DEVELOPMENT OF A THERAPEUTIC VACCINE AGAINST THE HEPATITIS C VIRUS

Gustaf Ahlén

Stockholm 2007
Cover picture:
Expression of the hepatitis C virus non-structural 3 protein in a mouse *tibialis anterior* muscle seven days post immunization and *in vivo* electroporation. NS3-protein was detected in a histological section using a rabbit anti-NS3 antibody.

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In loving memory of my mother

In loving memory of my mother
ABSTRACT

The hepatitis C virus (HCV) infection is a major cause of liver disease and it is estimated that 170 million people worldwide are chronically infected by HCV. There are no protective or curable vaccines available for HCV, however treatment consisting of interferon-α and ribavirin is curative in 45-75% of the chronic infected patients, depending on the viral genotype. The antiviral treatment has the lowest efficacy for patients infected by genotype 1. Today’s treatment regimens are associated with many side effects. Thus, new antiviral treatment regimens and/or vaccines are in urgent need. The majority of infected individuals develop a chronic disease and the reason for the high rate of persistence is in part explained by the highly genetic variability of HCV. Thus, development of a therapeutic vaccine against HCV should therefore be targeted against a region of the HCV genome with a limited genetic variability. We have based our development of a genetic vaccine on the non-structural (NS) 3 and NS4A proteins. The NS3 protein performs essential functions in the viral life cycle including protease and helicase activities. The NS3 co-factor, NS4A, is important for NS3 to stabilize the protein complex and to fully utilize its functions. Previous studies have shown that NS3/4A when delivered as a genetic vaccine induce both humoral and cellular immune responses in mice. We now investigated if we could further enhance the immunogenicity of the NS3/4A DNA vaccine. Codon optimization (co) of the NS3/4A DNA gene resulted in an enhanced immunogenicity explained by the increase of NS3/4A-protein expression. Due to the lack of small animal models to study HCV, we have generated a mouse model with transient expression of HCV proteins in the liver. By using this model we could show that peripherally vaccine-primed T cells could enter the liver, recognize and eradicate NS3/4A expressing hepatocytes, a prerequisite for a functional therapeutic vaccine against HCV. The NS3/4A protein has recently been shown to interfere with the innate immunity through cleavage of Cardif (also known as IPS-1, MAVS, VISA), resulting in suppression of the interferon response within the infected cell. We found that cleavage of Cardif by NS3/4A also occur in murine cells making it possible to study the effect of this interaction also in mouse models with hepatic expressing the NS3/4A-protein. The effects that NS3/4A exert on the innate immunity do not seem to affect the adaptive immunity, since NS3/4A-protein expression did not prevent clearance of transiently transfected hepatocytes in vivo. This helps to explain why escaping the adaptive immunity through mutations should be beneficial for HCV. To better understand the relationship between immune escape and viral fitness we studied the immunodominant human HLA-A2-restricted epitope at residues 1073-1081 of NS3. Despite that the epitope is immunodominant, only a limited number of mutations occur within this epitope. We now show that the absence of mutations at some of these positions can be explained by a reduced protease activity and viral replication, which reduce the viral fitness. DNA vaccines have been shown to induce promising immune responses in animal models. However, so far DNA vaccines have not been found to prime effective immune responses when tested in primates / humans. The explanation for this is at least partly explained by the poor uptake of the plasmid DNA in humans. We therefore evaluated different delivery methods of our DNA vaccine using transdermal delivery by the gene gun or by intramuscular delivery in combination with in vivo electroporation (EP). These studies revealed that the coNS3/4A vaccine primed the broadest immune responses when delivered with in vivo EP. Importantly, although NS3/4A can block the response to dsRNA, these signal pathways are not activated during DNA immunizations which helps to explain the effectiveness of the DNA-based coNS3/4A vaccine. The coNS3/4A vaccine was evaluated in a toxicological study in rabbits which showed that the vaccine had an acceptable safety profile and biodistribution when administred using in vivo EP. Finally, the current coNS3/4A DNA vaccine delivered using in vivo electroporation was recently approved by the Swedish Medical Products Agency to enter a clinical trial, which will be the first DNA vaccine delivered in combination with in vivo electroporation against an infectious disease in humans.

Keywords: HCV, NS3, DNA vaccine, electroporation

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LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to by their roman numbers [I-V].

I. Frelin L, Ahlén G, Alheim M, Weiland O, Barnfield C, Liljeström P and Sällberg M.
   *Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus nonstructural 3/4A gene.*

II. Ahlén G, Nyström J, Pult I, Frelin L, Hultgren C and Sällberg M.
    *In vivo clearance of hepatitis C virus nonstructural 3/4A-expressing hepatocytes by DNA vaccine-primed cytotoxic T lymphocytes.*
    *Journal of Infectious Diseases* (2005), 192, 2112-2116.

    *Relation between viral fitness and immune escape within the hepatitis C virus protease.*

    *Cleavage of the mouse Cardif/IPS-1/MAVS/VISA does not inhibit T cell-mediated elimination of hepatitis C virus non-structural 3/4A-expressing hepatocytes.*
    Submitted.

    *In vivo electroporation enhances the immunogenicity of hepatitis C virus nonstructural 3/4A DNA by increased local DNA uptake, protein expression, inflammation, and infiltration of CD3+ T cells.*
    *Journal of Immunology* (2007), 179, 4741-4753.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>co</td>
<td>codon optimized</td>
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<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>ds</td>
<td>double stranded</td>
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<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ER</td>
<td>endoplasmatic reticulum</td>
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<tr>
<td>EP</td>
<td>electroporation</td>
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<tr>
<td>gt</td>
<td>genotype</td>
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<td>HD</td>
<td>hydrodynamic</td>
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<td>hepatocellular carcinoma</td>
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<td>HCV</td>
<td>hepatitis C virus</td>
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<td>HLA</td>
<td>human leucocyte antigen</td>
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<td>IFN-α</td>
<td>interferon-alpha</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
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<tr>
<td>i.d.</td>
<td>intradermal</td>
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<tr>
<td>i.m.</td>
<td>intramuscular</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>NK</td>
<td>natural killer cell</td>
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<tr>
<td>NS</td>
<td>non-structural</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>pDNA</td>
<td>plasmid DNA</td>
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<tr>
<td>RdRp</td>
<td>RNA dependent RNA polymerase</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ss</td>
<td>single stranded</td>
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<tr>
<td>sTg</td>
<td>stably transgenic</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TLR</td>
<td>toll like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>tTg</td>
<td>transient transgenic</td>
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<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T-helper</td>
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1 INTRODUCTION TO HEPATITIS

The term hepatitis origins from the ancient greek, meaning inflammation of the liver, *hepar* is liver and *itis* refers to inflammation. There are several causes for hepatitis, viral or bacterial infections, toxins, chemicals, alcohol, drugs, autoimmune or metabolic disorders etc. To date, there are five human hepatitis viruses identified, hepatitis A, B, C, D and E. Other viruses that may cause hepatic infections are Cytomegalovirus (CMV), Epstein Barr virus (EBV), Herpes Simplex virus (HSV), Human herpesvirus 6 (HHV-6) and Varicella Zoster virus (VZV).

When hepatitis viruses enter the body, the virus will be transported by the bloodstream to the liver were it might cause an acute infection. The primary site for hepatitis viruses replication is the hepatocytes, representing about 75% of the cells in the liver. Elevated liver enzymes are a common feature of the clinical acute phase of a hepatitis infection, easily measured in the blood. Another feature are jaundice, or the yellowish tone of the skin, caused by the presence of bilirubin in the circulation. Other symptoms are fever, myalgia, nausea, fatique and vomiting. However, an acute infection with a hepatitis virus can also be asymptomatic. If the viral infection in the liver persists for more than 6 months it is regarded to be a chronic infection. Moreover, the chronic phase can vary from an asymptomatic to a symptomatic infection. The latter type is often associated with an active inflammation and a increased risk of developing cirrhosis and hepatocellular carcinoma (HCC). Albeit the hepatitis viruses all infect the liver and cause similar symptoms the viruses are quite different. The hepatitis A virus (HAV), discovered in 1973, is a single stranded (ss) RNA virus belonging to the *Picornaviridae* virus family [1]. HAV is transmitted by the faecal-oral route and has an incubation period of 2-6 weeks. The virus only causes acute infections and are often more symptomatic in adults as compared to children. It is believed that the HAV infection induces lifelong protection against re-infection. Both passive immunization using immunoglobulins or prophylactic vaccination is available. Hepatitis B virus (HBV) was discovered in 1965 and is a partially double stranded (ds) DNA virus classified into the *Hepadnaviridae* virus family [2]. HBV is transmitted through contaminated blood, sexual or vertical transmission and it is estimated that 350 million persons worldwide has a chronic infection. Only about 5% of infected adults develop a chronic infection but as many as 90% of infants become chronic carriers. This has been explained by the...
lack of a potent immune response and the potential ability of HBV to induce
immunological tolerance in the infant. Studies have shown that HBeAg (a secreted viral
protein that have shown not to be required for infection, assembly, nor replication of
HBV) can pass the placenta of the infected mother and enter the foetal circulation
resulting in HBV tolerance during neonatal development. A long-term chronic liver
disease is associated with development of cirrhosis and HCC. Immune modulating
treatment, interferon-α (IFN-α), in combination with anti-viral drugs, such as
lamivudine or adefovir, is used to control the chronic infection. Highly effective
prophylactic vaccines for HBV are available. The hepatitis C virus (HCV) was
discovered in 1989 and belongs to the Flaviviridae virus family [3]. The virus is a
blood-borne virus with a ssRNA genome causing chronic infections in 70-90% of those
infected. About 170 million persons worldwide are chronically infected with HCV and
no vaccine is yet available. Treatment involves interferon-α in combination with the
nucleoside analogue ribavirin. Like HBV, HCV also causes chronic infection that may
result in cirrhosis and HCC. HCV will be discussed in greater detail in chapter 2.
Hepatitis D virus (HDV), or the “deltavirus”, was discovered in 1977 and is an ssRNA
virus that is replication incompetent by itself [4]. HDV is dependent on the presence of
the HBV surface antigen to generate new infectious particles. It has therefore been
referred to as a satellite virus to HBV. HDV is transmitted either as a co-infection,
together with HBV, or as a super-infection of an already established HBV infection,
and generally causes a more severe hepatitis than HBV alone. It is estimated that 5% of
those infected by HBV are carriers of HDV. Treatment and prophylactics developed for
HBV are used for treatment. Finally, hepatitis E virus (HEV) is also an ssRNA virus
that was discovered in 1983 [5]. The virus, recently classified into the Hepeviridae
virus family, causes only acute hepatitis and is transmitted faecal-oral. There are no
treatment or preventive vaccine for HEV.
2 HEPATITIS C VIRUS

2.1 HISTORY

After development of serological tests to screen blood donors for HAV and HBV in 1970s it became clear that many cases of post-transfusion hepatitis could not be explained by these agents. This lead to the designation of a yet unknown hepatitis virus as causing “non-A, non-B” hepatitis [6, 7]. The disease was transmissible to chimpanzees and was confirmed to cause a persistent infection. In 1989 the genome of the infectious agent was first cloned and characterized, serological tests were developed and the cause for “non-A, non-B” hepatitis was named the hepatitis C virus (HCV) [3]. HCV was found to have an RNA genome with characteristics resembling the flaviviruses (Yellow fever virus, West-Nile virus and Dengue Fever) and pestiviruses (bovine viral diarrhea virus). HCV was therefore classified as a third separate genus hepacivirus in the Flaviviridae virus family [8]. Since the very early 1990s, all blood used for blood transfusion are screened for HCV and today transfusion associated transmission of HCV is rare in the developed countries.

2.2 HCV INFECTION

The hepatitis C virus is transmitted mainly through blood-to-blood contact with intravenous drug use as the dominating route of transmission today in developed countries. In developing countries nosocomial transmission of HCV is a not uncommon route of infection and may be vastly underestimated. Other more rare transmission routes are vertical (mother-to-child) and sexual transmission. The most predominant risk factor associated with HCV transmission is as mentioned intravenous drug use, other possible ways of transmission include tattooing, body piercing, blood transfusions and transplantation of organs. HCV infection can in approximately 20% of patients cause an acute infection, most often without any or with mild clinical symptoms such as jaundice, malaise and nausea [9]. Fulminant hepatitis (liver failure) has been reported during the acute phase but it is extremely rare [10]. Within a period of 15-150 days the infected person will develop a liver cell injury evidenced by elevated levels of serum alanine aminotransferase (ALT). HCV is self-limited in only 10-30 % of the cases, characterized by disappearance of serum HCV RNA and normalized liver
enzymes [11, 12]. The majority of infected individuals (70-90%) are unable to clear the viral infection within a 6-months period and will develop a chronic infection.

The chronic infection is characterized by fluctuating or persistently elevated ALT levels with an inflammation of the liver and a slowly progressing fibrosis (liver scarring). Most chronically infected individuals will have a mild to moderate liver disease with normal or almost normal liver function. Some will experience an active hepatitis with fibrosis and subsequent liver failure. Steatosis, or fatty liver, is another histological feature more commonly seen in chronic HCV infected individuals compared to the general population. In chronic HCV patients the prevalence of steatosis ranges from 40-86%. Except for general factors associated with hepatic steatosis such as high alcohol consumption or obesity, steatosis is more common in individuals infected with HCV genotype 3 [13]. Major factors associated with fibrosis progression are, male gender, alcohol consumption and older age at time of infection. It is also possible that the degree of steatosis influence the fibrosis progression whereas viral load do not seem to influence significantly [14]. Approximately 20 % of those chronically infected will develop cirrhosis within a 10-30 year period, and these patients have an elevated risk of developing hepatocellular carcinoma [15, 16]. Histological analysis of liver biopsies is an important tool to determine and follow the chronic liver disease and the progression of fibrosis and cirrhosis. Factors shown to affect the progression of the chronic disease (summarized in Figure 1) are many and are

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**Figure 1.** Flow chart of the clinical course of HCV infection and factors affecting the disease progress. Response rates to pegylated-interferon-α and ribavirin treatment. (NR=non-responder, SVR=sustained virological response).
dependent on both host and viral factors. HCV infection can be diagnosed using immunoassays such as enzyme immunoassay (EIA) or recombinant immunoblot assay (RIBA) by detection of anti-HCV. The diagnosis of HCV infection can also be confirmed by detection of HCV RNA using reverse transcriptase polymerase chain reaction (RT-PCR).

2.3 EPIDEMIOLOGY

It is estimated that 3% (170 million) of the world’s population are chronic carriers of HCV [17]. In developed countries the prevalence range from 0.1-2%, and in developing countries the prevalence is higher. Egypt has an exceptionally high prevalence ranging from 5 to as high as 20%. The high prevalence seen in Egypt is explained by the use of contaminated needles during mass-administration of parenteral antischistosomal therapy back in the 80’s [18]. It is estimated that 10 million Europeans are chronic carriers of HCV [19]. In Sweden approximately 40,000 cases of chronic HCV infection has been reported since 1990 when anti-HCV screening test for blood donors were introduced. In 2006 a total of 1977 cases of HCV were reported in Sweden with 57% related to intravenous drug use [20].

2.4 HCV GENOME

The hepatitis C virion is a spherical particle of approximately 55-65 nm [3, 21]. The genome consists of a single stranded positive sense RNA of approximately 9600 nucleotides, containing a single open reading frame (ORF). The ORF encodes a precursor poly-protein of 3010-3033 amino acids (aa) encoding the 10 viral proteins. The precursor protein is cleaved into the structural proteins core (c), envelope (E) 1, E2 and p7 and the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B [22-24].
Figure 2. Schematic illustration of the HCV genome, polyprotein processing and the protein membrane association in the endoplasmatic reticulum (ER).

