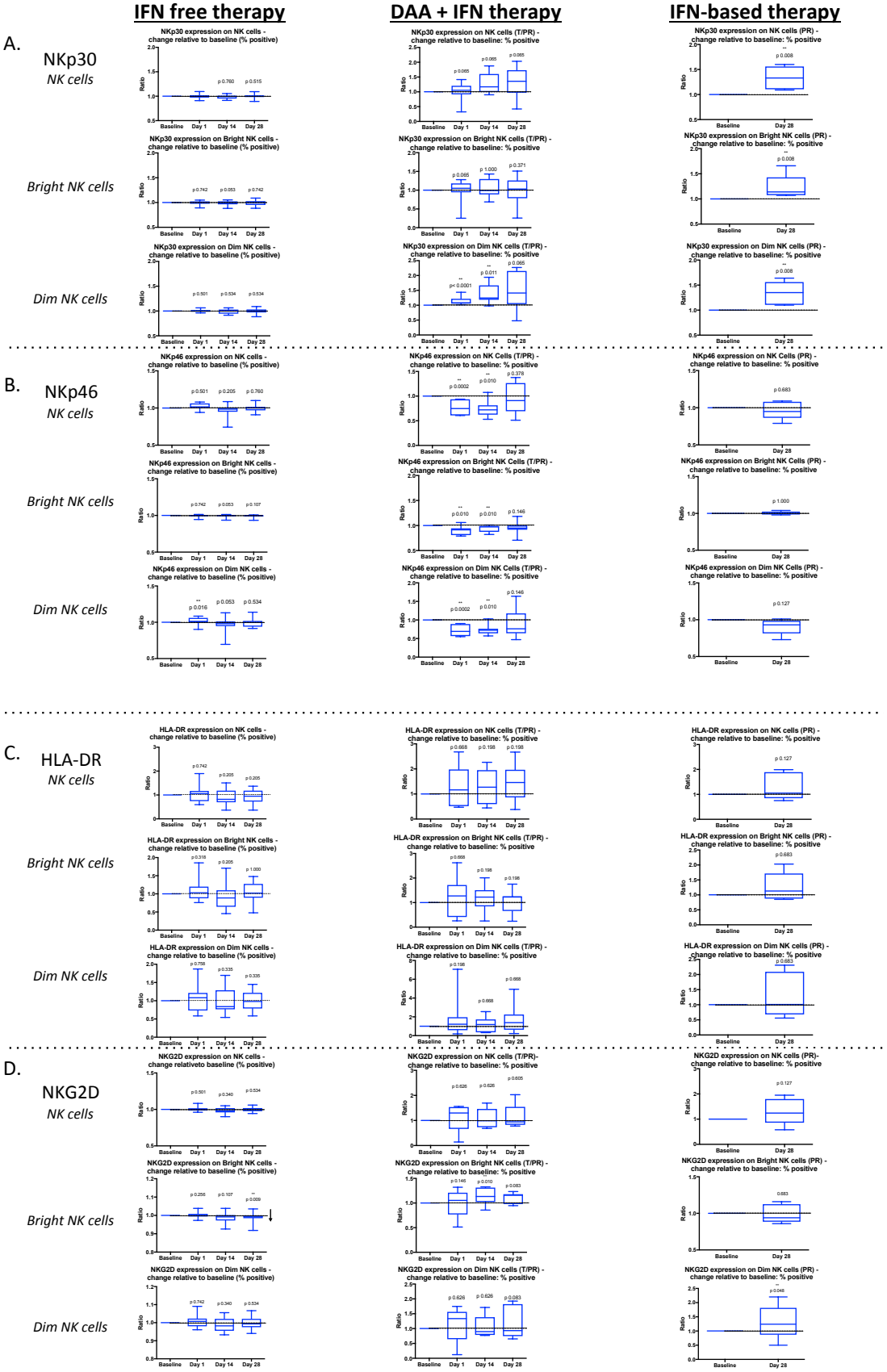


Figure 6.19 Pre-treatment mRNA levels of chemokines / cytokines according to IFNL4 genotype.

A trend to lower pre-treatment IP-10 mRNA expression was seen with the IFNL4 CC genotype with DAA therapy. Higher pre-treatment CXCL9 levels was associated with the IFNL4 CC genotype in those who received IFN-based therapy. A trend to higher pre-treatment MCP-1 mRNA expression was seen with the iFNL4 CC genotype when the entire CHC cohort was combined. (A) IP-10; (B) IL-8; (C) MCP-1; (D) CXCL9; (E) IL-10; (F) IL-6.


IFN free therapy
DAA + IFN therapy
IFN-based therapy
A.
NKp30
NK cells
Bright NK cells
Dim NK cells
B.
NKp46
NK cells
Bright NK cells
Dim NK cells
C.
HLA-DR
NK cells
Bright NK cells
Dim NK cells
D.
NKG2D
NK cells
Bright NK cells
Dim NK cells

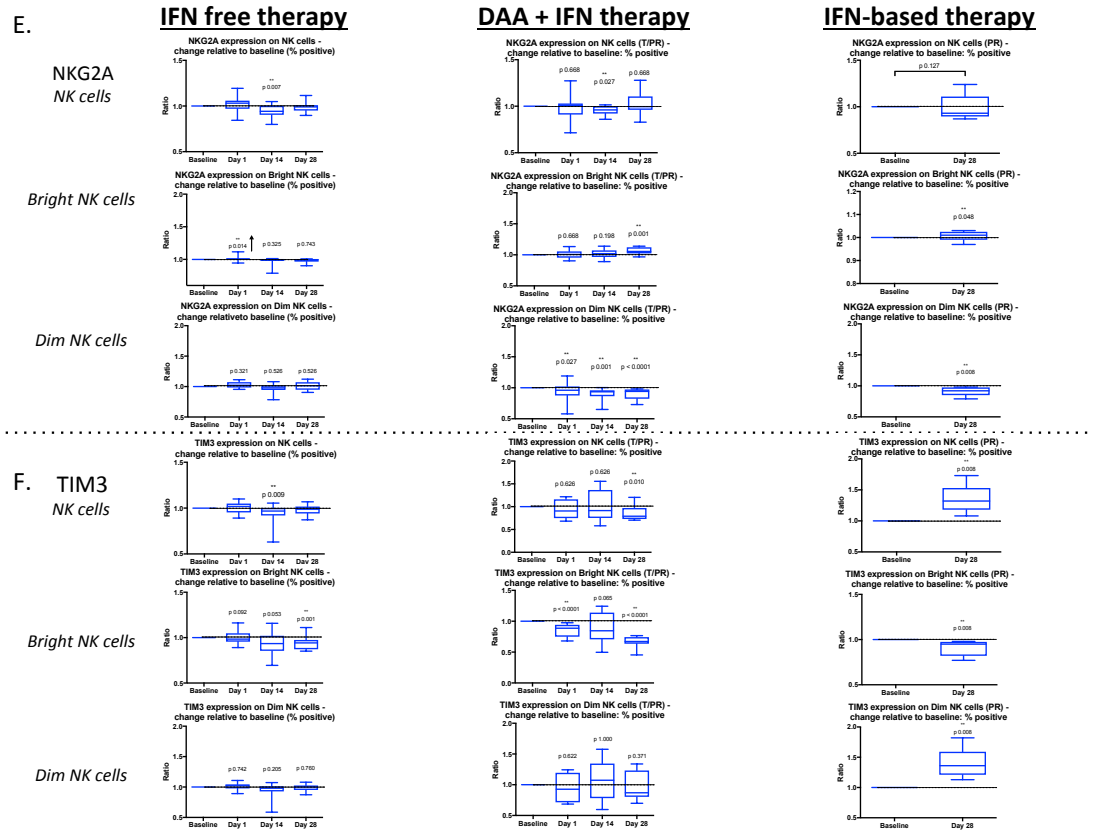


Figure 6.20 On-treatment changes in NK phenotype with antiviral therapy.
No change in NK expression of activation markers NKp30, NKp46, HLA-DR or NKG2D were observed with IFN-free therapy. Differences were seen with IFN-based therapy, as early as day-1 of treatment. (A) NKp30; (B) NKp46; (C) HLA-DR; (D) NKG2D; (E) NKG2A; and (F) TIM3 expression on NK cells, Bright NK cells and Dim NK cells.

7 Summary and Future Directions

7.1 Background

Chronic hepatitis C infection is associated with significant morbidity and mortality. It is a major public health concern with up to 71 million people chronically infected worldwide.[1] In the last decade we have witnessed the tremendous development of anti-viral therapy for HCV, with cure rates following DAA therapy approaching 100% and without the associated morbidity seen with IFN-based therapy. Despite these advances, there are important issues that remain controversial, including i) whether viral eradication is associated with long-term clinical benefits; and ii) what key biological mechanism is responsible for driving viral clearance in the context of viral suppression. In this thesis we set out to explore the long-term clinical impact of viral eradication, as well as the innate immunological host response to viral eradication, in people living with hepatitis C infection.

