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Review

Therapeutic vaccination against chronic hepatitis C virus infection

Peng Peng Ip [a], Hans W. Nijman [b], Jan Wilschut [a], Toos Daemen [a,*]

[a] Department of Medical Microbiology, Molecular Virology Section, University of Groningen, University Medical Center Groningen, The Netherlands
[b] Department of Gynecological Oncology, University of Groningen, University Medical Center Groningen, The Netherlands

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ABSTRACT

Approximately 170 million people worldwide are chronic carriers of Hepatitis C virus (HCV). To date, there is no prophylactic vaccine available against HCV. The standard-of-care therapy for HCV infection involves a combination of pegylated interferon-α and ribavirin. This therapy, which is commonly associated with side effects, has a curative rate varying from 43% (HCV genotype 1) to 80% (HCV genotype 2). In 2011, two direct-acting antiviral agents, telaprevir and boceprevir, were approved by the US Food and drug Administration and are now being used in combination with standard-of-care therapy in selected patients infected with HCV genotype 1. Although both drugs are promising, resulting in a shortening of therapy, these drugs also induce additional side effects and have reduced efficacy in patients who did not respond to standard-of-care previously.

An alternative approach would be to treat HCV by stimulating the immune system with a therapeutic vaccine ideally aimed at (i) the eradication of HCV-infected cells and (ii) neutralization of infectious HCV particles. The challenge is to develop therapeutic vaccination strategies that are either at least as effective as antiviral drugs but with lower side effects, or vaccines that, when combined with antiviral drugs, can circumvent long-term use of these drugs thereby reducing their side effects.

In this review, we summarize and discuss recent preclinical developments in the area of therapeutic vaccination against chronic HCV infection. Although neutralizing antibodies have been described to exert protective immunity, clinical studies on the induction of neutralizing antibodies in therapeutic settings are limited. Therefore, we will primarily discuss therapeutic vaccines which aim to induce effective cellular immune response against HCV.

Contents

* Corresponding author. Address: Department of Medical Microbiology, Molecular Virology, University of Groningen, University Medical Center Groningen, P.O. Box 30.001, HPC EB88, 9700 RB Groningen, The Netherlands. Tel.: +31 50 3632723; fax: +31 50 3638171.
E-mail address: c.a.h.h.daemen@umcg.nl (T. Daemen).

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1. Introduction

The World Health Organization (WHO) has estimated that about 3% of the world population is infected with hepatitis C virus (HCV) and approximately 170 million individuals are chronic carriers of HCV. In this group of chronic carriers, 5–10% are at risk of developing liver cirrhosis and liver cancer (1997). Each year, 3–4 million people are newly infected with HCV and 350,000 patients die from HCV-related disease. HCV infection is distributed worldwide (Fig. 1) with high rates of chronic infection in Egypt (22%) (Frank et al., 2000), Pakistan (4.8%) (Khattak et al., 2002) and China (3.2%) (Xia et al., 1996). The standard-of-care treatment for patients infected with HCV is a combination of pegylated interferon-α and ribavirin. This treatment is generally associated with side effects and is effective in 43% and 80% of the patients with genotype 1 and genotype 2 infections, respectively (Fried et al., 2002; Manns et al., 2001; Pearlman, 2004). A prophylactic or therapeutic vaccine against HCV is not yet available. Yet, it is known that immune responses against HCV do play a significant role in viral clearance. While patients recovering from an acute HCV infection mount an effective cellular and humoral immune response against HCV; in individuals with a chronic infection, T cell responses are often deficient and production of neutralizing antibodies is delayed.

An ideal therapeutic vaccine to treat patients with a chronic infection aims at the induction of both cellular and humoral immune responses. HCV-specific cellular immune responses to effectively clear HCV infected cells and humoral responses to reduce the amount of circulating HCV particles. And, humoral immune responses against HCV have been shown to also play a role in clearance of infected cells by antibody-dependent cellular cytotoxicity *in vitro* (Nattermann et al., 2005).