The cleavage of the structural proteins is performed by host cell signal peptidases, the non-structural proteins are cleaved by viral proteases, NS2-NS3 proteins by the NS2 protease and the cleavage downstream NS3 is mediated by the NS3 protease. The highly conserved 5’ untranslated region (UTR) of 341 nucleotides (nt) in length, contain an internal ribosome entry site (IRES) that is essential for initiation of translation of the viral RNA [25, 26]. It has also been shown that sequences within, and upstream of, the IRES are required for efficient replication of the virus [27]. The 3’UTR is composed of three regions of 200-235 nt in length. The middle section contains a 80 nt highly variable poly(UC) region. The first (52 nt), and the last part (98 nt) are conserved among genotypes [28, 29]. The core protein, located in the N-terminus of the HCV genome, forms the viral nucleocapsid. The amino acid sequence of core is highly conserved among different HCV strains as compared with other HCV
proteins. The C-terminal part of the core protein is hydrophobic and the N-terminal highly hydrophilic and basic. Core protein is primarily detected in the cytoplasm, in association with the endoplasmatic reticulum (ER) and in lipid droplets (LD) [30-32]. The core protein is a multifunctional protein involved in viral replication, maturation of viral particles and in the pathogenesis of the viral infection [33]. The two envelope glycoproteins E1 and E2 have important roles in the HCV lifecycle. They participate in the assembly of the infectious virus particle and are essential parts of the viral envelope. The E proteins are present on the surface of the virus and are necessary for viral entry into the host cell through binding to cellular receptors. The most variable region of the HCV genome is found in a parts of the E2 protein, referred to as hyper variable regions (HVRs) 1 and 2. The E1 and E2 are both C-terminal trans-membrane proteins present as a heterodimer formed in the ER [33-35]. The p7 is a small membrane protein composed of two trans-membrane domains. Although the function of p7 is unclear, studies in artificial lipid membranes show that p7 has ion-channel activity. It has also been shown that the p7 is needed for production of infectious virions in vivo [36, 37]. The NS2 is a trans-membrane protein forming at least three helices into the ER. NS2 participates in the protease activity responsible for the cleavage of the NS2-NS3 proteins [38]. The NS3 is a multifunctional protein with an N-terminal serine-protease domain of around 180 aa, and a C-terminal 442 aa domain with helicase/NTPase activities. The complete protease encompasses both the NS3 and the 54 aa co-factor NS4A. The NS4A protein is important for complete folding and membrane anchoring of the NS3 protease domain thereby optimizing the protease activity of NS3. The NS3/4A protease is responsible for polyprotein processing of the junctions downstream the NS3, an absolute essential activity for the generation of components of the viral RNA replication complex. The NS3 protein lacks a trans-membrane domain and therefore interacts with the central domain of NS4A to associate to the ER. The N-terminal part of NS4A containing a transmembrane sequence targets the complex to the ER membrane to enable interaction with the other membrane bound proteins in the replication machinery. NS4A also stabilizes the protease against proteolytic degradation [39-43]. The NS3 protease has also been shown to, in several ways, be involved in blocking the ability of the host cell to mount an innate antiviral response. These events are being discussed in detail in the section “Viral evasion strategies to HCV”. The C-terminal part of NS3 comprises the helicase-NTPase domain capable, in an NTP-dependent manner, of unwinding and strand separate RNA homoduplexes in a 3´to 5´ direction [42, 44]. The dual activities of the NS3 protein, paired with essential
functions for the viral persistence, are thought to contribute to the genetic stability of the protein. The NS4B is an integral membrane protein containing at least four transmembrane domains. NS4B is thought to be involved in the formation of a structured compartment, the membranous web, where the RNA replication takes place [45-47]. The NS5A protein, even though function not yet has been determined, is believed to be important for the viral replication [48]. NS5A has also been of interest due to its potential role in modulating the interferon response [49], and interaction with host cell antiviral signaling pathways (described in section “Viral evasion strategies to HCV”). Like most of the HCV non-structural proteins, also NS5B is membrane associated to the ER. NS5B is an RNA-dependent RNA polymerase (RdRp), and serves as the catalytic component of the HCV RNA replication complex [50, 51]. Recently another protein, known as ARFP (alternative reading frame protein) has been reported to be expressed from the core region due to a translational frame shift. So far the function for this protein is poorly understood. Studies have shown that it might have some type of functionally important RNA element since ARFP defective clones had low viremia and show minimal levels of liver damage in chimpanzee [52, 53].

2.5 GENETIC DIVERSITY

A common feature of single stranded RNA viruses, such as HCV, is that the genome often displays a high genetic variability. The high diversity is due to that the viral RdRp does not have proofreading activity, resulting in that erroneous nucleotide introductions are not corrected during replication. Hence, any new mutation will be incorporated in the progeny virus. Because of the high replication rate generating $10^{10}-10^{13}$ particles per day, the frequency of errors is high, resulting in about $10^3$ nucleotide substitutions/nucleotide/year. Subsequently, unlike the human immunodeficiency virus type-1 (HIV-1) where viral replication is generated by the host cell polymerases, each HCV infected cell has the ability to generate multiple viral species. Some mutations that accumulate during replication are synonymous, or silent, and will have no effect on the amino acid sequence but may have an impact on the secondary structure of the genomic RNA. Other mutations, non-synonymous will lead to a change in amino acid and subsequently the protein sequence leading to a unique viral variant, which may be of survival benefit for the virus. These kinds of mutations can also lead to the production of a defective viral genome that is lethal for the virus [54, 55]. HCV can be
classified into 6 different genotypes (gt), indicated by a number (1-6), and further divided into subtypes, indicated by a letter (a, b, c etc.), which represent subgroups of the most closely related viruses within an genotype. Genotypes differ from each other with 31-33% on nucleotide level, compared to subtypes that differ 20-25% [56, 57]. Another level of variability can be found within an individual host. These populations, termed quasispecies, represent closely related viral variants but with distinct genomes. The quasispecies display a variability of 1-9% in an infected host. The most conserved region found within the HCV genome is the 5′ untranslated-region with more than 90% identity between genotypes [25, 58]. Also core, NS3 and part of NS5B are well conserved, as compared to the other viral proteins [59, 60]. The most variable region is found within the HVRs of E2 were as much as 50% diversity exists among the different genotypes [25, 58]. Genotype 1 is the dominating genotype worldwide and represent >50% of all infections. Subtype 1a is dominating in America and Northern Europe, and 1b is extensively spread in Eastern and Western Europe. Genotype 2 is distributed worldwide. Also gt 3 are distributed worldwide but can be found more frequent in South Asia. Genotype 4 is found mainly in northern-Africa, gt 5 in South Africa, and gt 6 is found in Asia [61].

2.6 VIRAL LIFECYCLE

Due to lack of convenient animal models and, until just recently, efficient cell culture systems the HCV viral lifecycle is not fully understood. The HCV lifecycle can in a simplified way be divided into three different steps. A) Binding and entry into the host cell (1-2), B) translation and replication of the viral RNA (3-4) and C) viral assembly and release from the host cell (5-7).
2.6.1 A) Binding and entry into the host cell

The initial step in the viral lifecycle involves the attachment and receptor binding by the virus to the hepatocyte. Several receptors have been suggested to be involved in this process. The first co-receptor identified, CD-81, binds the E2 protein and has subsequently been shown to be important for viral attachment to the host cell, in the HCV cell culture (HCVcc) system [62, 63]. Another target for HCV attachment are the human scavenger receptor class B type I (SR-BI), also shown to be necessary for the viral entry [64]. Other molecules thought to be involved in the cell attachment are the lectins L-SIGN and DC-SIGN, glycosaminoglycans and low-density lipoprotein receptor (LDLr) [34, 64-66]. However, recently another receptor, Claudin-1, has been shown to be involved in the late stage of viral entry, after E2 binding to the co-receptor CD-81. Claudin-1 is an integral membrane protein, a tight junction highly expressed in the liver and necessary for the viral entry [67]. Besides the liver, HCV has been proposed to be present in extrahepatic compartments, such as peripheral blood mononuclear cells (PBMCs), but productive infections have not been found outside the
liver. Whether this is due to insufficient entry or replication is unclear but studies using HCVpp indicate no detectible entry into PBMCs [68]. After attachment, the virus enters the host cell by endocytosis and the release of the viral RNA genome into the cytoplasm is thought to be pH dependent.

2.6.2  B) Polyprotein processing and replication of the viral RNA

Immediately after the release of the viral RNA into the cytoplasm it acts as an mRNA. Translation of the viral proteins is initiated by the binding of the 5´-IRES to ribosomes. The translation generates the polyprotein that is co- and posttranslational cleaved into the 10 different proteins. The structural proteins are cleaved by host cell peptidases, the NS2/3 auto-protease cleaves the NS2-NS3 site and the NS3/4A is responsible for cleavage of all junctions between NS3-NS5B [33]. It was recently described that lipid droplets (LDs) are important for the viral replication. The core protein associates with LDs and recruits non-structural proteins and the replication complex to LDs-ER-associated membranes [31]. It is believed that newly translated NS5B RdRp replicate the genome by synthesis of a full-length negative strand RNA replication intermediate. This negative strand then serves as a template for synthesis of positive RNA strands, used for further translation, replication and RNA genomes for new virus particles. The replication is driven by the NS5B RdRp activity but other viral and host factors are important for a functional replication complex. The NS3/4A helicase/NTPase are involved in the RNA synthesis by unwinding and strand separation of the double stranded replication intermediates [42, 44]. Also, the NS4B might play an important function in the replication complex being be responsible for induction of the membranous web [46].

2.6.3  C) Viral assembly and release from the host cell

Much is still unclear on how the assembly and release of the virus particles occur. A crucial function of the core protein is the assembly of the viral nucleocapsid. Once the nucleocapsid is formed in the cytoplasm, it acquires an envelope as it buds through ER or other intracellular membranes. As seen in other members of the Flaviviridae family, HCV virus formation may occur, by interaction between core and the E1 and E2 proteins, followed by budding into the ER lumen. The mechanism how the viral RNA
associates with the viral core particle is still not determined, but the genome most likely interacts with the basic domain of the core protein. The progeny particles are thought to be released through the golgi complex via the secretory pathway before release from the host cell [33, 69].

2.7 HCV MODEL SYSTEMS

2.7.1 Experimental animals models

2.7.1.1 Chimpanzee
The possibility to study HCV in vitro has until recently not been possible. Except humans, chimpanzee is the only natural animal model susceptible for infection of HCV. Studies in chimpanzees have generated an extremely valuable understanding of HCV such as, viral transmission, replication and immune responses during the infection. Disease progress of HCV in chimpanzees is similar as in humans but the liver disease is milder and important differences may be present. Although chimpanzees are a valuable model system providing important information on HCV these experiments are associated with ethical issues, limitations in numbers of animals, and are highly costly to maintain. Therefore several attempts are being done to establish small animal models systems that can at least in part replace the use of chimpanzees but still emulate the in vivo situation.

2.7.1.2 The albumin promoter driven urokinase-type plasminogen activator (Alb-uPA) mice
One interesting mouse model system is the SCID/Alb-uPA. The Alb/uPA transgenic mouse, described in 1990 [70], express murine urokinase genes under the control of the albumin promoter. Transgene over expression of murine urokinase-type plasminogen activator (uPA) in these mice causes hepatocyte death, with hemorrhagic events due to defects in the coagulation system, but the liver also show a continuous regeneration. By crossing the Alb/uPA gene onto a severe combined immunodeficiency (SCID) background, a mouse model that tolerates xeno-transplantation of liver cells has been generated. Transplantation of human hepatocytes results in a chimeric liver with over 50% of the hepatocytes from human origin. The SCID/Alb-uPA mouse is susceptible for HCV infection with virus titers up to 10^6 copies/mL in blood [71]. This model system is applicable for studying anti-HCV drugs in vivo and has been used to study the
antiviral and side effects of NS3 protease inhibitors [72]. The major limitations of this system is that these mice are difficult to generate and due to immunodeficiency not useful from an immunological perspective.

2.7.1.3 Mice with germline integrated transgenes

Several transgenic mouse lineages have been generated to study the in vivo effect of individual or co-expression of HCV proteins. Core [73-77], core-E2 [78-81], core-p7 [82, 83], core-NS2 [84], E1-E2 [85], E2 [75], NS3/4A [86], NS4B [87], NS5A [88] and full-length [82, 89] stably transgenic (sTg) mice using different mouse strains and promoter constructs has been described. Only some major observations will be discussed here. Core Tg mouse lineages develop steatosis and HCC [73, 90], which are pathologies seen in chronically HCV patients. However, other groups report that no liver disease or change in pathology can be observed in their core sTg mice [74-76]. The reasons for these discrepancies are not clear but may be due to use of different mouse strains, haplotypes, or differences in core protein expression levels. Several other effects have been reported to be associated with core expression in mice, inhibition of the suppressor of cytokine signalling protein (SOCS)-1 expression [91], constitutive expression of signal transducers and activator of transcription (STAT)-3 [92], modulated sensitivity to Fas mediated apoptosis represent some of them [74]. The core-E2 sTg mice show similar pathology as the core sTg mice, ranging from no liver pathology to development of HCC [78-81]. In mice expressing only the E glycoproteins no effects have been observed [75, 85]. The NS3/4A Tg mice do not show any changes in the liver pathology, but alterations of hepatic immune cell subsets were observed [86]. These mice also had a reduced sensitivity to tumor necrosis factor (TNF)-α mediated liver disease. Recently the generation of a NS4B sTg mice was reported but data describing the effect of the protein is so far very limited [87]. The NS5A sTg mice do not develop any liver disease but seem to have, as the NS3/4A sTg mice, a reduced sensitivity to TNF-α induced apoptosis [88]. Finally we have the sTg mice expressing the full-length polyprotein of HCV developing steatosis and HCC [82]. Infection studies in these mice, using lymphocytic choriomeningitis virus (LCMV), demonstrate that these mice have an inhibition in STAT signalling in the liver resulting a weak IFN response against the viral infection [89]. The full-length polyprotein sTg mice infected with adenovirus are unable of eliminating the viral infection despite a normal T cell response. The failing in clearance was explained to be due to resistance of Fas mediated apoptosis of HCV expressing hepatocytes [93]. At last, induction of hepatic iron over-
load in these mice, a condition often observed in chronic HCV patients, revealed an increased risk of developing HCC [94]. The use of transgenic mice can, as shown here, generate important information on HCV proteins and their function but the relevance of the results needs to be interpreted with care with respect to the non-natural host. In many of these sTg models protein expression levels are low or maybe even absent, and in some cases maybe even higher than in the native HCV infection. It is also possible that the proteins are not expressed and processed in the same way as in the real situation. Further limitation using mice with a stable expression of HCV genes are that these mice often are immunologically tolerant making immunological studies representing the real infection difficult to interpret. Finally, stably transgenic mice are time consuming to generate.

2.7.1.4 Transiently transgenic (tTg) mice

Another model system useful in terms of studying intrahepatic immune responses against HCV proteins (or any other protein of interest) is the transiently transgenic mice. An important advantage using tTg instead of stable Tg mice in studies regarding HCV, except for the tolerance issue, is that each eradicated hepatocyte will be replaced by a non-Tg hepatocyte. In a stably Tg mouse each new hepatocyte will express the transgene. Thus, in this case the tTg mice may better represent the dynamics during a viral infection. The tTg mice are generated by an intravenous (i.v.) injection of a large volume in the tail vein consisting the plasmid DNA (pDNA) encoding the protein to be express in vivo. This technique was first described in 1999 and was termed a hydrodynamic (HD) injection. A hydrodynamic tail vein injection is a rapid injection of a large volume containing pDNA, which leads to a 10 to 40% transfection of hepatocytes [95]. Efficient uptake and expression of pDNA requires both a rapid injection (completed in less than 5-10 seconds) and the use of a large volume (1.6-2 mL in mice). The hydrodynamic pressure causes enlarging of the sinusoidal fenestrae resulting in enhanced extravasation of the liver and a higher permeability of the hepatocytes [96]. Using this model it has been shown that peripherally primed NS3-specific CTLs are able to enter the liver and eliminate HCV protein-expressing hepatocytes [paper II]. Hydrodynamic injection has also been used to generate a murine model for studying HBV replication by transient transfection of hepatocytes with a replication competent HBV genome [97]. The transient transgenic model system is versatile since many different genes and mouse lineages can easily be tested in
combination. A limitation using this model is the transient liver damage and subsequent elevation of ALT levels in serum caused by the HD injection.

2.7.2 In vitro systems

2.7.2.1 The subgenomic replicon system
An important step for studying HCV replication in cell culture became possible in 1999 when the replicon system was first described [98]. The replicon system is an artificial subgenomic self-replicating HCV RNA that partially mimics the replication cycle of HCV but without production of infectious particles. The bi-cistronic construct encodes the 5’UTR and the HCV IRES to translate the neomycin-phosphoryltransferase (Neo) gene, the encephalomyocarditis (EMCV) IRES to control the translation of the replicase complex, comprising the NS2- or NS3-NS5B and the 3’UTR. Upon transfection of replicons into human hepatoma derived cells (Huh-7 cells and treatment with the cellular toxin geneticin (G418) selection for positive self-replicating RNA clones can be selected due to Neo-mediated resistance to G418. It was soon shown that cell culture derived adaptive mutations occurred within most of the non-structural proteins that enhanced the RNA replication [99, 100]. Although some of these mutations effectively enhance the replication, two mutations within NS3 and one in NS5A introduced into an infectious clone was found to loose infectivity after intrahepatic transfection of a chimpanzee [101]. Further, although an RNA transcript consisting the NS5A mutation was infectious it reverted back to the original strain demonstrating that adaptive mutations seen in the cell culture system may attenuate infectivity in vivo [101]. To date, many different HCV replicons have been generated with other reporter genes. Firefly luciferase reporter allows for screening of high number of compounds in a fast and reproducible way [100]. The finding that some adaptive mutations that mediate efficient replication in vitro may result in non-replicative in vivo replicons, suggests that results from this system should be interpreted with care. These data also implies differences in the viral adaptation to the environments in vitro and in vivo. Regardless of these limitations, the replicon system serves as an extremely valuable tool to study HCV replication and also for screening and testing of new antiviral drugs against HCV, such as the NS3 helicase, NS3/4A protease and the NS5B polymerase, in vitro.
2.7.2.2  

**HCV pseudo-particles (HCVpp)**

HCV pseudo-particles are generated by transfection of cells with expression vectors encoding the E1/E2, retroviral core proteins, and retroviral packing components containing a green fluorescence protein (GFP) gene. Transfection of human 293T cells with these three expression vectors result in HCV pseudo-particles by the assembly of the E1 and E2 glycoproteins onto the retroviral core proteins derived from murine leukaemia virus (MLV). Transfected cells secrete viral pseudo-particles at an average of $10^5$ particles/mL, which can be used for infection studies of target cells. The efficacy of infection can be monitored by the GFP expression [102]. HCVpp’s are a tool to study functions related to the HCV E proteins and early events in the HCV infection, and to identify receptors involved in HCV attachment and entry into the host cell. This system also allows studies of HCV-specific neutralizing antibodies against the viral envelope glycoproteins.