7.2 Natural History

Natural history studies in CHC have suggested that approximately 20% of subjects will develop cirrhosis after 20-30 years, with an annual risk of hepatic decompensation and hepatocellular carcinoma estimated at 4% and 1.5% respectively thereafter. Whilst we assume that viral eradication will prevent such complications and improve long-term outcomes, good long-term data confirming this are lacking. Clinical trials evaluating anti-viral therapy for HCV used SVR or histological response at 12-24 months as a surrogate for clinical benefit, but did not evaluate long-term outcomes. In selected high-risk populations of subjects with advanced fibrosis or cirrhosis, SVR has been associated with improved overall and liver-related survival, as well as reduced rates of hepatic decompensation and hepatocellular carcinoma, with follow up periods of up to 3 to 8-years. However, there have been limited data demonstrating a clinical benefit from SVR in people with less advanced disease. In fact, a recent controversial Cochrane review concluded that there was no evidence supporting that DAAs reduce HCV-related morbidity or all-cause mortality.[55]

We have contributed to this debate by reporting on the long-term outcomes of a well-characterised cohort of CHC subjects, in whom paired liver fibrosis assessments were performed more than 10-years apart. At baseline assessment, the majority of our cohort had mild-moderate liver fibrosis (79%, n=109/131) with a significant proportion progressing to advanced fibrosis (21%, n= 27) after 10-years of infection. We were able to demonstrate that curative HCV therapy was associated with lower rates of fibrosis progression. Both virological cure and a shorter duration of infection prior to antiviral therapy were independent predictors of protection against liver fibrosis progression. Since the publication of our data a number of other large datasets have reported similar benefits to curative therapy.[224,225]

Our data support the early treatment of all people with CHC regardless of liver fibrosis stage, to prevent cirrhosis. Prevention of cirrhosis is important because people with cirrhosis retain a risk of HCC following viral eradication and require long term surveillance. The data are particularly relevant to jurisdictions where DAA treatment is restricted to people with advanced fibrosis or cirrhosis. Other important arguments to treat people with minimal liver disease include reducing transmission of hepatitis C among people who inject drugs, as well as reducing stigma and improved quality of life in these patients.[226,227] Indeed, the elimination of hepatitis C as a public health threat by 2030, in line with the WHO targets[228], will require active targeting of high risks populations to interrupt transmission.

7.3 Immunopathogenesis Of HCV Infection

In the remaining chapters of my thesis, I then planned a series of experiments to explore the underlying immunopathogenesis of HCV by looking at the host response to viral eradication, in both acute (spontaneous clearance) and chronic (antiviral therapy) HCV infection. We were particularly interested in the role of TLR signaling in viral eradication. HCV replicative intermediates have been shown to be sensed by cytoplasmic RIG-I and endosomal TLR3 in vitro, leading to stimulation of the interferon signaling pathway and downstream interferon stimulated gene (ISG) expression.[88,89] In vitro studies have also identified components of the HCV polyprotein that interact with the TLRs leading to induction of a pro-inflammatory response, specifically the HCV core

and NS3 protein stimulating TLR2 and the HCV NS5A protein stimulates TLR4.[92,93] Previous work in our laboratory has shown that patients with chronic hepatitis C display higher levels of TLR2/4 protein expression and signaling on peripheral blood monocytes, higher TLR2/4 gene expression in liver compared to healthy controls, and higher circulating levels of pro inflammatory cytokines.[97,98]

7.3.1 Immunopathogenesis of Acute HCV Infection

We first investigated markers of innate immunity in a well-characterised prospective cohort of subjects with acute / subacute HCV infection, and found that persistence of HCV infection was associated with high level reactivity of TLR4 and TLR7/8 signaling pathways in response to toll ligand-specific stimulation, as well as with higher levels of TLR4 expression on monocytes and NK cells. The data suggest that persistent HCV infection is associated with higher levels of TLR4/7 signaling and implicate TLR4/7 in the biological response to CHC. Further work will be necessary to determine whether this represents a direct antiviral response, or whether this reflects upregulation of TLR 4/7 secondary to intrahepatic inflammation. Regardless, TLR 4/7 signaling may represent therapeutic targets. Indeed, TLR7 agonists have previously been evaluated as a candidate for the treatment of HCV infection in clinical trials. It is unlikely given the current treatment landscape that this would be pursued, but TLR agonists may be relevant to other viral infections, including HBV. TLR8 agonists are now in phase 2 development for the treatment of chronic HBV infection. The data also presents TLR4 as a potential biomarker for predicting acute clearance of HCV. This would require further validation in a large, prospective longitudinal cohort study and clinically translate into individuals presenting with acute / subacute HCV infection being able to defer drug therapy, and its associated morbidity and cost.

7.3.2 Immunopathogenesis of Chronic HCV Infection

We then hypothesised that TLR signaling and expression may contribute to antiviral efficacy in the setting of potent interferon-free DAA therapy. To date, there are little data exploring the role of TLR signaling / expression in chronic HCV infection with the new DAAs, nor whether endogenous TLR signaling pathways are associated with the outcome of antiviral therapy.

In a cohort of CHC subjects with 'hard-to-cure' characteristics, we observed rapid down-regulation of peripheral monocyte TLR4, TLR2, TLR7 and TLR9 signaling activity, PBMC ISG expression, NK cell expression of the activation markers TRAIL and CD122, as well as circulating plasma chemokine / cytokine levels, with DAA-mediated viral suppression (Chapter 6). We tested for an association between innate immune activity at baseline and response to DAA therapy, and found a strong trend for PBMC TLR2, TLR4 and TLR7 signaling to be higher in patients who went on to achieve SVR following DAA treatment. The data implicates innate immunity as playing a key role in viral clearance in the setting of DAA therapy (Figure 7.1).

This higher level of TLR signaling was not explained by higher serum ALT levels, suggesting that this observation is not simply a reflection of intrahepatic inflammation associated with HCV infection. In contrast to the rapid down-regulation of peripheral innate immune markers seen with the DAAs, IFN-based therapy led to the early induction of peripheral TLR signaling, PBMC ISG expression, cytokine/chemokine expression and NK cell activation markers. Despite this induction of the innate immune response, HCV clearance rates are suboptimal with IFN-based therapy.

The data show that potent suppression of HCV replication reduces intrahepatic inflammation with reduction of markers of innate immune activity. This is the first detailed description of TLR signaling and innate immune activity in the setting of HCV infection. The data clearly implicate innate immunity in the chronic inflammatory response to HCV and raises a number of key questions:

i) Does this observation reflect a specific interaction between HCV and TLR signaling pathways, or rather a secondary bystander inflammation driven by an alternate inflammatory pathway?

ii) Is the TLR signaling pathway associated with direct anti-HCV activity and does TLR signaling directly promote viral clearance?

iii) Why is the innate immune activity observed not sufficient to clear HCV?

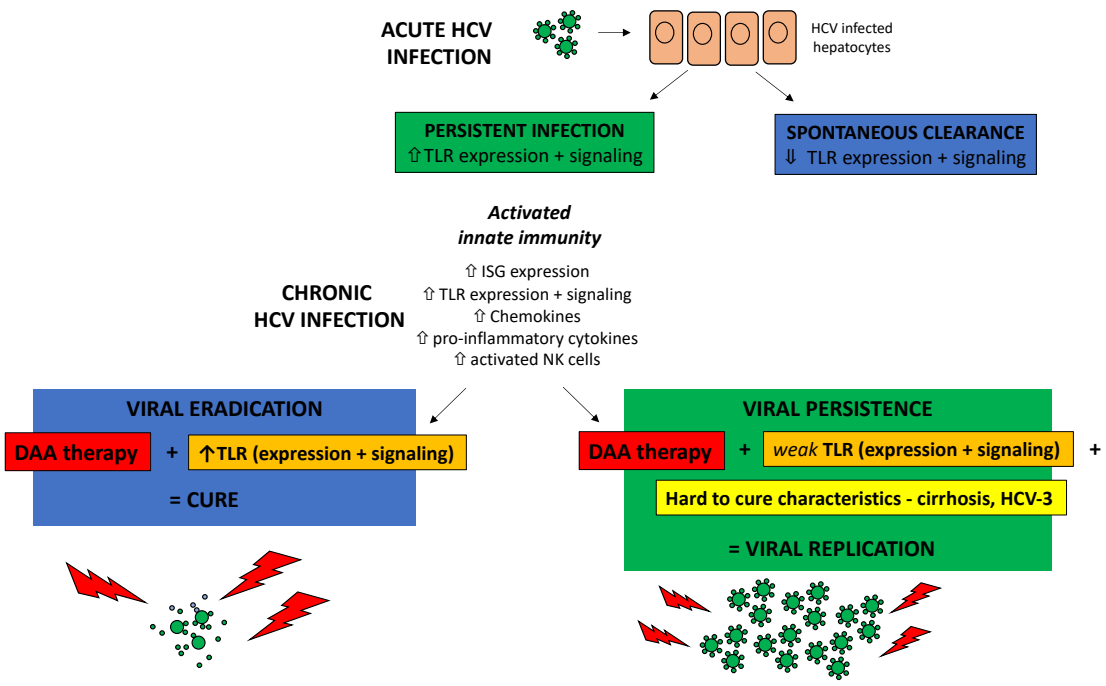


Figure 7.1 Proposed model implicating the role of innate immunity in viral clearance in the setting of DAA therapy

We acknowledge that over the course of this thesis, the treatment of HCV has advanced rapidly from IFN-based therapy being the backbone of treatment associated with suboptimal response, to highly effective DAA regimens with cure rates of >95% in adherent patients becoming standard of care. Despite this, the data support further evaluation of the role of TLR signaling and innate immunity in hard-to-cure populations receiving the DAAs, including subjects with Gt 3 HCV and DAA non-responders; or as adjuvant treatment to shorten overall treatment duration. TLR agonists have been investigated in HCV. As noted above, a small molecule TLR7 agonist was shown in humans to have an IFN-dependent anti-viral effect against HCV.[205] This candidate was stopped due to toxicity in the face of the rapid developments in efficacy with DAA therapy.