Several approaches are currently being studied to develop vaccines that induce or reactivate a robust T cell response. Yet, so far studies on the induction of neutralizing antibodies in therapeutic settings are limited. In this review, we will therefore summarize different preclinical vaccine candidates, vaccine formulations, approaches and clinical studies which aim to induce HCV-specific cellular immune responses in patients with a chronic HCV infection (see Table 1). However before reviewing these vaccination strategies we will, in Section 2, briefly describe the virology and pathology of HCV and the standard-of-care treatment for HCV patients. Next, in Section 3, we will address immune mechanisms in acute HCV-infected patients that either result in clearance of the virus or result in chronic disease. In Section 3 we will also address the problems and challenges associated with the development of prophylactic and therapeutic HCV vaccines.

2. Hepatitis C: virology, pathogenesis and current therapy

2.1. Virology

HCV was first identified by Houghton and colleagues as non-A, non-B hepatitis virus in 1989 (Choo et al., 1989). HCV is a small enveloped, positive-sense single-stranded RNA virus belonging to the family *Flaviviridae* in the genus Hepacivirus. The 9.6 kb viral RNA genome is composed of one open reading frame encoding a polyprotein of 3000 amino acid residues, and a 5′ and 3′ UTR. HCV enters cells through receptor-mediated endocytosis. Several cellular receptor proteins for HCV have been identified. Among them are tight-junction proteins occludin and claudin-1, scavenger receptor class B type I, and the tetraspanin CD81 (Evans et al., 2007; Pileri et al., 1998; Ploss et al., 2009; Scarselli et al., 2002). Since HCV particles are associated with lipoproteins such as LDL and VLDL (Gastaminza et al., 2008; Ye, 2007), binding of viral particles may be initiated by interaction of the lipoprotein on the viral particles and their corresponding receptors expressed on host cells (Burlone and Budkowska, 2009), followed by recognition through other cellular receptors. Recently, Sainz and coworkers demonstrated that, besides the scavenger receptor B type I and the LDL receptor, also the Niemann-Pick C1-like cholesterol adsorption receptor is involved in HCV entry (Sainz et al., 2012). Upon entry into the cytoplasm, the viral RNA genome is translated to a polyprotein that is post-translationally cleaved by viral and cellular proteases into three major structural viral proteins, a small membrane polypeptide p7 and six non-structural proteins (nsPs). The structural proteins are core (C), envelope (E) 1 and 2, and the nsPs are NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 2). HCV encodes a small protein, F (or ARFP, for "alternative reading frame protein"), which is produced by a ribosomal frame shift within the core gene (Branch et al., 2005). The function of each of the viral proteins is discussed in reference (Lindenbach and Rice, 2005).

To date, at least seven genotypes and 50 subtypes of HCV have been isolated worldwide and the presence of HCV quasispecies can be detected in HCV-infected individuals. HCV has a high production rate of 10^{12} particles per day with a half-life of 2.5 h. With the high error rate of the viral RNA-dependent RNA polymerase, the mutation rate of the HCV genome has been estimated to be 1.92×10^{-3} base substitutions per site per year (Ogata et al., 1991). This mutation rate is higher than that of human immunodeficiency virus (HIV) type 1 which is 3.4×10^{-5} base substitutions per site per year (Mansky and Temin, 1995). These frequent mutations result in production of viral variants which may not only improve replication fitness but also hampers the host immune system to recognize and clear the virus (Cox et al., 2005).

Table 1
Recent clinical studies relating to therapeutic vaccination against HCV infection.

