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DEVELOPMENT OF VACCINES AND MOUSE MODELS FOR CHRONIC HEPATITIS C VIRUS INFECTION

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To My Family

ABSTRACT

Chronic hepatitis C virus (HCV) infection is a major causative agent for severe liver disease and cancer worldwide. Globally, it is estimated that approximately 185 million people are infected with HCV and 130-170 million of these are chronic carriers of the virus (1, 2). The HCV infection is one of the major causes of liver disease and the infection is characterized by a slow and silent progression. Patients infected with HCV have an increased risk of developing fibrosis, cirrhosis and hepatocellular carcinoma. Importantly, this infection is today considered curable in the majority of individuals receiving the recently introduced direct-acting antiviral (DAA) therapy (3-5). Therefore the urgency for a HCV vaccine has reduced. However, only 10% of all chronic HCV carriers receive any treatment due to high cost of treatment and the majority of the chronic carriers live in resource-poor countries. Hence, there is still an urgent need to prevent the spread of HCV through a vaccine. Today, no prophylactic or therapeutic vaccines are available. However, numerous vaccines have been developed and tested for efficacy in clinical trials (6-9). A few HCV vaccines are currently being tested for both protective and therapeutic effects (10). A key feature of patients with chronic HCV is their lack of functional T cell responses to HCV (11-13). Interestingly, a recent study showed that DAA induced cure of HCV rapidly restores at least partly HCV-specific T cell responses. However, these responses do not seem to protect against reinfection (14, 15). Thus, it may be of importance to broaden the post-cure T cell responses through vaccination to reduce the risk of reinfection. This will save significant costs and reduce HCV associated morbidity and mortality.

In this thesis we have evaluated our in-house therapeutic DNA vaccine in 12 patients with chronic HCV infection. The phase I clinical trial was performed in treatment naive HCV genotype 1 patients, receiving four monthly vaccinations in the deltoid muscles with 167, 500, or 1,500 μg codon-optimized HCV nonstructural (NS) 3/4A-expressing DNA vaccine delivered by *in vivo* electroporation (EP). This first-in-man therapeutic HCV DNA vaccine study with a DNA vaccine delivered by *in vivo* EP showed a good tolerability and safety with no severe adverse events. In addition, a transient immune activation and transient reductions in serum HCV RNA levels was seen in a few patients at the time of the vaccinations. Thus, DNA-based vaccines may be explored as a therapeutic or prophylactic tool in hepatitis C.

There are many ways to make a DNA vaccine more potent to get a good effect and fully utilize the vaccines effect. One approach is to use molecular adjuvants to obtain the most potent immune modulatory effect of a DNA vaccination. Other methods to increase the immunogenicity of the DNA vaccine can also be to make the delivery method more effective, like EP and *in vivo* intracellular injection (IVIN) device. The importance here is to be able to activate and reactivate the dysfunctional T-cells. Therefore have we made our DNA vaccine more potent where we have included a new molecular adjuvant for genetic vaccines based on sequences from the non-human stork hepatitis B virus core genes. It has previously been shown that HBcAg can act as an adjuvant and can be expressed as a recombinant protein. We here used avian stork HBcAg because avian HBcAg and human HBcAg only share around 40% sequence homology. Full-length and fragmented stork HBcAg gene-sequences were added to an HCV non-structural (NS) 3/4A gene (NS3/4A-stork-HBcAg). This addition

enhanced priming of HCV-specific IFN-γ and IL-2 responses in wild-type (wt)- and NS3/4A-transgenic (Tg) mice, the latter with dysfunctional NS3/4A-specific T cells. In addition, the NS3/4A-stork-HBcAg vaccine also primed NS3/4A-specific T cells in human hepatitis B e antigen (HBeAg)-Tg mice with dysfunctional T cells to HBcAg and HBeAg. We also found that repeated immunizations boosted expansion of IFN-γ and IL-2-producing NS3/4A-specific T cells in wt- and NS3/4A-Tg mice. Importantly, NS3/4A-stork-HBcAg-DNA induced *in vivo* long-term functional memory T cell responses, whose maintenance required CD4+ T cells.

To study therapeutic vaccines and therapies for HCV there is a need for a simple small animal model. Currently there is no immuno-competent small animal model supporting HCV RNA replication, which has hampered studies of HCV-specific immune responses. We therefore aimed at developing an immuno-competent mouse model that allows *in vivo* growth of a syngeneic mouse hepatoma cell line harboring an autonomously replicating sub genomic HCV replicon of the genotype (gt) 2 JFH1 isolate (16). In this tumor model we can by different approaches study the functional immunity to HCV. By challenging the mice with the hepatoma cells we can measure the tumor growth and compare the volumes between vaccinated and naïve mice. Even though no virus particles are generated in this model, we have been able to show presence of HCV RNA through quantitative PCR and by *in situ* hybridization. To characterize a protective T cell response we first mapped cytotoxic T lymphocyte (CTL) epitopes within HCV NS3/4A gt2a. Using these epitopes we were able to quantify the number HCV-specific CTLs post immunization. We found that a NS3/4A-gt2a-based DNA vaccination protects against tumor growth, although this was dependent on an optimal vaccination. Importantly, challenge of naive or vaccinated mice with the HCV replicon resulted in a poor activation, or boosting of HCV-specific T cells. In contrast, challenge with a stably NS3/4A-expressing hepatoma cells resulted in a potent T cell activation and boosting in naive and vaccinated mice, respectively. Thus, the presence of HCV RNA replication seems to impair immunogenicity of HCV antigens. This mimics the human infection.

In conclusion, we have in a phase I clinical trial shown that DNA vaccine can be immunogenic in humans. However, the study also suggested that the immunogenicity needed improvement. We therefore developed new molecular adjuvants that greatly improved immunogenicity in a host with a dysfunctional immune response to HCV. Finally, we developed a new mouse model with replication of a subgenomic HCV RNA replicon. This model should be useful for evaluation of new HCV vaccines.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hepatit C (HCV) är ett virus som infekterar leverceller och som orsakar en kronisk infektion i 80 % av fallen. Kronisk HCV infektion ökar risken för allvarlig leverskada och levercancer. Infektionen är oftast symptomfri men de infekterade kan i akutskedet få influensa likdanande besvär, gulsot, trötthet, illamående eller magsmärtor. Det finns mellan 130-170 miljoner HCV infekterade individer i världen, varav ungefär 40 000 finns i Sverige. Spridningen av HCV identifierades via kontaminerat blod 1989. Låginkomstländer är de som drabbas hårdast, då det ofta finns brister i kontroll av blod och blodprodukter och bristande sterilisering av medicinska instrument. Under tidigt 90-tal introducerade Sverige hepatit C screening, vilket har lett till en bra kontroll av smittan. Idag sker framför allt spridning av viruset mellan intravenösa missbrukare. Andra smittvägar är möjliga, men är mindre vanliga, som till exempel piercing och tatueringar med kontaminerad utrustning som smittokälla. En anledning till varför HCV är så svår att behandla är dess höga mutationsfrekvens, vilket innebär att den kontinuerligt förändrar sina egenskaper så pass att det kan undvika immunförsvaret utan att påverka sina egenskaper att infektera nya leverceller. Det finns en rad olika behandlingar i form av virushämmande läkemedel. Sedan 90-talet har stora framsteg skett inom läkemedels- och behandlingsutvecklingen, och till en början med interferon-α (IFN-α), en kroppsegen substans, och ribavirin (RBV). RBV är en antiviral substans. Patienterna behandlades i 12, 24 eller upp till 48 veckor. Denna behandling förde med sig en rad svåra biverkningar såsom blodbrist, depression, trötthet, muskelvärk och diarré. När man började behandla patienter med kronisk HCV infektion på tidigt 1990-tal användes bara IFN-α och endast 6 % av patienterna kunde botas. En kombinationsbehandling med en mer effektiv (pegylerat) IFN-α och RBV kom i slutet av 1990-talet och varvid omkring 45 % kunde botas. Omkring 2012 kom den första virushämmaren, då kunde man använda trippelterapin IFN-α, RBV och virushämmaren, då botade man ca 75 % av patienterna. Idag används en kombination av två till tre virushämmare och nu botas omkring 95 % av patienterna som får den behandlingen. Behandlingstiden har kortas ner, och oftast räcker 12 veckors behandling som ges oralt. Trots den nya behandlingens effektivitet har den en rad nackdelar. Priset per behandling är mycket hög, vilket leder till att endast de rikaste delarna av världen får tillgång till behandlingen. Idag får bara omkring 10 % av världens HCV infekterade tillgång till någon behandling. Det finns också flera patientgrupper som inte kan ta dessa läkemedel på grund av risk för resistensutveckling. Det är även ovisst hur man ska behandla patienter som inte svarar på de nya virushämmande läkemedlen. Studier föreslår att de som botats med de nya läkemedlen inte har skydd mot återinfektion av HCV. I dagsläget finns det inget profylaktiskt (skyddande) eller terapeutisk (behandlande) vaccin mot HCV, därför arbetar vi med att utveckla nya behandlingar som på ett effektivt sätt kan aktivera immunförsvaret hos HCV infekterade individer. Detta för att patienterna med hjälp av sin egen immunitet skall göra sig av med virussjukdomen. Vi försöker utveckla behandlande genetiskt vaccin som kan aktivera den infekterade individens immunförsvar vilket delvis har blivit försvagat på grund av den kroniska HCV infektionen. Det finns en rad fördelar med ett HCV vaccin, man kan tänkas få ett visst skydd mot reinfektion, och ett vaccin är sannolikt billigare än att behandla med virushämmande läkemedel. Detta torde i sin tur leda till att fler infekterade runt om i världen kan få tillgång till behandlingen. Det finns alltid risker med genetisk vaccination, där tillförande av genetiskt material kan leda till biverkningar. Målet med genetiska vaccin är att kunna aktivera/återaktivera kroppens eget

immunförsvar, där man hoppas på en så balanserad immunaktivering att infektionen kan läka ut. Vår behandling är baserad på en viruskomponent (i.e. det icke-strukturella proteinet (NS)3/4A) som är jämförelsevis stabil hos viruset. Detta komplex har viktiga funktioner för att HCV ska kunna producera nya viruspartiklar. Vi har utvecklat en behandlingsmetod där vi använder genen (arvsmassan) for HCV NS3/4A proteinet och använder det som vaccin. Vaccinet består av ett cirkulärt DNA (plasmid) som innehåller genen för NS3/4A. Genom att odla DNA plasmid i bakterier kan man isolera stora mängder DNA plasmid, som man sedan i sin tur renar från bakterierna och kan ge till människa. Vaccinet ges intramuskulärt (i muskeln) i kombination med en metod som kallas för *in vivo* elektroporering (EP). DNA plasmid i lösning injiceras i någon del av kroppen, i detta fall i deltoideusmuskel i överarmen. Genom EP blir muskelcellernas cellmembran mer genomsläppligt och således fås ett bättre upptag av vaccin DNAt. När DNA vaccinet väl är inne i cellkärnan kommer NS3/4A genen att uttryckas till proteiner vilket efterliknar när en individ blivit infekterad av ett virus eller en bakterie, och immunförsvaret blir aktiverat. Både de immunceller (T-mördar celler) som har hand om dödandet av infekterade celler och de immunceller som hjälper till att rikta immunförsvaret (T-hjälpar celler) aktiveras av DNA vaccinet. Vårt arbete har gått ut på att bland annat utvärdera ett DNA vaccin i patienter med kroniskt HCV infektion. Detta var den första studien i världen med ett behandlande DNA vaccin som ges med *in vivo* EP till HCV patienter. Vaccinationen var säker utan allvarliga biverkningar. Vi kunde också se en viss effekt av DNA vaccinationen med tillfällig immun aktivering och en möjlig effekt på HCV RNA nivåer hos ett fåtal patienter. Dock visade studien att man sannolikt behöver en starkare immunaktivering för att uppnå en tydlig behandlingseffekt. Vi har därför vidareutvecklat vårt vaccin genom att tillsätta nya komponenter (adjuvant) som ökar aktiveringen av immunförsvaret. Ett adjuvant som har visat sig fungera väl för vårt DNA vaccin var tillsatsen av en gen från ett fågelvirus (stork hepatit B virus). Genom att använda denna gen i kombination med vår vaccin-gen har vi kunnat få en betydligt starkare aktivering av immunförsvaret vilket förbättrar möjligheterna till att immunförsvaret kan utplåna HCV. Den djurmodell som ligger närmast en infektion i människa, är HCV infektion i schimpans. Här kan man studera både HCV infektionen och immunförsvaret. Idag är det förbjudet att använda schimpans då det finns många etiska aspekter samt höga kostnader. Idag finns det musmodeller där HCV infektion kan studeras, men de är tyvärr begränsade av att dessa saknar ett intakt immunsystem. Detta gör att man inte kan studera infektionen på ett optimalt sätt. Vi har därför utvecklat en musmodell där immunförsvaret är intakt och som ger oss möjligheten att studera hur HCV replikationen påverkar immuniteten. Vi har genererat levercancerceller som med ett självreplikerande HCV RNA producerar alla icke-strukturella protein NS2 till NS5B och växer ohämmat. När cellerna injiceras under huden på mössen kan vi studera hur dessa ”tumörer” växer under olika förhållanden. Mössen kan antingen vaccineras med vårt DNA vaccin eller vara ovaccinerade. På så sätt kan man jämföra storleken på ”tumörerna” och se om vaccinering skyddar mot ”tumörväxt” jämfört med de ovaccinerade mössen. Sammanfattningsvis syftar vår forskning till att ta fram skydd och behandling mot HCV infektion som enkelt skall kunna ges till så många patienter som möjligt. Ett genetiskt vaccin är billigt att producera och har en bra hållbarhet vilket möjliggör transport och enklare förvaring. Dessa egenskaper medför möjlighet att skapa ett behandlande vaccin som kan användas på bred front i så väl höginkomstländer som låg- och medelinkomstländer.

LIST OF SCIENTIFIC PAPERS

I. Ola Weiland, Gustaf Ahlén, Helmut Diepolder, Maria-Christina Jung, **Sepideh Levander**, Michael Fons, Iacob Mathiesen, Niranjan Y Sardesai, Anders Vahlne, Lars Frellin and Matti Sällberg. **Therapeutic DNA vaccination using in vivo electroporation followed by standard of care therapy in patients with genotype 1 chronic hepatitis C.** *Molecular Therapy, sep 2013, Vol.21 no 9, 1796-1805.*

II. **Sepideh Levander**, Matti Sällberg, Gustaf Ahlén and Lars Frelin
A non-human hepadnaviral adjuvant for hepatitis C virus-based genetic vaccines. *Vaccine, April 2016* (pii: S0264-410X(16)30171-2).

III. **Sepideh Levander**, Fredrik Holmström, Lars Frelin, Gustaf Ahlén, Daniel Rupp, Gang Long, Ralf Bartenschlager, and Matti Sällberg. **T cell-mediated protection against hepatitis C virus in a syngeneic transplantation mouse model.** *Manuscript.*

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LIST OF ABBREVIATIONS

aa	Amino acid
ALT	Alanine aminotransferase
APC	Antigen presenting cell
CTL	Cytotoxic T lymphocytes
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DAA	Direct acting antivirals
ER	Endoplasmic reticulum
EP	Electroporation
HBcAg	Hepatitis B core antigen
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HLA	Human leucocyte antigen
i.m.	Intramuscular
IFN	Interferon
IL	Interleukin
IVIN	*In vivo intracellular* injection
MHC	Major histocompatibility complex
NK	Natural killer cell
NS	Non-structural
ORF	Open reading frame
pDNA	Plasmid DNA
RBV	Ribavirin
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid
SOC	Standard of care
SVR	Sustained virologic response
TCR	T cell receptor
Tg	Transgene
Th	T helper
TLR	Toll like receptor

1 INTRODUCTION TO HEPATITIS

The liver is located in the upper part of the abdomen and is the largest of the organs inside the human body. The liver functions as the body's filter and as a factory of vital products which makes it impossible to live without. When the liver does not function correctly almost all other organs in the body are affected. A huge amount of blood is pumped through the liver since toxins and waste products needs to be removed quickly (17). Hepatitis means inflammation of the liver. There are several different reasons why inflammation occurs in the liver. It could be due to toxic compounds and chemicals, such as consuming a large amount of alcohol, autoimmune diseases, pharmaceutical drugs, metabolic disorders, and viral or bacterial infections. As of today five hepatitis viruses have been identified, the A, B, C, D and E viruses. These viruses all infect liver cells, the hepatocytes. Approximately 75% of the cells in the liver are hepatocytes. Most likely in all these infections it is the body´s immune response to the infected cells that cause the liver disease. When the virus is not cleared by the immune system and the inflammation is maintained over time and becomes more severe, the liver will become fibrotic and loose the characteristics of a liver. When a person has had a persistent infection for more than 6 months, it is considered a chronic infection. As more time passes with the infection, the liver cells are replaced by scar tissue, which will interfere with the ability to function properly. Ultimately an infected person may develop cirrhosis or liver cancer.