The data here may also be potentially relevant to improved vaccine design. Moving forward, the field is now focused on elimination of HCV in line with the targets set by WHO, with particular emphasis on harm reduction and development of new models of care for the treatment of people who inject drugs. Prevention of HCV remains an ongoing challenge, with little progress made in the last few decades, particularly in contrast to the progress made with DAA therapy. Vaccine development

has been challenged by the HCV error prone replication cycle rapidly evades humoral and T-cell immune responses, limited funding where the industry has prioritized DAA therapy, and difficulties associated with designing a clinical trial to demonstrate vaccine efficacy funding and clinical challenges. An NIH-funded study in the US investigating a HCV vaccine, recently found that it was ineffective in preventing chronic HCV.[229] TLR agonists have been used as vaccine adjuvants, and our data suggest this approach may be particularly relevant to HCV vaccine candidates. This warrants further evaluation.

Finally, the data in this thesis reinforce the relevance of TLR signaling to the immunopathogenesis of chronic viral infections. TLR signaling has been linked to the pathogenesis of HIV, HBV, RSV and numerous other viruses.[208,230,231,225] As an integral part of the antiviral response to viruses, engagement of the TLR pathway through the development of therapeutic agents. HCV has previously been identified to target innate immune pathways as an immune evasion strategy *in vitro.*[221] The data in this thesis support that TLR signaling is relevant to the HCV life cycle and host-virus interaction. A deeper mechanistic understanding of how the innate immune system targets viral pathogens, including how the relationship to induction of adaptive immunity, may inform future therapeutic strategies for fighting viral infections.

Bibliography

1. Abbie. GLOBAL HEPATITIS REPORT,2017. World Health Organization. 2017 Apr 28;:1–83.

2. Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J Viral Hepat. 2006 Jan;13(1):34–41.

3. Gumber SC, Chopra S. Hepatitis C: a multifaceted disease. Review of extrahepatic manifestations. Annals of Internal Medicine. 1995 Oct 15;123(8):615–20.

4. The Kirby Institute. Hepatitis B and C in Australia Annual Surveillance Report Supplement 2016. The Kirby Institute, University fo NSW. 2016 Sep 28;:1–56.

5. Antonelli A, Ferri C, Pampana A, Fallahi P, Nesti C, Pasquini M, et al. Thyroid disorders in chronic hepatitis C. Am J Med. 2004 Jul 1;117(1):10–3.

6. The Kirby Institute. Monitoring hepatitis C treatment uptake in Australia. 2018 Jul 26;(9):1–11.

7. Tran A, Quaranta JF, Benzaken S, Thiers V, Chau HT, Hastier P, et al. High prevalence of thyroid autoantibodies in a prospective series of patients with chronic hepatitis C before interferon therapy. Hepatology. 1993 Aug;18(2):253–7.

8. White DL, Ratziu V, El-Serag HB. Hepatitis C infection and risk of diabetes: a systematic review and meta-analysis. Journal of Hepatology. 2008 Nov;49(5):831–44.

9. Sievert W, Altraif I, Razavi HA, Abdo A, Ahmed EA, Alomair A, et al. A systematic review of hepatitis C virus epidemiology in Asia, Australia and Egypt. Liver international : official journal of the International Association for the Study of the Liver. 2011;31 Suppl 2:61–80.

10. Poynard T, Leroy V, Cohard M, Thevenot T, Mathurin P, Opolon P, et al. Meta-analysis of interferon randomized trials in the treatment of viral hepatitis C: effects of dose and duration. Hepatology (Baltimore, Md). 1996 Oct;24(4):778–89.

11. Department of Health and Aging. National Hepatitis C Testing Policy. Hepatitis C subcommittee of the Ministerial Advisory Committee on AIDS, Sexual Health and Hepatitis. Australia; 2007 Aug 28;:1–60.

12. Dusheiko G, Main J, Thomas H, Reichard O, Lee C, Dhillon A, et al. Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled

study. Journal of Hepatology. 1996 Nov;25(5):591–8.

13. Bradshaw D, Matthews G, Danta M. Sexually transmitted hepatitis C infection: the new epidemic in MSM? Current opinion in infectious diseases. 2013 Feb;26(1):66–72.

14. Brok J, Gluud LL, Gluud C. Ribavirin monotherapy for chronic hepatitis C infection: a Cochrane Hepato-Biliary Group systematic review and meta-analysis of randomized trials. The American journal of gastroenterology. 2006 Mar 31;101(4):842–7.

15. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. New England Journal of Medicine. 1998 Nov 19;339(21):1485–92.

16. Zhang J, Randall G, Higginbottom A, Monk P, Rice CM, McKeating JA. CD81 Is Required for Hepatitis C Virus Glycoprotein-Mediated Viral Infection. Journal of virology. 2004 Jan 13;78(3):1448–55.

17. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). The Lancet. 1998 Oct 30;352(9138):1426–32.

18. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, et al. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science. 1998 Oct 1;282(5386):103–7.

19. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. 2002 Oct 1;21(19):5017–25.

20. Zeuzem S, Schmidt JM, Lee JH, Rüster B, Roth WK. Effect of interferon alfa on the dynamics of hepatitis C virus turnover in vivo. Hepatology (Baltimore, Md). 1996 Jan 31;23(2):366–71.

21. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. The Lancet. 2001;358(9286):958–65.

22. Evans MJ, Hahn von T, Tscherne DM, Syder AJ, Panis M, Wölk B, et al. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature. 2007 Apr 12;446(7137):801–5.

23. Fried MW, Shiffman ML, Reddy KR, Smith C. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. The New England journal of

medicine. 2002;347(13):975–82.

24. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, et al. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature. 2009 Feb 11;457(7231):882–6.

25. Hadziyannis SJ. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C. Annals of Internal Medicine. 2004;140(5):346–55.

26. Lai C-K, Jeng K-S, Machida K, Lai MMC. Hepatitis C virus egress and release depend on endosomal trafficking of core protein. Journal of virology. 2010 Nov;84(21):11590–8.

27. Zhu H, Elyar J, Foss R, Hemming A, Hall E, Lecluyse EL, et al. Chapter 6 Characterization of HCV Quasispecies in the Hypervariable Region 1 (HVR1) by In Vitro Translation and Mass Spectrometry. Tang H, editor. Hepatitis C: Methods and Protocols, Second Edition. Totowa, NJ: Humana Press; 2009;510(2):427–38.

28. Farci P, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, et al. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science. 2000 Apr 14;288(5464):339–44.

29. Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology (Baltimore, Md). 2014 Jan;59(1):318–27.

30. Bowden DS, Berzsenyi MD. Chronic hepatitis C virus infection: genotyping and its clinical role. Future Microbiol. 2006 Jun;1(1):103–12.

31. Petruzziello A, Marigliano S, Loquercio G, Cozzolino A, Cacciapuoti C. Global epidemiology of hepatitis C virus infection: An up-date of the distribution and circulation of hepatitis C virus genotypes. World J Gastroenterol. 2016 Sep 14;22(34):7824–40.

32. Loomba R, Rivera MM, McBurney R, Park Y, Haynes-Williams V, Rehermann B, et al. The natural history of acute hepatitis C: clinical presentation, laboratory findings and treatment outcomes. Alimentary pharmacology \& therapeutics. 2011;33(5):559–65.

33. Kamal SM. Acute hepatitis C: a systematic review. The American journal of gastroenterology. 2008 May;103(5):1283–97–quiz1298.

34. Mosley JW, Operskalski EA, Tobler LH, Buskell ZJ, Andrews WW, Phelps B, et al. The course of hepatitis C viraemia in transfusion recipients prior to availability of antiviral therapy. J Viral Hepat. 2008 Feb;15(2):120–8.

35. Westbrook RH, Dusheiko G. Natural history of hepatitis C. Journal of

Hepatology. European Association for the Study of the Liver; 2014 Nov 1;61(Supplement):S58–S68.

36. Yano M, Kumada H, Kage M, Ikeda K, Shimamatsu K, Inoue O, et al. The long-term pathological evolution of chronic hepatitis C. Hepatology. 1996 Jun;23(6):1334–40.

37. Xu F, Moorman AC, Tong X, Gordon SC, Rupp LB, Lu M, et al. All-Cause Mortality and Progression Risks to Hepatic Decompensation and Hepatocellular Carcinoma in Patients Infected With Hepatitis C Virus. Clin Infect Dis. 2016 Jan 8;62(3):289–97.

38. Cammà C, Bruno S, Di Marco V, Di Bona D, Rumi M, Vinci M, et al. Insulin resistance is associated with steatosis in nondiabetic patients with genotype 1 chronic hepatitis C. Hepatology. 2006 Jan;43(1):64–71.

39. Rubbia-Brandt L, Fabris P, Paganin S, Leandro G, Male P-J, Giostra E, et al. Steatosis affects chronic hepatitis C progression in a genotype specific way. Gut. 2004 Mar;53(3):406–12.

40. Adinolfi LE, Gambardella M, Andreana A, Tripodi MF, Utili R, Ruggiero G. Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. Hepatology. 2001 Jun;33(6):1358–64.

41. Poynard T, McHutchison J, Manns M, Trépo C, Lindsay K, Goodman Z, et al. Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. YGAST. 2002 May;122(5):1303–13.

42. Cammà C, Di Bona D, Schepis F, Heathcote EJ, Zeuzem S, Pockros PJ, et al. Effect of peginterferon alfa-2a on liver histology in chronic hepatitis C: a meta-analysis of individual patient data. Hepatology. 2004 Feb;39(2):333–42.

43. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet. 1997 Mar 22;349(9055):825–32.