2.7.2.3  

**HCV cell culture system (HCVcc)**

In 2005, the first cell culture system for *in vitro* replication and productive infection was established for HCV. A genotype 2a strain (JFH1) isolated from a Japanese patient with fulminant hepatitis was shown to replicate and secrete viral particles in Huh-7 cells [63], and was immediately improved using a sub-clones of the Huh-7 cells, termed Huh-7.5 and Huh-7.5.1, that possess an inactivating mutation in RIG-I disturbing the interferon response [63, 103, 104]. The JFH1 clone was infectious in chimpanzee causing a transient expression but without any obvious hepatitis or immune response [63]. Since the discovery of the JFH1 the system has been further optimized. The first chimeric clone created, the JFH1 genome with structural genes and NS2 from another gt 2a strain (J6), was shown to be infectious in Huh-7.5 cells. This could not be achieved with the full-length J6 clone [103]. This J6/JFH1 clone was also able to establish long-term infections in chimpanzees and was infectious in Alb-uPA SCID mice [105]. Several other variants of the infectious clone have been generated, based on the non-structural genes from JFH1 in combination with core-NS2 from the same gt (2a) or other, 1a or 1b [106-108]. Human hepatocellular liver carcinoma (HepG2) cells, usually not susceptible to HCV, can be infected when expressing CD81, confirming the importance of the receptor in the HCV infection [103]. Recently it was shown that cell culture adapted variants of JFH1 occur (with mutations both in structural and non-structural genes), yielding higher titres of infectious particles with enhanced spread of
infection *in vitro*. Interestingly, a mutation within NS5A adapted *in vitro* was reverted to the wild type sequence in Alb-uPA SCID mice, showing impaired fitness *in vivo* [108]. The HCVcc system is surely a great system to analyse HCV replication and a tool in the development of antivirals and vaccines. However, there are some important limitations using the HCVcc system as it is today. The infectious clone is isolated from a fulminant hepatitis patient, a quite rare event in HCV. In addition, only this clone has been shown to infect these cell lines questioning the relevance of both the particular clone and the cells. Another important detail is that the system is based on a gt 2 strain and not on the globally dominant gt 1, which is the most difficult to treat. Finally, the *in vitro* system cannot reflect the true *in vivo* situation, such as pressures exerted outside the infected cell.

2.8 IMMUNE RESPONSES IN HCV INFECTION

The first defence against a viral infection is the innate immune responses. Anatomic barriers, the skin and mucous membranes must be penetrated to allow the infectious agent to reach the blood flow and subsequently the liver. In the liver the innate immune responses are represented by, natural killer (NK) cells, natural killer T (NKT) cells, Kupffer cells (liver macrophages) and a rapid IFN response exerted by the infected hepatocytes. The innate immune responses are followed by the activation of adaptive immune responses including CD4+, CD8+ T cells and B-cells. To survive the host immune responses HCV has adapted several ways to down regulate the host innate responses as well as to evade the selective pressure from the host specific immune response.

2.8.1 Innate immune responses

When the hepatitis C virus enters the liver and the infection start the innate immunity is activated and reacts through several different mechanisms to combat the viral infection. At a cellular level, NK and NKT cells are important players in the early response against a viral infection. This is also most probably true in respect to HCV infections since NKT cells are abundant in the liver. NK and NKT cells recognize infected cells in an antigen independent manner controlled by activating and inhibiting receptors on the NK cell, or by the interaction between CD1 (or other yet unknown ligands) and the T
cell receptor on NKT cells, and perform cytotoxic lysis of infected cells by releasing granules containing perforin and proteases (granzymes). The antigen independent recognition of abnormalities in infected cells, such as down regulation of MHC class I molecules, trigger the cytolytic activity by the NK cell. NK and NKT cells also produce large amounts of the type II class interferon-γ (IFN-γ) cytokine, a potent antiviral and immune regulatory cytokine promoting recruitment of inflammatory cells. The production of IFN-γ and tumor necrosis factor-α (TNF-α) from NK and NKT cells function as a link between the innate and the adaptive immunity by the stimulation of dendritic cell (DC) maturation. This is of vital importance for antigen presenting cells (APCs) towards activation of the specific immunity. The activation of early type I interferons, IFN-α and IFN-β, is an important part in the early intracellular defence system against the viral infection. It is shown that mice lacking IFN-α/β fail to clear or control viral infections [109]. The production of dsRNA intermediates during HCV replication activate type I IFN genes. The host cell recognizes dsRNA either via the Toll like receptor 3 (TLR3) [110], or in a TLR-3 independent way (Figure 4). The latter pathway represented by the cytoplasmatic sensors of viral RNA, the retinoic acid inducible gene-I (RIG-I) [111] and melanoma differentiation associated gene 5 (MDA5) [112, 113].

Figure 4. Simplified illustration of the innate immune activation during HCV infection, described in “innate immune system” (2.8.1). In red, HCV protein interactions with the innate immune response, described in section “impaired innate immune responses to HCV” (2.8.3.1).
Upon RIG-I (or MDA-5) activation, CARD adaptor inducing IFN-β (Cardif) [114] also known as interferon β promoter stimulator 1 (IPS-1) [115], mitochondrial antiviral signalling (MAVS) [116], virus induced signalling adaptor (VISA) [117], bind the CARD domain of RIG-I and stimulate activation of TANK binding kinase-1 (TBK-1) and IκB kinase ε (IKKε). These in turn phosphorylate interferon regulatory factor-3 (IRF-3) [118-120]. Phosphorylated IRF-3 homo-dimerize and translocate into the nucleus where it recruits transcriptional co-factors (p300 and CREB-binding protein (CBP)) that induce production of IFN-β. Next, IFN-α/β is recognized by type I interferon receptor (IFNAR) resulting in a positive feedback loop of both IRF-3/7 and activation of multiple IFN-inducible genes [121, 122]. The viral dsRNA can also be recognized by TLR3. Activated TLR3 recruits Toll/IL-1 receptor domain containing adaptor-inducing IFN-β (TRIF), which will lead to downstream activation of nuclear factor kappa B (NFkB). This will activate inflammatory cytokine genes and induce phosphorylation of IRF-3/7 that promote IFN-β production [123]. Interferon-α and β are both able to bind and activate the IFNAR receptor resulting in Janus kinase (JAK)-STAT signalling. Janus kinases, JAK-1 and TYK-2, phosphorylate signal transducers and activator of transcription (STAT)-1 and STAT-2. Upon phosphorylation STAT molecules dimerize and together with IRF-9 form the IFN-stimulated gene factor 3 (ISGF-3) capable of binding interferon stimulatory response element (ISRE) promoter motifs inducing IFN-stimulated genes (ISGs) that have antiviral activity against HCV. Examples of these are, protein kinase R (PKR) production which leads to block of mRNA translation in the infected cell [124, 125], and 2′-5′ oligoadenylate synthetases (OAS) that activate RNaseL which in turn degradades RNA in the cell [126].

2.8.2 Adaptive immune responses

Adaptive immune responses are composed of humoral (antibody producing B-cells, e.g. plasma cells) and possibly more important in HCV infections, cellular immune responses (CD4+ T helper (TH) cells and CD8+ cytotoxic T lymphocytes (CTLs). A cell with an ongoing infection produces viral proteins that are processed the same way as an endogenous protein. Like cellular proteins, some viral proteins will be processed in the cytosol by the proteasome into 8 to 10 amino acid peptides. Peptides are then transported into the endoplasmatic reticulum by the transporters associated with antigen
processing (TAP) were the peptide can be recognized and bind to major histocompatibility complex (MHC; in humans, human leukocyte antigen [HLA]) class I molecules, which are subsequently transported to the cell surface. Foreign peptides processed through the endogenous pathway are presented on MHC class I of the infected cell can then be recognized by CD8+ T cells, expressing the T cell receptor (TCR) and the CD8 molecule, resulting in signalling cascades in the CTL. These may lead to maturation and activation or to effector functions.

Another way of immune cell activation goes through the exogenous pathway. This occurs when antigen-presenting cells (APCs) (dendritic cells, macrophages and B-cells) engulf infected apoptotic or necrotic cells or cell debris, or viral proteins. The viral exogenous antigens are then processed in endocytic compartments into 13-18 aa long peptides that associate with MHC class II molecules which are then exposed at the cell surface. Peptides that are presented on MHC class II are identified by CD4+ T cells, which express TCR and the CD4 molecule, which activates the T helper cell. CD4+ T cells perform several important functions including direct activation of macrophages, antigen specific B cells and production of cytokines that activate CD8+ cells [127].

When APCs present viral antigens processed through the exogenous pathway and presented on MHC class II molecules to CD4+ T-helper lymphocytes. These become activated and differentiate into the T_{H1} or the T_{H2} type cells, generally characterised by different cytokine production and different functions (Figure 5). The T_{H1}-like cytokines interleukin (IL)-2, IL-12, IFN-γ and TNF-α stimulates cellular immune responses by favouring activation of CD8+ and NK cells. A T_{H2}-like differentiation results in production of IL-4, IL-5, IL-6 and IL-10 cytokines stimulating maturation of the humoral immune responses resulting in activation of B cells [128-130]. CD8+ T cells recognize antigens processed through the endogenous pathway and presented on MHC class I on infected cells. An activated CTL recognizing a foreign peptide on MHC I result in activation of granule exocytosis pathway or Fas pathway to induce apoptosis of the infected cell. CTLs are capable to release perforin that forms pores in the target cell membrane, which permits entry of granzymes that induce caspase cascades resulting in apoptosis. CTLs can also use Fas ligand (Fas-L) expressed on the CTL to bind to Fas on the infected cell. This results in activation of Fas death domains and recruitment of Fas associated protein with death domain (FADD), which in turn induces caspase cascades and eventually apoptosis [131, 132]. CD8+ T cells can also
be primed by cross-presentation (cross-priming) of HCV antigens by DCs. This occurs when APCs acquire exogenous antigens or cell debris from infected dying or dead cells, process the antigen in the cytosol or endosomal compartment and present them by the endogenous MHC class I pathway [133]. CD8+ T cells do not only perform the classical CTL activity, CD8+ T cells also perform important non-cytolytic effector functions including the secretion of cytokines such as IFN-γ and TNF-α that can inhibit viral replication without killing the infected cell [134].

Figure 5. Schematic illustration of hepatitis C virus immune response activation and effector mechanisms of humoral and cellular immune responses.

Several studies demonstrate that a strong, multi-specific and a sustained HCV specific CD4+ T cell response is associated with viral clearance during acute HCV infection, whereas the corresponding response is weak in persistently infected patients [135-138]. CD4+ T cell responses have been found to be directed mainly against core, NS3, NS4 and NS5, and often seem to target the same immunodominant epitopes within the NS3 [139, 140]. Patients with self-limited HCV often have a strong CD4+ T cell proliferation with IL-2 and IFN-γ production indicating predominant Th1-like response, whereas Th2 responses are seen in patients that develop a chronic infection [141]. This is consistent with studies showing that patients with self-limiting HCV infection have a vigorous and multi-specific CD8+ T cell response with an intrahepatic expression of
When chimpanzees with memory T cell responses against several HCV proteins were re-infected years after clearance of the primary infection they had in contrast to a prolonged course of infection normally seen during a primary infection a viremia that was terminated within 2 weeks. Furthermore, antibody depletion studies of CD4+ or CD8+ T cells in re-infected chimpanzees revealed that CD8+ T cells are the major effector cells mediating protective immunity, since control of viremia could not be achieved without a CD8+ immune response. However, low level of viremia was detected in the absence of a CD4+ T cells despite the presence of functional CD8+ T cells. Thus CD4+ T cells seems to be important for the CD8+ T cell function in order to keep the evolution of viral escape mutations under control and to resolve the HCV infection [146-148]. Thus, both CD4+ and CD8+ T cells are essential for the control of the HCV infection.

A primary HCV infection also results in antibody production to several HCV proteins in the infected patient, usually detectible after 7 weeks of infection. However, as compared to the chronic infection these responses are weak and of a much narrower epitope specificity [149]. However, broad antibody responses seem to be important for controlling the HCV infection, even in the chronic phase of the infection. This is evidenced by that HCV patients with hypogammaglobulinemia have a rapid progression of the liver disease, with a poor response to interferon treatment [150]. However, in the same type of antibody deficient patients it has been shown that an early activation of T cell responses through antiviral therapy in the acute phase are maintained over long time [151]. The importance of antibodies regarding clearance of the acute infection is not clear, although studies in chimpanzees have shown that antibodies do not seem to protect from infection of heterologous or homologous strains [152]. The reason for this is not clear but could possibly be related to that the HCV particles present in the circulation is coated by host derived proteins.

2.8.3 Viral evasion strategies to HCV

2.8.3.1 Impaired innate immune responses to HCV

HCV interferes with the innate immunity in several ways to impair the IFN responses against the viral infection (Figure 4). The NS3/4A protease has the ability to block RIG-I activation and translocation of IRF-3 [153]. The mechanism behind this is the
specific cleavage of the c-terminal part of Cardif resulting in release of the protein from the mitochondria and thereby inhibiting its function. This cleavage has been observed in human cell lines but also in liver biopsies from chronically infected patients [154, 155]. Moreover, the cleavage of Cardif seems to occur also in murine cells [Paper IV]. NS3/4A further interferes with the host cell immune response by cleavage of TRIF, which will interrupt TLR-3 mediated activation of the IRF-3 IFN pathway and also affect NFκB signalling [156]. Also the HCV core protein seem to be able to affect the IFN signalling through activation of a JAK/STAT signalling adaptor protein, suppressor of cytokine signalling protein-3 (SOCS-3), which down regulate the IFN response [157, 158].

Transgenic mice with stable expression of NS3/4A in the liver are resistant to lethal doses of TNF-α, an observation possibly favouring development of chronicity that may represent a new evasion strategy conferred by NS3/4A [86]. Moreover, the NS5A protein has also been described to have an important role in the escape from antiviral action of IFN. The proposed NS5A IFN sensitivity determining region (ISDR) has been shown to correlate with responsiveness to IFN therapy [159]. This finding has been widely debated. NS5A also inhibit 2′5′oligoadenylate synthetase (OAS) and thereby interferes with the IFN activity in an ISDR independent way [126]. Both NS5A and the glycoprotein E2 have been described to bind to PKR, a molecule involved in the viral IFN response [124, 125]. HCV also seem to inhibit the interferon signalling through up regulation of protein phosphatase 2A (PP2A), a serine/threonine phosphatase involved in several cellular processes including signal transduction, apoptosis and stress responses [160]. Over expression of PP2A, seen in chronically infected livers, result in hypomethylation of STAT1 and in increased binding of protein inhibitor of activated STAT1 (PIAS1) to STAT1 and a reduced activation of ISGs [161].

Hepatitis C virus also has an impact on the NK cell population that might contribute to explain HCV surveillance. Chronic HCV patients often have reduced frequencies of NK cells correlating with low levels of IL-15 (important in NK stimulation) in serum [162].

2.8.3.2 Impaired adaptive immune responses to HCV

As described earlier, the majority of HCV infected individuals will develop a chronic HCV infection. Various mechanisms have been suggested to be involved in the impaired adaptive immune responses to clear the acute HCV infection. These include, T cell failure, exhaustion, dysfunction, viral escape mutations and many more. Some of the most evident will be discussed here. Several studies have shown that HCV infected
patients that develop a chronic infection have weak HCV specific CD4+ and CD8+ T cell responses both in the acute phase and during the chronic infection [129, 144, 148]. However, if this depends on that CD8+ T cells were not primed (failure) or if the responses were primed but then vanished (exhaustion) is not easy to define. In a study on health care workers accidentally exposed to the HCV, patients that failed to mount strong T cell responses developed chronic disease [145]. However, patients with initially strong CD4+ and T\(_{H1}\) responses, that later loose their functional T cell responses during the acute phase has been observed to results in progression to a chronic state [136]. The mechanism for T cell exhaustion is not fully clear but data suggest that the inhibitory receptor, programmed death 1 (PD-1), is involved and puts the CD8+ T cells in an exhausted state. HCV specific CD8+ T cells express high levels of PD-1 and are often seen in chronically infected individuals. Antibodies blocking the interaction between PD-1 and its ligand PD-L1, in vitro, result in enhanced T cell responses further confirming PD-1 as a specific mechanism for T cell exhaustion [163, 164]. These data are also consistent with observations on PD-1 in HIV patients [165]. Moreover, for another chronic viral disease, adoptive transfer of HBV specific CTLs into HBV transgenic mice treated with a blocking PD-L1 antibody resulted in a delayed suppression and instead an increase of IFN-\(\gamma\) producing CTLs in the liver [166].