1.1 HEPATITIS A VIRUS

Hepatitis A virus (HAV) is spread through fecal-oral ingestion and causes acute infections and is mostly symptomatic. HAV was identified through electron microscopy in 1973 (18). The infection induces lifelong protection against reinfection and it never becomes chronic. The virus is a single stranded (ss) RNA virus belonging to the *Picornaviridae virus* family (19). There are both passive immunization (immunoglobulins) and an efficient prophylactic vaccine available (20).

1.2 HEPATITIS B VIRUS

Hepatitis B virus (HBV) was discovered in 1965 and is transmitted through contaminated blood, blood products, sexual contacts, and vertical transmission from mother to child (21). It is estimated that 2 billion people have been infected and that around 400 million people are currently chronically infected by HBV (22, 23). HBV has a partially double stranded circular DNA genome and belongs to the *Hepadnaviridae virus family.* There are eight different genotypes of human HBV and they differ from each other by more than 8% (22, 24). Chronic HBV is treated with interferon-α (IFN-α) or reversed transcriptase inhibitors such as entecavir and tenefovir. HBV infection is preventable by an effective prophylactic vaccine (25).

1.3 HEPATITIS C VIRUS

Hepatitis C virus (HCV) is mainly transmitted through contaminated blood or blood products. HCV was discovered in beginning of 1990 and was identified in serum from a non-A non-B hepatitis patient (26, 27). HCV is the most common cause of chronic liver disease, and it is

estimated that around 130-170 million people worldwide are chronically infected (28). HCV is a RNA virus that belongs to the *Flaviviridae* virus family. HCV has seven major genotypes that differ from each other by approximately 30-35% (29-31).

1.4 HEPATITIS D VIRUS

Hepatitis D virus (HDV) was discovered in the late 70s and is only able to infect patients as a co-infection or super-infection with HBV. HDV is transmitted through contaminated blood or blood products. HDV has a circular single stranded RNA molecule and uses the hepatitis B surface antigen (HBsAg) from HBV as its envelope. Although many examples of this type of incomplete virus exist in plants, HDV is the only one known in humans (22, 32). Chronic infection with HDV gives rise to a severe form of hepatitis and can lead to fulminant hepatitis. The treatment for HDV is limited to a 24 month IFN-α therapy that only cure 25% of treated patients (33).

1.5 HEPATITIS E VIRUS

Hepatitis E virus (HEV) was discovered in 1983. HEV lacks an envelope and has a positive sense, single stranded RNA genome. HEV is similar to HAV genome and capsid structure (34, 35). HEV is mostly spread through contaminated (fecal) water and food and causes self-limited acute hepatitis. The HEV acute infection only requires symptomatic treatments since almost all infected individuals can clear the infection. For persistent HEV infections in immunosuppressed patients the treatment with pegylated interferon-α leads to sustained clearance of the virus (36).

2 HEPATITIS C VIRUS

2.1 HISTORY

The origin of HCV is not known as HCVs RNA genome is easily degraded when not stored properly, there are no blood samples older than 50 years stored to test. One can speculate that HCV probably has been around for thousands of years before evolving into the current strains (37). In 1973 and 1963 blood tests were developed to identify hepatitis A and B, however there were many unidentified hepatitis cases referred to as non-A, non-B hepatitis (26). The disease was transmissible to chimpanzees and could cause persistent infection. In 1989 HCV was identified. The first antigen, HCV NS4, could be used to identify HCV infected subjects by detecting specific antibodies. Many features of HCV were later identified through research that gave important answers such as transmission routes, the size and the structure of the virus (38, 39). This was the first time in history that scientists identified a virus using only molecular biology (38). Since the 1990s all blood products are screened for HCV and as of today the transfusion associated HCV infection is very rare in high-income countries. When the genome of HCV was cloned it was found to have a single stranded positive sense RNA molecule of 9.6 kb. Since HCV genome showed the same characteristics in assembly model and the envelope protein as the yellow *fever, West –Nile virus and the Dengue fever,* HCV were put in the new genus of hepaciviruses, within the family of *flaviviruses* (26, 39).

2.2 EPIDEMIOLOGY

Approximately 130-170 million people around the world are chronically infected with HCV. Persistence of HCV infection is associated with liver cirrhosis, hepatocellular carcinoma and liver failure (40, 41). Transmission of HCV infection is mainly through blood transfusions from unscreened blood donors, intravenous drug use (IDU), unsafe therapeutic injections and other health-care related procedures. IDU seems to be the most prominent mode of HCV transmission in high-income countries. Unsafe therapeutic injections and other health-care related procedures have been responsible for the spread of HCV in low- and middle-income countries (40). Around 25% of the people who get infected with HCV spontaneously clear the infection. The remaining 75% infected with HCV progress to chronic HCV infection. Generally chronic HCV progress slowly in the initial two decades, however it can accelerate during this time as a result of factors such as, genotype, age, heavy alcohol intake and HIV co-infection (41, 42). HCV is classified into 7 different major genotypes (gt) and further divided into numerous subtypes (a, b, c, etc.). HCV prevalence is highest in Egypt with 11 million people infected (around 10% prevalence). As Egypt had problems during the 1950-1980s with the parasite *Schistosoma mansoni* the government initiated a large campaign to control the parasite. By intravenous injections of tartar emetic treatment under unsterile conditions a large part of the population was exposed and became infected with HCV (43). In Egypt gt 4 is the dominant variant of HCV. China has around 30 million people with HCV (2% prevalence) with gt 1b being dominant. India has 18 million people infected (2% prevalence), gt 3 and 1 is dominant, and Pakistan has 9 million people infected (3% prevalence), gt 3 is dominant (41, 42). The majority of high-income countries, North America, Northern and Western Europe, Australia and Japan have low prevalence's of HCV (<2%). In Europe 8 million people are infected with HCV. High prevalence rates (>2.5%) in Romania, Russia and Italy, and low prevalence rates (<1%) Netherlands, Norway and the UK

(40, 41). HCV gt 1 is the most prevalent genotype worldwide, representing almost 50% of all HCV cases. Approximately one-third of these are in East Asia. Genotype 3 is the next most prevalent worldwide, around 30% of all HCV cases. Around 20% of all HCV cases are related to genotypes 2, 4 and 6. Less than 1% of all HCV cases are related to genotype 5. Genotypes 1 and 3 are dominant in most countries, regardless of the economic status, whereas the large proportion of genotypes 4 and 5 are found in lower-income countries (40, 42). At least 40,000 individuals are estimated to be chronic HCV carriers in Sweden (44).

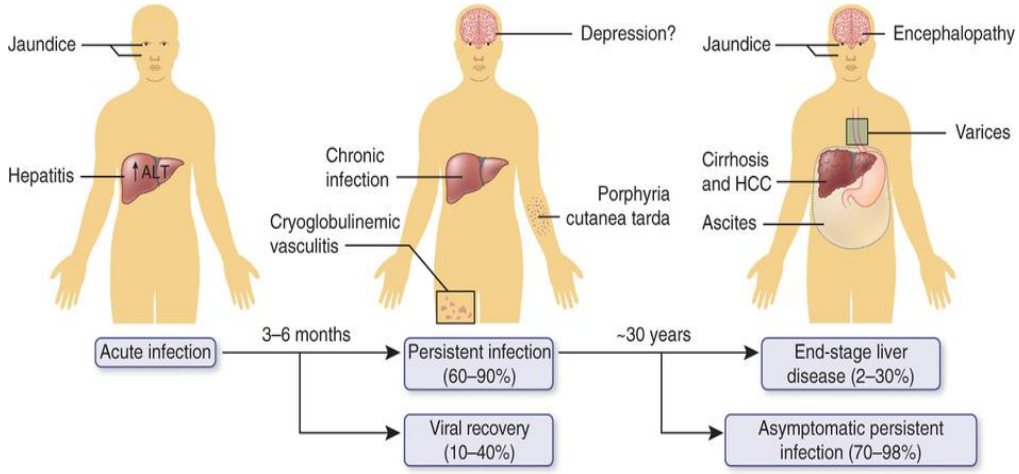


Figure 1. *Illustrates the different stages of HCV disease burden. From acute to chronic infection, causing persistence in 80% of all cases. Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [Nature Publishing Group], advance online publication (1) copyright (2013).*

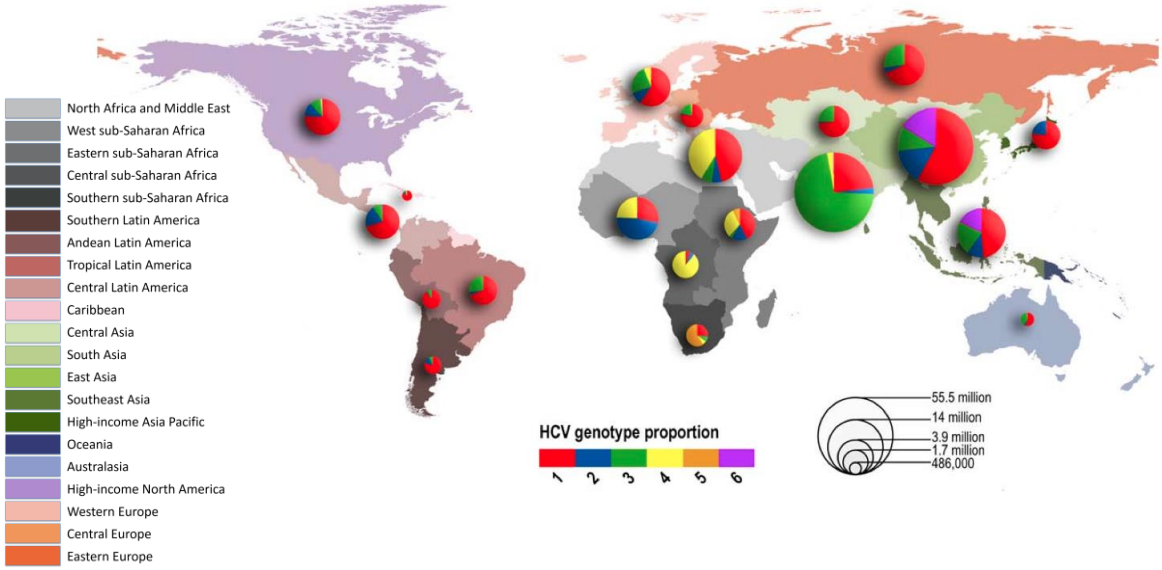


Figure 2. *The global prevalence of each genotype by Global Burden of disease (GBD) region. Size pie charts are proportional to the number of seroprevalent cases. Adapted from (40).*

2.3 HCV GENOME

HCV is an enveloped, spherical, single stranded positive sense hepatotropic RNA virus, and belongs to the *Flaviviridae* family. The genome is approximately 9.6kb in length and encodes for a polyprotein of around 3000 amino acids (Figure 3). HCV has seven different genotypes, the genotypes genetic diversity is 30-35%, subtypes are 20-25% genetically diverse, quasispecies are 1-9% genetically diverse (31, 45, 46). The genome encodes the structural proteins core and envelopes (E) 1 and E2, the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Figure 3). The structural proteins are important for assembly of viral particles while the non-structural proteins are important for viral replication and protein processing. The envelope proteins are required for viral entry into hepatocytes (46, 47).

The core protein (21kDa) is a capsid-forming protein, with regulatory functions in translation, RNA replication and particle assembly. E1 (35kDa) and E2 (70kDa) are trans membrane glycoproteins of the viral envelope, and is part of the adsorption and receptor-mediated endocytosis. P7 (7kDa) forms an ion-channel in the endoplasmic reticulum and is essential for the formation of infectious virions. NS2 (21kDa) is one part of the NS2-3 protease, which catalyzes cleavage of the polyprotein precursor between NS2 and NS3. NS3 (70kDa) is a serine protease that forms complexes with NS4A and cleaves HCV structural proteins downstream of NS3 into their respective individual proteins. Also, NS3 has an ATPase/helicase activity, which participates in binding and unwinding of the viral RNA. NS4A (6kDa) anchors the NS3 protein to the intracellular membranes and forms the NS3/NS4A complex with protease activity. The function of NS4B (27kDa) is crucial in inducing the membranous web at the ER lumen during HCV RNA replication. NS5A (56kDa) is a phosphoprotein that has been suggested to contains an IFN-α sensitivity-determining region (ISDR) (48). NS5B (66kDa) encodes the error prone viral RNA-dependent RNA polymerase (RdRp). (46, 47)

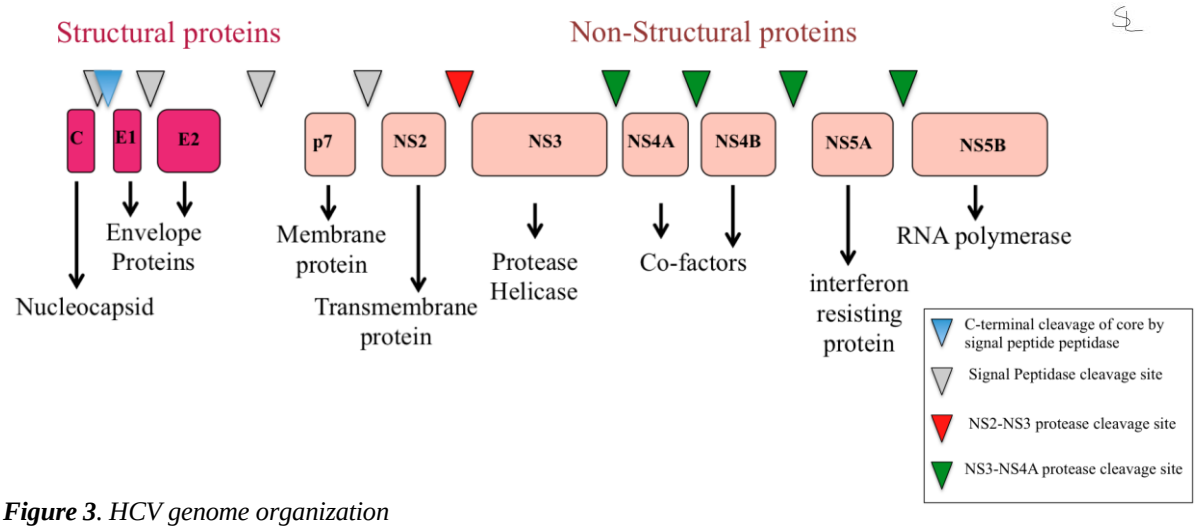


***Figure 3**. HCV genome organization*

2.4 GENETIC DIVERSITY

Since HCV is a single stranded RNA virus it displays high genetic variability, which is explained by the viral RdRp lacking proofreading activity. The lack of proofreading activity may cause an incorrect nucleotide introduction. There are some regions in the genome that display more mutations than other regions, such as the "hypervariable" region (HVR) of E2 and parts of NS5A. The reason why HCV effectively evades the immune system is due to the high mutation rate and can therefore escape the immune system and detection. The HCV genotypes differ 30-35% in nucleotide sequence from each other. However, within genotypes there are also differences with some viral proteins being more variable compared to others. For example E1 and E2 show a higher degree of variability whereas the core protein and some of the non-structural proteins, such as NS3, show less variability. The 5´UTR region is the most conserved region of HCV, which has important functions in the replication and translation process. The seven genotypes of HCV have many closely related subtypes that differ from each other by approximately 20-25% in nucleotide sequences (49, 50).