44. Gordon SC, Bayati N, Silverman AL. Clinical outcome of hepatitis C as a function of mode of transmission. Hepatology. 1998 Jul 31;28(2):562–7.

45. García-Álvarez M, Pineda-Tenor D, Jiménez-Sousa MA, Fernández-Rodríguez A, Guzmán-Fulgencio M, Resino S. Relationship of vitamin D status with advanced liver fibrosis and response to hepatitis C virus therapy: a meta-analysis. Hepatology (Baltimore, Md). 2014 Nov;60(5):1541–50.

46. Freedman ND, Curto TM, Lindsay KL, Wright EC, Sinha R, Everhart JE. Coffee consumption is associated with response to peginterferon and ribavirin therapy in patients with chronic hepatitis C. Gastroenterology. Elsevier Inc; 2011;140(7):1961–9.

47. Modi AA, Feld JJ, Park Y, Kleiner DE, Everhart JE, Liang TJ, et al. Increased caffeine consumption is associated with reduced hepatic fibrosis. Hepatology (Baltimore, Md). 2010 Jan;51(1):201–9.

48. Huang H, Shiffman ML, Friedman S, Venkatesh R, Bzowej N, Abar OT, et al. A 7 gene signature identifies the risk of developing cirrhosis in patients with chronic hepatitis C. Hepatology. 2007 Aug;46(2):297–306.

49. Curto TM, Lagier RJ, Lok AS, Everhart JE, Rowland CM, Sninsky JJ, et al. Predicting cirrhosis and clinical outcomes in patients with advanced chronic hepatitis C with a panel of genetic markers (CRS7). Pharmacogenet Genomics. 2011 Dec;21(12):851–60.

50. Lee WM, Dienstag JL, Lindsay KL, Lok AS, Bonkovsky HL, Shiffman ML, et al. Evolution of the HALT-C Trial: pegylated interferon as maintenance therapy for chronic hepatitis C in previous interferon nonresponders. Control Clin Trials. 2004 Oct;25(5):472–92.

51. Di Bisceglie AM, Shiffman ML, Everson GT, Lindsay KL, Everhart JE, Wright EC, et al. Prolonged therapy of advanced chronic hepatitis C with low-dose peginterferon. The New England journal of medicine. 2008 Dec 4;359(23):2429–41.

52. Pearlman BLB, Traub NN. Sustained virologic response to antiviral therapy for chronic hepatitis C virus infection: a cure and so much more. Clin Infect Dis. 2011 Mar 31;52(7):889–900.

53. Lens S, Torres F, Puigvehi M, Mariño Z, Londoño M-C, Martinez SM, et al. Predicting the development of liver cirrhosis by simple modelling in patients with chronic hepatitis C. Alimentary pharmacology \& therapeutics. 2016 Jan 31;43(3):364–74.

54. Lee HJ, Yeon JE, Yoon EL, Suh SJ, Kang K, Kim HR, et al. Long-term follow-up of chronic hepatitis C patients treated with interferon-alpha: risk of cirrhosis and hepatocellular carcinoma in a single center over 10 years. Intervirology. 2015;58(1):14–21.

55. Jakobsen JC, Nielsen EE, Feinberg J, Katakam KK, Fobian K, Hauser G, et al. Direct-acting antivirals for chronic hepatitis C. Cochrane Database Syst Rev. 2017 Jun 6;6:CD012143.

56. Evon DM, Stewart PW, Amador J, Serper M, Lok AS, Sterling RK, et al. A comprehensive assessment of patient reported symptom burden, medical comorbidities, and functional well being in patients initiating direct acting antiviral therapy for chronic hepatitis C: Results from a large US multi-center observational study. PLoS ONE. 2018;13(8):e0196908.

57. Cacoub P, Renou C, Rosenthal E, Cohen P, Loury I, Loustaud-Ratti V, et al. Extrahepatic manifestations associated with hepatitis C virus infection. A

prospective multicenter study of 321 patients. The GERMIVIC. Groupe d"Etude et de Recherche en Medecine Interne et Maladies Infectieuses sur le Virus de l"Hepatite C. Medicine. 2000 Jan;79(1):47–56.

58. Lunel F, Musset L, Cacoub P, Frangeul L, Cresta P, Perrin M, et al. Cryoglobulinemia in chronic liver diseases: role of hepatitis C virus and liver damage. YGAST. 1994 May;106(5):1291–300.

59. de Sanjose S, Benavente Y, Vajdic CM, Engels EA, Morton LM, Bracci PM, et al. Hepatitis C and non-Hodgkin lymphoma among 4784 cases and 6269 controls from the International Lymphoma Epidemiology Consortium. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2008 Apr;6(4):451–8.

60. Giordano TP, Henderson L, Landgren O, Chiao EY, Kramer JR, El-Serag H, et al. Risk of non-Hodgkin lymphoma and lymphoproliferative precursor diseases in US veterans with hepatitis C virus. JAMA : the journal of the American Medical Association. 2007 May 9;297(18):2010–7.

61. Kawamura Y, Ikeda K, Arase Y, Yatsuji H, Sezaki H, Hosaka T, et al. Viral elimination reduces incidence of malignant lymphoma in patients with hepatitis C. Am J Med. 2007 Dec;120(12):1034–41.

62. Gisbert JP, García-Buey L, Pajares JM, Moreno-Otero R. Prevalence of hepatitis C virus infection in porphyria cutanea tarda: systematic review and meta-analysis. Journal of Hepatology. 2003 Oct;39(4):620–7.

63. Maylin S, Peignoux MM, Moucari R, Boyer N, Ripault M-P, Hatem DC, et al. Eradication of Hepatitis C Virus in Patients Successfully Treated for Chronic Hepatitis C. YGAST. 2007 Dec 31;135(3):821–9.

64. Swain MG, Lai MY, Shiffman ML, Cooksley WGE, Zeuzem S, Dieterich DT, et al. A Sustained Virologic Response Is Durable in Patients With Chronic Hepatitis C Treated With Peginterferon Alfa-2a and Ribavirin. YGAST. 2009 Dec 31;139(5):1593–601.

65. Poordad F, McCone J, Bacon BR, Bruno S, Manns MP, Sulkowski MS, et al. Boceprevir for untreated chronic HCV genotype 1 infection. The New England journal of medicine. 2011 Mar 31;364(13):1195–206.

66. Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, Bzowej NH, et al. Telaprevir for previously untreated chronic hepatitis C virus infection. The New England journal of medicine. 2011;364(25):2405–16.

67. Jacobson IM, Dore GJ, Foster GR, Fried MW, Radu M, Rafalsky VV, et al. Simeprevir with pegylated interferon alfa 2a plus ribavirin in treatment-naive patients with chronic hepatitis C virus genotype 1 infection (QUEST-1): a phase 3, randomised, double-blind, placebo-controlled trial. The Lancet. 2014 Aug 1;384(9941):403–13.

68. Manns M, Marcellin P, Poordad F, de Araujo ESA, Buti M, Horsmans Y, et al. Simeprevir with pegylated interferon alfa 2a or 2b plus ribavirin in treatment-naive patients with chronic hepatitis C virus genotype 1 infection (QUEST-2): a randomised, double-blind, placebo-controlled phase 3 trial. Lancet. 2014 Aug 2;384(9941):414–26.

69. Strader DB, Seeff LB. A brief history of the treatment of viral hepatitis C. Davis M D GL, editor. Clinical Liver Disease. 2012 Mar 6;1(1):6–11.

70. Feld JJ, Jacobson IM, Hezode C, Asselah T, Ruane PJ, Gruener N, et al. Sofosbuvir and Velpatasvir for HCV Genotype 1, 2, 4, 5, and 6 Infection. The New England journal of medicine. 2015 Dec 31;373(27):2599–607.

71. Afdhal N, Zeuzem S, Kwo P, Chojkier M, Gitlin N, Puoti M, et al. Ledipasvir and Sofosbuvir for Untreated HCV Genotype 1 Infection. The New England journal of medicine. 2014 May 15;370(20):1889–98.

72. Afdhal N, Reddy KR, Nelson DR, Lawitz E, Gordon SC, Schiff E, et al. Ledipasvir and Sofosbuvir for Previously Treated HCV Genotype 1 Infection. The New England journal of medicine. 2014 Apr 17;370(16):1483–93.

73. Zeuzem S, Ghalib R, Reddy KR, Pockros PJ, Ben Ari Z, Zhao Y, et al. Grazoprevir-Elbasvir Combination Therapy for Treatment-Naive Cirrhotic and Noncirrhotic Patients With Chronic Hepatitis C Virus Genotype 1, 4, or 6 Infection: A Randomized Trial. Annals of Internal Medicine. 2015 Jul 7;163(1):1–13.

74. Rockstroh JK, Nelson M, Katlama C, Lalezari J, Mallolas J, Bloch M, et al. Efficacy and safety of grazoprevir (MK-5172) and elbasvir (MK-8742) in patients with hepatitis C virus and HIV co-infection (C-EDGE CO-INFECTION): a non-randomised, open-label trial. Lancet HIV. 2015 Aug;2(8):e319–27.

75. Sulkowski MS, Gardiner DF, Rodriguez-Torres M, Reddy KR, Hassanein T, Jacobson I, et al. Daclatasvir plus Sofosbuvir for Previously Treated or Untreated Chronic HCV Infection. The New England journal of medicine. 2014 Jan 16;370(3):211–21.

76. Feld JJ, Kowdley KV, Coakley E, Sigal S, Nelson DR, Crawford D, et al. Treatment of HCV with ABT-450/r–Ombitasvir and Dasabuvir with Ribavirin. The New England journal of medicine. 2014 Apr 24;370(17):1594–603.