Another possible mechanism of immune evasion is dysfunction of T cells. This involves impairment of CD8+ T cells affecting their proliferative capacity, ability to exhibit cytotoxic activity, and cytokine (TNF-\(\alpha\) and IFN-\(\gamma\)) secretion upon stimulation [167, 168]. Quite recently the role of regulatory T cells have been demonstrated to suppress the IFN-\(\gamma\) producing CD8+ T cells in chronically infected patients. This study observed that the number of regulatory T cells in the liver were two times higher in the chronically infected patients as compared to healthy controls [169]. The reason for the higher number of regulatory T cells is not clear but might be an immunological response to suppress the liver damage caused by elimination of infected hepatocytes.

One of the major evasion strategies to circumvent the adaptive immune responses has been suggested to be escape by mutations. The high viral replication rate and the error prone RdRp generates a tremendous amount of mutations promoting immune escape. Some escape variants involve mutations in anchor residues in the MHC molecule resulting in peptides that can no longer be presented by the infected cell [170]. Some mutations affecting the proteasomal processing resulting in impaired epitope presentation [171]. Other mutations may not affect the antigen presentation but rather
residues reducing the recognition of the antigen by the T cell receptor [172]. This describes the factors that determine the occurrence of escape mutations, the viral factors (viral fitness) versus the host responses (T cell repertoire or TCR diversity) [148, 173]. There are several studies showing a clear correlation between persistent infection and aa substitutions within CD8+ T cell epitopes and non-mutated epitopes in individuals that clear infection [174-176]. However, some epitopes are highly conserved since they do not allow for escape mutations due to a reduced viral fitness [paper III] [177].

2.9 TREATMENT

There is currently no vaccine for HCV but treatment is available that can cure a chronic HCV infection. Both viral (genotype, quasispecies diversity, viral load) and host (gender, age, fibrosis etc.) factors (Figure 1), influence the outcome of treatment [178, 179]. The current recommended treatment for HCV is a combination therapy of pegylated interferon alpha (peg-IFN-α) and ribavirin [180, 181]. Two peg-IFNs are approved for treatment, peg-IFN-α-2a (Pegasys; Roche) and peg-IFNα-2b (peg-Intron; Schering-Plough). The current recommendations are therapy with peg-IFN-α and ribavirin for 48 weeks in patients infected with genotype 1 and a shorter 24-week treatment for patients infected with genotypes 2 and 3. Patients with gt1 are recommended 180µg/week IFN and 1000-1200mg/day of ribavirin whereas non-gt 1 patients receive a lower dose of ribavirin (800µg/day) [180]. In clinical trials, sustained virological response (SVR) can be achieved in 70-80% of non-gt 1 patients whereas only 45% of gt 1 patients have a SVR (Figure 1). SVR is defined as persistent absence of serum HCV RNA for at least six months after cessation of therapy. However, patients that lack an early virological response (EVR), defined as at least a 2-log drop of viral load during first 12 weeks of treatment, have a very small chance of viral clearance and may consider therapy termination. Treatment is recommended for patients with chronic HCV infection with detectable HCV RNA in serum, elevations in ALT levels, histological evidence of progressive liver disease and no other severe co-existing conditions [180]. Even if today’s available treatment cures many chronically infected individuals, the treatment have some significant drawbacks. Many patients experience severe side effects, such as hematological toxicity, depression and hemolytic anemia, and the therapy is expensive (35,000 USD for 48 weeks treatment). Thus, new approaches such as antivirals or vaccines are needed.
2.9.1 Interferon-α

IFN-α is a cytokine with known important functions in the innate immune responses toward viruses. IFN-α binds to IFN sensitive receptors at the cell surface and signal through the JAK-STAT signalling pathway. This induces several interferon stimulatory genes (ISGs) leading to direct effects in the infected cell by promoting RNA degradation and inhibition of protein synthesis, but it also results in activation of immune cells. The currently used interferons have a polyethylene glycol molecule (peg) attached to the IFN-α molecule (peg-IFN-α), which prolongs the serum half-life of the antiviral cytokine. In an approach to further improve the responses and prolong the half-life of IFN-α, albumin-IFN-α, which is a recombinant human IFN-α-2b genetically fused to recombinant human serum albumin has been developed and is currently evaluated. This compound shows similar antiviral activity and tolerability as peg-IFN-α, which may reduce the treatment from once a week to every second week [182].

2.9.2 Ribavirin

Ribavirin is a nucleoside analogue with a broad activity against viral pathogens. The mechanism of ribavirin is not fully understood but several effects have been described. These include, immune modulation of the T_H responses favouring a T_H-1 phenotype, inhibition of inosine monophosphate dehydrogenase leading to GTP depletion, direct inhibitory effects on the RdRp and/or mutagenesis resulting in reduced virion infectivity [178, 183]. Several approaches have been done to reduce the haemolytic anemia associated with ribavirin treatment. Trials have been made to develop a pro-drug of ribavirin (Taribavirin) that becomes activated in the liver. Phase II studies have shown that the SVRs are the same as with ribavirin but with less extensive anemia [184].

2.10 TREATMENTS UNDER DEVELOPMENT AGAINST HCV

Even if the use of peg-IFN-α in combination with ribavirin as therapy against HCV has cured several patients the treatment has many disadvantages. The treatment is associated with severe side effects and the efficacy in treating genotype 1 patients is
low. Therefore much effort is being made on trying to improve the current treatment or to develop other new therapies. Here, a brief overview of some of the different strategies and compounds that are currently under development is given.

2.10.1 HCV specific inhibitors

2.10.1.1 Protease inhibitors

NS3/4A comprises as previously described a multifunctional protein exerting serine protease activity essential for viral replication. Several specific inhibitors of the NS3 protease enzymatic activity are under development or in clinical trials. Some have shown to be effective with a rapid decrease in viral load but toxicity and resistance mutations have been reported.

BILN 2061 (Boehringer-Ingelheim), a peptide domain inhibitor of the NS3/4A protease was the first inhibitor for HCV protease to reach a clinical trial. The very first clinical trial showed proof of concept with a 2-3-log reduction in viral load in all patients after 48 hrs of treatment. The inhibition was transient as viral load rebound in all patients to the same level as before treatment 6-13 days after cessation of therapy [185]. Interestingly, a single mutation (Asp 168) conferred resistance in vitro using the replicon system, indicating that these type of compounds are associated with escape mutations, at least when used as mono-therapy [186]. However, due to cardiotoxicity in animals further development of this compound was stopped.

VX-950 or Telaprevir (Vertex Pharmaceuticals) is also a peptidomimetic inhibitor of the NS3 protease, but it is using a different mode of binding compared to BILN 2061 [186]. In contrast to BILN 2061, VX-950 has some activity also against gt 2 proteases [187]. Replicons carrying the major in vitro resistance mutation to BILN 2061 remained sensitive to VX-950, to which another mutation (Ala 156) conferred resistance [186]. A recent clinical phase II study of previously untreated gt 1 patients showed a reduction in viral load of 4-logs using VX-950 alone, and a 5.5-log reduction in combination with peg-IFN-α after 15 days of treatment. No major side effects were reported. Twelve weeks after the end of treatment 8/8 patients treated with VX-950 and peg-IFN, 5/8 VX-950 alone and 1/8 peg-IFN-α alone had undetectable levels of HCV RNA [188]. However, a rapid development if viral resistance remains a major problem.

A phase 2b trial on IFN non-responding gt 1 infected patients is currently ongoing.

SCH 503034 or Boceprevir (Schering Plough) is another NS3 protease inhibitor that has shown to have a potent activity both in the replicon system and in clinical trials
In a study combining SCH 503034 and peg-IFN-α on gt 1 previously non-responders a 2-2.5-log reduction in serum RNA was observed with no significant changes in side effects [190]. The compound is now in a phase II clinical trials.

2.10.1.2 Helicase inhibitors
Attempts are also being made to develop specific inhibitors against the NS3 helicase. Limited data on NS3 helicase inhibitors are reported and so far none of the candidate drugs has reached clinical trials.

2.10.1.3 Polymerase inhibitors
Another protein suitable for specific inhibitors is the NS5B region encoding the HCV RdRp, essential for the HCV replication. There are several possible binding sites on the HCV polymerase and a number of different nucleoside analogues are under development. One of them, NM283 or Valopicitabine (Idenix/Novartis) is an oral pro-drug nucleoside analogue that upon intracellular phosphorylation becomes an active inhibitor of the viral polymerase [191, 192]. A 48-week Phase II trial show a slight reduction in viral load when delivered in combination with standard treatment (peg-IFN-α and ribavirin) compared only to standard treatment in non-responding gt 1 patients. Due to the minimal efficacy of the combination therapy, non-responders will probably require two or more complementary agents for an optimal clinical outcome [193]. Discontinued treatment due to adverse events, mostly related to gastrointestinal intolerance has been reported when using NM283. The clinical development of NM283 has recently been terminated.

2.10.1.4 NS4A antagonists
When most of the researches are focused on protease and/or polymerase inhibitors, Achillion pharmaceuticals instead are developing a NS4A antagonist. ACH-1095 is believed to bind to NS4A and thereby interrupting the interaction between NS3 and NS4A. This should impair the generation and/or function of the replication complex. This drug inhibits HCV replication in cell based replicon system assays that have developed resistance to protease and polymerase inhibitors. The NS4A antagonist may either be used in combination with current standard treatment or in combination with other antivirals. Achillions first candidate drug ACH-806 significantly reduced HCV viral load in a human proof of concept trial but due to high levels of serum creatinine...
further development of this compound was stopped [194]. As with the protease inhibitors, also NS4A antagonists seem to be associated with adverse events.

A positive step forward would be if these types of compounds, alone, as a cocktail or in combination with currently available treatment, could remain safe and effective, which over time could shorten the treatment period. Resistance regarding individual drugs or cross-resistance between drugs needs to be studied to enable the most effective treatment regimen.

### 2.10.2 Vaccines against HCV

The studies reported in this thesis aim at developing a therapeutic vaccine against chronic HCV infections caused by genotype 1 as an initial target. There are currently several therapeutic vaccines under development against HCV using a wide variety of vaccine strategies. The composition of some of these vaccines, which have entered clinical trials will be discussed here and are summarized in table 1.

<table>
<thead>
<tr>
<th>Therapeutic vaccines against HCV under development</th>
</tr>
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<tbody>
<tr>
<td><strong>Product</strong></td>
</tr>
<tr>
<td>IC41</td>
</tr>
<tr>
<td>Results: Preliminary Pla data, low but significant drop in viral load after 8 i.d. injections.</td>
</tr>
<tr>
<td>NN-0101</td>
</tr>
<tr>
<td>Results: E1 specific immune responses monitored but no significantly effect. Vaccine development terminated.</td>
</tr>
<tr>
<td>HCV-E1E2</td>
</tr>
<tr>
<td>Results: PI, E1/2 vaccine trial discontinued due to severe local and systemic advents.</td>
</tr>
<tr>
<td>GI-5005</td>
</tr>
<tr>
<td>Results: Plb, Safe. Cellular immune responses detectible in 41% of patients. 1-log viral load reduction at most.</td>
</tr>
<tr>
<td>TG4040</td>
</tr>
<tr>
<td>Results: Pre-clinical data, CD8 &amp; CD4 responses in mice.</td>
</tr>
<tr>
<td>ChronVac-C</td>
</tr>
<tr>
<td>Results: Pre-clinical data, No toxicity. Potent induction of T cell responses in mice and rabbits.</td>
</tr>
<tr>
<td>PEV2A/PEV2B</td>
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<tr>
<td>Results: No data released.</td>
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</table>

Table 1. Therapeutic vaccine candidates currently in clinical trials against HCV. P=phase.

Intercell AG has developed a peptide based therapeutic vaccine (IC41) composed of 8 synthetic peptides in combination with a poly-L-arginine (IC30) adjuvant, resulting in slow release of peptides and has also been reported to serve as a TLR9 agonist. The synthetic peptides in the vaccine are derived from HCV gt 1 core, NS3 and NS4. The vaccine has passed phase I clinical trials and is currently in a second phase II trial.
Preliminary results in chronically infected gt 1 patients receiving 8 intradermal (i.d.) injections of IC41 indicate a small, but significant, therapeutic effect with a 0.2-log reduction (40%) of viral load compared to baselines prior to treatment [195, 196]. Final results of the phase IIb study have not yet been presented.

Another therapeutic vaccine candidate, INN-0101, developed by Innogenetics recently finished a second phase II clinical trial. The vaccine, recombinant E1 delivered in combination with alum emulation adjuvant, have in previous studies shown to be safe and able to induce E1 specific immune responses. A first phase Ila clinical trial using histological controls showed a slower development of fibrosis than expected. However, in a recently concluded phase IIb study including 122 chronic HCV patients no statistical difference in reduction of liver damage could be observed between treated and placebo patients. Innogenetics therefore decided to discontinue further development of the E1 vaccine candidate [197, 198].

Novartis Vaccines are developing a recombinant E1/E2 vaccine. A first phase I clinical trial proved that the E1/E2 combination is immunogenic in humans. However, in a recent clinical trial the E1/E2 complex was delivered with two adjuvants, MP59 and CpG. MP59 is an oil-in-water emulsion based on squalene, and CpG is a TLR9 inducer stimulating innate immune responses and DC maturation. A phase I safety study using 3 different doses of CPG7909 in healthy volunteers was recently halted due to severe adverse events including malaise, myalgia and arthralgia [199]. Specific immunity of the vaccine has not yet been presented and the future of the vaccine is not known.

Novartis vaccines also develop a polyprotein vaccine comprising NS3-NS5B [200].

Globeimmune develops a therapeutic HCV vaccine based on a fusion protein consisting parts of core and NS3. The vaccine uses Globeimmune’s patented Tarmogen (targeted molecular immunogen) technology, heat-killed recombinant yeast expressing HCV fusion protein sequences. Pre-clinical studies in mice revealed that the vaccine induce specific CTL responses with a Th1 cytokine profile capable of inhibit growth of NS3-protein expressing tumor cells. Preliminary results from an ongoing phase I/II study show safety and tolerability, cellular immune responses is detectible in 41% of patients, and with an up to 1-log reduction in viral load [201, 202].

Only one HCV vaccine is currently in a clinical trial that uses a viral vector for delivery. The vaccine TG4040 developed by Transgene uses the non-replicative modified vaccinia Ankara (MVA) virus expressing NS3, NS4 and NS5B from a gt 1b viral strain. CD8 T cell responses including specific CTLs with IFN-γ production as well as CD4 T cell responses has been monitored in mice. The vaccine is currently in a
phase I/II clinical trial consisting of 15 chronically infected patients given 3 injections of 3 different doses ($10^6$, $10^7$, $10^8$ pfu). A second phase I/II on patients who have relapsed peg-IFN-α and ribavirin treatment is initiated [203, 204].

A DNA based vaccine delivered using in vivo electroporation has been developed in collaboration between Karolinska Institutet, Tripep AB, and Inovio Biomedical Corporation [paper V]. The vaccine consists of a synthetic codon optimized (co) NS3/4A gene isolated from a gt 1a infected patient and has been shown to induce both humoral and cellular immune responses in mice [paper I]. Priming of CTL responses capable to eradicate NS3/4A-protein expressing cells in mice livers in vivo [paper II, IV] with further enhanced protein expression and immunogenicity when delivered in combination with in vivo electroporation [paper V]. Furthermore, no toxicity or unexpected biodistribution was observed in rabbits [paper V]. The vaccine is currently in a phase I/II clinical trial consisting of 12 chronically gt 1 infected treatment naïve patients, with the primary endpoint being safety and tolerability but immune responses and the viral load will also be monitored.

Finally, Pevion Biotech is currently in a phase I clinical trial, comprising 30 healthy volunteers, using virosome based vaccine delivery of synthetic HCV peptides (sequences used not publicised). Two different patented virosomes for T cell priming, PeviTER (CTL) and PeviPRO (Th) will primary be tested for safety, but the immunogenicity of the vaccine will also be analyzed [205].
3 GENETIC VACCINES

Live vaccines, based on weakened (attenuated) forms of the infectious organism, are the most effective in priming a protective immune response. Live attenuated vaccines have been used with varying success for both bacterial (tuberculosis) and viral (smallpox, measles, mumps, polio) diseases. This type of vaccines, causing a mild infection induces broad humoral and cellular immune responses that often induces lifelong protection against the pathogen. A key factor seems to be the viral replication that activates a series of pathogen activated molecular patterns (PAMPs), such as the activation of various TLRs. However, due to safety concerns such as the risk of reversion back to a wild type strain or possibility of integration of genes into the host genome, these types of vaccines may be less suitable for diseases such as HCV, HIV and influenza. Other strategies, using inactivated or subunit vaccines often fail to induce potent cellular immune responses, which are needed for controlling these infections. Therefore, efforts are being made on developing genetic vaccines using replication deficient viral vectors as delivery vehicles of vaccine genes. Another strategy is the use of plasmid DNA, often referred to as naked DNA, delivered into host cells to make them produce the vaccine proteins themselves. The factors influencing an effective vaccine are not only based on the delivery system, also other factors such as delivery method, inherent properties and immunogenicity of the antigen, number of immunizations, dosing and adjuvants are also important to direct and induce a desirable immune response.