2.5 VIRAL LIFECYCLE

The infection of HCV is a dynamic process that has a high rate of replication. As we know the virus preferentially infects hepatocytes. The viral capsid is released after the virus has attached to the hepatocyte and fused with a cell membrane (45, 46). In the cytoplasm HCV mRNA is translated into polyproteins on the ribosome and processed into viral proteins. Viral replication, packaging and assembly take place in the cytosol on the membranous web structures that is derived from the endoplasmic reticulum (ER). Lastly, the virion is matured and released (Figure 4) (45, 50).

2.5.1 Entry and uncoating

The initial hepatocyte-binding is thought to occur through the LDL receptor and glycosaminoglycans (GAG) before E1 and E2 interacts with the co-receptors SR-B1 and CD81. Binding to Claudin-1 (CLDN1) and occluding (OCLN) initiates the next step. (51). Uptake occurs through clathrin-mediated endocytosis and fusion is encountered in endosomes. Primary translation occurs when the HCV genome is released into the cytoplasm (45, 51).

2.5.2 Translation and polyprotein processing

The positive strand HCV RNA is used as the template for HCV polyprotein translation. The translation is initiated via the IRES in the 5'NTR. The HCV polyprotein are associated with the endoplasmic reticulum (ER), which means that replication, and assembly occurs in association with the ER. HCV structural proteins are released from the polyprotein by the host ER signal peptidase. Additional processing is mediated by a signal peptide peptidase at the C terminus of the capsid protein. Cellular signal peptidase mediates the cleavage between p7 and NS2. NS2 to NS5B are released from the polyprotein after cleavage by HCV protease NS2-3 and NS3-4A (51-53).

2.5.3 RNA replication

HCV RNA replication takes place in the ER, and is initiated by NS4B and NS5A in the membranous web, consisting of small vesicles enclosed in a membranous matrix where the membrane-associated multiprotein complex is formed. This multiprotein complex consists of the nonstructural HCV proteins. The environment is rich in cholesterol and fatty acid, which influences the membrane fluidity and modulation of HCV replication. NS5B RdRp catalyzes replication; the positive strand RNA is used as a template for the synthesis of the negative strand RNA, which will then be used as a template for the production of the positive strand RNAs. The positive strand is also then used for the translation of the polyprotein. (51, 52)

2.5.4 Assembly and release

The host cell lipid synthesis regulates the assembly and release process. There are two models suggested, one where the mature core protein relocates from ER membranes to cytoplasmic lipid droplets (cLDs), after cleavage by the host peptidase. CLDs that are highly mobile along microtubules, transports core protein from sites translation and replication to the assembly sites. The other suggested model is that nucleocapsid formation is initiated at the surface of cLDs and the delivery of viral RNA is mediated from the replicase (or NS5A) that remains bound to the ER membrane (51, 54). Maturation and release of the HCV particle is tightly linked to the very-low-density lipo-proteins (VLDLs) containing the apolipoprotein B (apoB). The nucleocapsid is transferred to the precursors of VLDL particles, the Luminal lipid droplets (luLDs), which fuse with apoB-containing pre-VLDL particles to form the lipoviroparticles (LVPs) and exit through the Golgi. (54, 55)

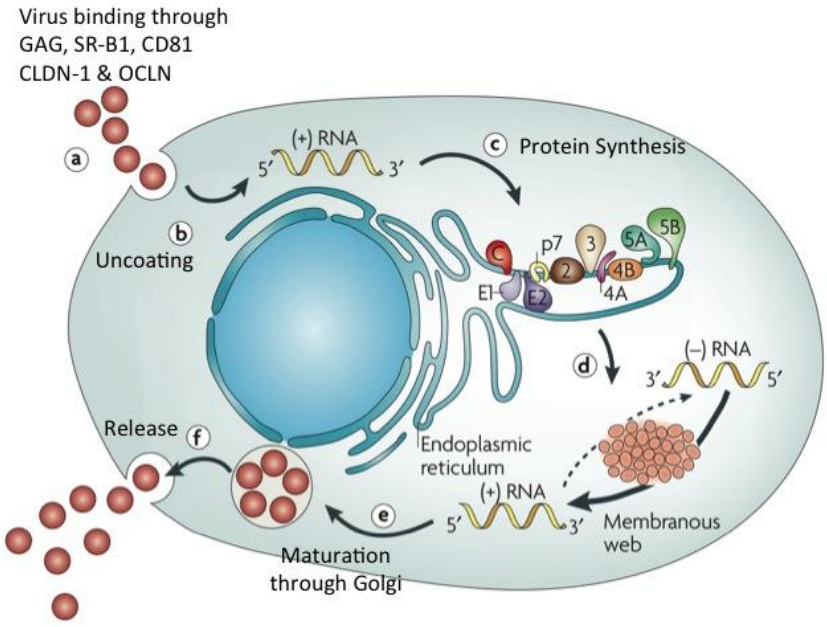


Figure 4. *HCV Life cycle. The hepatocyte binding occurs via the receptors GAG, SR-B1, CD81, CLDN-1 and OCLN (a), uncoating and release into the cytoplasm (b), Protein synthesis (c), RNA replication (d), packaging and assembly (e), virion maturation and release (f). Adapted with permission from Macmillan Publishers Ltd: [Nature Publishing Group] (45), copyright (2007)*

HCV MODEL SYSTEMS

2.6 *IN VITRO* SYSTEMS

It has been difficult to do molecular studies of HCV replication since no good cell culture systems and no easy and simple animal models have been available. The only animal model to use was the chimpanzee model, which had many drawbacks, as they are expensive, surrounded by ethical issues, difficult to breed and work with (56, 57). This has hampered the study of HCV for the development of new therapies such as vaccines (57). In 1997 the initial breakthrough came, HCV clones that were infectious in chimpanzees were generated. However these viral genomes did not produce virus in cell culture (58). It did not take long for different surrogate systems to develop, which made it easier to understand the different steps of HCV life cycle (57).

2.6.1 The HCV subgenomic replicon system

Lohmann and colleagues established an efficient cell culture system in 1999. This system was based on the transfection of a cloned viral consensus genome sequence (56, 59). They used high amount of HCV RNA from a chronically infected patient and cloned the total liver RNA isolates from the patient who had undergone a liver transplantation (60). They further amplified the complete HCV open reading frame (ORF) in two overlapping fragments. The fragment had several clones. They were analyzed and an isolate-specific consensus sequence belonging to genotype 1b was created. Selectable replicons were constructed that had a neomycin (G418) resistance gene so only those cells that support HCV replication could grow. The experiments were performed with the human hepatoma cell line Huh-7. Lohmann defined the structure of selectable HCV RNAs replicating autonomously and found high levels in a human hepatoma cell line. A detailed analysis of HCV replication, pathogenesis, and evolution in cell culture was possible (61). Herein the viral RNAs could be generated in unlimited quantities and the viral genome could be manipulated for genetic analysis of HCV functions that are important for replication. The replicon system allowed researchers to have a cell based assay system in order to evaluate antivirals. The replicon system generates no infectious particles due to the lack of structural proteins, however many mutations will occur randomly, which will lead to the emergence of variants with higher replicative fitness. As of today there is a replicon system for the study of all the different HCV genotypes (1, 56).

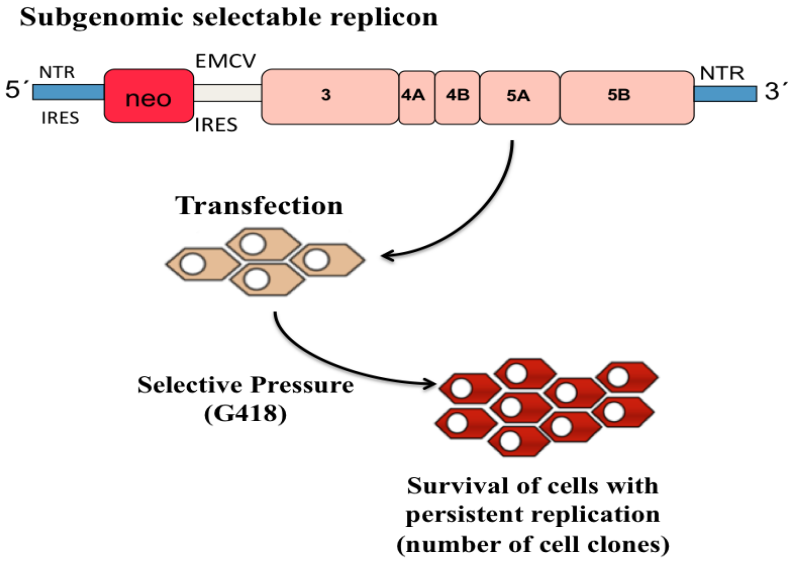


***Figure 5**. Cell culture model for HCV replication based on cloned viral isolates. Adapted with permission from (38). Copyright (2014) American Chemical Society.*

2.6.2 HCV pseudo particles (HCVpp)

HCVpp is a very successful model for investigating the early steps of HCV infection the process of HCV entry; this model consists of defective retroviral particles that express the HCV envelope glycoproteins on their surface. A kidney cell line was transfected with different plasmids encoding different HCV proteins together with a retroviral genome that contains a reporter gene (e.g. luciferase or GFP). The entry of the generated particles will deliver the capsid into the cytoplasm of the target cell, followed by integration of the viral genome into the host cell genome. This model system enables researchers to validate HCV attachment factors and receptors, the mechanism of virus internalization. This is an efficient way to study HCV cell entry. (59, 62, 63)

2.6.3 Cell culture derived HCV (HCVcc)

In the history of hepatitis C it has been a struggle and difficult to develop selectable drugs and efficient vaccines due to the poor virus propagation in cell culture. For unknown reasons infectious viral particles are not produced even though the subgenomic replicons replicate efficiently in cell culture (16). Fulminant hepatitis is the state when a person gets hepatic encephalopathy in a period of 8 weeks of the early symptoms of hepatitis. This state is very rare, where basically all hepatocytes are infected with HCV. In 2001 a patient from Japan was diagnosed with fulminant hepatitis based on clinical and virological parameters. The entire genome of HCV was recovered and compared with strains isolated from six different chronic hepatitis C patients. Based on those data it was concluded that the HCV strain (JFH-1, Japanese Fulminant Hepatitis 1) was of genotype 2a (16, 64). In 2005 the JFH-1 genotype 2a strain clone made it possible to study HCV in tissue culture since it was shown to replicate efficiently *in vitro* (16). The genome did not only replicate efficiently in human hepatoma cell line (Huh7) but also showed secretion of infectious viral particles. The secreted virus could also infect chimpanzees as well as mice transplanted with human hepatocytes. This cell culture system is a powerful tool for studying the viral life cycle and for the development of antiviral strategies. This system has made it easier to propagate HCV *in vitro* and made it

possible to better understand the HCV biology, for example the identification of novel entry factors. This system has made it possible to study assembly, virus release and the biophysical properties of the HCV virion (59, 65).

2.7 *IN VIVO* MODELS

2.7.1 Chimpanzee model

The chimpanzee was for a long time the only animal model for infectious HCV studies and this model is naturally susceptible to HCV infection, which has been a great advantage for our knowledge and understanding of HCV biology. Since the chimpanzees genome are 98% identical to humans, it has been a very useful tool to study the different aspects of HCV, such as the study of immune response and the evaluation of vaccine candidates. However the clinical course of HCV infection in chimpanzees and humans are not identical, the HCV pathogenesis is milder in chimpanzees than in humans, chronicity occurs only in 30-40% of infected animals while it can be up to 85% in humans. Chimpanzees do not get the same signs of liver disease or tissue damage as humans do, this is why it makes it much more difficult to study cirrhosis and hepatocellular carcinoma (HCC). Humans and chimpanzees do not share HLA class I and there are also differences in MHC class II. Other limitations with this animal model are the availability of chimpanzees, the cost for acquiring and maintaining animals is very high, and the ethical considerations has been a major issue to use this animal model. The use of chimpanzees for biochemical and behavioral research is forbidden in Europe since the new European directive 2010/63 (41, 59, 66).

2.7.2 Genetically humanized mouse models

As described before the human receptors CD81 and OCLN are important for viral entry, which makes it difficult to use wild type mice as a model system for HCV research, since wild type mice lack these receptors. Many mouse models for liver studies has been established e.g. uPA-SCID, MUP-uPA, and FRG, where repopulation with human primary hepatocytes is taken place after liver injury has been established. Currently, there are mouse models that support HCV entry and make it easier to examine and evaluate this process. These mouse models express human CD81 and OCLN, via adenoviral gene delivery, or by transgenic expression (59). Persistent HCV infection has so far only been achieved in immunodeficient mice (68) where the sustained viremia could be monitored more than 12 months after infection.(67-69).

2.7.3 HCV transgenic mice

The transgenic mouse model of HCV expression is an effective way to study the pathogenic effects of the HCV core protein and the envelope glycoproteins. With the transgenic (Tg) model, the expression of viral proteins in inbred mice, that holds a well-characterized immune system, can allow detailed studies of the viral proteins *in vivo*. It has been difficult to draw reliable conclusions when it comes to the pathogenicity of the HCV structural proteins, since in some studies the chronicity due to the core protein of HCV (genotype 1a) gave similar result as in HCV infected individuals. In other studies pathogenesis could not be seen over the course of 18 months. Wakita and colleagues examined the immune response to HCV structural proteins by using the Cre/loxP system to express the core, E1

and E2 proteins in Tg mice. Core protein was detected in serum seven days after transgene activation and serum alanine aminotransferase (ALT) levels increased. Anti-core antibody appeared 14 days after activation of the transgene. When CD4+ and CD8+ T cells were depleted, the ALT normalized the increase and the pathological changes in the liver, which suggests that the immune response rather than the HCV proteins mediated liver injury. Other problems that one can encounter when working with Tg mice model systems, is that very high level gene expression and inappropriate tissue expression can lead to artifacts that are not relevant to the natural infection (70-72). Frelin and colleagues generated a stable NS3/4A transgenic mouse in order to study the effects of HCV NS3/4A *in vivo*. A full-length wild type HCV genotype 1a NS3/NS4A gene with a functional protease was fused to the mouse major urinary protein (MUP) promoter. The expression of the NS3/4A proteins was restricted to the liver. Expression exerted notably effects in the liver, like altering the intrahepatic immune cell subsets. Also, NS3/4A expression *in vivo* impairs the TNF-α pro-apoptotic signaling pathway, which could explain why HCV can establish chronic infections. (73)

2.7.4 Transiently transgenic mice

Transiently transgenic mice allows us in an efficient way to express exogenous genes in animals, this technique provides tools for gene function studies, treatment of diseases and for obtaining gene products (74). The technique was described for the first time in 1999 and was referred to as hydrodynamic injection (75) This is a good and simple model, its very effective and versatile. The delivery of DNA, RNA and proteins has been an experimental success in the transiently transgenic mouse model. (76). Transiently transgenic mice can be generated in any mouse strain by an intravenous injection in the tail vein. The principle of this method is based on 5-10 seconds injections of a large volume, 1.6-2 ml of plasmid DNA solution in the tail vain (74). The injection leads to a high pressure in the liver and thus causes enlargement of the endothelial cells and increased permeability of the hepatocytes where around 10-40% of the liver cells are transfected (77). This model is valuable for studies of transient expression of proteins in the liver. This model can be used to study clearance and functionality of the immune response. This is a useful tool to evaluate vaccines and vaccine efficacy. By using this model the target-specific cytotoxic T cells primed can enter the liver and eradicate the target-expressing hepatocytes (78).