77. Ferenci P, Bernstein D, Lalezari J, Cohen D, Luo Y, Cooper C, et al. ABT-450/r–Ombitasvir and Dasabuvir with or without Ribavirin for HCV. The New England journal of medicine. 2014 May 22;370(21):1983–92.

78. Zeuzem S, Jacobson IM, Baykal T, Marinho RT, Poordad F, Bourlière M, et al. Retreatment of HCV with ABT-450/r-ombitasvir and dasabuvir with ribavirin. New England Journal of Medicine. 2014 Apr 23;370(17):1604–14.

79. Poordad F, Hezode C, Trinh R, Kowdley KV, Zeuzem S, Agarwal K, et al. ABT-

450/r-ombitasvir and dasabuvir with ribavirin for hepatitis C with cirrhosis. New England Journal of Medicine. 2014 May 21;370(21):1973–82.

80. Foster GR, Afdhal N, Roberts SK, Bräu N, Gane EJ, Pianko S, et al. Sofosbuvir and Velpatasvir for HCV Genotype 2 and 3 Infection. The New England journal of medicine. 2015 Dec 31;373(27):2608–17.

81. Nelson DR, Cooper JN, Lalezari JP, Lawitz E, Pockros PJ, Gitlin N, et al. All-oral 12-week treatment with daclatasvir plus sofosbuvir in patients with hepatitis C virus genotype 3 infection: ALLY-3 phase III study. Hepatology (Baltimore, Md). 2015 Apr;61(4):1127–35.

82. Asselah T, Reesink H, Gerstoft J, de Ledinghen V, Pockros PJ, Robertson M, et al. Efficacy of elbasvir and grazoprevir in participants with hepatitis C virus genotype 4 infection: A pooled analysis. Liver international : official journal of the International Association for the Study of the Liver. 2018 Sep;38(9):1583–91.

83. Asselah T, Kowdley KV, Zadeikis N, Wang S, Hassanein T, Horsmans Y, et al. Efficacy of Glecaprevir/Pibrentasvir for 8 or 12 Weeks in Patients With Hepatitis C Virus Genotype 2, 4, 5, or 6 Infection Without Cirrhosis. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2018 Mar;16(3):417–26.

84. Forns X, Lee SS, Valdes J, Lens S, Ghalib R, Aguilar H, et al. Glecaprevir plus pibrentasvir for chronic hepatitis C virus genotype 1, 2, 4, 5, or 6 infection in adults with compensated cirrhosis (EXPEDITION-1): a single-arm, open-label, multicentre phase 3 trial. The Lancet infectious diseases. 2017 Oct;17(10):1062–8.

85. Haziot A, Chen S, Ferrero E, Low MG, Silber R, Goyert SM. The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. J Immunol. 1988 Jul 15;141(2):547–52.

86. Eisenächer K, Steinberg C, Reindl W, Krug A. The role of viral nucleic acid recognition in dendritic cells for innate and adaptive antiviral immunity. Immunobiology. 2006 Dec 31;212(9-10):701–14.

87. Barton GM, Kagan JC, Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nature immunology. 2005 Dec 31;7(1):49–56.

88. Loo Y-MY, Owen DMD, Li KK, Erickson AKA, Johnson CLC, Fish PMP, et al. Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc Natl Acad Sci U S A. 2006 Apr 10;103(15):6001–6.

89. Li K, Li NL, Wei D, Pfeffer SR, Fan M, Pfeffer LM. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded

RNA intermediates. Hepatology (Baltimore, Md). 2012 Mar;55(3):666–75.

90. Dolganiuc A, Oak S, Kodys K, Golenbock DT, Finberg RW, Kurt-Jones E, et al. Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. YGAST. 2004 Nov;127(5):1513–24.

91. Chang S, Dolganiuc A, Szabo G. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. J Leukoc Biol. 2007 Sep;82(3):479–87.

92. Sato K, Ishikawa T, Okumura A, Yamauchi T, Sato S, Ayada M, et al. Expression of Toll-like receptors in chronic hepatitis C virus infection. Journal of Gastroenterology and Hepatology. 2007 Sep 30;22(10):1627–32.

93. Machida K, Cheng KTH, Sung VMH, Levine AM, Foung S, Lai MMC. Hepatitis C Virus Induces Toll-Like Receptor 4 Expression, Leading to Enhanced Production of Beta Interferon and Interleukin-6. Journal of virology. 2005 Dec 26;80(2):866–74.

94. Zhang Y, El-Far M, Dupuy FP, Abdel-Hakeem MS, He Z, Procopio FA, et al. HCV RNA Activates APCs via TLR7/TLR8 While Virus Selectively Stimulates Macrophages Without Inducing Antiviral Responses. Sci Rep. Nature Publishing Group; 2016 Jun 29;:1–13.

95. Fischer J, Weber ANR, Böhm S, Dickhöfer S, Maadidi El S, Deichsel D, et al. Sex-specific effects of TLR9 promoter variants on spontaneous clearance of HCV infection. Gut. 2017 Oct;66(10):1829–37.

96. Arnaud N, Dabo S, Akazawa D, Fukasawa M, Shinkai-Ouchi F, Hugon J, et al. Hepatitis C virus reveals a novel early control in acute immune response. PLoS pathogens. 2011 Oct;7(10):e1002289.

97. Riordan SM, Skinner NA, Kurtovic J, Locarnini S, McIver CJ, Williams R, et al. Toll-like receptor expression in chronic hepatitis C: correlation with pro-inflammatory cytokine levels and liver injury. Inflamm Res. 2006 Jul;55(7):279–85.

98. Berzsenyi MD, Roberts SK, Preiss S, Woollard DJ, Beard MR, Skinner NA, et al. Hepatic TLR2 & TLR4 expression correlates with hepatic inflammation and TNF-α in HCV & HCV/HIV infection. J Viral Hepat. 2011 Nov 30;18(12):852–60.

99. Wang J-P, Zhang Y, Wei X, Li J, Nan X-P, Yu H-T, et al. Circulating Toll-like receptor (TLR) 2, TLR4, and regulatory T cells in patients with chronic hepatitis C. APMIS. 2010 Apr;118(4):261–70.

100. Shehata MAH, El-Enein AA, El-Sharnouby GA. Significance of toll-like receptors 2 and 4 mRNA expression in chronic hepatitis C virus infection. Egypt J Immunol. 2005 Dec 31;13(1):141–52.

101. Dolganiuc A, Garcia C, Kodys K, Szabo G. Distinct Toll-like receptor expression in monocytes and T cells in chronic HCV infection. World Journal of Gastroenterology. 2006 Feb 28;12(8):1198–204.

102. He Q, Graham CS, Mangoni ED, Koziel MJ. Differential expression of toll-like receptor mRNA in treatment non-responders and sustained virologic responders at baseline in patients with chronic hepatitis C. Liver International. 2006 Oct 31;26(9):1100–10.

103. Villacres MC, Literat O, DeGiacomo M, Du W, Frederick T, Kovacs A. Defective response to Toll-like receptor 3 and 4 ligands by activated monocytes in chronic hepatitis C virus infection. J Viral Hepat. 2008 Jan 31;15(2):137–44.

104. Bigger CB, Brasky KM, Lanford RE. DNA Microarray Analysis of Chimpanzee Liver during Acute Resolving Hepatitis C Virus Infection. Journal of virology. 2001 Aug 1;75(15):7059–66.

105. Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR, Luxon BA, et al. Intrahepatic Gene Expression during Chronic Hepatitis C Virus Infection in Chimpanzees. Journal of virology. 2004 Nov 24;78(24):13779–92.

106. Lau DT-Y, Fish PM, Sinha M, Owen DM, Lemon SM, Gale M. Interferon regulatory factor-3 activation, hepatic interferon-stimulated gene expression, and immune cell infiltration in hepatitis C virus patients. Hepatology (Baltimore, Md). 2008 Feb 29;47(3):799–809.

107. Brodsky LI, Wahed AS, Li J, Tavis JE, Tsukahara T, Taylor MW. A novel unsupervised method to identify genes important in the anti-viral response: application to interferon/ribavirin in hepatitis C patients. PLoS ONE. 2007;2(7):e584.

108. Ishibashi M, Wakita T, Esumi M. 2’,5’-Oligoadenylate synthetase-like gene highly induced by hepatitis C virus infection in human liver is inhibitory to viral replicationin vitro. Biochem Biophys Res Commun. 2009 Dec 31;392(3):397–402.

109. Arnaud N, Dabo S, Maillard P, Budkowska A, Kalliampakou KI, Mavromara P, et al. Hepatitis C virus controls interferon production through PKR activation. PLoS ONE. 2009 Dec 31;5(5):e10575–5.

110. Wang C, Pflugheber J, Sumpter R, Sodora DL, Hui D, Sen GC, et al. Alpha Interferon Induces Distinct Translational Control Programs To Suppress Hepatitis C Virus RNA Replication. Journal of virology. 2003 Apr 1;77(7):3898–912.

111. Taylor DR, Puig M, Darnell MER, Mihalik K, Feinstone SM. New Antiviral Pathway That Mediates Hepatitis C Virus Replicon Interferon Sensitivity through ADAR1. Journal of virology. 2005 Apr 27;79(10):6291–8.

112. Helbig KJ, Lau DT-Y, Semendric L, Harley HAJ, Beard MR. Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector. Hepatology (Baltimore, Md). 2005 Aug 31;42(3):702–10.