3.1 VIRAL VECTORS

The use of viruses for delivery of vaccine genes has shown to be very effective and the use of viral vectors is widely used and has been evaluated in several clinical trials. Recombinant viral vectors, based on RNA or DNA viruses, differ in their capacity to carry foreign genes, their efficacy to deliver genes into host cells and their expression levels. Several different genes have been delivered using a variety of recombinant viral vectors from Adenoviruses [206, 207], Adeno-associated viruses [208], Alphaviruses [209], Poxviruses [209, 210] and others. Except from potent cellular priming by viral vector vaccines, several of the RNA genomes often also generate RNA intermediates that are potent inducers of innate immune responses through activation of toll-like
receptors that trigger interferon responses. Even though viral vector-based systems have shown to be effective and relatively safe, there are issues with large scale manufacturing and maintaining the genetic stability of the vaccine. The antigenic properties and immune response against the viral vectors can on one hand be favourable in the immune priming but may also be a limitation due to pre-existing immunity in the host due to previous infection or vaccination. Thus, viral vectors are often not effective for repeated immunizations and more often viral vectors are used to boost an immune response after priming using naked DNA.

3.2 DNA VACCINES

In 1990, the discovery that naked plasmid DNA (pDNA) when injected into mouse muscle generated an in vivo expression of the plasmid encoded reporter gene [211]. This suggested that a pDNA could be used to express a foreign gene in a cell in vivo and capable to induce an immune response against the protein encoded by the gene. After this breakthrough, there were soon reports showing that pDNA was able to induce immune responses against several infectious pathogens. After injection of pDNA encoding influenza A nucleoprotein in mice, nucleoprotein specific CTLs could be detected and protection from challenge with a heterologous influenza virus strain was observed [212]. Other reports confirmed that DNA vaccines were able to induce both cellular and humoral immune responses, which further supported the possibilities of DNA vaccination [213-215]. The effort in development of DNA vaccines has since then been made for several pathogens, cancer, autoimmune disorders and allergy. The first DNA vaccine against an infectious disease entering a human clinical trial was consisting of the env and rev genes of HIV-1 [216] and was soon followed by other clinical trials testing safety and host immune responses. To date, there is no approved DNA vaccine for use in humans but two for animals, against West Nile virus infections in horses and against infectious hematopoietic necrosis in salmons. There are several reasons why no DNA vaccine has been approved for humans, where the main reason is lack of potent immunogenicity. Even if strong and protective immune responses were achieved in small animals, the immune responses observed in larger animals including humans have been quite low. This is probably explained by the poor uptake and expression of the injected pDNA into the target cells. Therefore, much focus has been set to find new techniques to deliver the DNA in a more efficient way. Also, several
other enhancements in addition to the delivery route, such as further optimization of the vaccine construct or the use of adjuvants to enhance the immune priming are under investigation. These parameters will be discussed later. The use of DNA vaccines has several advantages compared to traditional killed or live attenuated vaccines. First, DNA vaccines have so far been considered safe as compared to live attenuated vaccines. This general perception safety regarding DNA vaccines may well change when the efficiency of DNA vaccination improves, and becomes immunogenic in humans. Inactivated viruses and recombinant proteins may also be safe, but are often much less efficient in inducing CTL responses since priming of this response requires endogenous production of the antigen. One concern has been the risk of integration of the delivered DNA into the host genome. Studies have so far shown that this event is much lower than spontaneous genome mutations, suggesting that this may not be a major concern in reality [217-219]. Some side effects have been reported. Most have been associated with local irritation and inflammation at the injection site. Second, DNA vaccines are able to induce both humoral and cellular immune responses resulting in a broad immune response against infectious viruses in several animal models. Third, the costs of developing and producing DNA vaccines is quite inexpensive which raise hopes that even developing countries can benefit from this type of vaccines. Fourth, the high stability of pDNA and ability for storage at room temperature further simplifies the handling and worldwide distribution of the vaccine.

### 3.3 IMMUNE RESPONSES FOLLOWING DNA IMMUNIZATION

When the plasmid DNA is introduced into the body it directly transfects or is taken up by muscle, skin or mucosal cells depending on injection site, or by circulating professional APCs (Figure 6). Once inside the cell nucleus, the eukaryotic promoter driven gene will be transcribed and translated into the vaccine protein. This endogenous expression of the protein and the host cells presentation of vaccine antigens is a major advantage with DNA vaccine priming since it mimics a natural infection, which is the natural way of immune activation. A direct transfection of a professional APC, such as a dendritic cell will process the antigen through the proteasome and present antigen peptides on MHC class I molecules for activation of CD8+ T cells [220, 221]. Almost all nucleated cells are able to present foreign antigens on MHC class I, thereby being targets for CD8+ CTLs. However, the expression of MHC class I on non-professional
Antigens released from protein expressing, or apoptotic muscle cells or keratinocytes, is taken up or phagocytosed by APCs and degraded and presented on MHC class II for CD4+ immune activation. These exogenous antigens can also be cross-presented (cross-priming) on MHC class I after processing through the endogenous pathway [222]. However, a recent study suggests that transfected muscle cells, upon stimulation, express necessary co-stimulatory molecules and directly present endogenously produced antigens to immune cells [223].

**Figure 6.** Illustration of immune priming by DNA vaccines. CD8+ T cell activation (1) direct-priming or (2) cross-priming and (3) CD4+ T cell activation.

### 3.4 PLASMID BACKBONE

The traditional DNA vaccine construct consists of a bacterial backbone and a transcription unit (Figure 7). The transcription unit consists of a eukaryotic promoter, the most commonly used is the cytomegalovirus (CMV) immediate-early promoter. The promoter is followed by a Kozak sequence and the gene(s) of interest and a
polyadenylation signal, important for stability and half-life of the messenger RNA (mRNA). The bacterial backbone contains a selection marker (antibiotic resistance gene) and an origin of replication of the plasmid.

Figure 7. Schematic organization of a plasmid DNA vaccine. Bacterial backbone, consisting elements important for production in bacterial cells. Transcription unit, sequences for expression of gene of interest in host cell.

The design of the plasmid can in many ways be modified to regulate and enhance the expression level of the gene transcript. One way is the choice of the promoter. The most commonly used promoter is the human CMV promoter or the simian virus 40 (SV40) promoter. The CMV promoter seems to be more efficient than SV40 in promoting a gene expression and immune priming against vaccine antigens. However, both these two promoters have been shown to be down regulated by IFN-\(\gamma\), which is activated as a result of the DNA vaccine [224, 225]. An alternative that might target expression and enhance the efficacy is the use of tissue specific promoters. Also the polyadenylation sequence used may affect the messenger RNA potency. The polyA terminal has importance for mRNA stability, which affect antigen expression. One of the most commonly used sequences is derived from bovine growth hormone (BGH).

Another factor that has been shown to enhance both the gene expression levels and the immunogenicity of DNA vaccines is codon optimization [226]. Different organisms have different preferred codon usage. Subsequently, a higher gene expression might be achieved by replacing each viral codon with the most commonly used codons in highly expressed human genes. Higher immunogenicity due to codon optimization has been reported for several viral antigens including the structural gag gene (HIV) [227, 228] and the NS3/4A gene (HCV) [paper I].
DNA vaccines naturally contain immune stimulatory CpG motifs adding an adjuvant effect to the vaccine. Unmethylated phosphodiesterlinked cytosine or guanine (CpG) motifs, predominant in bacterial DNA as compared to vertebrates, trigger activation pattern recognition receptor TLR9 that induces innate immune responses in B cells and DCs. This increases the antigen uptake and promotes production of Th1 proinflammatory cytokines and chemokines promoting adaptive immune responses against the plasmid antigen [229, 230].

3.5 DELIVERY METHODS

Several different delivery methods for pDNA administration has been developed and tested in animal models and/or in clinical trials. The optimal delivery for DNA vaccines may differ between different animals and between different antigens. An enhanced uptake of the DNA seems to be required to be able to induce immune priming in larger animals and humans. Different antigens may also require different delivery methods or priming sites to be able to induce the type of immune responses desired.

3.5.1 Intramuscular (i.m.) injection

The traditional ways to deliver DNA vaccines are i.m. injection of pDNA in water or phosphatase buffer saline (PBS). Using this technique myocytes will passively take up the plasmid. In smaller animals, such as mice, also the (hydrodynamic) pressure in the muscle using a disproportionately high volume further enhances the pDNA uptake [231]. Although myocytes will produce the plasmid antigen they are poor antigen presenters and may lack features such as co-stimulatory molecules enriched on APCs. Instead, vaccine proteins produced mainly by the myocytes will be taken up by migrating APCs and presented on MHC class I (cross priming) and on MHC class II molecules to T cells in lymph nodes [133]. However, DNA vaccines delivered using regular i.m. injections have shown very poor efficacy and immunogenicity in higher primates and therefore other strategies are needed to improve the immune responses induced by the DNA vaccines in humans.
3.5.2 Gene gun (GG) immunization

One way shown to enhance the immunogenicity of the vaccine antigen is to deliver the DNA vaccine by intradermal (i.d.) particle bombardment using a gene gun [232, 233]. This involves coating of pDNA onto gold particles and by using the gene gun device and helium gas pressure the pDNA-gold particles are delivered into the epidermis of the skin. Using intradermal gene gun delivery a 100-fold less DNA compared to regular i.m. needle injection is needed to induce the same level of immune responses [234]. This can partly be explained by that the skin is enriched in Langerhans cells, which are professional APCs, which may be directly transfected by the DNA coated gold particles. Needle injections typically induce predominantly TH1 responses with high levels of IgG2a and rather low IgG1 levels in mice whereas gene gun immunization has a bias towards TH2 or a mixed TH1/TH2 response [235] [paper I]. A DNA vaccine using this gold-particle epidermal delivery method for influenza in humans was recently found to be both safe and to induce specific antibody responses [236]. In respect to cost effectiveness gene gun immunization uses small amounts of DNA but instead it requires gold. Also the stability of the DNA-gold complexes may have limitations.

3.5.3 Biojector

Another needle free delivery method, the Biojector, uses jet injection (high-pressure stream) driven by carbon dioxide (CO2) of the DNA in liquid into the skin or the muscle. Due to the needle free injection accidental needle stick injuries can be avoided. This technique can be used to deliver drugs, proteins or genetic vaccines. Administration of the protein based hepatitis A virus vaccine (Havrix) by Biojector resulted in higher antibody titers compared to needle injection [237]. For DNA vaccines, reduced, similar or enhanced immune responses have been detected as compared to needle injection in both small animals and primates [238, 239].

3.5.4 Microneedles

Different approaches of needle arrays technology to deliver and enhance the uptake of DNA vaccines in the skin are under development. The general idea using microneedles is to by minimum invasive means deliver the DNA into the skin in a non-painful way.
Microneedles can either be used only to create holes in the skin were after DNA is administrated or the microneedles are pre-coated with the compound to be delivered [240, 241].

3.5.5 Electroporation (EP)

The phenomena of electroporation to transfer genes into cells were described in the 1980s [242, 243]. *In vivo* electroporation is a method that uses electrodes to apply electrical pulses to the injection site of the pDNA, thereby enhancing plasmid uptake. The principle behind this treatment of the injection site is that EP generates transient pores in the cell membrane thereby allowing for a better uptake of larger molecules such as pDNA [244]. In addition to the permeabilization of the muscle fibre it is thought that the DNA uptake is promoted by migration and cellular uptake occur due to electrophoresis [245]. Recently, another study suggested that electroporation uptake involve permeabilization and passive diffusion of DNA through the permeabilized membrane rather than an electrophoresis effect *in vivo* [246]. Optimal electroporation parameters (voltage, pulses, duration e.g) are important to create membrane permeability and transfection of cells without causing extensive cell death. In addition, *in vivo* EP causes an inflammatory response generating infiltration of immune cells to the treated area that may be important in generating a strong immune response using DNA vaccination [247][paper V]. *In vivo* EP of muscle tissue have been shown to enhance the transfection efficiency of pDNA resulting in a increased pDNA uptake as compared to regular i.m. immunization [248, 249]. This technique has been shown to be useful to deliver DNA vaccines both in the skin and in muscle tissue. Both humoral and cellular immune responses using various different DNA vaccines have shown to be effectively enhanced by *in vivo* EP in several animals such as, mice, rabbits, guinea pigs, sheep, rhesus macaques and pigs [244, 250-254][paper V]. In macaques immunized with pDNA encoding multiple HIV proteins with *in vivo* EP, a 10-40 fold, increase in cellular immune responses was primed as compared to animals receiving 5-fold higher doses without EP [255]. In another vaccine study in macaques, pDNA encoding a HBV antigen delivered using EP enhanced both humoral and cellular immune responses [256]. For HCV, electroporation in combination with an NS3-NS5B DNA vaccine induced more potent CD4+ and CD8+ cellular immunity than naked DNA in both mice and macaques [257]. Our own studies using a NS3/4A DNA vaccine
further confirms the potency of EP, a 100-fold less DNA delivered with EP induce a comparable activation of antibodies, CD4+ and CD8+ T cells, and IFN-γ production [254][paper V]. The delivery method itself may provide an adjuvant effect. One reason for the greatly enhanced immunogenicity seen using electroporation except the enhanced uptake of the pDNA may be the tissue damage caused by the electric pulses. As mentioned, the electric pulses cause tissue damage resulting in inflammation at the site of injection that effectively induces the recruitment of immune cells to the area [247, 258] [paper V]. The reduced amount of pDNA needed using EP delivery is a major advance, which is also necessary to make these vaccines realistic in humans. The debate about integration using DNA vaccines raises new concerns when combined with electroporation. Higher integration rate of a host-derived gene has been shown using EP [255, 259]. However, with respect to vaccine genes, the cells that are most likely to have integration are the cells with a high uptake of pDNA. Though, these will also express high levels of antigens and should therefore be effectively cleared by the host T cell response. Several studies are now ongoing to further address this concern. Finally, it should be noted that electroporation is generally perceived as a bit more painful than a standard i.m. injection.

3.5.6 Prime-boost regimens

In most cases immune responses primed by a single dose is not sufficiently strong to be protective. Repeated administrations, boosting, can in these cases be a way to enhance the immune activation. Plasmid DNA vaccines have when using homologous boosting, so far been found to induce rather poor immunogenicity in humans. Viral vector delivery has been more effective in priming a strong immune response. However, if the same viral vector is used in a homologous boost, the immune response to the vector itself might lead to a rapid clearance of the vector by the immune system. This may result in a weakened response to the vaccine antigen. Therefore, heterologous prime-boost strategies, using two different vaccine compositions, have shown to be an alternative approach. An initial vaccination using plasmid DNA to prime a broad immune response followed by a effective boosting using a viral vector seem to be the most effective approach and have been demonstrated effective for HIV [260, 261], HCV [210, 262] and other diseases. For vaccines with the intention to induce strong antibody responses, priming using pDNA followed by a boost with recombinant protein
might be more effective. Although, the most ideal vaccines would of course be to use homologous immunizations with a competent adjuvant, to avoid delivery, manufacturing and distribution issues using several different vaccine approaches. The use of *in vivo* electroporation is a promising approach to prime an effective immune response using pDNA vaccines and could hopefully be used in homologous vaccinations. This may also reduce production costs.

### 3.6 ADJUVANTS

Naked DNA vaccines (or protein based vaccines) by themselves are not very immunogenic and after a regular needle injection the degree of inflammation is low. Therefore, an additional immune stimulation, an adjuvant, can be used to amplify or enhance the effect of the DNA vaccine. Adjuvants are defined as compounds that increase and/or modulate the immunogenicity of an antigen. The term adjuvant is itself derived from the Latin *adjuvare* with the meaning, to help. The most commonly used adjuvant in experimental models to enhance the immunogenicity of protein-based vaccines is Freund’s complete adjuvant (CFA). It consists of killed mycobacteria in a water-in-oil emulsion that will prolong the distribution of the antigen and cause an inflammatory response recruiting immune cells to the injection site. CFA is not approved for use in humans, instead aluminium (alum) phosphate is widely used as an adjuvant for protein or subunit vaccines. The mechanism behind is not fully clear but may include a slow release and recruitment of APCs. Alum predominately induces Th2 responses and has been used to enhance antibody responses towards DNA vaccines [263, 264]. After aluminium, emulsions are the most frequently used adjuvants. Emulsions contain two immiscible phases of oil-in-water (o/w) or water-in-oil (w/o). An o/w emulsion MF59 has been evaluated to use as vaccine adjuvant to enhance immunogenicity of several antigens such as influenza, HIV, HBV and HCV. The mechanism of action of MF59 is not clear, but seems to stimulate recruitment of immune cells, preferably macrophages resulting predominantly in a Th2 response with increased levels of IL-5 and 6 [265]. MF59 has now been approved for human use.