2.7.5 Genetically modified mice

Since wild type mice cannot be infected by HCV, researchers have come up with ways to genetically manipulate/modify mice. Rudolph Jaenisch bred the first genetically modified (GM) mice in 1974. The use of GM mice has exploded in the past decades. There are two ways to create a genetically modified mouse, first: the injection of a DNA sequence containing a gene of interest into a fertilized egg of a pregnant mouse (pronuclear injection), this is done when you want to add additional genes to the mouse genome. Second, mouse embryonic stem cells can be modified, using human DNA, then injecting them into the pre-embryo (blastocyst) of a pregnant mouse. This is common when you want to “knock-out” a single gene in the mouse genome (79, 80). Wild type mice cannot be infected by HCV, knocking down or complementing the mouse with human exogenous factors vital for the viral life cycle can manipulate the mouse genetics. The human entry factors CD81 and

OCLN are essential for entry of HCV into mouse hepatocytes. In a first mouse model that recapitulates the entire virus life cycle mice had their innate immunity impaired and transgenic for the human entry factors. The mice were susceptible to HCV infection. Replication and virus production was seen for two months (80).

2.7.6 Tupaia belangeri

Tupaia (Tree shrew) has shown to be susceptible to HCV infection, which leads to a transient HCV viraemia that causes to mild hepatitis during the acute phase of infection, HCV infection is not able to develop to the chronic phase. Due to the lack of tools to study the host responses and the difficulty in breeding Tree shrews makes HCV research difficult in this model. (59)

3 THE IMMUNESYSTEM AND HCV

Only 20-40% of the HCV infected persons will clear an acute HCV infection. There are genetic determinants that are associated with viral clearance. The polymorphism that affects both the innate and adaptive immune system seems to play a central role in understanding the immunology of HCV (81). HCV has evolved different mechanisms to utilize and control cellular molecules and pathways to evade elimination by the innate and adaptive immunity. The functionality and the subset of natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells (DCs), macrophages and T cells is disturbed when HCV causes chronicity. When the balance between immune evasion and antiviral immune response is disorganized the liver become chronically inflamed. (82)

3.1 THE INNATE IMMUNE RESPONSE

The liver has a large population of the macrophage cells named Kupffer cells. The liver also has high number of NK cells, NKT cells and DCs. When the liver is infected by HCV the infected hepatocytes and other cells react by secreting type I IFNs. Type I IFNs induces cell death of the infected hepatocytes, and activates an antiviral state in the neighboring uninfected cells. The innate immune cells such as Kupffer cells, DCs, NK cells and NKT cells is also activated. The activated innate immune cells multiply the antiviral response by releasing pro-inflammatory cytokines and chemokines. This induces the activation of liver-resident immune cells and the recruitment of immune cells from the periphery. Since DCs are able to migrate from the site of infection to lymphoid tissue the DCs have a key function in being a bridge between innate and adaptive immunity. DCs prime naïve T cells by presenting the processed viral antigen, which in turn will lead to induction of virus-specific T and B cell response. (82, 83)

3.1.1 Natural Killer (NK) cells

NK cells kill virally infected cells and tumor cells without the major histocompatibility complex (MHC). NK cells are important for liver immunology since their frequency in the liver is much higher than in the peripheral blood (84). NK cells are regarded either as a more mature subset or a less mature subset. The more mature subset has higher cytotoxic potential with both secreting cytotoxic granules and activation of death receptors such as Fas receptor and the tumor necrosis factor (TNF)-related apoptosis-induced ligand receptor. The less mature, NK cells have an immuno-modulatory function and they secrete cytokines such as

granulocyte-macrophage colony-stimulating factor (IFN-c), interleukin (IL)-10, IL-13, tumor growth factor (TGF)-β and TNF-α. NK cells function is regulated by the interplay between the stimulatory and inhibitory receptors. HCV core protein is able to impair NK cell activity via p53-dependent up-regulation of TAP1 which leads to the expression of MHC class I on the surface. When MHC class I is expressed on infected hepatocytes, the NK cells cannot mediate killing. The dysfunction of NK cells is critically involved in the establishment of chronic HCV infection. The inhibitory receptor CD94/NKG2A in combination with the enhanced release of IL-10 and TGF-β by NK cells reduces the capacity to activate DCs and skews the Th1/Th2 balance toward a Th2 response favoring T cell exhaustion and HCV chronicity (82, 85, 86).

3.1.2 Natural killer T (NKT) cells

Natural killer T (NKT) cells are unique in their way because they co-express T cell receptors (TCRs) and NK cell markers. They are abundant in liver and there is a decrease of frequency when HCV becomes chronic. NKT cells have both immuno-regulatory and effector functions. NKT cells recognize glycolipids that are presented by MHC class 1b and express either a highly restricted or diverse TCR repertoire. Upon activation NKT cells secrete cytokines, such as IL-4, IFN-γ and TNF-α, Fas ligand and activate other cell types such as DCs and NK cells. This suggests that both DCs and NK cells are involved in both clearances of infected cells and immune-mediated liver damage. Even though NKT cells may play a beneficial role in HCV clearance the number of activated NKT cells in the liver of chronic HCV patients is correlated with the degree of hepatocellular damage and the onset of fibrosis (87). This suggests that NKT cells are also involved in the destructive effects mediated by immune cells during chronic liver inflammation (82, 88, 89).

3.1.3 Dendritic cells (DCs)

DCs are activated through antigen uptake and stimulation by inflammatory cytokines. They secrete chemokines, cytokines and IFNs, which results in recruitment of inflammatory infiltrates to the site of infection. DCs migrate to the secondary lymphoid organs where they prime T cells and initiate virus-specific T cell responses. The two major DC subsets in humans are the myeloid DCs (mDCs), they mainly secrete IL-10 and IL-12 and express Toll-like receptors (TLR) 3 and 8, and the plasmacytoid DCs (pDCs), they produce IFN-α and express TLRs 7 and 9. It is of importance that the DCs are functional in order to avoid progression to chronic HCV. It has been shown that only patients who are able to increase the number of circulating mDCs during acute HCV infection are capable of eradicating the virus (90). The HCV infected patients that do not show any changes in mDC numbers are more prone to develop viral persistence (91). mDCs ability to stimulate CD4+ T cells and IL-12 production is impaired in chronic HCV patients, while their capacity to produce IL-10 increases, which in turn will lead to an immunosuppressive environment (90). The number of pDCs is significantly reduced in mice with liver-specific NS3/4A-protein expression (56, 82, 90, 92).

3.1.4 Macrophages (Kupffer cells)

Kupffer cells (KCs) act both as phagocytes and antigen presenting cells. They are involved in the clearance of pathogen-derived particles and toxins, in the killing of pathogens and tumor cells but also contribute to tissue damage during chronic inflammation (94). The KCs are activated when they express high levels of CD80, CD40 and MHC class II. When KCs are activated they come in close contact with CD4+ cells and form a KC-T cell cluster (56). By expressing a variety of TLRs KCs can easily sense viral pathogens and respond by secreting inflammatory cytokines such as TNF-α. Studies done *in vivo* showed that mice with liver-specific NS3/4A-protein expression have increased levels of CCL2 and TNF-α and hence enhanced number of macrophages in the liver. The CCL2-mediated recruitment of macrophages to the liver that is initiated by NS3/4A increases the level of TNF-α. It has been shown that galectin-9 is involved in the expansion of Tregs, reduction of CD4+ effector T cells and the apoptosis of HCV-specific CD8+ cells. Galectin-9 is part of the galectin family of carbohydrate- binding proteins abundant in the liver. They are in very high levels in serum and they are increased in patients with chronic HCV patients. Galectin-9 seems to be part of the activation of the innate immune response and the down regulation of Th1 responses (93). The inhibitory receptors PD-1 and TIM-3 that are expressed on macrophages are overexpressed and associated with impairment in the production of IL-12, which is very important for both the innate and adaptive immune response. Hence, this will in turn lead to disturbing the differentiation of T cells and the secretion of IFN-α and IFN-γ (56, 82, 94, 95).

3.2 THE ADAPTIVE IMMUNE RESPONSE

The adaptive immune response is characterized by cellular and humoral recognition of specific viral epitopes. MHC-class I present epitopes to CD8+ cytotoxic T cells and MHC-class II present epitopes to CD4+ T helper cells. CD8+ T cells are responsible for the elimination of infected cells through perforin-mediated cytolysis or activation of the death receptor pathways (96). CD4+ T helper I (Th1) cells activate CD8+ cells by secreting IFN-γ, IL-2 and TNF-α. Th2 cells are important for B cell activation and antibody secretion through the production of IL-4, IL-5, IL-6 and IL-13. B cells release antibodies when activated by Th2 signals; the antibodies bind to free virus and lyse the infected cells. The antibodies neutralize the virus so it no longer is capable of infecting host cells. Antibodies can also work together, a process called agglutination, which makes the virus an easier target to detect. Antibodies can also activate phagocytosis, where the antibody that has bound to a virus binds to the Fc receptors on a phagocytic cell and triggers engulfment and the virus is destroyed. (97). When HCV infects the liver there is a production of IFN-β and IFN-α, this induces KCs to secrete the cytokine CCL3 (inflammatory response), which will recruit NK cells. This induces DC activation by cell-cell contact and production of IFN-γ and TNF-α. This will lead to the activation of B and T cells in secondary lymphoid organs by mature DCs, which is necessary in order to get an effective antigen specific adaptive immune response. There are unfortunately no detectable neutralizing antibody titers in the acute HCV patients and the antibody development is very delayed and the existing antibody response does not correlate with viral clearance. In most chronic HCV patients the neutralizing antibodies recognize epitopes of the viral envelope E2 protein. The antibodies are ineffective in terminating the

ongoing HCV infection and that can be explained by the rapid viral evolution involving a selection of viral quasispecies, which escapes the reactive HCV-specific antibodies (82, 98).

3.2.1 Activation of T cells

During a viral infection, the host cell produces viral proteins that are processed the same way as an endogenous protein. Proteins from intracellular pathogen, such as viruses, are degraded by the proteasome. The peptides are transported by the transporter associated with antigen processing (TAP), a protein that spans the membrane of the rough endoplasmic reticulum (ER), into the lumen of the ER. The peptides are loaded onto MHC class I molecules and delivered to the cell surface to interact with CD8+ T cells, which become activated and subsequently kill infected cells. In the exogenous pathway, the extracellular pathogens are taken up by phagosomes and peptides are directly loaded onto the MHC class II molecule and transported to the cellular membrane to interact with CD4+ T cells. This interaction is important for further activation of antibody producing B cells. However exogenous antigen can also be presented on the MHC class I to CD8+ T cells by cross-presentation. Macrophages and dendritic cells (DCs) presents engulfed exogenous antigens via the MHC class I pathway. The antigens are released from the vesicle to the cytosol and enter the endogenous pathway for the presentation on MHC class I molecule for the interaction with CD8+ T cells (Figure 6) (99-101).

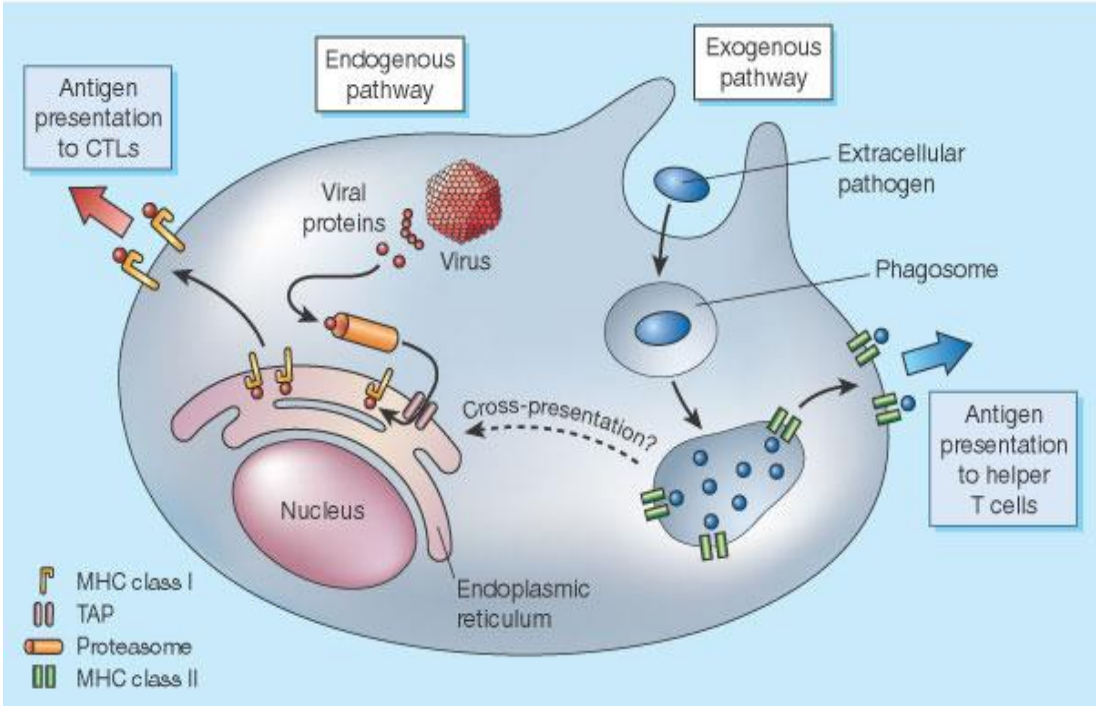


***Figure 6**. Illustration of the antigen processing and presentation pathways via the endogenous pathway, the exogenous pathway and by cross-presentation. Adapted by permission from Macmillan Publishers Ltd: [Nature Publishing Group] (99) copyright (2003).*

3.2.2 T cell response in HCV infection

Liver injury is indicated by increased alanine aminotransferase (ALT) levels in the blood. Spontaneous clearance of HCV infection is associated with a robust, sustained and multi-specific CD4+ and CD8+ T cell response (82). The patients that can control the HCV

infection have a diversity of virus-epitopes that are recognized by CD4+ T cells. By studying patients it has been shown that the diversity of virus-epitopes that are recognized by CD4+ T cells were much higher in patients that could control the HCV infection. Even though that CD4+ T cells are important for control, CD8+ T cell response is required for viral clearance. CD4+ T cells in peripheral blood mononuclear cells (PBMCs) that was taken from acutely infected HCV patients indicated that viral clearance was correlated to a Th1 CD4 T cell profile, production of IFN-γ and IL-2, as compared to patients having Th2 CD4 T cell profile, production of IL-4 and IL-10, who progressed to chronic HCV infection (82, 102-104). The T cells (CD4 and CD8) from patients with chronic HCV infection shows impaired effector function with reduced proliferative response, with abolished peptide-specific cytotoxicity and decreased IFN-γ production (82). Since the HCV RNA dependent RNA polymerase lacks proofreading there are many genetically different variants (quasispecies) being generated. Hence, no proofreading ability and a high replication rate ensures that HCV adapts rapidly to the immunological pressure by selecting variants that are better fit from the pre-existing quasispecies reservoir (82, 105). The liver receives blood from both the systemic circulation and the intestine, a unique organ in that way because it is continuously exposed to antigens and endotoxins derived from the intestine and to avoid constant immune activation, intrahepatic immune cells exists in a state of active tolerance (106). This means that the stimuli the T cells gets leads to Fas-mediated T cell apoptosis, the T cells that are activated have low expression of CD25 which results in low IL-2 production. In HCV infection where hepatocytes are the primary site of infection, premature death of the activated HCV-specific T cells will lead to the abolishment of the antiviral T cell repertoire, which will favor T cell dysfunction and HCV persistence (107, 108). When HCV becomes chronic it is characterized by continuous exposure of T cells to high levels of HCV antigens, which will activate HCV specific T cells in a dysfunctional way (82). IL-2 production and the proliferative capacities are lost, as well as the TNF-α and the IFN-γ production. CD4+ T cell function play a critical role in sustaining virus-specific CD8+ T cells during a chronic viral infection that is why the absence of functionally CD4+ T cells, where the response is weak and is characterized by reduced IL-2 production the result is T cell exhaustion and chronic infection (102, 106, 109, 110). Several reports have indicated that the frequency of CD4+/CD25+ Tregs is increased in chronic HCV patients (111). Tregs expand during the acute phase, maintain their number during the chronic phase and decrease their number to the level of healthy controls when the patients are cured from the HCV infection (111). IL-10 and TGF-β secretion are important for HCV-specific Treg function, and the recruitment of immune cells into the liver is dependent on the release of specific chemokines, the increase of CCL17 and CCL22 has recently been reported to be important (56, 82).