113. Helbig KJ, Eyre NS, Yip E, Narayana S, Li K, Fiches G, et al. The antiviral protein viperin inhibits hepatitis C virus replication via interaction with nonstructural protein 5A. Hepatology. 2011 Oct 28;54(5):1506–17.

114. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature. 2011 Apr 28;472(7344):481–5.

115. Thomas E, Gonzalez VD, Li Q, Modi AA, Chen W, Noureddin M, et al. HCV Infection Induces a Unique Hepatic Innate Immune Response Associated With Robust Production of Type III Interferons. Gastroenterology. 2012;142(4):978–88.

116. Marukian S, Andrus L, Sheahan TP, Jones CT, Charles ED, Ploss A, et al. Hepatitis C virus induces interferon-lambda and interferon-stimulated genes in primary liver cultures. Hepatology (Baltimore, Md) [Internet]. 2011 Nov 30;54(6):1913–23. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3219820\&tool=pmcentrez\&rendertype=abstract

117. Park H, Serti E, Eke O, Muchmore B, Prokunina-olsson L, Capone S, et al. IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. Hepatology (Baltimore, Md). 2012 Dec;56(6):2060–70.

118. Sarasin-Filipowicz M, Oakeley EJ, Duong FHT, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(19):7034–9.

119. O'Brien TR, Pfeiffer RM, Paquin A, Lang Kuhs KA, Chen S, Bonkovsky HL, et al. Comparison of functional variants in IFNL4 and IFNL3 for association with HCV clearance. Journal of Hepatology. 2015 Nov;63(5):1103–10.

120. Urban TJ, Thompson AJ, Bradrick SS, Fellay J, Schuppan D, Cronin KD, et al. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. Hepatology (Baltimore, Md). 2010;52(6):1888–96.

121. Dill MT, Duong FHT, Vogt JE, Bibert SEP, Bochud P-Y, Terracciano L, et al. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. Gastroenterology. Elsevier Inc; 2011;140(3):1021–31.

122. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, et al.

Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology. Elsevier Inc; 2010;139(2):499–509.

123. Naggie S, Osinusi A, Katsounas A, Lempicki R, Herrmann E, Thompson AJ, et al. Dysregulation of innate immunity in hepatitis C virus genotype 1 IL28B-unfavorable genotype patients: impaired viral kinetics and therapeutic response. Hepatology (Baltimore, Md). 2012;56(2):444–54.

124. Chen L, Borozan I, Sun J, Guindi M, Fischer S, Feld J, et al. Cell-Type Specific Gene Expression Signature in Liver Underlies Response to Interferon Therapy in Chronic Hepatitis C Infection. YGAST. Elsevier Inc; 2010 Mar 1;138(3):1123–3.

125. Meissner EG, Wu D, Osinusi A, Bon D, Virtaneva K, Sturdevant D, et al. Endogenous intrahepatic IFNs and association with IFN-free HCV treatment outcome. The Journal of clinical investigation. 2014 Jul 1;124(8):3352–63.

126. Alao H, Cam M, Keembiyehetty C, Zhang F, Serti E, Suarez D, et al. Baseline Intrahepatic and Peripheral Innate Immunity are Associated with Hepatitis C Virus Clearance During DAA Therapy. Hepatology (Baltimore, Md). 2018 Apr 27.

127. Neuman MG, Benhamou J-P, Malkiewicz IM, Akremi R, Shear NH, Asselah T, et al. Cytokines as predictors for sustained response and as markers for immunomodulation in patients with chronic hepatitis C. Clinical Biochemistry. 2001 May;34(3):173–82.

128. Neuman MG, Benhamou JP, Malkiewicz IM, Ibrahim A, Valla DC, Martinot-Peignoux M, et al. Kinetics of serum cytokines reflect changes in the severity of chronic hepatitis C presenting minimal fibrosis. J Viral Hepat. 2002 Feb 28;9(2):134–40.

129. Oyanagi Y, Takahashi T, Matsui S, Takahashi S, Boku S, Takahashi K, et al. Enhanced expression of interleukin-6 in chronic hepatitis C. Liver International. 1999 Nov 30;19(6):464–72.

130. Mihm U, Herrmann E, Sarrazin U, Wagner von M, Kronenberger B, Zeuzem S, et al. Association of serum interleukin-8 with virologic response to antiviral therapy in patients with chronic hepatitis C. Journal of Hepatology. 2004 Apr 30;40(5):845–52.

131. Nelson DR, Lim HL, Marousis CG, Fang JW, Davis GL, Shen L, et al. Activation of tumor necrosis factor-alpha system in chronic hepatitis C virus infection. Digestive diseases and sciences. 1997 Dec;42(12):2487–94.

132. Polyak SJ, Khabar KSA, Rezeiq M, Gretch DR. Elevated Levels of Interleukin-8 in Serum Are Associated with Hepatitis C Virus Infection and Resistance to Interferon Therapy. Journal of virology. 2001 Jul 1;75(13):6209–11.

133. Yoneda S, Umemura T, Katsuyama Y, Kamijo A, Joshita S, Komatsu M, et al. Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis C patients. The Journal of infectious diseases. 2011 Apr 15;203(8):1087–95.

134. de Souza-Cruz S, Victória MB, Tarragô AM, da Costa AG, Pimentel JPD, Pires EF, et al. Liver and blood cytokine microenvironment in HCV patients is associated to liver fibrosis score: a proinflammatory cytokine ensemble orchestrated by TNF and tuned by IL-10. BMC Microbiol. 2016 Jan 7;16:3.

135. Darling JM, Aerssens J, Fanning G. Quantitation of pretreatment serum interferon-gamma–inducible protein-10 improves the predictive value of an IL28B gene polymorphism for hepatitis C treatment response. Hepatology (Baltimore, Md). 2011;53(1):14–21.

136. Riva A, Laird M, Casrouge A, Ambrozaitis A, Williams R, Naoumov NV, et al. Truncated CXCL10 is associated with failure to achieve spontaneous clearance of acute hepatitis C infection. Hepatology (Baltimore, Md). 2014 Aug;60(2):487–96.

137. Dolganiuc A, Chang S, Kodys K, Mandrekar P, Bakis G, Cormier M, et al. Hepatitis C virus (HCV) core protein-induced, monocyte-mediated mechanisms of reduced IFN-alpha and plasmacytoid dendritic cell loss in chronic HCV infection. J Immunol. 2006 Nov 15;177(10):6758–68.

138. Dolganiuc A, Kodys K, Kopasz A, Marshall C, Do T, Romics L, et al. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. J Immunol. 2003 May 31;170(11):5615–24.

139. Shiina M, Rehermann B. Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function. Hepatology (Baltimore, Md). 2008 Feb;47(2):385–95.

140. Polyak SJ, Khabar KSA, Paschal DM, Ezelle HJ, Duverlie G, Barber GN, et al. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. Journal of virology. 2001 Jun 30;75(13):6095–106.

141. Khakoo SI. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science. 2004;305:872.

142. Paladino N, Flores AC, Marcos CY, Fainboim H, Theiler G, Arruvito L, et al. Increased frequencies of activating natural killer receptors are associated with liver injury in individuals who do not eliminate hepatitis C virus. Tissue Antigens. Blackwell Publishing Ltd; 2007 Apr;69(Suppl 1):109–11.

143. Ahlenstiel G, Titerence RH, Koh C, Edlich B, Feld JJ, Rotman Y, et al. Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an

interferon-alfa-dependent manner. Gastroenterology. Elsevier Inc; 2010;138(1):325–35.e1–2.

144. Ahlenstiel G, Edlich B, Hogdal LJ, Rotman Y, Noureddin M, Feld JJ, et al. Early changes in natural killer cell function indicate virologic response to interferon therapy for hepatitis C. Gastroenterology. Elsevier Inc; 2011;141(4):1231–9, 1239.e1–2.

145. Golden-Mason L, Bambha KM, Cheng L, Howell CD, Taylor MW, Clark PJ, et al. Natural killer inhibitory receptor expression associated with treatment failure and interleukin-28B genotype in patients with chronic hepatitis C. Hepatology (Baltimore, Md). 2011;54(5):1559–69.

146. Serti E, Chepa-Lotrea X, Kim YJ, Keane M, Fryzek N, Liang TJ, et al. Successful Interferon-Free Therapy of Chronic Hepatitis C Virus Infection Normalizes Natural Killer Cell Function. YGAST. Elsevier, Inc; 2015 Jul 1;149(1):190–2.

147. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009;461(7262):399–401.

148. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nature genetics. 2009;41(10):1105–9.

149. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nature genetics. 2009;41(10):1100–4.

150. Rauch A, Kutalik ZAN, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology. Elsevier Inc; 2010;138(4):1338–45, 1345.e1–7.

151. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 2009;461(7265):798–801.

152. Tillmann HL, Thompson AJ, Patel K, Wiese M, Tenckhoff H, Nischalke HD, et al. A polymorphism near IL28B is associated with spontaneous clearance of acute hepatitis C virus and jaundice. Gastroenterology. Elsevier Inc; 2010;139(5):1586–92, 1592.e1.

153. Conjeevaram HS, Fried MW, Jeffers LJ, Terrault NA, Wiley-Lucas TE, Afdhal N, et al. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. Gastroenterology. 2006;131(2):470–7.

154. Muir AJ, Bornstein JD, Killenberg PG. Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. The New England journal of medicine. 2004;350(22):2265–71.

155. McHutchison JG, Lawitz EJ, Shiffman ML, Muir AJ, Galler GW, McCone J, et al. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. The New England journal of medicine. 2009;361(6):580–93.