Liposomes, spherical membranes of phospholipids, have been shown to protect DNA from degradation and function as a delivery vehicle facilitating transfer of pDNA into
cells by fusion. Liposome formulations can enhance both humoral and cellular immune responses of pDNA immunogens [266].

A large number of cytokines, chemokines or co-stimulatory molecules have been evaluated as DNA vaccine adjuvants in animal models but so far only a few have been tested in humans. Expression of cytokines from plasmids or injected as recombinant proteins simultaneously to the DNA vaccine has been shown to be effective. Using cytokines, specific immune cells can be triggered and the immune responses can be stimulated in a desired direction. Plasmid expressed cytokines can either be used to enhance stimulation of T cells or to recruit APCs. However, most cytokines are involved in a complex regulation of each other and the induction of one cytokine might up or down regulate the effect of others. Furthermore, as seen in treatment using interferons, the half-life of cytokines is short and therefore might need to be modified. IL-2 has a well-characterized ability to activate both T cells and NK cells and are therefore a choice as an adjuvant. Plasmid expressed IL-2 has shown to stimulate both humoral and cellular immune responses for DNA vaccines for both HBV and HIV [267, 268]. Attention has also been focused on IL-12 because of its ability to promote Th1 responses. Several studies have reported plasmid expression of IL-12 to further induce cellular responses to DNA vaccines encoding HIV-1 genes [269, 270]. Several other molecules such as colony-stimulating factors (GM-CSF) for recruit APCs and activation of T cells [271, 272], or chemokines such as macrophage inflammatory protein (MIP-1α, RANTES) are being tested with DNA vaccines. Also the use of plasmid co-stimulatory molecules, B7 and CD40 improving the priming of T cells, has been addressed as adjuvant strategies.

Another attractive adjuvant approach is the use of toll-like receptor ligands to trigger the innate immune responses and stimulate for the adaptive immune responses. A key feature in the innate immunity is the pattern-recognition receptors (PPRs) that can bind different types of molecules expressed by different pathogens. The best studied PPRs are the toll-like receptors (TLRs), of which 10 are identified in humans [123]. There are TLRs specific for extracellular components as well as for intracellular parasites. TLR-9 detects unmethylated CpG, common in genomes of bacteria and viruses. For DNA vaccines CpG motifs that trigger TLR-9 innate immune responses has an immunostimulatory effect, activating inflammatory cytokines and type I interferons. The use of synthetic CpG-oligodeoxynucleotides has been shown to cause
inflammatory responses and stimulate immunity [273, 274]. In a clinical study, CpG co-administered with the HBV vaccine Engerix-B, more rapid and improved antibody responses were obtained [275]. dsRNA, an important inducer of type I IFN signalling can be mimicked using a synthetic dsRNA analogue polyinosine-deoxycytidylic acid (poly(I:C)) to activate TLR-3 [110]. Flagellin, a protein from the flagella used for bacteria motility, is a potent activator of innate immune responses recognized by TLR-5. Adjuvant stimulation of TLR-5 using recombinant flagellin has been shown to induce both humoral and cellular immune responses in combination with pDNA [276].
4 AIMS OF THE STUDY

The overall aim of this thesis was to develop a hepatitis C virus non-structural 3/4A protein based therapeutic DNA vaccine and to characterize immune responses raised by this vaccine.

Specific aims in individual papers I-V.

I. To improve the immunogenicity of an HCV NS3/4A-based genetic vaccine.

II. To establish a murine model system to study entry of functional NS3/4A-specific T cell responses in the liver.

III. To study the relation between immune escape mutations and viral fitness within immunodominant CTL epitopes of the NS3 protease.

IV. To study effects of the NS3/4A protein on the innate and adaptive immune responses.

V. To characterize the effects of \textit{in vivo} electroporation for delivery of a codon optimized NS3/4A DNA-based vaccine.
5 COMMENTS ON MATERIALS AND METHODS

5.1 MICE AND CELL LINES

Inbred mice of different genetic backgrounds, BALB/c (H-2^d), C57BL/6 (H-2^b) and gene deficient or knockout (-/-) mice such as, B-cell^-/- (μMT, H-2^b), CD4^-/- (H-2^b), CD8^-/- (H-2^b), Fas^-/- (H-2^b), IFN-γR2^-/- (H-2^b), Perforin^-/- (H-2^b) and TLR4^-/- (H-2^b) were obtained from commercial vendors, the animal facility at the Department of Microbiology, Tumor biology and Cell biology (MTC), Karolinska Institutet or the Unit for Embryology and Genetics, Karolinska Institutet. Transgenic C57BL/6 (H-2^b) mice with hepatic expression of HCV NS3/4A were generated at the Unit for Embryology and Genetics, Karolinska Institutet. Inbred transgenic HHD-HLA-A2.1 (HHD^+ H-2D^b/- βm^-/-) mice were kindly provided by Dr F Lemonier (Institute Pasteur, Paris, France). All animal experiments were approved by the ethical committee for animal experimentation at Karolinska Institutet.

Cell lines used throughout this work, BHK-21 (Baby Hamster Kidney), HEK293 (Human Embryonic Kidney), HepG2 (human Hepatoblastoma), EL-4 (H-2^b) (mouse lymphoma), EL-4-NS3/4A (H-2^b), RMA-S (H-2^b) (lymphoma mutant), RMA-S (HHD^+ H-2D^b/-) SP2/0-Ag14 (H-2^d) (mouse myeloma), and SP2/0-NS3/4A (H-2^d). RMA-S is a transporter associated with antigen processing (TAP) deficient cell line. Its MHC class I molecules can be stabilized when exposed to specific peptides [277].

5.2 RECOMBINANT PROTEINS AND PEPTIDE ANTIGENS

A variety of different NS3-derived MHC class I peptides have been used, most importantly, H-2^b-restricted; GAVQNEVTL, HLA-A2-restricted; CINGVCWTV (1073-1081), LLCPAGHAV (1169-1177), TGSPISTSYT (1287-1296), KLVALGINGV (1406-1415), YLVAYQATV (1590-1598). Numbers within parenthesis indicates the amino acid location of the HCV full-length genome. Additional HBV core H-2^b-restricted; MGLKFRQL MHC class I peptide, the TPPATRPPNPAPIL T cell helper peptide and the CMV pp65 NLVPMVATV peptide, have been used in these experiments. Peptides were kindly synthesized by Dr M Levi (Tripep AB, Huddinge, Sweden) using an automated synthesizer [278].
Recombinant NS3 helicase (rNS3) protein was kindly produced and provided by Darrell L Peterson, Department of Biochemistry, Commonmonwealth University, VA, USA.

5.3 DNA VECTORS FOR IMMUNIZATIONS

There are several reasons for using the HCV NS3 gene as a DNA vaccine target. First, the NS3 protein is one of the few proteins within the HCV genome that has a limited genetic variability [59, 60]. Second, NS3 is a multifunctional protein with important enzymatic functions [39-42]. Third, it is a relatively large protein, thereby increasing the possibility that the protein contains multiple T cell epitopes. Fourth, and most importantly, several studies have shown that NS3-specific CD4+ and CD8+ T cell responses are important for control and clearance of HCV infection [139-141, 143].

Inclusion of the NS3 co-factor NS4A is important to stabilize the NS3/4A-protein complex, which also results in an enhanced immunogenicity [279, 280]. All NS3-genes were derived from an HCV genotype 1a isolate. For immunization, wild type (wt) NS3, wtNS3/4A, mutant (mut) NS3/4A or coNS3/4A were delivered and expressed from the pVAX1 vector (Invitrogen). The generation of wtNS3-pVAX1, wtNS3/4A-pVAX1 and mutNS3/4A-pVAX1 has previously been described [279]. The mutNS3/4A plasmid contains a disrupted cleavage site between NS3 and NS4A resulting in an uncleaved NS3/4A fusion protein. To further enhance the intrinsic immunogenicity of the NS3/4A complex a synthetic codon optimized version, coNS3/4A-pVAX1, using the most commonly used codons in highly expressed human genes, was generated [paper I].

5.4 VIRAL VECTORS

Recombinant suicidal Semliki forest virus (SFV) vectors expressing wtNS3/4A or coNS3/4A were used as an approach to enhance expression levels and immunogenicity of the NS3 genes. These SFV replicons encode the non-structural proteins (nsp1-4) of SFV and the NS3/4A antigen from HCV. The structural proteins have been replaced by the antigen of interest (wtNS3/4A or coNS3/4A) resulting in a replication deficient virus that is only infective once but without risk of production of new viral particles. Upon infection the genomic sense RNA immediately translate an antisense RNA to
generate the replication complex. The SFV replicase then uses the anti sense RNA as a template for amplification of the subgenomic NS3/4A sense mRNA and subsequently NS3/4A-protein. The rapid mRNA production by the replicase complex results in amplification of mRNA until the cells lyses [281, 282].

![Diagram](image)

**Figure 8**: Simplified schematic picture of the SFV-NS3/4A replicon system. SFV structural proteins have been replaced by HCV NS3/4A. Synthesis of subgenomic HCV NS3/4A RNA is initiated at a promoter site in the (-) RNA intermediate.

### 5.5 IMMUNIZATION PROTOCOLS

Mice were immunized with NS3-based vaccines as pDNA, peptides, recombinant proteins or recombinant SFV particles to generate immune responses against the vaccine antigen. Groups of mice (5-10 mice/group) were used in all experiments. Intramuscular (i.m) immunizations of pDNA were done in the tibialis anterior (TA) muscle by needle injection using 0.5-100 µg pDNA alone, or in combination with *in vivo* electroporation. pDNA was also delivered by transdermal gene gun immunization, on shaved mouse abdomen, using 2-4 µg pDNA. rSFV particles were immunized subcutaneously (at the base of the tail) using $1 \times 10^7$ virus particles diluted in PBS. rNS3 protein or peptides, in incomplete Freund's adjuvant, were given subcutaneously at the base of the tail. Where indicated, mice were boosted once or twice with monthly intervals.

**Gene Gun (GG)**

The Gene gun (Bio-Rad Laboratories, Hercules, CA, USA) (described in 3.5.2; delivery methods) was used for transdermal immunization of NS3-based vaccine candidates. Plasmid DNA was coated onto 1µm gold particles according to manufacturer’s
In vivo electroporation (EP)

In vivo electroporation was performed using the MedPulser® electroporation system (Inovio Biomedical Corporation, San Diego, CA, USA) (Figure 9). Immediately after a standard i.m. immunization in the TA muscle with 0.5-50 µg of coNS3/4A-pVAX1 (or pVAX1 empty vector alone) diluted in 50 µL PBS, the injection area was electroporated. In mice, a 0.5 cm array of a two needle-electrode applicator device (for larger animals and humans four needle-electrodes) was used. Two electrical pulses, with a one second spacing, which generated an electric field of 246 V/cm during 60 milliseconds, were used in all immunizations with electroporation.

Figure 9: Left; Inovio’s MedPulser DNA electroporation device. Right; Applicator unit for mice with a disposable 2 electrode tip.

5.6 DETECTION OF LYTIC CTLS, AND IFN-γ PRODUCING CTLS AND Tʜ CELLS

5¹ Cr-release assay

Cytotoxic T lymphocytes primed by NS3-based vaccines were detected using a standard ⁵¹ Cr-release assay. Two weeks after (last) immunization mice were sacrificed and splenocytes were in vitro re-stimulated for five days in presence of a NS3 MHC class I peptide. Lytic activity was then measured by the ⁵¹ Cr-release assay with
radioactive labelled target cells (RMA-S) coated with the CTL peptide. During four hours of incubation, effector cells (e.g. NS3-specific CTLs) were allowed to specifically kill target cells, resulting in release of radioactive chromium into the supernatant. Specific CTL activity could thereafter indirectly be measure in a γ-counter. In some experiments cells with stable expression of the NS3/4A protein were used, instead of peptide loaded cells, both during re-stimulation and as targets during the 51Cr-release assay. The use of transfected target cells is favourable since it more mimics the natural situation with endogenous processing of the antigen peptides. Unfortunately, the fine specificity of the CTLs is not known, and a lower CTL activity is often observed. The latter might be explained by that fewer antigen peptides are presented to the effector cells during the re-stimulation. Exogenously loaded peptides generate a higher CTL activity and can also be favourable when studying individual CTL epitopes. On the other hand, the system of using exogenously loaded peptides is more artificial and may not always represent the true in vivo situation, but is very useful when characterizing lytic CTL activity since the sensitivity is very high. Thus, if possible, CTLs should be determined by both approaches.

**ELISPOT**

Another method used in this work to study immune priming is the quantification of IFN-γ production by HCV NS3-specific T cells in an enzyme linked immunosorbent spot (ELISPOT) assay [283, 284]. The IFN-γ producing CTLs or T\(_H\) cells were detected after in vitro recall using NS3 MHC class I peptide or rNS3 protein in spleenocyte and/or lymph node cultures. In brief, IFN-γ secreted during a 36-48 hour antigen re-stimulation is captured by coated anti-IFN-γ antibody and can after removal of cells be detected using a biotinylated antibody and visualized with avidin-HRP, resulting in coloured spots. Spots were then counted using an automatic spot reader. 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.

5.7 **GENERATION OF TRANSIENTLY TRANSGENIC MICE**

To be able to study intrahepatic immune responses primed by our DNA vaccine candidates we established a mouse model with transient expression of the NS3/4A
protein in the liver. Transiently transgenic mice are generated by a hydrodynamic (HD) injection of 1.6-2.0 mL of Ringer solution containing 50-100 µg of pDNA injected in the mouse tail-vein (within a period of 5-10 seconds). The HD injection results in perfusion of the liver and the hydrodynamic pressure increase the permeability of the cells, allowing for efficient uptake of the pDNA into the hepatocytes [95, 96]. Using this technique, protein expression can be detected from hours up to weeks depending on the specific antigen used.

**Figure 10: Immunization schedule to study immune responses towards transient protein expression of NS3/4A-protein in a mouse liver.**

The transiently transgenic mouse model was used to study whether NS3-specific T cells primed in the periphery (intramuscularly or transdermally) were able to enter the liver, and to recognize and kill hepatocytes expressing the NS3/4A protein. Various types of mice were vaccinated as described 14 days prior the HD injection. NS3-protein expression from the injected pDNA, which was taken up by hepatocytes was monitored from one hour up to weeks after HD challenge by immuno-precipitation-Western blot, immunohistochemistry, or by *in vivo* imaging.

**5.8 DETECTION OF HCV NS3 PROTEIN**

*Immunoprecipitation (IP) and Western blot (WB)*

To detect NS3-protein in mice livers or mice and rabbit muscle tissues, the NS3-protein was immunoprecipitated from lysed tissue using protein A sepharose coupled to a polyclonal anti-NS3 antibody, followed by SDS-PAGE gel, electrotransfer and Western blot analysis. Using this approach we could monitor expression of NS3/4A-protein in transiently transgenic mouse livers and clearance of expressing cells due to vaccine induced immune responses. This technique was also used for kinetic analyses of NS3/4A protein expression in mice and rabbit muscle tissue after i.m. immunization with or without electroporation.


Real time in vivo imaging

Real time in vivo imaging can be used to study ongoing processes using a bioluminescent reporter gene, which emits light when expressed in a living organism. By measuring emitted light by a charge coupled device (CCD) camera, a bioluminescence signal can be captured by a computer and analyzed using computer software. Real time in vivo imaging was used for two purposes in the present studies. First, using in vivo imaging and a luciferase reporter plasmid, we studied protein expression and the bio-distribution of protein expression within the whole body of mice after an i.m. injection. The technique was also applied to follow clearance of protein expression in liver of mice to determine the kinetics of functional intra-hepatic immune responses primed by DNA vaccines. Since a cell that takes up one plasmid most likely also will take up a co-injected plasmid, we found that it was possible to generate hepatocytes that in vivo co-expressing NS43/4A and the luciferase reporter genes in transiently transgenic mouse livers. In vivo imaging is beneficial to use for several reasons. The possibility to follow the in vivo situation in individual mice both reduces the number of animals needed but also strengthens data and reduces variability. Luciferase protein expression was measured using in vivo imaging equipment (IVIS® Imaging System 100 Series, Xenogen Corporation, CA, USA) 5-15 minutes after an intraperitoneal (i.p.) injection of 3 mg of the luciferase substrate luciferin.