4 TREATMENTS FOR HCV

4.1 ANTIVIRAL THERAPY

Interferon (IFN) is signaling proteins released by the host cell upon the presence of a pathogen. Two IFNs have for many years been important in the treatment regime for HCV, type I IFNs and IFN-α. A virus-infected cell will release IFNs causing the nearby cells to heighten their anti-viral defenses. IFNs are cytokines, molecules that is released so that the cells can communicate with each other in order to trigger the protective defenses of the immune system to eradicate the pathogens (112). When IFNs are released the cells produce a

large amount of the enzyme protein kinase R (PKR), which will in turn lead to reduced protein synthesis within a cell. In addition, IFNs will induce the production of many other proteins encoded by interferon-stimulated genes (ISGs) that have a role in the defense against pathogens (113). Major histocompatibility complex (MHC) class II and I are also up regulated in order to increase the presentation of viral peptides to the helper T cells (Th) and the cytotoxic T cells (CTLs) (114). IFNs interact with their specific receptors, and activate signal transducer and activator of transcription (STAT) complexes. STATs regulate the expression of specific immune system genes. STATs activate the Janus kinase-STAT (JAK-STAT) signaling pathway. The association between JAKs and IFN receptors results into IFN-stimulated gene factor 3 (ISGF3) complex is formed. The complex then travels to the nucleus and binds to the promoter, IFN-stimulated response element (ISRE), of a certain gene, IFN stimulated genes (ISGs) (113, 114). IFN-α has been given as subcutaneous injections since late 1980s in the treatment for chronic HCV with around 10% sustained virological response (SVR). In 1991 Weiland and colleagues showed the antiviral activity of combining IFN and ribavirin (RBV) to Flaviviruses (115). RBV is a guanosine analog (e.g. a nucleoside inhibitor), used to stop viral RNA synthesis and viral mRNA capping. RBV interferes with RNA metabolism that is required for viral replication. The activity of RBV, given orally, was synergic with IFN-α and had an increased therapeutic response up to 40%, after 48 weeks of treatment (116). In the year of 2000, the pegylated form of IFN-α (peg-IFN) was available for the patients. The drug had an increased half-life, which provided a new breakthrough. Pegylated IFN-α in combination with RBV had a SVR of around 60% (117, 118). It is likely that RBV exerts a number of different effects in HCV therapy.

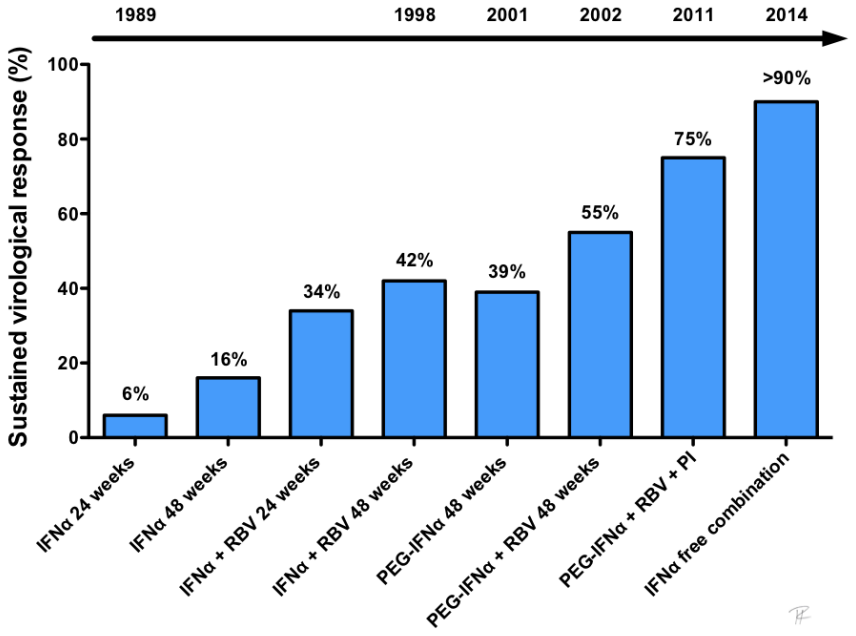


Figure 7*. Treatment success rate over the years. Adapted with permission from(119)*

4.1.1 Direct acting antivirals

Thanks to *in vitro* models of HCV replication, the screening for antivirals became possible. In 2011, new and promising direct-acting antivirals (DAAs) become available (120). The DAAs target HCV encoded proteins and disrupt the viral life cycle. (121). The protease inhibitors (PIs) Boceprevir and Telaprevir in combination with peg-IFN and RBV were the first DAAs that were approved for the treatment of HCV gt1 infections. This combination treatment increased the SVR to around 70% (46, 122). Even though the efficacy of the treatment was increased the problem with adverse events remained. The IFN regime in clinical practice ended when the improved DAAs were incorporated into the antiviral cocktail, they had minimal adverse events. Today the DAAs are more effective, have shorter course of treatment and have a cure rate over 90% in different clinical studies (121). There are four classes of DAAs, the NS3/4A serine PIs, NS5A inhibitors, NS5B polymerase inhibitors (nucleoside/nucleotide analogs and non-nucleoside agents) and cyclophilin inhibitors. DAAs have made HCV treatment more effective and tolerable. However one must have in mind that the treatment regime is genotype depended (121). For genotype 2 it is recommended to have a combination treatment of Sofosbuvir (SOF) (NS5B inhibitor) and RBV, with the possible addition of peg-IFN-α depending on the outcome of the treatment (123). For genotype 3 the recommended treatment regime is SOF and Daclatasvir (NS5A inhibitor) with or without RBV (123). HCV is a very diverse virus, with 10^{10} to 10^{12} new virions produced per day in a patient and has an error rate of 10^{-3} to 10^{-4} mutations per nucleotide per genomic replication (124-127). Some of the different variants, quasispiecies, may have a survival advantage since the accumulated mutations may lead to an on-going selection of the variants that are best fit (128). This is why, today, there is an increased problem with drug resistance and treatment failure in patients. Even though a patient may initially respond to a treatment there is a risk of relapse due to the different virus variants that are not detectable at the end of treatment (121).

4.1.2 NS3/4A protease inhibitors

The NS3/4A protease activity is essential for HCV viral replication, NS3/4A PIs inhibits the enzymatic activity that is needed for the cleavage of the HCV polyprotein into the non-structural proteins and the viral replication is inhibited (46, 122). Telaprevir and Boceprevir were the first PIs approved in 2011, in a reversible reaction they bind covalently to the catalytic site of the NS3 protease and interfere with formation of the HCV polyprotein, thereby blocking HCV replication. Telaprevir and Boceprevir are used for HCV genotype 1 and have been used in combination with peg-IFN and RBV (129). Other PIs include simeprevir, Paritaprevir and Asunaprevir (123). Asunaprevir is available in Japan (130). Simeprevir is a PI that is a macrolytic non-covalent inhibitor of the enzyme. It has similar resistance profile as Telaprevir and Boceprevir but Simeprevir is a more improved PI with improved tolerability, increased genotypic activity, and reduced daily dosing schedules (46, 129). Simeprevir is used in combination with Daclatasvir and SOF (131).

4.1.3 NS5A inhibitors

The NS5A protein is involved in viral replication, assembly and release of HCV particles (132, 133). It has three domains that are involved in RNA replication and virion assembly. When NS5A is inhibited the viral replication is blocked, hence the viral protein release is inhibited (46). Current NS5A inhibitors are Daclatasvir, Ledipasvir, and Ombitasvir (134). Elbasvir and Veltapasvir are being studied in phase III clinical trials in combination with NS3 inhibitor Grazoprevir and SOF (134).

4.1.4 NS5B polymerase inhibitors

NS5B encodes the RNA dependent RNA polymerase (RdRp), responsible for viral replication of the viral genome to make negative strand template and transcription of daughter copies of the genome (135). The two classes of polymerase inhibitors are nucleoside polymerase inhibitors (NPI) and non-nucleoside polymerase inhibitors (NNPI) (46). RdRps structure is highly conserved across all HCV genotypes, thereby making these agents efficacious against all seven HCV genotypes (136).

Nucleoside polymerase inhibitors (NPI) are inhibitors that are activated through phosphorylation within the hepatocytes, and compete with the nucleotide substrates that are incorporated into the RNA chain, resulting in the chain termination during RNA replication of the viral genome (137). SOF is the first NPI to become available, in clinical trials of SOF/PEG-IFN/RBV; patients achieved SVR rates of 92% (136). This group of DAA has been shown to achieve high cure rate in an IFN-α free treatment in combination with Ledipasvir and Simeprevir. SOF seems to have an effect on many of the HCV genotypes (121, 138)

Non-nucleoside polymerase inhibitors (NNPI) interact with RNA-dependent RNA-polymerase and prevent conformational changes in the enzyme that is crucial for its function (139). These inhibitory agents are Dasabuvir, and Beclabuvir. Beclabuvir has been reported to work in a combination study with Daclatasvir and Asunaprevir (140). Dasabuvir is administrated in combination with Ombitasvir, and Paritaprevir (140-142).

4.1.5 Cyclophilin A (CypA) inhibitors

CypA plays an important role in transmembrane transportation and viral replication. It has been shown that CypA is crucial for HCV replication by interacting with NS5A, NS5B and NS2. These inhibitors prevent the HCV from assembly and replication by binding to cyclophilin A. They are host-targeting agents with potent activity against HCV. They work against many of the HCV genotypes and have a high barrier to resistance. There are 3 CypA inhibitors; Alisporivir (ALV) is in clinical phase III (143). NIM811 and SCY-635 is in development. These inhibitors prevents CypA recruitment into the replication complex, interfering with NS5B polymerase activity, neutralizing NS5A activities, neutralizing NS2 activities, and inhibiting the assembly and release of viral particles (144-146).

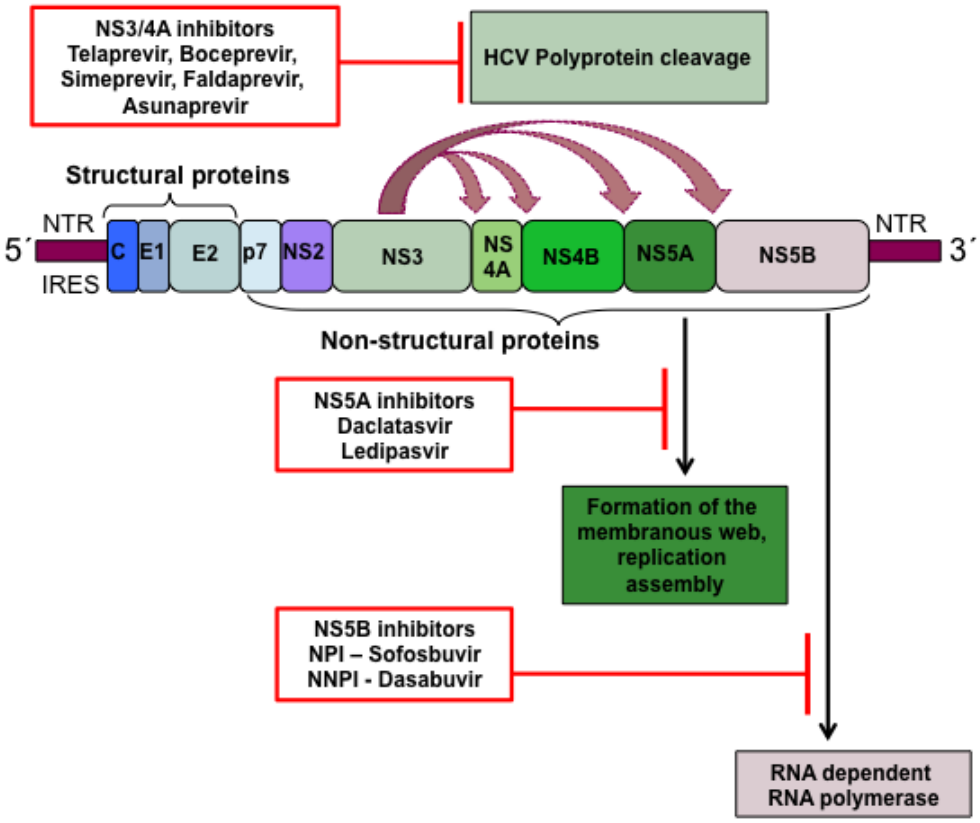


Figure 8*. The enzymatic function of the non-structural proteins has been the basis of DAAs (outlined in red). Adapted from (121).*

4.2 HCV VACCINE

There are two ways of immunization, passive or active immunization. Active immunization is when an un-immunized individual is immunized, by exposure to the pathogenic agent. There are two types of active immunization, one is natural and the other is artificial. When an individual is exposured to for example the influenza virus a natural active immunization has occurred. Artificial immunization is when introducing parts of the pathogen to the individual in order to obtain protection (147-150). There are activated and inactivated vaccines. The activated vaccines are attenuated, which is the weakened versions of the original pathogen agent. Inactivated vaccines are such as the influenza vaccines; chemicals, heat or radiation destroys the pathogenic quality of the virus. Naked DNA vaccines are still in experimental stage of development, where a specific part of the pathogens DNA can be used to stimulate the immune system. There are recombinant vector vaccines that produce the antigen of interest, which is displayed on the surface to stimulate the immune system (150-152). In order to get an effective and optimal immune response post immunizing, the activation of both innate and adaptive immune responses are essential. A potent activation of both humoral and cellular responses is also important for efficient priming of effector and memory cells. There are two types of vaccines, the prophylactic and the therapeutic vaccine. Prophylactic vaccines aim to activate strong, specific and effective immune responses, which is mainly focused on B cells (antibodies) that neutralize the virus. It is preferred that the immune activation is remembered (immunological memory) which means that the B cells are very important (150). The memory of the immune system is of vital importance for a vaccine to

be able to rapidly recall the immune response when an individual is infected by a pathogen. Therapeutic vaccine is aiming to activate an already existing sub-optimal or exhausted immune response. The focus of a therapeutic vaccine is to activate the robust, strong, specific and effective immune response, which are mainly the cytotoxic T cells (CTLs). The CTLs, when matured and activated, eradicates the infected cells and with the immunological memory prevents the host from being re-infected (119, 153, 154). There are several prophylactic and therapeutic vaccines under development for HCV (Figure 8). Although a cure for HCV is available, drug cocktails are expensive, and may be associated with side effects and resistance. Thus, it is therefore important to develop a global vaccine against HCV, given the high incident of infection around the world and the low percentage that have access to the drugs. Only around 10% of the world's chronically infected HCV patients are able to use any HCV treatment and even fewer have access to the latest DAAs. Hence, only the high-income countries that have access to the newest drugs. To induce neutralizing antibodies one approach is to use the envelope glycoproteins E1/E2 as target in a prophylactic HCV vaccine. Another method is to target HCV proteins that are conserved, such as some of the non-structural proteins. This could give a broader T-cell response (154, 155). In addition, it has been shown that neutralizing antibodies during the acute phase of infection correlate with resolution of HCV infection compared to the patients who proceed to chronic HCV (155, 156, 157). Another approach can be to use whole, killed virus. Akazawa et al. have shown that when HCV where inactivated, in cell culture, the virions were capable of inducing cross-genotype neutralizing antibodies to allow protection against HCV infection in mouse (150, 158). Okarios Inc. has been using replication-defective Modified Vaccinia Ankara (MVA) and chimpanzee-derived adenovirus 3 vectors, where they have tested the delivery of NS3, NS4A, NS4B, NS5A and NS5B genes of genotype 1b (7, 159). What is critical for therapeutic vaccines is to be able to activate and recruit dysfunctional HCV-specific or naive T cells to the liver. The efficacy of therapeutic vaccines can probably be improved if patients first are treated with the existing DAAs to reduce the viral load (154, 160). In figure 9 all the current therapeutic vaccine projects are listed, here some of them will be discussed. In order to activate $CD4^+$ Th and $CD8^+$ CTLs specific for the HCV non-structural proteins, Transgene has used the Modified Vaccinia Ankara strain as vector. The vector expresses the HCV NS3, NS4A, NS5A, and NS5B genes. In a phase I clinical trial, 6 out of 15, patients had a reduction in viral load after vaccination (161). By vaccinating prior to treatment with IFN-α an RBV increased the early virological response from 30% to 60%. Unfortunately significant side effects have been reported (154, 162, 163) We have been using another approach, the direct delivery of a DNA plasmid encoding the HCV NS3/4A proteins by using electroporation (165) .