156. Dev AT, McCaw R, Sundararajan V, Bowden S, Sievert W. Southeast Asian patients with chronic hepatitis C: the impact of novel genotypes and race on treatment outcome. Hepatology (Baltimore, Md). 2002;36(5):1259–65.

157. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nature immunology. 2003;4(1):69–77.

158. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nature immunology. 2003;4(1):63–8.

159. Witte K, Gruetz G, Volk H-D, Looman AC, Asadullah K, Sterry W, et al. Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines. Genes and immunity. 2009;10(8):702–14.

160. Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology. 2006;131(6):1887–98.

161. Robek MD, Boyd BS, Chisari FV. Lambda Interferon Inhibits Hepatitis B and C Virus Replication. Society. 2005;79(6):1–5.

162. Muir AJ, Shiffman ML, Zaman A, Yoffe B, la Torre de A, Flamm S, et al. Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. Hepatology (Baltimore, Md). 2010;52(3):822–32.

163. McGilvray I, Feld JJ, Chen L, Pattullo V, Guindi M, Fischer S, et al. Hepatic Cell-Specific Gene Expression Better Predicts HCV Treatment Outcome than IL28B Genotype. Gastroenterology. AGA Institute American Gastroenterological Association; 2012.

164. Prokunina-olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nature genetics. Nature Publishing Group; 2013;45(2):164–71.

165. Hamming OJ, ska-Dyla ETN, Vieyres G, Dijkman R, rgensen SEJO, Akhtar H, et al. Interferon lambda 4 signals via the IFN. EMBO J. Nature Publishing Group; 2013 Oct 29;32(23):3055–65.

166. Pawlotsky JM. Diagnostic tests for hepatitis C. Journal of Hepatology. 1999;31 Suppl 1:71–9.

167. Christie JM, Healey CJ, Watson J, Wong VS, Duddridge M, Snowden N, et al. Clinical outcome of hypogammaglobulinaemic patients following outbreak of acute hepatitis C: 2 year follow up. Clinical and experimental immunology. 1997 Sep 16;110(1):4–8.

168. Logvinoff C, Major ME, Oldach D, Heyward S, Talal A, Balfe P, et al. Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(27):10149–54.

169. Zibert A, Meisel H, Kraas W, Schulz A, Jung G, Roggendorf M. Early antibody response against hypervariable region 1 is associated with acute self-limiting infections of hepatitis C virus. Hepatology. 1997 May;25(5):1245–9.

170. Dittmann S, Roggendorf M, Dürkop J, Wiese M, Lorbeer B, Deinhardt F. Long-term persistence of hepatitis C virus antibodies in a single source outbreak. Journal of Hepatology. 1991 Nov;13(3):323–7.

171. Pestka JM, Zeisel MB, Bl a ser E, Sch u rmann P, Bartosch B, Cosset F-LIC, et al. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(14):6025–30.

172. Kaplan DE, Sugimoto K, Newton K, Valiga ME, Ikeda F, Aytaman A, et al. Discordant role of CD4 T-cell response relative to neutralizing antibody and CD8 T-cell responses in acute hepatitis C. YGAST. 2007 Feb;132(2):654–66.

173. Lavillette D, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, et al. Human Serum Facilitates Hepatitis C Virus Infection, and Neutralizing Responses Inversely Correlate with Viral Replication Kinetics at the Acute Phase of Hepatitis C Virus Infection. Journal of virology. 2005 Apr 27;79(10):6023–34.

174. Dowd KA, Netski DM, Wang X-H, Cox AL, Ray SC. Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. Gastroenterology. AGA Institute American Gastroenterological Association; 2009;136(7):2377–86.

175. Meunier J-C, Engle RE, Faulk K, Zhao M, Bartosch B, Alter H, et al. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. Proc Natl Acad Sci U S A. 2005 Mar 22;102(12):4560–5.

176. Osburn WO, Snider AE, Wells BL, Latanich R, Bailey JR, Thomas DL, et al. Clearance of hepatitis C infection is associated with the early appearance of broad neutralizing antibody responses. Hepatology (Baltimore, Md). 2014 Jun;59(6):2140–51.

177. Keck Z-Y, Li SH, Xia J, Hahn von T, Balfe P, McKeating JA, et al. Mutations in hepatitis C virus E2 located outside the CD81 binding sites lead to escape from broadly neutralizing antibodies but compromise virus infectivity. Journal of virology. 2009 Jun;83(12):6149–60.

178. Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. The Journal of experimental medicine. 2001 Nov 19;194(10):1395–406.

179. Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, et al. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. Proc Natl Acad Sci U S A. 2002 Nov 26;99(24):15661–8.

180. Missale G, Bertoni R, Lamonaca V, Valli A, Massari M, Mori C, et al. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. The Journal of clinical investigation. 1996;98(3):706–14.

181. Tsai SL, Liaw YF, Chen MH, Huang CY, Kuo GC. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. Hepatology. 1997 Feb;25(2):449–58.

182. Takaki A, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, et al. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med. 2000 May;6(5):578–82.

183. Day CL, Lauer GM, Robbins GK, McGovern B, Wurcel AG, Gandhi RT, et al. Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection. Journal of virology. 2002 Dec;76(24):12584–95.

184. Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, et al. Analysis of successful immune responses in persons infected with hepatitis C virus. The Journal of experimental medicine. 2000;191(9):1499–512.

185. Grüner NH, Gerlach TJ, Jung MC, Diepolder HM, Schirren CA, Schraut WW, et al. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. J Infect Dis. 2000 May;181(5):1528–36.

186. Urbani S, Amadei B, Fisicaro P, Tola D, Orlandini A, Sacchelli L, et al. Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. Hepatology. 2006 Jul;44(1):126–39.

187. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. Journal of virology. 2003 Apr;77(8):4911–27.

188. Li K, Foy E, Ferreon JC, Nakamura M, Ferreon ACM, Ikeda M, et al. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(8):2992–7.

189. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature. 2005;437(7062):1167–72.

190. Otsuka M, Kato N, Moriyama M, Taniguchi H, Wang Y, Dharel N, et al. Interaction between the HCV NS3 protein and the host TBK1 protein leads to inhibition of cellular antiviral responses. Hepatology. 2005 May;41(5):1004–12.

191. Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, et al. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J. 2003 Mar;17(3):488–90.

192. Lin W, Kim SS, Yeung E, Kamegaya Y, Blackard JT, Kim KA, et al. Hepatitis C Virus Core Protein Blocks Interferon Signaling by Interaction with the STAT1 SH2 Domain. Journal of virology. 2006 Aug 29;80(18):9226–35.

193. Heim MH, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. Journal of virology. 1999 Oct;73(10):8469–75.

194. Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, et al. Expression of hepatitis c virus proteins inhibits interferon α signaling in the liver of transgenic mice. YGAST. 2002 Dec 31;124(5):1465–75.

195. Melén K, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. Journal of Medical Virology. 2004 Jul 31;73(4):536–47.

196. Duong FH, Filipowicz M, Tripodi M, La Monica N, Heim MH. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. YGAST. 2003 Dec 31;126(1):263–77.

197. de Lucas S, Bartolome J, Carreno V. Hepatitis C virus core protein down-regulates transcription of interferon-induced antiviral genes. J Infect Dis. 2005 Jan 1;191(1):93–9.

198. Taguchi T. Hepatitis C virus NS5A protein interacts with 2“,5-”oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent manner. The Journal of general virology.

2004 Apr 1;85(4):959–69.

199. Gale MJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, et al. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology. 1997 Apr 14;230(2):217–27.

200. Pflugheber J, Fredericksen B, Sumpter R, Wang C, Ware F, Sodora DL, et al. Regulation of PKR and IRF-1 during hepatitis C virus RNA replication. Proc Natl Acad Sci U S A. 2002 Apr 2;99(7):4650–5.

201. Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science. 1999 Jul 2;285(5424):107–10.

202. Xagorari A, Chlichlia K. Toll-like receptors and viruses: induction of innate antiviral immune responses. Open Microbiol J. 2008;2:49–59.

203. Chen Yi Mei SLG, Burchell J, Skinner N, Millen R, Matthews G, Hellard M, et al. Toll-like Receptor Expression and Signaling in Peripheral Blood Mononuclear Cells Correlate With Clinical Outcomes in Acute Hepatitis C Virus Infection. The Journal of infectious diseases. 2016 Jun 9;214(5):739–47.

204. Lau DT-Y, Negash A, Chen J, Crochet N, Sinha M, Zhang Y, et al. Innate Immune Tolerance and the Role of Kupffer Cells in Differential Responses to Interferon Therapy Among Patients With HCV Genotype 1 Infection. Gastroenterology. 2013 Feb;144(2):402–12.

205. Bergmann JF, de Bruijne J, Hotho DM, de Knegt RJ, Boonstra A, Weegink CJ, et al. Randomised clinical trial: anti-viral activity of ANA773, an oral inducer of endogenous interferons acting via TLR7, in chronic HCV. Alimentary pharmacology \& therapeutics. 2011 Aug;34(4):443–53.

206. Holmes JA, Nguyen T, Ratnam D, Heerasing NM, Tehan JV, Bonanzinga S, et al. IL28Bgenotype is not useful for predicting treatment outcome in Asian chronic hepatitis B patients treated with pegylated interferon-α. Journal of Gastroenterology and Hepatology. 2013 Apr 25;28(5):861–6.

207. Hernández JC, Stevenson M, Latz E, Urcuqui-Inchima S. HIV Type 1 Infection Up-Regulates TLR2 and TLR4 Expression and Function in Vivoand in Vitro. AIDS Research and Human Retroviruses. 2012 Oct;28(10):1313–28.