5.9 STATISTICAL ANALYSIS

Statistical comparisons were performed using the Statview 5.0 and Excel:mac software packages for Macintosh. Parametrical data were compared using Student’s t-test (Statview and Excel) and non-parametrical data were compared using the Mann-Whitney U-test (Statview). Frequencies were compared using Fisher’s exact test (Statview). Kinetic of tumor growth in groups of mice were compared using the area under the curve (AUC) and the values were compared using analysis of variance (ANOVA, Statview).
6 RESULTS

6.1 CODON OPTIMIZATION AND mRNA AMPLIFICATION ENHANCES THE IMMUNOGENICITY OF NS3/4A [PAPER I]

In the first paper we evaluated different ways to enhance the protein expression levels and the immunogenicity of HCV NS3/4A-based genetic vaccines. This was achieved by using two separate strategies, codon optimization (co) of pDNA, or mRNA amplification using the Semliki forest virus (SFV) system.

The coNS3/4A plasmid generated >11-fold higher expression-levels of the NS3-protein, as compared to the wild type (wt) NS3/4A plasmid in transient transfection of HepG2 cells. Both codon optimization and mRNA amplification enhanced the humoral immune responses evidenced by higher levels of NS3-specific antibody titres, as compared to the wtNS3/4A construct. Codon optimization did not alter the T\textsubscript{H1}-like profile of the NS3/4A-gene and both when the SFV construct where delivered subcutaneous, and the coNS3/4A gene where delivered by gene gun immunization it resulted in a mixed T\textsubscript{H1}/T\textsubscript{H2} phenotype. Both codon optimization and mRNA amplification resulted in a quicker priming of CTLs as compared to the wt gene. Also the \textit{in vivo} functionality of the CTLs was significantly improved, as determined by a tumor challenge model where efficiency is measured as an inhibition of growth of an NS3/4A-expressing tumor cell line. \textit{In vivo} protection against tumor growth was found to be dependent on CD8\textsuperscript{+} T cells, and independent of both B cells and CD4\textsuperscript{+} T cells. Protective immunity against tumor growth was analyzed and detected up to six months after a single immunization using both the codon optimized NS3/4A plasmid and SFV vaccines, which indicates priming of memory T cell responses.
Figure 11: In vivo protection against tumor growth in C57Bl/6 (H-2b) mice 6 month after a single immunization of coNS3/4A-pVAX1 or wtNS3/4A-SFV. Also shown, tumor growth in non-vaccinated mice.

More importantly the ability of the genetic vaccine to work therapeutically was tested by immunization of mice 6 and 12 days after tumor challenge. A rapid priming of CTLs was confirmed since tumors were significantly smaller in vaccinated mice as compared to the control groups.

In conclusion, codon optimization of the wtNS3/4A gene or expression of the wt gene using the SFV vector system improved both immunogenicity and protein expression levels of the NS3/4A vaccine.

6.2 IN VIVO CLEARENCE OF NS3/4A EXPRESSING HEATOCYTES

In the second paper we tried to answer one of the most important questions regarding a therapeutic vaccine against chronic HCV infections; can the vaccine primed T cells enter the liver and eliminate hepatocytes expressing HCV proteins? We therefore established a transiently transgenic mouse model, which allowed studies of the intra-hepatic immune responses primed by the coNS3/4A DNA vaccine whereby we could specifically address this question. Transiently Tg mice are generated by an intravenous hydrodynamic (HD) tail vein injection of a large volume (1.6-2 mL) at high speed (less than 5-10 seconds) containing a plasmid expressing NS3/4A. Further, the perfusion of the liver promotes hepatocyte up-take of the plasmid, followed by protein-expression of
the gene product. Consistent with the observations in paper I, did we see a higher and prolonged intrahepatic protein-expression of NS3 when using the coNS3/4A gene compared to the wtNS3/4A gene or the wtNS3 gene alone.

When the coNS3/4A gene was injected, NS3-protein was detected in up to 30% of the hepatocytes after 24-72 hrs, and the protein could be detected in the liver for at least two weeks. Using these transiently Tg mice, we could show that T cells primed by transdermal vaccination indeed entered the liver and cleared NS3/4A-expressing hepatocytes evidenced by the following scenario. Prior to a HD challenge a functional CTL activity could be detected in the spleen of immunized mice with a 40% lysis of peptide-loaded RMA-S cells at a 60:1 effector:target (E:T) ratio. At 48 hrs after the HD challenge the presence of NS3-protein was lower when detected by western blot analysis in most of the immunized mice as compared to controls. Also, a statistically significant lower numbers of NS3-expressing cells were noted in the immunized mice as compared to controls, evidenced by immunohistochemical staining. Interestingly, at the 48-hour time-point the CTL activity in the spleens from the same immunized mice was almost undetectable with an average lysis below 10% and half of the mice were considered as negative. No difference in CD3+ T cell infiltration in the liver could be detected at this time-point. At the 72 h time-point post HD challenge, almost no hepatic NS3/4A-protein could be detected either by western blot or IHC analysis in the immunized mice. At the same time-point, the number of CD3+ T cells was statistically higher in the immunized mice as compared to the non-vaccinated mice. Also at the same time point, a CTL activity with an average of 20% lysis was starting to reappear in the spleens of the immunized mice. To test whether the clearance was mediated by CTLs, CD8-/- mice were studied using the same approach. We could by this approach show that CD8-/- mice were not able to clear NS3/4A protein expressing hepatocytes.
In conclusion, this study shows that vaccine primed NS3/4A-specific CD8+ CTLs are effectively activated in the periphery, which are able to enter the liver and to eliminate HCV protein-expressing liver cells. This is a prerequisite for a functional therapeutic vaccine.

6.3 RELATIONSHIP BETWEEN IMMUNE ESCAPE AND VIRAL FITNESS [PAPER III]

The hepatitis C virus has evolved several ways to circumvent the immune system both with respect to the innate and adaptive immune response. Mechanisms for immune evasion of the adaptive immune responses are not fully clear but one evasion strategy used by HCV is to positively select for viruses with mutations within immunodominant epitopes. This may escape CTL recognition and thereby promote persistence of the infection. Therefore, in the third paper, the overall aim was to study the natural variability within the NS3 gene and try to explain why some immunodominant epitopes under a selective pressure do not accumulate mutations. The NS3/4A gene of 48 clones from five HCV chronically infected patients were analysed and sequence analysis revealed that the variability within NS3/4A was limited, and in particular within the immunodominant epitope at residues 1073-1081 of the NS3 protease.

To study the immunodominance within HLA-A2-restricted epitopes, mice transgenic for the human HLA-A2 molecule were primed using either gene gun immunization of pDNA or by expression of the NS3/4A vaccine using the SFV system. In vitro stimulation using peptides corresponding to known epitopes revealed that the immunodominant CTL activity was directed against the HCV NS3 protease at residues 1073-1081. HLA-A2 interaction with the 1073-1081 epitope was analyzed in detail using Alanine (Ala) substitution peptide analogues. Ala substitution at positions 1074, 1079 or 1081 reduced the binding to HLA-A2 molecule in a peptide stabilisation assay, suggesting that such mutations would result in a reduced presentation and stability of the HLA-A2-peptide complex. Furthermore, CTL recognition was reduced in HLA-A2 vaccine-primed mice against peptides with Ala substitutions at positions 1075, 1077 and 1079, suggesting that these mutations affect the recognition by the T cell receptor. Thus, we found that introduction of Ala substitutions at five positions within the
immunodominant NS3-derived 1073-1081 epitope affected CTL recognition, either by reduced HLA-A2 binding or TCR recognition.

Next, to address the conserved nature of this epitope, we tested whether mutations within the epitope affected the fitness of the NS3/4A protease. We generated constructs that harboured the introduced mutations. These mutant genes were then expressed and tested by a transcription and translation assay. This revealed that the protease activity of NS3 was reduced by mutations at positions 1074 and 1079. To confirm that these mutation affected viral fitness in the context of complete polyprotein processing the HCV replicon system was used. This confirmed that some mutations at positions 1074, 1075, 1076 and 1079 reduced the level of RNA replication, as a consequence of a less active protease, and subsequently the viral fitness was indeed reduced.

Thus, our data indicate that the variability of immunodominant epitopes is limited by the viral fitness, which help to explain why certain escape variants do not appear as a major viral species in infected humans.

6.4 THE INHIBITION OF THE INNATE IMMUNITY BY NS3/4A DO NOT INHIBIT VIRAL CLEARANCE BY THE ADAPTIVE IMMUNE RESPONSE [PAPER IV]

Not only the adaptive immunity are affected by HCV. Several mechanisms disturbing the innate immune response have been reported, some of them described in section “2.8.3.1; Impaired innate immune responses to HCV”. One important mechanism is the cleavage of the Cardif adaptor molecule (also known as IPS-1, MAVS or VISA) involved in the RIG-I response to the presence of dsRNA [153, 156]. This cleavage results in impaired interferon and apoptotic responses, presumably promoting viral persistence. Since most of the vaccine studies have been performed in mice, it is important to determine whether NS3/4A can impair the responses to dsRNA also in murine cells. If this turns out to be the case, new angles of the role of NS3/4A can be studied in vivo in mice. In the fourth paper we therefore investigated if the reported interference by NS3/4A with Cardif (in the innate immune response) also occurs in murine cells and if this had any affects on the adaptive immunity. We first found that our genotype 1a coNS3/4A gene reduced the interferon response in transfected human (HEK293) tumor cells after stimulation with synthetic dsRNA, poly(I:C). Second, we
also found that NS3/4A reduced the NFκB response to synthetic dsRNA in transiently transfected NS3/4A murine (SP2/0) cells. Importantly, to test if this was true also in non-tumor cells and in an *in vivo* situation, transgenic mice with stable hepatic expression of the NS3/4A-protein were used. Consistent with the *in vitro* findings did we see a reduced activation of NFκB and IRF-3 responses in hepatic nuclear fractions from these mice after poly(I:C) treatment, as compared to wt mice. This suggest that NS3/4A interferes with the host immune responses not only in human cells but also in murine cells. These findings led us to ask the question whether murine (m) Cardif may be cleaved by NS3/4A. We could show that this is probably true since the apperence of a truncated mCardif was seen in the presence of NS3/4A in an *in vitro* transcription and translation assay. The cleavage of mCardif was further confirmed by co-transfection of HepG2 cells with mCardif and different NS3/4A genes. Cleavage of Cardif was only observed in presence of a functional NS3/4A complex but not in any of the controls, such as empty expression vector, expression of HBV core or a NS3/4A construct with a disrupted cleavage site between NS3 and NS4A. Thus, also murine Cardif is cleaved by HCV NS3/4A.

Since NS3/4A affects murine cells in a similar way as human cells, it is of interest to study if the modulating effect by NS3/4A also affects the *in vivo* clearance of the NS3/4A-protein by the adaptive immune response. We used real-time monitoring by *in vivo* imaging of transiently NS3/4A- and/or Firefly luciferace (FLuc) transgenic mouse livers.

*Figure 13:* Study design for monitoring in vivo clearence of NS3/4A-protein expressing hepatocytes by real-time *in vivo* imaging.

This revealed that the presence of NS3/4A-protein did not prevent clearance of double-transgenic hepatocytes in naive, or NS3/4A- or FLuc-DNA vaccinated mice. Also, consistent with the observations in paper II, a statistically significant difference in
clearance was observed from 48 hrs post HD challenge in NS3/4A pre-vaccinated mice as compared to non-immunized mice. Moreover, we studied clearance of hepatic NS3/4A-protein expression in various knock-out mice in order to investigate the role of the deleted gene. Our results suggest that clearance of NS3/4A-protein expressing hepatocytes requires the classical mediators of virus infected cell killing including CD8+, IFN-γ, perforin and FasL, but not CD4+ or Toll-like receptor-4.

In conclusion, it is well documented that NS3/4A interferes with the host innate immune response in human tumor cells. However, this interference does not clearly affect the NS3/4A-specific adaptive immunity. These results help to explain why HCV has to evade the host immune response through various mechanisms such as mutations in immunodominant CTL recognition epitopes and not only through disruption of the innate immunity.

6.5 EFFICIENCY AND SAFETY DELIVERY OF THE coNS3/4A-VACCINE USING IN VIVO ELECTROPORATION [PAPER V]

As previously stated, the major obstacle in using DNA vaccines in human is the poor delivery of the DNA by standard techniques. We therefore continued our evaluation of delivery techniques that may work well in large animals and humans. In the fifth paper we investigate whether the efficiency of the NS3/4A DNA-based vaccine could be further improved by administering the vaccine through in vivo electroporation (EP). A major concern regarding DNA-based vaccination in humans is the very poor uptake of pDNA into human cells. This subsequently results in a poor immune response. The in vivo EP technique has shown promising results in recent studies regarding an enhanced uptake of the vaccine [248, 249]. Directly after an i.m. injection of the pDNA intramuscularly, the injection site is treated with two short electrical pulses. This generates small transient pores in the cell membrane of the muscle cells, which will enhance the uptake of the pDNA.

To study if in vivo EP could further enhance the immunogenicity of our coNS3/4A vaccine groups of mice were immunized one, two or three times with different doses with or without EP. Mice were also immunized and the site of injection was analyzed for presence of NS3-protein, inflammation and infiltration of immune cells. From these
studies we could show that *in vivo* EP primed equally or stronger immune responses when using a 100-fold lower dose of pDNA. Moreover, using the same dose as the control (50 μg i.m.) or a 10-fold lower dose, the EP primed mice had stronger both humoral and cellular immune responses. Furthermore, *in vivo* EP increased and prolonged the NS3/4A-protein expression levels, caused local tissue damage and showed a statistically significant increased infiltration of CD3+ T cells at the site of injection. These factors most likely collectively contributed to an enhanced NS3/4A-specific antibody response, CTL activity and an improvement in IFN-γ producing T-helper and CTLs.

One concern using DNA vaccines is the risk of integration of DNA into the host genome. The use of *in vivo* EP that promote uptake of pDNA might result in a prolonged persistence of pDNA in the cells and thereby an enhanced risk of integration. Therefore, we studied the persistence of plasmid DNA in mice up to 60 days after a second monthly immunization. From this study, we could conclude that EP treatment did not promote DNA persistence since the control group had similarly low levels of pDNA at this time-point. At 60 days post immunization the pDNA was cleared or almost cleared in all groups of mice. The coNS3/4A vaccine was also analyzed for biodistribution of the injected pDNA in both mice and rabbits. DNA was detected mainly in the injected TA muscle, the neighbouring quadriceps muscle and the contra lateral TA and quadriceps muscle, independently of EP delivery. Protein expression was restricted to the injection muscle and was cleared within 28 days, confirmed in rabbits by IP-WB (Figure 14) or in mice using a luciferase reporter gene and *in vivo* imaging.

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<th>Day after injection</th>
<th>Right TA (site of injection)</th>
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<tr>
<td></td>
<td>Mean % of positive control</td>
<td>No. of positive of tested</td>
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<td>1</td>
<td>60±41</td>
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<td>3</td>
<td>78±54</td>
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<td>7</td>
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<td>14</td>
<td>14±13</td>
<td>2/6</td>
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<td>28</td>
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*Figure 14:* Detection of NS3/4A-protein expression in rabbit tibialis anterior (TA) muscles determined by immunoprecipititation and western blot after a single injection of 70μg coNS3/4A followed by in vivo EP. (Positive control, coNS3/4A-pVAX1 transiently transfected BHK-21 cells). All values below 20% of positive control were considered as negative.
Both an acute and a repeated toxicological study were performed in rabbits to address if the coNS3/4A vaccine delivered by in vivo EP had any unfavourable effects. The only pathology observed was restricted to the site of injection, both with and without injection of NS3/4A, with some degree of degeneration, inflammation, fibrosis and regeneration of the muscle tissue. Although the degree of histological change was clearly higher when coNS3/4A was delivered, the pathology seen using only EP show that in vivo EP per se causes local tissue damage and an inflammatory response. Finally, repeated immunizations of high doses of coNS3/4A DNA did not result in any persisting tissue damage at the injection site.

In summary, these data suggest that the coNS3/4A DNA vaccine can be delivered in a safe way using in vivo electroporation resulting in an improved immunogenicity by increased plasmid uptake, protein expression, local inflammation, and infiltration of CD3+ T cells.
7 DISCUSSION

The hepatitis C virus is a global health problem and a major causative agent of liver disease. It is estimated that approximately 3% of the world’s population (~170 million people) are chronically infected with HCV. Most infections (70-90%) become chronic of which 45 to 75%, depending of viral genotype, respond to current state-of-the-art antiviral therapy. Among patients that respond poorly to treatment, the majority are infected with the genotype 1 strain. This patient group have an increased risk of developing severe liver disease, such as fibrosis, cirrhosis and HCC. The viral genome shows a high genetic variability, which allows new immune escape variants to occur. An ideal HCV vaccine candidate should therefore be targeted towards the most genetically stable regions of the genome. The non-structural (NS) 3 gene, encoding both protease and helicase enzymatic activities, is most likely due to its pivotal role during viral replication one of the most well conserved regions within the HCV genome. The NS3 co-factor NS4A is needed for NS3 to stabilize the NS3/4A complex to fully utilize the functionality of the NS3 protein. Development of NS3-based therapeutic vaccines should therefore benefit by the inclusion of NS4A, which would strengthen the immunogenicity of the NS3-protein [279].