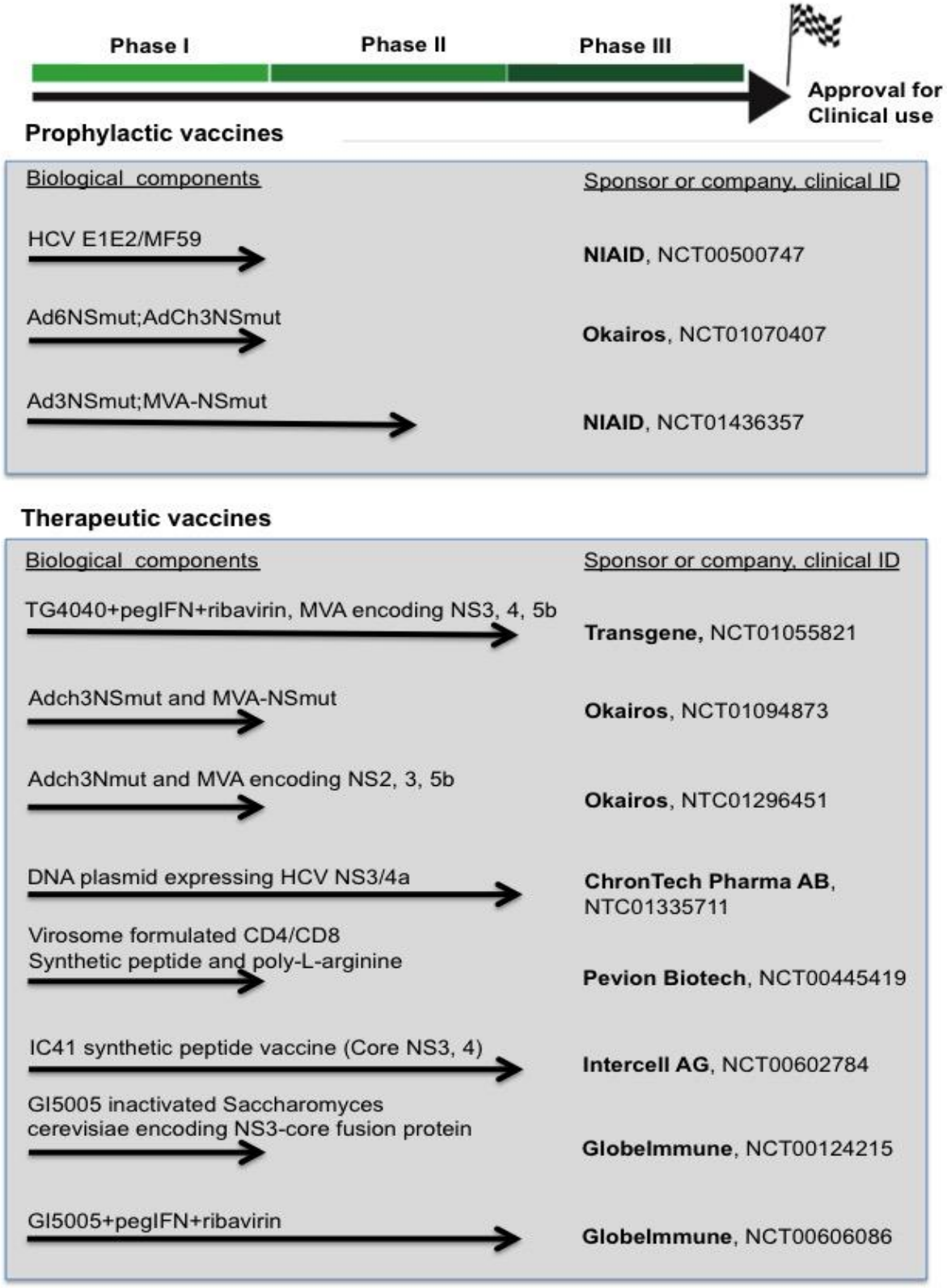
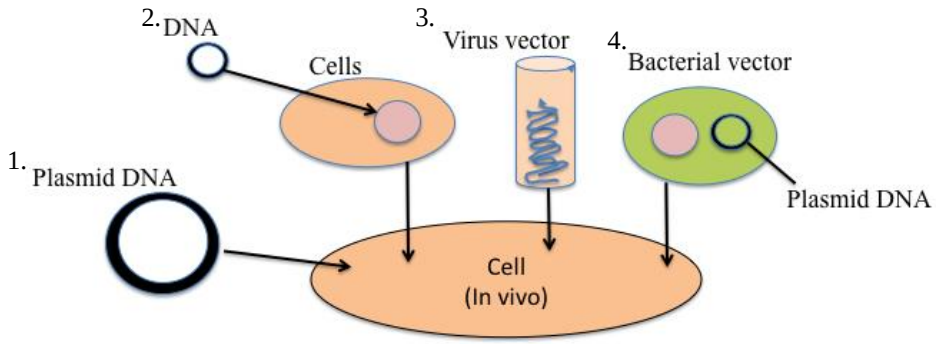


Figure 9. *A summery of the potential HCV vaccines in clinical development. The biological component(s) of the vaccine is listed on top of the arrow. Company conducting the trial is listed at the end of arrow along with clinical ID number (http://www.clinicaltrials.gov). Adapted from(154)*

5 GENETIC VACCINES

In 1990 Felgner and colleagues showed that a plasmid DNA could directly enter a mammalian cell when injected *in vivo* and synthesize the protein they encode (76, 166). There are various methods of gene delivery (Figure 10), alternatively a virus or bacteria can be modified such that it is no longer virulent but contains a gene encoding the desired antigen. DNA vaccines are however the simplest approach consisting of a plasmid encoding only the antigen of interest (166). The various methods of gene delivery are shown in (Figure 10). DNA vaccines are the simplest approach consisting of a plasmid encoding only the antigen **(1)** (Figure 10). Transfection is the process of deliberately introducing nucleic acids into cells. Plasmid DNA are stable and cheap to produce and easy to get good purity and with minimal risk of replication or viral incorporation. It is an efficient and safe method for delivering genetic materials into cells. This method is simple and versatile. In transfection of DNA plasmids the pores of the cell is transiently open and the plasmid can go through the "holes" of the cell membrane, which allow uptake of the material. Transfection can be carried out through different methods such as EP or hydrodynamic injection **(2)** (Figure 10) (167). A virus or bacteria can be modified that it is no longer virulent. No replication occurs but contains the genes that are encoding for the desired proteins **(3.4)** (Figure 10) (166)



10. *Illustrates the different methods of gene delivery.*

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5.1 DNA VACCINES

Successful *in vivo* transfection of mammalian cells following injection of purified DNA was first reported over 40 years ago. However its potential was not realized until the 1990 when Wolff and colleges demonstrated that a reporter gene encoding an enzyme protein could be expressed in murine skeletal muscle *in vivo* and the tissue retained its transgenic biological activity for up to 60 days after inoculation (145). Tang *et al*., (1992) demonstrated that mice injected with plasmid DNA encoding human growth hormone elicited antigen-specific antibody responses (168). The genes that encodes for the protein of interest is identified and isolated from the pathogen, then inserted into a plasmid that will carry the gene into the living system such as mice. DNA immunization can be administered in two different ways in general either with needle injection into different tissues, where the most effective way is intramuscular injection (in the muscle) (Figure 11), or with intradermal injection, in the

dermis, one of the layers of the skin, which is highly vascularized and contains a large amount of immune cells. This type of immunization is very effective and provokes strong antigen-specific Th1-baised, humoral and cellular immune responses (169-173). The DNA (plasmid) that is carrying the gene of interest is directly transfecting the muscle cell and the cells will start to shed exogenous antigens, this will in turn elicit the immune response (174-176). When the encoded polypeptides are synthesized the immune response is stimulated. The difference between gene therapy and DNA vaccination is; with DNA vaccination integration into the genome does not occur. DNA vaccines are designed to have a local, short-term expression of the target antigen (152).

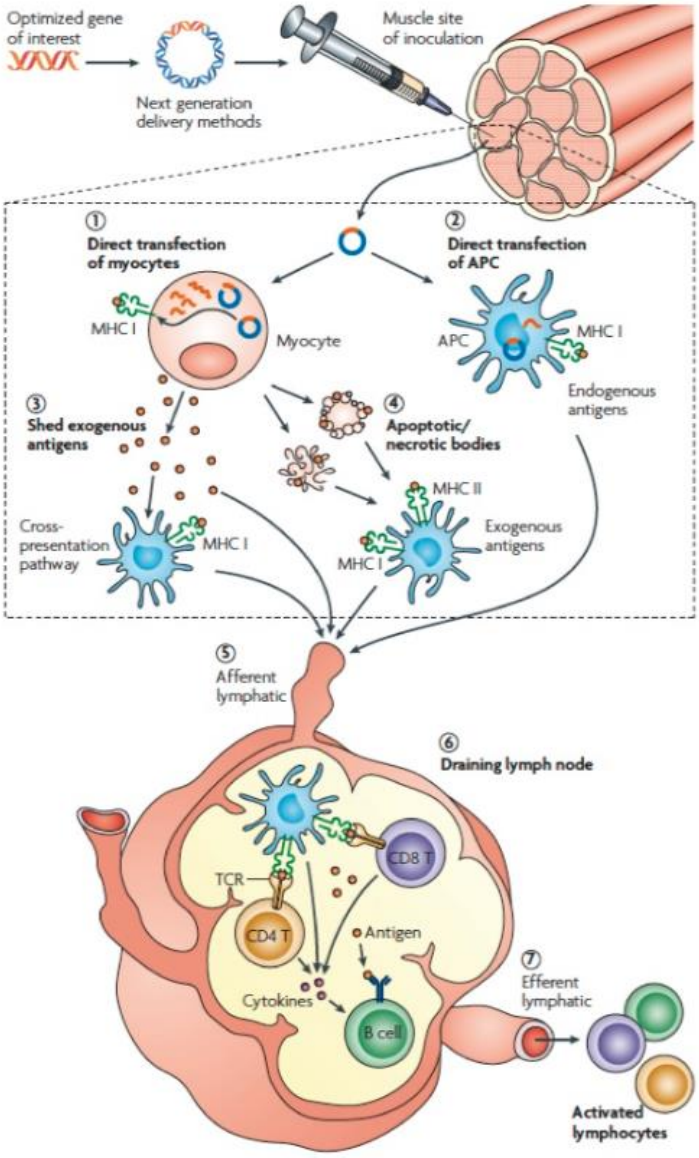

Figure 11. *Illustrates the cellular and humoral immunity by DNA immunization. From the optimized gene of interest to muscle inoculation and the transfection of the different celltypes. Here it is also illustrated the direct and cross presentation of antigens in the draining lymph node. Adapted with permission from Macmillan Publishers Ltd: [Nat Rev Genet.] (177), Copyright (2008).*

Important advantages of DNA vaccination are, it's cheaper to produce, easier to transport and use, easier to store, safer than live attenuated vaccines and they affect not only the humoral immunity (antibodies) but also the cellular immunity (T-cells). Other advantages of DNA vaccines are that they are highly specific and the expressed antigen is subjected to the same glycosylation and post-translational modifications as during a natural viral infection (177). In order to prime a strong immune response adequate adjuvants can be necessary to use (178, 179). What also needs to be improved in a DNA vaccine setting is to develop simpler and more efficient delivery methods (179). Plasmid immunization can have a significant affect on the cellular immunity, depending on the mode and site of gene delivery, the dose of plasmid and the administration of booster injections (180, 181). In order to design an immunogenic DNA vaccine one needs to really understand the correlation between the immune system and protection. Antigen presenting cells (APCs) are the cells that usually present the antigen that is produced after DNA immunization, either through MHC I or II molecules. These processed antigens are presented to CD4+ and CD8+ T cells respectively. There are several ways T-lymphocytes can be activated by DNA immunization: **(1)** transfection of professional APCs, **(2)** antigen presentation that is mediated directly through the muscle cells (myocytes), **(3)** cross priming, the muscle cell is transfected and produces the antigen but then the antigen is taken up by a professional APC which is then responsible for activating the CTL (182, 183). Currently the focus of DNA vaccine research is on developing different approaches to improve the DNA platform. The improvements can include the optimization of the antigens the plasmid is encoding, in order to increase antigen expression such as codon optimization (184). As of today there are about 43 clinical trials evaluating DNA vaccines, where the majority is investigating vaccines for HIV and cancers. Other trials include the investigation for human papillomavirus, malaria, influenza and hepatitis B and C viruses (184, 185). However in the current trials there is a lack of long-term follow up. The availability of data from randomized clinical trials that contains biochemical responses, progression and survival results will provide us with the evidence for DNA vaccinations potentials (186).

6 DNA DELIVERY METHODS

6.1 INTRAMUSCULAR INJECTION (IM)

In order to activate the immune system in a preferred way DNA vaccines can be delivered by different means and techniques. There are several aspects to have in mind to increase the uptake of DNA into the cell to enhance the expression of the desired antigen. How effective a technique is depends on the tissue and species delivered to. In order to get an optimal immune activation it is of importance to know that different antigens may need to be delivered by different methods to give rise to an optimal immune activation (187). Intramuscular injection is the procedure when DNA plasmid is delivered to the muscle cells, where they passively take up the DNA. The antigen of interest is expressed and the protein will be processed and presented on the MHC I molecule on the surface of the myocytes to the T cells. An alternate mode of presentation can be through the APCs, which migrate to the lymph nodes and present the antigen on MHC II. A third mode of presentation can be through cross presentation on MHC I to the T cells (75, 188).

6.2 ELECTROPORATION (EP)

Electroporation (EP) is mostly used in combination with IM injection, where the uptake of the DNA can be enhanced and in turn increase the expression of the antigen of interest. Many have discussed the theory behind *in vivo* EP and it is proposed that DNA enters the cell in a passive manner through the pores created by the EP. It has also been debated weather or not the DNA is transported into the cells through the pores due to the electric field e.g. the electrophoretic effect. Electro transfer of drugs and DNA differs from each other since DNA is to large to passively diffuse through the pores while drugs can be passively transported through the pores into the cells (189). It seems that the electric field is indeed important for the transfection of the cells (190). Also, since DNA is negatively charged, permeabilization of the cell membrane and the electric field are both important for DNA uptake. There are certain EP pulse patterns that can be used in order to optimize the DNA delivery and use EP as an adjuvant in order to enhance the immune response (191). The inflammation and cell death that occurs after EP gives rise to a strong immune activation. Depending on what type of tissue and species are used, the voltage, number of pulses, and duration of pulses can be optimized to fit the procedure (192, 193). It is very important to have in mind when cells have high uptake of DNA there is a high expression of the vaccine antigen and in turn, these cell, will more efficiently be eradicated by the primed immune response (193). In order to increase the uptake of DNA into the cells many groups are using IM in combination with *in vivo* EP. This has shown an increase uptake of the DNA vaccine, which in turn has lead to the possibility of using a lower DNA dose. The DNA dose is important so that a realistic dose may be administrated to humans (194, 195). With this method both the humoral and cellular responses has been improved, this has also been shown in several animal models (193, 196). To further increase the immunogenicity of vaccine DNA vaccines, Ahlén and colleagues developed a new device called *in vivo* intracellular injection (IVIN) device that could be used in combination with *in vivo* EP. In short, the technology is based on a device with multiple needles with several holes along the needle shafts. The IVIN device is delivering the DNA vaccine to an isolated area using a high-speed injection, which more efficiently transfects the myocytes, which in turn will lead to an increased expression of the DNA vaccine encoded antigen (197, 198).