208. Ma Z, Cao Q, Xiong Y, Zhang E, Lu M. Interaction between Hepatitis B Virus and Toll-Like Receptors: Current Status and Potential Therapeutic Use for Chronic Hepatitis B. Vaccines. 2018 Mar;6(1):6.

209. Gilead S. Safety, Tolerability and Antiviral Activity of GS-9688 in Virally-Suppressed Adults With Chronic Hepatitis B [Internet]. University of Mayland, Institute of Human Virology, Baltimore, Maryland, United States|Auckland

Clinical Studies Limited, Auckland, New Zealand; 2018. Available from: https://ClinicalTrials.gov/show/NCT03491553

210. Janssen HLA, Brunetto MR, Kim YJ, Ferrari C, Massetto B, Nguyen A-H, et al. Safety, efficacy and pharmacodynamics of vesatolimod (GS-9620) in virally suppressed patients with chronic hepatitis B. Journal of Hepatology. 2018 Mar;68(3):431–40.

211. Askarieh G, Alsio A, Pugnale P, Negro F, Ferrari C, Neumann AU, et al. Systemic and Intrahepatic Interferon-Gamma-Inducible Protein 10 kDa Predicts the First-Phase Decline in Hepatitis C Virus RNA and Overall Viral Response to Therapy in Chronic Hepatitis C. Hepatology (Baltimore, Md). 2010;51:1523–30.

212. Serti E, Park H, Keane M, O'Keefe AC, Rivera E, Liang TJ, et al. Rapid decrease in hepatitis C viremia by direct acting antivirals improves the natural killer cell response to IFNα. Gut. 2017 Apr;66(4):724–35.

213. Golden-Mason L, Mcmahan RH, Kriss MS, Kilgore AL, Cheng L, Dran RJ, et al. Early and late changes in natural killer cells in response to ledipasvir/sofosbuvir treatment. Hepatol Commun. 2018 Apr;2(4):364–75.

214. Wang X-X, Luo B-F, Jiang H-J, Cong X, Jin Q, Ma D-L, et al. Recovery of natural killer cells is mainly in post-treatment period in chronic hepatitis C patients treated with sofosbuvir plus ledipasvir. World J Gastroenterol. 2018 Oct 28;24(40):4554–64.

215. Spaan M, van Oord G, Kreefft K, Hou J, Hansen BE, Janssen HLA, et al. Immunological Analysis During Interferon-Free Therapy for Chronic Hepatitis C Virus Infection Reveals Modulation of the Natural Killer Cell Compartment. The Journal of infectious diseases. 2015 Jul 28.

216. Carlton-Smith C, Holmes JA, Naggie S, Lidofsky A, Lauer GM, Kim AY, et al. IFN-free therapy is associated with restoration of type I IFN response in HIV-1 patients with acute HCV infection who achieve SVR. J Viral Hepat. 2018 May;25(5):465–72.

217. Asselah TT, Bieche II, Narguet SS, Sabbagh AA, Laurendeau II, Ripault M-PM, et al. Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C. Audio, Transactions of the IRE Professional Group on. 2008 Mar 31;57(4):516–24.

218. Chen L, Borozan I, Feld J, Sun J, Tannis L-L, Coltescu C, et al. Hepatic Gene Expression Discriminates Responders and Nonresponders in Treatment of Chronic Hepatitis C Viral Infection. Gastroenterology. 2005;128(5):1437–44.

219. Feld JJJ, Nanda SS, Huang YY, Chen WW, Cam MM, Pusek SNS, et al. Hepatic gene expression during treatment with peginterferon and ribavirin: Identifying molecular pathways for treatment response. Hepatology. 2007 Oct 31;46(5):1548–63.

220. Burchill MA, Golden Mason L, Wind-Rotolo M, Rosen HR. Memory re-differentiation and reduced lymphocyte activation in chronic HCV-infected patients receiving direct-acting antivirals. J Viral Hepat. 2015 Dec;22(12):983–91.

221. Rehermann B. Hepatitis C virus versus innate and adaptive immune responses : a tale of coevolution and coexistence. The Journal of clinical investigation. 2009;119(7):1745–54.

222. Taylor MW, Tsukahara T, Brodsky L, Schaley J, Sanda C, Stephens MJ, et al. Changes in gene expression during pegylated interferon and ribavirin therapy of chronic hepatitis C virus distinguish responders from nonresponders to antiviral therapy. Journal of virology. 2007;81(7):3391–401.

223. Liang Y, Ishida H, Lenz O, Lin T-I, Nyanguile O, Simmen K, et al. Antiviral suppression vs restoration of RIG-I signaling by hepatitis C protease and polymerase inhibitors. Gastroenterology. 2008 Nov;135(5):1710–2.

224. Rao H-Y, Li H, Chen H, Shang J, Xie Q, Gao Z-L, et al. Real-world treatment patterns and clinical outcomes of HCV treatment-naive patients in China: an interim analysis from the CCgenos study. Journal of Gastroenterology and Hepatology. 2017 Jan 4;32(1):244–52.

225. Cordero-Ruiz P, Carmona-Soria I, Rodríguez-Téllez M, Caunedo-Alvarez A, Quezada-Pacheco RH, Flores-Cucho A, et al. Long-term follow-up of patients with chronic hepatitis C treated with α-interferon and ribavirin antiviral therapy: clinical and fibrosis impact of treatment response. European journal of gastroenterology \& hepatology. 2017 Jul;29(7):792–9.

226. Hellard M, Rolls DA, Sacks-Davis R, Robins G, Pattison P, Higgs P, et al. The impact of injecting networks on hepatitis C transmission and treatment in people who inject drugs. Hepatology (Baltimore, Md). 2014 Dec;60(6):1861–70.

227. Younossi Z, Papatheodoridis G, Cacoub P, Negro F, Wedemeyer H, Henry L, et al. The comprehensive outcomes of hepatitis C virus infection: A multi-faceted chronic disease. J Viral Hepat. 2018 Nov 6;25(1 Suppl):6–14.

228. ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000 Feb 29 - . Identifier NCT01436357, Staged Phase I/II Hepatitis C Prophylactic Vaccine; 2011 Sept 19. Available from: https://clinicaltrials.gov/ct2/show/NCT01436357

229. Organisation WH. COMBATING HEPATITIS B AND C TO REACH ELIMINATIONBY 2030. World Health Organization. 2016 May 11;:1–24.

230. Hernández JC, Arteaga J, Paul S, Kumar A, Latz E, Urcuqui-Inchima S. Up-regulation of TLR2 and TLR4 in dendritic cells in response to HIV type 1 and coinfection with opportunistic pathogens. AIDS Research and Human

Retroviruses. 2011 Sep 30;27(10):1099–109.

231. Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, Tripp RA. Involvement of Toll-Like Receptor 4 in Innate Immunity to Respiratory Syncytial Virus. Journal of virology. 2001 Nov 15;75(22):10730–7.

Appendices

Appendix A - ABBREVIATIONS

CBA	Cytometric bead array
cDNA	Complimentary DNA
CLDN	Claudin
CHC	Chronic HCV infection
DAA	Direct-acting antivirals
DMSO	Dimethyl sulfoxide
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
EOT	End of treatment
FACS	Fluorescence-activated cell sorting for flow cytometry
FCS	Fetal calf serum
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B infection
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCV-1	Hepatitis C virus Genotype 1
HCV-2	Hepatitis C virus Genotype 2
HCV-3	Hepatitis C virus Genotype 3
HCV-4	Hepatitis C virus Genotype 4
HCV-6	Hepatitis C virus Genotype 6
HIV	Human immunodeficiency virus

HLA-DR	Human Leukocyte Antigen – DR isotype
IFNL3	Interferon-λ-3
IFLN4	Interferon-λ-4
IFN	Interferon
IFNRF	Interferon regulatory factor
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IP-10	Interferon-γ-induced protein 10
IRES	Internal ribosomal entry site
ISG	Interferon stimulated gene
JAK	Janus kinase
KIR	Killer immunoglobulin-like receptor
kPa	Kilopascals
LDL	Low density lipoprotein
LOWESS	Locally weighted scatterplot smoothing
LSM	Liver stiffness measurement
MAVS	mitochondrial antiviral signaling protein
MCP-1	Monocyte chemo-attractant protein-1
mRNA	Messenger RNA
NF-κB	Nuclear factor κB
NI	Nucleoside/nucleotide analogue inhibitors
NK	Natural Killer

NNI	Non-nucleoside analogue inhibitors
OAS	Oligoadenylate synthetase
PAMP	Pattern- associated molecular patterns
PBS	Phosphate buffered saline
pDCs	Plasmacytoid dendritic cell
PegIFN	Pegylated interferon-α
PCR	Polymerase chain reaction
PBMCs	Peripheral blood mononuclear cells
PKR	Protein kinase R
PR	Pegylated interferon-α + Ribavirin
PRoD	Ombitsavir, ritonavir boosted paritaprevir, dasabuvir (250mg)
PRR	Pattern Recognition Receptors
RIG-I	Retinoic acid induced gene-I
RBV	Ribavirin
SC-B1	Scavenger receptor class B1
SNPs	Single nucleotide polypmorphisms
SOCS1	Suppressor of cytokine signaling-1
STAT	Signal transducers and activators of transcription
SVR	Sustained virological response
Th1	T helper 1
Th2	T helper 2
TIM3	T cell immunoglobulin mucin 3
TLR	Toll-like receptor
TNF-α	Tissue necrosis factor-alpha
T/PR	Telaprevir / Pegylated interferon-α + Ribavirin

TRAIL	TNF-related apoptosis-inducing ligand
UTR	Untranslated region