Two different approaches were evaluated aiming to optimize the immunogenicity of the NS3/4A-based vaccine. First, mRNA amplification using a SFV replicon system, and second a codon optimization of the complete DNA gene. Both these approaches increased the expression levels of the NS3-protein, which help to explain the enhanced immunogenicity [paper I]. Moreover, SFV infected cells, with a rapid mRNA amplification of the vaccine gene results in a stress response, which shuts down the host cell protein synthesis and eventually lead to cell death by apoptosis. The presence of dsRNA intermediates during the SFV replication cycle results in activation of interferon responses, which may contribute to the enhanced immunogenicity. However, NS3/4A has been shown to specifically inhibit the host cell response to dsRNA by cleavage of the adaptor molecule Cardif thereby blunting the interferon response. Notably, expression of the codon optimized NS3/4A gene in the SFV replicon system did not result in any obvious enhancement of immune priming, suggesting that the system is saturated with respect to NS3/4A mRNA amplification and therefore we did not see any increase in the potency of the specific immunity (unpublished data). This
can possibly be explained by the fact that the mRNA amplification cannot be further enhanced in the infected cell. Alternatively, that the NS3/4A protein expressed by the cell blunted the dsRNA-induced IFN responses, which impaired the adjuvant effect of the vector. Thus, it is possible that viral vectors based on RNA viruses may be less optimal for the delivery of HCV NS3/4A. This should be investigated further.

Codon optimization of the DNA vaccine resulted in a quicker and enhanced priming of both humoral and cellular immune responses compared to the wtNS3/4A DNA vaccine. In fact, codon optimization has been shown to improve the expression for several DNA vaccine genes often with an improved immunogenicity. With respect to HCV, it is a bit surprising that the genome has not fully adapted to the codon usage in human cells. However, this may be speculated that HCV has found an optimal codon usage resulting in an optimal level of translation in order not to upset the host too much. There are several factors that favour the use of plasmid DNA (pDNA) as compared to viral vectors as vaccine platforms for HCV. DNA vaccines are easily manufactured, more stable facilitating distribution under simple storage conditions and show a good safety profile. However, the use of viral vectors have been shown to induce stronger immune responses compared to naked DNA vaccines, partly explained by the uptake of the gene of interest, and activation of innate immune responses and pathogen-recognition receptors. Even if DNA vaccines have been shown to be able to induce protective immune responses in animal models, these vaccines have so far failed to impress in humans. Albeit this, a licensed DNA based vaccine against West Nile virus for horses and a promising trend in studies reporting priming of protective immune responses in non-human primates proves that DNA vaccines will be a viable option also in humans. Still, a more effective delivery route of the pDNA delivery or a potent adjuvant is needed that can contribute to an improvement of the vaccination.

Since one of the most limiting factors for efficient priming in humans is the poor uptake of the pDNA into the cells after a regular i.m. immunization, we evaluated different delivery routes for our coNS3/4A DNA vaccine. Both gene gun immunization and in vivo electroporation (EP) resulted in an effective priming of CD8+ and CD4+ T cell responses in mice. Transdermal delivery using gene gun immunization was shown to be effective using 25-50 times lower doses as compared to intramuscular immunizations. This enhanced efficacy is probably explained by direct delivery of the pDNA into the cell. Also, it is known that the skin epidermis is rich in professional antigen presenting cells (e.g. Langerhans cells) which could be directly transfected,
resulting in both direct presentation to CD4+ and CD8+ T cells and cross presentation. The effect of EP was possibly even more impressive, since 100-fold less pDNA (0.5 µg) was equal or even better to priming achieved by regular i.m. immunization (50 µg). Also, a 10-fold lower dose (5 µg) administered using EP was clearly superior to i.m. injection alone. Interestingly, a 10-fold higher dose (50 µg vs 5 µg) in combination with EP did not remarkably further improve the humoral nor the cellular immune responses implying that the optimal dosing in mice should be around 5 µg. This suggests that the optimal dosing needs to be evaluated specifically when testing the vaccine in humans. The reduced amount of pDNA required when using either GG or EP delivery to induce effective cellular immune responses in mice are promising for vaccination studies in larger animals and humans. Priming of antibody responses was quick and clearly detectible already after a single immunization using EP both in mice and rabbits, repeated immunizations generated high antibody titres. Using transdermal delivery by the gene gun, two immunizations were needed to generate detectable titres. Even if the humoral immune response is not the primary aim for the NS3/4A-based vaccines, these data are good indicators of vaccine immunogenicity. In this case such data favoured EP delivery since it seemed to more easily generate a detectible humoral immune response.

A regular i.m. immunization generally results in limited uptake of the pDNA into the muscle cells and high doses are often needed to prime measurable immune responses. The explanation for the increased immunogenicity achieved when using electroporation can to a certain extent be explained by the more efficient uptake of the pDNA into the cell. This will result in an enhanced expression of the vaccine gene, an improved protein expression and thereby better immune priming. Another factor favouring in vivo EP is the adjuvant effect by the electric forces causing a local inflammatory response. This effectively recruits immune cells, such as granulocytes, macrophages and antigen presenting cells to the area of vaccine production. We believe that the increased infiltration of immune cells into the injection area contribute to a rapid and improved priming observed by using in vivo EP. The early and massive infiltration of primed CD3+ T cells to the injected muscle further supports this statement. The potential risk of causing permanent damage to the muscle fibres using EP does not seem to be an issue. Histological examination of the injection site one month after a fifth immunization at the same site in rabbits using EP, revealed a healing tissue with less pathological changes than seen two weeks after a single injection. Another concern
by using DNA vaccines and especially with improved delivery techniques such as electroporation is the persistence of pDNA and the risk of integration of the genetic material into the host cell genome. The major aim of using electroporation is to enhance pDNA uptake into cells and thereby improve the immune response. However, a relative rapid elimination of pDNA is to be expected if a potent immune priming is achieved. The ideal situation would be if there were a high presence of pDNA and vaccine-protein in the transfected cells, which results in efficient presentation of vaccine-peptides on MHC class I and II molecules. This will induce rapid priming and subsequently also a quick elimination of vaccine-protein expressing cells, hence, also the cells with a high level of pDNA uptake. We could show a rapid elimination of detectible pDNA the first week after immunization and most of the pDNA is gone within two weeks. After repeated immunizations almost all plasmid is still cleared within 60 days from the last injection and only very low copy numbers can be detected when high doses have been used, independently of the use of EP. This clearance profile is consistent with what has been reported in other studies using DNA vaccines with or without in vivo EP [255, 285]. Furthermore, if the high level of pDNA taken up into a cell should favour integration one could also argue that the higher expression of the vaccine gene within this cell makes it an even more favourable target for the primed immune response and a rapid elimination of the cell is therefore most probable. However, in the case of integration, the NS3/4A-protein has not shown to cause any spontaneously liver disease in transgenic mice expressing NS3/4A in the liver. We have followed mice with intrahepatic NS3/4A-protein expression for up to 20 months without any indications of spontaneous liver pathology [86]. In addition, it is well known that long-term expression of any HCV proteins does not cause permanent liver damage, since when the virus have been cleared in a previous infected individual, the liver will start to heal.

It has been suggested that one reason for insufficient control and clearance of HCV infection is due to exhaustion and/or dysfunction of the existing T cells in the liver and that the liver is not the optimal place for immune cell priming. Using various in vitro techniques we have monitored both humoral and cellular immune responses including activity of CTLs and IFN-γ producing CD4+ and CD8+ T cells. The general idea with a therapeutic vaccine is to activate T cells outside the liver and thereby create a complementing T cell repertoire to add on to the already existing, and most likely impaired, T cell response, of the infected liver. A peripheral priming of HCV-specific T
cells will hopefully result in a better activation of both CD4+ and CD8+ T cells against NS3/4A. To be able to study the in vivo situation and if the immune responses primed in the periphery by the coNS3/4A DNA vaccine can enter an infected liver we used a surrogate model, mice with a transient expression of NS3/4A in the liver. Although these livers cannot represent the true infectious situation this model is useful to study peripheral priming of immune responses against viral proteins expressed in the liver. In the transient transgenic (tTg) mouse model [paper II, IV, V], or in the tumor challenge model [paper I], we could show that our vaccine-primed T cells are functional in vivo and capable to eradicate NS3/4A-protein expressing cells. Using the tTg model system we could also study different mechanisms involved in the immune priming towards the liver. We could show the importance of an effective CD8+ T cell response, in this case surprisingly independently on the presence of CD4+ T cells, to eliminate NS3/4A expressing cells both expressed from tumor cells [paper I] or in hepatocytes [paper II, IV]. It should be noted that this does not exclude the role of CD4+ T cells in controlling the infection or for the priming of memory CTLs. The importance of IFN-γ produced by activated antigen-specific CTLs in the response against virus-infected cells is well documented [134, 142, 143]. We can show that IFN-γR2 is needed for the elimination of infected cells in the liver. It was interesting to note that elimination of IFN-γR2/- hepatocytes was impaired in vivo despite the presence CTLs with a potent lytic activity in vitro. Moreover, we did not detect any significant increase in infiltration of CD3+ T cells in immunized IFN-γR2/- mouse livers. This supports the previously proposed importance of IFN-γ for the recruitment of immune cells to the infected organ [286].

Several studies have shown that HCV has evolved different mechanisms to down-regulate the innate immune responses within the infected cell. The NS3 protease has previously been reported to cleave Cardif (IPS-1, MAVS, VISA) in vitro, a signal transducer in the response to dsRNA, and thereby impairing the interferon and apoptotic response in the infected cell. We could confirm that cleavage of Cardif also occurs in murine cells and that NS3/4A impairs intracellular signalling after activation by dsRNA [paper IV]. This observation suggests that it should be possible to study the effect of NS3/4A on the innate immunity in vivo in various mouse models. It seems that NS3/4A can affect the innate immunity also in an in vivo situation, since poly(I:C)-treated NS3/4A-sTg mice have reduced levels of nuclear IRF-3 and NFκB. The reason for also studying NFκB responses is that NFκB is involved in both TLR3-dependent
and independent (RIG-I and MDA5) sensing of dsRNA. It has been shown that NS3/4A also cleave TRIF, which is activated through the TLR3 dependent recognition of dsRNA. Hence, reduced nuclear translocation of NFκB in this case can be a further indicator of NS3/4A interference with the innate immunity. However, the presence of NS3/4A does not seem to have a major effect on the adaptive immunity since NS3/4A-protein expressing hepatocytes are effectively eliminated in the tTg mice. It seems instead likely that HCV has evolved other mechanisms to circumvent the immune response in the infected host. The possibility for HCV to escape from the adaptive immunity by the accumulation of mutations within immunodominant epitopes, and thereby escape the immune response was studied. One would expect that the virus would select for mutations within such regions and thereby escape the immune response primed by the vaccine. Therefore, we studied this particular question in detail by introducing mutations within one of the most immunodominant epitopes of the NS3-protein, the HLA-A2 epitope at residues 1073-1081 [paper III]. HLA-A2 is one of the most common alleles in Caucasians. By introducing amino acid substitutions within the 1073-1081 epitope we found that specific mutations at residues 1074, 1079 and 1081 affected HLA-A2-peptide binding. The recognition by the T cell receptor was reduced when residues 1075, 1077 and 1079 had been changed. Mutations at four of these residues (1074, 1075, 1076 and 1079) also affected the protease activity and the replication of HCV replicon RNA. This suggests that the possibility for extensive variations within the immunodominant NS3 epitope 1073-1081 is limited, due to cost to viral fitness. Genetic variability within this region drastically reduced the viral fitness thereby impairing the virus ability to positively select strains carrying escape mutations at these positions. This clearly shows that the viral fitness may be a limiting factor for viral immune escape. However, the limited variability of NS3 cannot only be explained exclusively by reduced viral fitness. Single mutations introduced at every position within the NS3 helicase were surprisingly well tolerated and only some reduced the protease activity [177]. Therefore, antiviral drugs such as protease inhibitors or vaccines should be designed in a way that they target these regions were escape mutations will occur less frequently.

In summary, the herein described studies indicate that naked NS3/4A-DNA is preferably delivered using in vivo electroporation, which induce a broad immune response evidenced by splenic and lymphatic IFN-γ producing CD4+ and CD8+ T cell responses with lytic activity and active clearance in the liver. Furthermore,
biodistribution and toxicology studies of the vaccine show a promising safety profile even with high doses and repeated immunizations in combination with EP. The coNS3/4A vaccine, described in detail herein, delivered in combination with *in vivo* electroporation has recently been approved for a phase I/II testing in humans. This will, to our knowledge, be the first study in the world in which a DNA vaccine against an infectious disease delivered intramuscularly in combination with *in vivo* electroporation is tested in humans. It is also, to our knowledge, the first DNA vaccine against HCV to be tested in humans. The study consists of a total of 12 treatment naive genotype 1-infected patients that will receive 4 monthly injections of the vaccine using 3 different doses (167, 500 or 1500 µg). The study will primarily assesses safety and tolerability, but the secondary aim is to analyze cellular and humoral immune responses to HCV NS3-protein and possible effects on the viral load.
8 GENERAL CONCLUSIONS

- Codon optimization of the NS3/4A gene or mRNA amplification using the Semliki forest virus system enhanced both expression levels and the immunogenicity of NS3/4A.

- A NS3/4A transiently transgenic mouse model was generated and can be used to study vaccine efficiency and T cell mediated clearance of NS3/4A-protein expressing hepatocytes.

- Some immune escape mutations within the immunodominant HLA-A2 restricted HCV NS3 epitope at residues 1073-1081 are not positively selected for due deleterious effects in the viral fitness.

- Although NS3/4A interfere with the innate host cell response, it does not appear to affect clearance of NS3/4A-protein expressing hepatocytes by the adaptive immune response.

- *In vivo* electroporation enhanced the immunogenicity of the NS3/4A-DNA vaccine by increased DNA uptake, protein expression, inflammation and infiltration of CD3+ T cells.
The hepatitis C virus (HCV) is an infection primarily transmitted by contaminated blood and has the liver as its primary target. It is estimated that 170 million people are carriers of HCV. In around 80% of all infections the host immune response is unable to defeat the virus. This leads to persistence of the virus, and over time to a chronic liver disease. Several years of chronic HCV infection may result in cirrhosis, and in the worst case, development of liver cancer. There are no vaccines available either to protect from or to cure a HCV infection. However, there are antiviral therapies available consisting of interferon-alpha and ribavirin that now can cure around 50% of all infections. However, the therapy is tough, expensive, and has substantial side effects. We are therefore working on development of a therapeutic vaccine against HCV, aimed as a new treatment regimen for chronic infections. The HCV genome is highly variable, which probably is one of the main reasons that the virus is capable to evade the host immune system and persist in the liver. With that in mind, a vaccine should therefore be targeted against a region of the virus that does not change too much to be able to activate an effective anti-viral immune response in most patients. The non-structural (NS) 3 protein of HCV is one such region. As we show herein, a single immune escape mutation within this protein can affect the function of the protein, which reduces the viral replication (or fitness). Since HCV NS3 plays key roles in the virus life cycle by assisting during replication, this finding could help to explain why this is one of the least variable regions of the HCV genome. The NS3 protein associates with NS4A to be fully functional. NS4A is a cofactor of the NS3 protein, which targets the protein-complex to intracellular membranes. Several studies have shown that specific T cell responses against the NS3 protein are important to be able to combat the virus and to clear the viral infection. To activate both killer and helper T cells we developed a genetic vaccine, meaning a vaccine consisting of the gene coding for the vaccine. When the genetic vaccine is introduced into a human or animal, the hosts own cells will take up the gene and produce the vaccine. We developed a DNA-based vaccine on the NS3/4A-region from a genotype 1 virus. By humanizing the gene for NS3/4A we were able to improve performance and to enhance immune responses against the NS3/4A-protein in immunized mice. Despite that DNA vaccines often perform well in small animals they are generally ineffective in humans since when DNA is injected in to a human muscle the cells do not take up the DNA. By using a technique called in vivo electroporation it is possible to increase the uptake of the DNA vaccine into the muscle cells, which is where the production of the vaccine takes place. The electroporation generates electrical pulses that open pores in the muscle cells, allowing the DNA to be taken up. This results in an increased vaccine production and a stronger immune response. As we could show herein, electroporation acts as a vaccine adjuvant that creates a mild inflammation at the site of injection. This helps immune cells to find the vaccine and to activate the immune system. A key function of a vaccine against HCV is that the activated immune cells should enter the liver and fight the infection. Since only humans and chimpanzees can naturally be infected by HCV, we established a mouse model where HCV NS3/4A-protein could be transiently produced in the mouse liver. Studies in this model revealed that the NS3/4A-DNA vaccine could activate immune cells that killed liver cells producing NS3/4A-protein. Safety studies in mice and rabbits showed, that intramuscular injection of the DNA vaccine using in vivo electroporation activated strong immune responses and was safe. The developed NS3/4A vaccine herein is currently tested in, to our knowledge, the worlds first phase I/II clinical trial using a DNA vaccine delivered by in vivo electroporation to treat chronic HCV infections in humans.
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13 APPENDIX [PAPER I-V]