6.3 ADJUVANTS

Adjuvants are often used in vaccine compositions to help induce a stronger immune response to the vaccine antigen. The most common adjuvant is Alum (aluminum potassium sulfate) (199). It is not fully clear how alum works but it seems to stimulate CD4+ T cells and the humoral immune response (200). MF59 is an adjuvant that is used for the induction of antibody responses for HCV, HIV and influenza vaccines. MF59 is the first oil-in-water adjuvant that is said to create a local environment at the injection site resulting in recruitment of immune cells (201). Adjuvants such as Toll-like receptors is also being used, they are considered to induce a rapid activation of the innate immunity by inducing production of cytokines, important for type I IFN response and co-stimulatory molecules which will then in turn lead to effective adaptive immunity (202). Cytokines can also be used as adjuvants; they can be used to help the activation and priming of a specific cell types. Cytokines can be given as a plasmid DNA and or as a recombinant protein. This addition of cytokines may improve

the activation of T cells and the recruitment of APCs. In HCV studies there are some cytokines more interesting than others, such as IL-2, IL-12 and IL-21. IL-2 is important for immunological memory and clonal expansion of T-cells. IL-2 can both enhance the humoral and cellular responses. IL-12 has the ability to promote a Th1 skewed immune response and when co-expressing IL-12 improve the immune response to DNA vaccination (197, 203). It is interesting that the levels of IL-21 are elevated in patients that resolve their acute HCV infection. IL-21 has the capacity to induce proliferation and effector function of T cells (204). Cytokines in general needs to be chosen with great care depending on what type of immune response and immune cells to be activated (205). Since the half-life of cytokines in general is short it may need to be prolonged. IFN-α was pegylated to prolong the half-life, delivery of cytokine genes as DNA plasmids results in the continuous expression of the cytokine as long as the vaccine gene is expressed (205, 206). Antigens have the ability to enhance immune activation; one of the antigens that can do this is the hepatitis B core antigen (HBcAg). The core antigen has shown to be a strong inducer of TLR-7. HBcAg can act as an adjuvant to enhance the immune response. In paper II we have shown that stork HBcAg can be used to enhance the immune response. One advantage of using stork core is that it will not cross react with human HBcAg and can therefore be used as an adjuvant for patients that has previously been exposed to the virus or have a chronic HBV infection (203, 207, 208).

AIMS OF THE STUDY

Paper I

- To investigate if therapeutic HCV NS3/4A-DNA vaccination in patients with chronic HCV infection is safe and immunogenic.

Paper II

- To investigate whether the immunogenicity of a clinically evaluated HCV NS3/4A-DNA vaccine could be improved by modifying the vaccine antigen, addition of adjuvants and the use of a new delivery technology.

Paper III

- To establish a robust and reproducible immuno-competent mouse model with HCV RNA replication, which allow for evaluation of HCV vaccines in the presence of HCV RNA replication and studies of immune escape.

7 COMMENTS ON MATERIALS AND METHODS

7.1 HUMAN SUBJECTS

A total of 12 patients with confirmed chronic HCV infection of a known duration of more than six months were included in the study, 5 females and 7 males, with an age range between 29-60 years. All had genotype 1 infection and were treatment naïve. The individuals had acquired HCV infection through blood transfusion, intravenous drug use (IVDU), and sporadic transmission including sexual transmission. The majority of patients had mild fibrosis (209).

7.2 CLINICAL TRIAL DESIGN

The primary aims of the trial were safety, immunogenicity, and effect on the serum levels of HCV RNA, respectively. The patients were divided in three dose groups and they received four monthly vaccinations. Two weeks were allowed between the enrolments of each patient to monitor safety. Patients were admitted to hospital for eight hours after the first vaccination and then phoned back at 24 hours. The local reaction at the site of vaccination was recorded during the first week. Venous blood was sampled before each vaccination, six hours after, and then every second week until treatment week 36. A final sample was taken 24 weeks after last vaccination at week 12. These samples were tested for blood biochemistry, hematology, and HCV RNA. Peripheral blood mononuclear cells (PBMC) for analysis of immune responses were isolated at screening, week zero, two weeks after each vaccination, and at week 39.

7.3 MICE

All mice in this study have been used for immunological studies and had a H-2^b background (table 1). The mice were obtained either from Charles River, Sulzfeld, Germany, the Jackson Laboratory, Bar Harbor, ME, or bred in-house (Karolinska Institutet, Division of Comparative Medicine, Clinical Research Center, Karolinska University Hospital, Huddinge, Sweden). All mice were 6-12 weeks old at the start of the experiments and were bred and maintained at Karolinska Institutet, Division of Comparative Medicine (AKM), Clinical Research Centre, Karolinska University Hospital Huddinge according to the Ethical Committee for animal research at Karolinska Institutet. The animals were caged at five to ten mice per cage and fed with a commercial diet with free access to food and water. All NS3/4A-Tg mice were genotyped by PCR to verify the transgene. HBeAg-Tg mice were genotyped by analyzing the presence of secreted HBeAg in serum samples by a commercially available ELISA. The methods were carried out in accordance with the approved guidelines.

Name of strain	Background	Haplotype
Wild type	C57BL/6J	H-2^b
CD4-/-	C57BL/6J	H-2^b
CD8-/-	C57BL/6J	H-2^b
NS3/4A-Tg	C57BL/6J	H-2^b
HBeAg-Tg	C57BL/6J	H-2^b

Table 1. List of mouse strains used in this thesis

7.4 CELL LINES

The murine H-2b-restricted Hep56.1D hepatoma cell line was originally obtained from Cell Line Services (CLS) (210) used in **paper III.** Hep56.1D cells containing a HCV subgenomic RNA replicon of genotype 2a were designated Hep56-sgJFH-cl3 (HCV replicon) and Hep56-sgJFH-cl10 (21-10) has been described previously (211). Hep56.1D cells were stably transfected by a functional NS3/4A protease complex gt2a gene by standard protocols (NS3/4A hepatoma) and used as a control cell line. All cell lines expressed similar levels of NS3. The cells were analysed for the antigen expression in the absence of selection (G418). Since G418 cannot be used in the *in vivo* setting we wanted to make sure the replicon cells expressed the replicon also in the absence of selective pressure. A total of 5 million parental, replicon or stably cells were used for inoculation into H-2^b mice. Inoculations were always performed in the right flank of mice. In **Paper II** HepG2 cells were used for transient transfection and western blot analysis in order to detect expression of NS3-protein.

7.5 PEPTIDES AND PROTEINS

Until today, no H-2^b restricted T cell epitopes has been reported for the genotype 2a strain of HCV. Thus, we decided to identify T cell epitopes in the HCV JFH-1 infectious clone (genotype 2a). In **paper III**, 75 20-mer peptides with 10 aa overlap corresponding to the full-length NS3/4A-JFH-1 were used for stimulation of splenocytes obtained from NS3/4A immunized mice. Two CTL epitopes were identified and were used to determine specific immune responses in immunized and tumor challenge experiments in **paper III**. In **Paper I and II**, a total of 135 15-mer peptides covering the full-length HCV NS3/4A genotype 1a were used. In order to perform detailed studies of HCV- and HBV-specific $CD4^+$ and $CD8^+$ T cell responses, previously identified H-2^b-restricted CTL and T helper (Th) epitopes were used. Recombinant NS3 protein (gt1 and gt2) was for stimulation of splenocytes obtained

from NS3/4A immunized mice. Chicken egg albumin (OVA) and Concanavalin A (ConA) were used as controls in both **paper II and paper III.** Phytohemagglutinin (PHA) was used as control in **paper I.**

7.6 DNA PLASMIDS FOR IMMUNIZATIONS

A full-length codon-optimized (co) HCV NS3/4A genotype 2a gene (JFH-1) (**paper III)** was generated synthetically and cloned into the pVAX1 backbone (Figure 12a). All plasmids were grown in *E.coli* and purified for *in vivo* injection. Five codon-optimized fusion constructs of NS3/4A and stork HBcAg (co-Stork-HBcAg) were made synthetically **(paper II)** and inserted into the pVAX1 vector. The five separate fusion constructs were C2.1, C2.2, C2.3, C2.4, and C2.5 (Figure 12b).

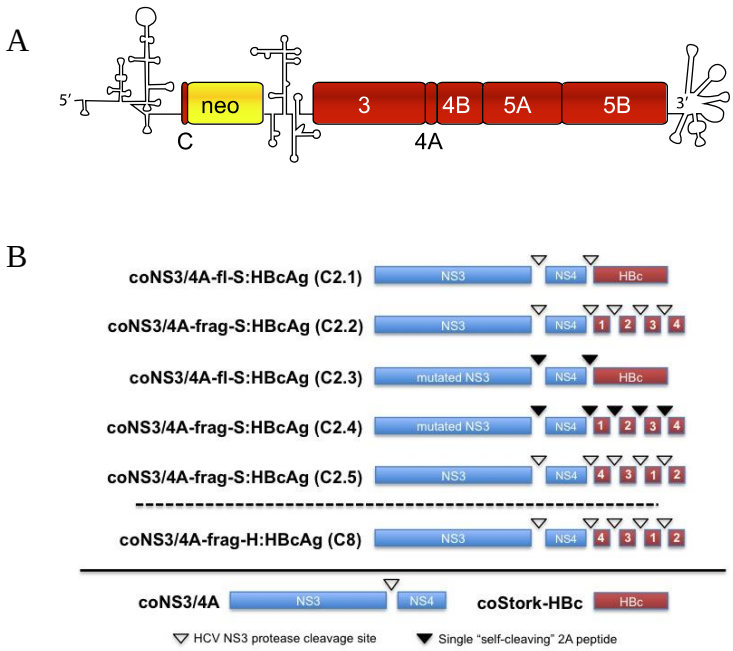

Figure 12. *(A) The replicon system is based on the transfection of cloned viral consensus genome replicons that was constructed to transduced neomycin (G418) resistance only to those cells that support HCV replication. (B) Design of NS3/4A-HBcAg fusion genes.*

7.7 IMMUNIZATION PROTOCOL

In **paper I** the T cell assays were performed on frozen PBMC (212). The proliferation assay was performed by ^{3}H-thymidine incorporation and for IFNγ-production by ELISpot assay (212). Peptides corresponding to known human leukocyte antigen (HLA) class I epitopes and overlapping peptide to the full-length NS3/4A sequence of the vaccine were generated by standard techniques (213). The following peptide pools were generated: 1: CD4 peptide pool (6 peptides), 2: CD8 peptide pool, HLA-A2 (7 peptides), 3: CD8 peptide pool, HLA-A non-A2 (9 peptides), 4: CD8 peptide pool, HLA-B and HLA-C (9 peptides), 5: nine peptide pools containing 15 overlapping peptides of HCV NS3/4A. In **paper II and III** groups (5-10 mice/group) of female C57BL/6J (wt), CD4-/-, CD8-/-, NS3/4A-Tg, and HBeAg-Tg C57BL/6J mice were used. Immunization was performed intramuscularly (i.m.) in the *tibialis cranialis* (TC) muscle using a regular needle and syringe or using the intracellular injection device *(IVIN)* device directly followed by

electroporation (EP) (214, 215). The mice were boosted at monthly intervals and were immunized one or two times. Analysis of HCV NS3/4A-specific immune responses were performed at two-weeks (**paper II and III**) or six months (**paper II**) post last immunization. Determination of HCV NS3-protein expressing liver cells *in vivo* was performed in groups of immunized and non-immunized mice. Six months after the last immunization (**paper II**), mice were given a hydrodynamic injection of the coNS3/4A plasmid, exactly as described previously (77).

7.8 *IN VIVO* CHALLENGE WITH HCV REPLICON AND NS3/3A EXPRESSING HEP56 CELLS

In vivo challenge with HCV replicon cells or the NS3/4A hepatoma cell was performed in naïve and immunized mice two weeks post last immunization. The hepatoma cells were inoculated subcutaneously in the right flank of the mouse. The kinetics of the tumor growth was determined by measuring the tumor volumes through the skin using a sliding caliper every second or third day (216). HCV replicon tumors were also monitored for luciferase activity using *in vivo* imaging after injection of luciferin.

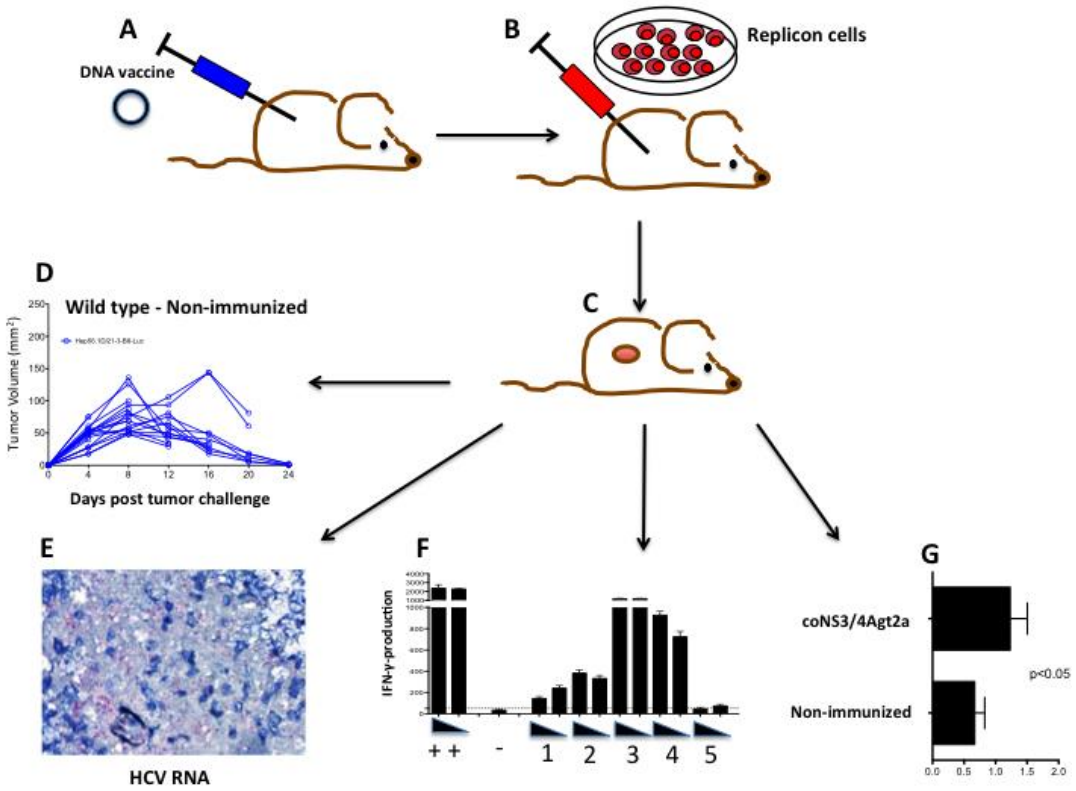

Figure 13. *Mice are immunized by i.m immunization (A). After 2-4 weeks Hep56.1D-replicon (Hep56.1D-rep) cells are harvested and inoculated subcutaneous into the right flank of the mouse (B). Tumor growth is measured every two to three days for 2-4 weeks (D). Every two to three days the tumor tissue is harvested, sectioned and using an in situ hybridization assay we determine presence of HCV RNA (E). Every two to three days or at the end of the experiment a NS3-specific IFN-γ ELISpot assay and quantification of NS3-specific CD8+ T cells using Dimer X technique.*

7.9 ELISPOT ASSAY

The ELISpot assay was used in **paper I, II, and III** to quantify IFN-γ-production of HCV NS3-specific T cells after immunization. Human PBMC (**paper I**) or splenocytes (**paper II, and III**) were obtained from immunized individuals or obtained from groups of immunized and/or tumor challenged mice that were pooled. Immediately after thawing peripheral blood mononuclear cells (PBMCs) or harvesting of splenocytes, the mean number of HCV NS3-specific IFN- γ spot forming cells was determined. The use of ELISpot to detect cytokines is widely used due to high reproducibility and sensitivity and has become a well-accepted tool for immune studies both in animals and humans.

7.10 QUANTIFICATION OF HCV NS3 GT2A-SPECIFIC CD8+ T CELLS

The frequency of NS3-specific CD8+ T cells was analyzed by *ex vivo* staining of splenocytes using the recombinant soluble mouse Dimeric Mouse H-2D [b]:Ig (BD Biosciences) as described previously (217).

7.11 STATISTICAL ANALYSIS

Statistical comparisons were performed using the GraphPad InStat 3 for Macintosh (version 3.0b; GraphPad Software, San Diego, CA) and Microsoft Excel 2008 for Macintosh (version 12.2.8; Microsoft, Redmond, WA). Growth kinetics of tumor cells in vaccinated and naive mice were compared using the area under the curve (AUC, Excel) and by analysis of variance (ANOVA). Group means were compared using either Student's t-test or Mann-Whitney. Nonparametrical data were compared using the Fisher’s exact test, the Mann–Whitney U test and Wilcoxons matched pairs test (InStat 3).

8 RESULTS

8.1 THERAPEUTIC DNA VACCINATION USING IN VIVO ELECTROPORATION FOLLOWED BY STANDARD OF CARE THERAPY IN PATIENTS WITH GENOTYPE 1 CHRONIC HEPATITIS C [PAPER I]

The safety and tolerability of DNA vaccination delivered by intramuscular immunization in combination with *in vivo* electroporation (EP) were investigated. We evaluated the pain and major adverse events after immunization and EP. The 12 subjects receiving EP described it as "leaving a small feeling of having been hit". We concluded that immunization with DNA followed by EP was safe and tolerable. Next we showed that DNA vaccination induced a transient HCV-specific T-cell activation. Due to high levels of pre-existing antibodies, the mean endpoint titers did not change significantly during the vaccination. However, when looking at the optical densities, we found an early transient effect of the vaccination. This increase was noticed in the two lowest dose groups. The HCV specific T cell response was determined by measuring the number of IFN-γ-producing spots. Our data showed an increase after the two first vaccinations comparing to week 0. Four subjects in the lowest dose group and one in the highest dose group showed detectable IFN-γ responses compared to week 0. There was a significant change in IFN-γ response before compared to after vaccination, when stimulation was performed with peptide pools. This suggests that the vaccination at least transiently enhanced the T cell response in patients with chronic HCV infection. In five patients, we have also shown a reduction in viral load. This was seen in the two highest dose groups and lasted from 2 to more than 10 weeks. We could however not associate the IL-28B CC genotype with T-cell responses. Vaccination prior to SOC treatment seemed to be favorable since five out of eight patients showed a rapid viral response. Six out of eight patients had a complete early virological response and sustained virological response (SVR), which is unusually high compared to patients only treated with SOC.

8.2 A NON-HUMAN HEPADNAVIRAL ADJUVANT FOR HEPATITIS C VIRUS-BASED GENETIC VACCINES [PAPER II]

We have previously shown that the addition of heterologous gene sequence enhances the antigen-specific vaccine response in the presence of dysfunctional antigen-specific T cell responses (208). The addition of stork HBcAg gene-sequences to our NS3/4A-DNA vaccine, co-expression of IL-12, and delivery using the IVIN device significantly improved the immunogenicity. Avian hepadnaviruses, such as stork HBV, are genetically different from human HBV. Stork and human HBcAg proteins show less than 40% homology, hence there is no evident epitope sequence homology between stork and human HBcAg. HCV-specific immune responses were significantly enhanced in wild type and NS3/4A-transgenic mice compared to previously evaluated DNA vaccines. Herein, we generated fusion genes expressing HCV NS3/4A and full-length or fragmented stork (s) HBcAg. All constructs were confirmed to have the correct size and cleavage pattern. The fusion constructs were expressed and processed to the expected proteins. By the inclusion of murine (m) IL-12 we could further improve the immunogenicity of the DNA vaccines in immunized mice. We also evaluated the fusion plasmids in combination with mIL-12 using the IVIN device followed by EP. In both wild type and NS3/4A-Tg mice the vaccine with the active protease and fragmented sHBcAg (C2.2) induced the strongest immune response

determined by IFN-γ and IL-2 production. When NS3/4A-specific T cells were primed in host with dysfunctional HBV-specific T cells, we found that the stork containing plasmid primed significantly stronger IFN-γ producing T cells compared to the same plasmid containing human HBcAg. In addition, this allowed us to immunize mice using lower vaccine doses with retained immunogenicity. Also, repeated immunization (up to five immunizations) showed improved immunogenicity in both wild type and NS3/4A-transgenic mice. This was especially the case with NS3/4A-specific IL-2 responses in wild type mice. Importantly, we found that NS3/4A-DNA immunization induced long-term memory T-cell responses that were functional for at least 6-months post immunization.

8.3 T CELL-MEDIATED PROTECTION AGAINST HEPATITIS C VIRUS IN A SYNGENEIC TRANSPLANTATION MOUSE MODEL [PAPER III]

In paper III we established an immuno-competent mouse model, which is based on syngeneic transplantation of H-2^b-restricted Hep56 cells containing a self-replicating subgenomic HCV replicon RNA of genotype (gt) 2a. Herein, we explored this mouse model for evaluation of HCV vaccines in the presence of HCV RNA replication and studies of immune escape. We could show that the replicon cells could grow and maintain HCV antigen expression in the absence of selection for at least 4 weeks (data not shown). We found that after challenge, all cell lines generated palpable tumors with volumes peaking around day 8-16 post inoculation. Tumor growth was correlated with an accumulation of inflammatory cells and central necrotic areas, associated with a decrease of HCV RNA and luciferase DNA copy numbers. The infiltration of CD3 cells was more invasive in the non-vaccinated compared to the vaccinated mice. The infiltration was almost completely prevented when tumor growth was controlled by an early HCV specific T cell response. Thus, we know now that in this model the HCV RNA replication is associated with a strong inflammatory response, which is consistent with the human HCV infection. An early HCV specific T cell response can prevent a strong inflammatory response and the expansion of HCV replicon cell *in vivo*. The early role of genotype-specific HCV-specific T cells that are controlling HCV RNA levels is lost at later time points. DNA vaccination resulted in a robust NS3/4A(gt2a)-specific CD4+ and CD8+ T cell response protecting wild-type, but not HCV NS3/4A(gt1a)-transgenic (Tg) mice against HCV replicon cell tumors suggesting cross-genotypic tolerance in NS3/4A(gt1a)-Tg mice. HCV replicon cells were reduced in the presence of early T cells both in wild type and in Tg mice. In order for us to have a better understanding of the protective T cell response we identified two H-2^b restricted NS3/4A gt2 specific cytotoxic T lymphocyte epitopes. Mice immunized with NS3/4A gt2 DNA were protected against replicon cell induced tumors. This was in contrast to the non-immunized mice that were not able to control the tumor growth. Hep56 cells stably expressing gt2a NS3/4A primed and boosted a stronger CTL-dominated response, despite comparable levels of NS3-protein. This was also confirmed by the Dimer X technology where the expansion of epitope-specific CTLs could be quantified. The HCV replicon cells only weakly recalled detectable CTL responses, whereas Hep56 cells stably expressing gt2a NS3/4A recalled prominent CTL responses. Hence, the weaker response primed by the replicon cells supports the fact that the HCV replication is poorly immunogenic, and therefore very similar to the human HCV infections. In addition, the protection against the replicon tumors was found to be dependent on both CD4+ and CD8+ T cell responses (data not shown).

9 DISCUSSION

HCV is a virus characterized with a high genetic variability. Some of the HCV proteins impair the host response so that the host immune system is incapable of fighting the infection. This may be seen as a dysfunctional immunity. The specific T cell response is most likely dysfunctional when the infection becomes chronic. One way to overcome this dysfunction may be therapeutic vaccination. Through therapeutic vaccination dysfunctional HCV-specific T cells can be re-activated, or new T cells may become activated. Today, a treatment for chronic HCV does exist. The DAAs have a cure rate up to 95%, which is a revolution in the HCV history. However, cure through DAA treatment do not give rise to immune memory (e.g. no evident protection against re-infection). Thus, there are several issues we face with the DAAs; only a fraction (10%) of the worlds HCV chronic infected individuals has access to the treatments. Due to the high cost of the antivirals only parts of the wealthiest countries in the western part of the world have access to these DAAs. All individuals with chronic HCV infection cannot be treated with the DAAs, due to individual aspects such as, HCV genotype and drug resistance issues. However, the fact still remains that although the DAAs have a high cure rate, a prophylactic or therapeutic vaccination is still of importance in order to protect against new infections and to provide patients with post-cure HCV-specific immune protection. One approach can be to use the DAAs together with vaccination in order to overcome the hurdles that exist today. Therapeutic DNA vaccination is of importance not only because the different aspects such as the low cost, easier to manufacture and better storage possibilities, but importantly it also gives rise to cellular immunity. Therefore we have made a first-in-man phase I clinical trial of HCV DNA vaccination with intramuscular delivery of DNA in combination with *in vivo* EP. In addition, after the therapeutic vaccination, the patients received SOC treatment. We wanted to investigate if HCV vaccination was safe in patients infected with chronic HCV and if vaccination had any effect on the immune response. Our data in **paper I** suggests that therapeutic vaccination delivered by intramuscular immunization in combination with EP was safe, immunogenic and possibly had transient effects on the viral replication. We noted that 75% of the vaccinated patients were cured from their HCV infection after SOC treatment.

In order to prime potent immune response using a DNA vaccine the use of, molecular adjuvants are an important key factor. To improve the immunogenicity of DNA vaccines several different parameters can be adjusted such as addition of adjuvants, modifications of the vaccine-antigen, and use of different delivery technologies, in order to better activate or re-activate the dysfunctional T cells during a chronic infection. In **paper I,** we found out that the DNA vaccine was not immunogenic enough to activate immune responses that could clear the HCV infection. Therefore, in **paper II**, we tried to make the DNA vaccine more immunogenic. It has previously been shown that HBcAg from human HBV can act as a molecular adjuvant. HBcAg is a unique protein since it is presented by B-cells through cross-linking and causes Toll-like receptor 7 (TLR-7) activation. However to overcome the problem of having pre-existing immune responses to human HBcAg, to which 2 billion people have been exposed and another 350 million people living with chronic HBV infection, we instead chose stork HBcAg that has similar properties, but only a 40% sequence homology with human HBcAg. Moreover, the use of human HBcAg may cause unexpected and undesired effects such as fulminant hepatitis. Hence, a safe adjuvant is desired. Thus, in **paper II**, we used stork HBcAg, which is highly immunogenic, and stork HBcAg and human

HBcAg do not share the same HLA/MHC class I and II epitope sequences. This would minimize the risk of cross-reactivity between individuals previously exposed to human HBV. In addition, by using mIL-12 as an adjuvant, IVIN and EP as delivery methods we could further improve the immunogenicity of the DNA vaccine. We found that fragmented stork HBcAg was more effective and improved T-cell priming to HCV NS3/4A. We could effectively prime CTLs specific for HCV NS3/4A without the induction of CTLs specific to human HBcAg. Repeatable immunization at monthly intervals was found to further boost the immunogenicity of the vaccine. We have shown, in **paper II**, that the use of stork HBcAg gene sequence is effective, simple, and safe. This vaccine approach and design may also be used and applied to other vaccine antigens. As mentioned above, specific immune responses can be re-activated by immunizing a host with an optimal immunogenic composition.

In order to study HCV therapeutic vaccines and therapies, one needs a small and reproducible animal model. Today there is no small animal model with an intact immune system that allows for such studies. An animal model with an intact immune system is essential in order to be to study all aspects of the immune system. In **paper III,** we have generated and established an immuno-competent mouse model based on a mouse hepatoma cell line with HCV RNA NS2-NS5B subgenomic replicon replication. The HCV replicon cells, like the HCV infection in the liver, attract unspecific immune cells that cannot control the infection, in our case tumor growth. In this study we have shown that an early and broad specific CD4 positive and CD8 positive T cell response is needed in order to prevent the expansion of HCV replicon cells and tumor formation. This resembles the events in patients who spontaneously clear the acute HCV infection. Interestingly, the HCV replicon cells seem to be less immunogenic *in vivo*, which mimic the inhibitory effects of the HCV replication itself. We have shown that mice challenged with the replicon cells after vaccination are protected against tumor growth. Even though the replicon cells lack many features of the human HCV infection such as release of infectious particles, and difficulties in tracing HCV RNA replication, the replicon cells mimic key events in human HCV infection; they harbor HCV RNA replication simultaneous with an inflammatory infiltrate. Thus, we have established a new simple mouse model in which we may understand the immune responses to liver cells with HCV RNA replication. This model will enable us to study our DNA vaccines in a reproducible small animal with an intact immune system in the presence of HCV RNA replication. In conclusion, we have performed the first-in man therapeutic HCV DNA vaccine study using *in vivo* EP, which was safe with no severe adverse events and showed immunogenicity. We then improved the immunogenicity of our DNA vaccines by using avian HBcAg, mIL-12, IVIN and EP. All these changes have significantly enhanced the priming of HCV NS3/4A specific CTLs. Last but not least, we have developed an immuno-competent mouse model that allow for studies of HCV immunity and RNA replication. We have shown that DNA vaccination protects against tumor growth in mice, RNA levels seems to correlate with the inflammatory infiltration and immune response seen in tumor tissues.

10 GENERAL CONCLUSION

- ✓ DNA immunization followed by *in vivo* EP was safe and tolerable in patients with chronic HCV infection and induced a transient HCV-specific T-cell activation in patients with chronic HCV infection.

- ✓ The stork HBcAg acts as an effective adjuvant for HCV-derived antigens and induced a strong immune response determined by IFN-γ production. The mIL-12 could further improve the immunogenicity of the DNA vaccine in immunized mice.

- ✓ The HCV replicon cells could grow and maintain HCV antigen expression in the absence of selection. A DNA vaccination resulted in NS3/4A(gt2a)-specific CD4+ and CD8+ T cell response generating protection against HCV replicon cell tumor growth.

- ✓ HCV RNA replication could coexist with a inflammatory infiltrate, mimicking key aspects of the human infection.

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مادر و دوست عزیز من
کلمه یا جمله را نمیتوانم پیدا کنم که بگوایم چقدر در زندگی من مهم بودی و هستی و چقدر من دوستت دارم .از تو تشکر میکنم برأی زندگی که دارم تو برأی من یك فرشته بودی و هستی فرشته ای که در کنار من بودی و محافظ من بودی.
من از تو تشکر میکنم برأی أین مادر و همسری که امروز هستم.
برأی من افتخار بزرگی هست که مادري مثل تو دارم و برأی من باعث افتخار است که دختر تو هستم.
مادر عزیزم تو هر روز و همیشه فرشته نجات من بودی و هستی.
کلمه و جمله ای پیدا نمیکنم که بگویم چقدر دوستت دارم
مادر عزیزم دوستت دارم

To my father **Mokhtar,** thank you for pushing and challenging me, for inspiring me to be the best I can be. Thank you for all the years you were by my side. I wouldn't be here if it wasn't for you.

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