Delivery system	Commercial name	HCV antigen	HCV genotype	HLA restriction	Study phase	Adjuvant	Participants[A]	Clinical outcome	References or ClinicalTrials.gov Identifier
Peptides	IC41 (Intercell AG, Vienna; Verena)	Core 23–44; core 132–140; NS3 1073–1081; NS3 1248– 1261; NS4 1764–1786	1	HLA-A*0201	Phase I	+Poly-L-arginine	c, d	Induction of HCV-specific central memory as well as effector $CD8^+$ T cells in healthy subjects. Increase of HCV-specific $CD8^+$ T cells with a decline of $CD45RA^+$ effector memory cells in some but not all patients	Schlaphoff et al. (2007)
					Phase II	±Poly-L-arginine	c, d	Increase of $CD4^+$ T cell proliferation (67%); increase of IFN-γ ELIspot (42%); >1 log declines of HCV serum RNA (3 of 60 patients)	Klade et al. (2008)
					Phase I	+Poly-L-arginine and ±Imiquimod	a	Increase of $CD4^+$ T cell proliferation (60%); increase of tetramer-binding and IFN-γ $CD8^+$ ELIspot (70%)	Firbas et al. (2010)
	None	E1 213–221; E2 488–496; NS3 1081–1090; NS5A 2132–2140	1b	$HLA-A24^+$	Phase I	Montanide ISA5I VG	c	Increase of peptide-specific CTL activity and IgG. Decrease of HCV serum level (3 of 12 patients)	Yutani et al. (2007)
	None	C 35–44(HLA-A2-restricted CTL epitope)	1a, 1b, 2a, 3a	HLA-A2, HLA-A24, HLA-A26, HLA-A31, HLA-A33	Phase I	Montanide ISA5I VG	b, c	Increase of peptide-specific CTL activity (15 of 25 patients), Increase of peptide-specific IgG (15 of 22 patients). >1 log declines of HCV serum RNA (2 of 25 patients)	Mine et al. (2004)
DNA	CIGB-230	pIDKE2 expressing Core, E1 and E2 protein and recombinant HCV core proteins, Co120	1b, Cuban isolated	None	Phase I	None	c	Increase of specific T cell proliferative response and T cell IFN-γ ELIspot (73%). Improved or stabilized liver histology (>40%)	Alvarez-Lajonchere et al. (2009)
		CIGB-230 and recombinant anti-Hepatitis B vaccine (Heberbiovac HB, Cuba)	1b, Cuban isolated	None	Phase I	None	c	Induction of specific $CD4^+$ T cells response (42.8%)	Castellanos et al. (2010)
	ChronVac-C® + standard-of-care	NS3/4A	1	None	Phase I and II	None	b	Phase I: sero-conversion of HCV RNA (5 of 6 patients)	NCT01335711
Viral vector	TG4040	MVA virus carrying NS3, NS4 and NS5B	1	None	Phase I	None	b, d	Decline in serum HCV RNA (6 of 15 patients)	Habersetzer et al. (2011)
	HCVac (TG4040 + standard-of-care)	MVA virus carrying NS3, NS4 and NS5B	1	None	Phase II	None	b	Ongoing	NCT01055821
	None	Ad6 or Chimpanzees adenoviral (ChAd3) vector carrying complete NS proteins	1b	None	Phase I	None	a	Induction of long-lived $CD4^+$ and $CD8^+$ T cells response with central and effector memory phenotypes	Barnes et al. (2012)
Yeast	GI-5005	Core-NS3 fusion protein	1a and 1b	None	Phase Ib	None	b, c, d	Decline in serum HCV RNA	NCT00124215
	GI-5005 + standard-of-care	Core-NS3 fusion protein	1a and 1b	None	Phase II	None	b, c	Ongoing	NCT00606086
Dendritic cells	None	HCV CTL epitopes: core 132–140; core 35–44; core 177–187; NS3 1406–1415; NS4B 1807–1816; NS4B 1851–1859; universal Th epitopes	1	HLA-A2	Phase I	Pam_2Cys	c	All patients generated *de novo* specific-IFN-γ response	Gowans et al. (2010)

[A] a, healthy subjects; b, HCV-infected patients without (or refused) the standard-of-care therapy; c, HCV-infected patients not responding to the standard-of-care therapy; d, HCV-infected patients relapsing from the standard-of-care therapy.

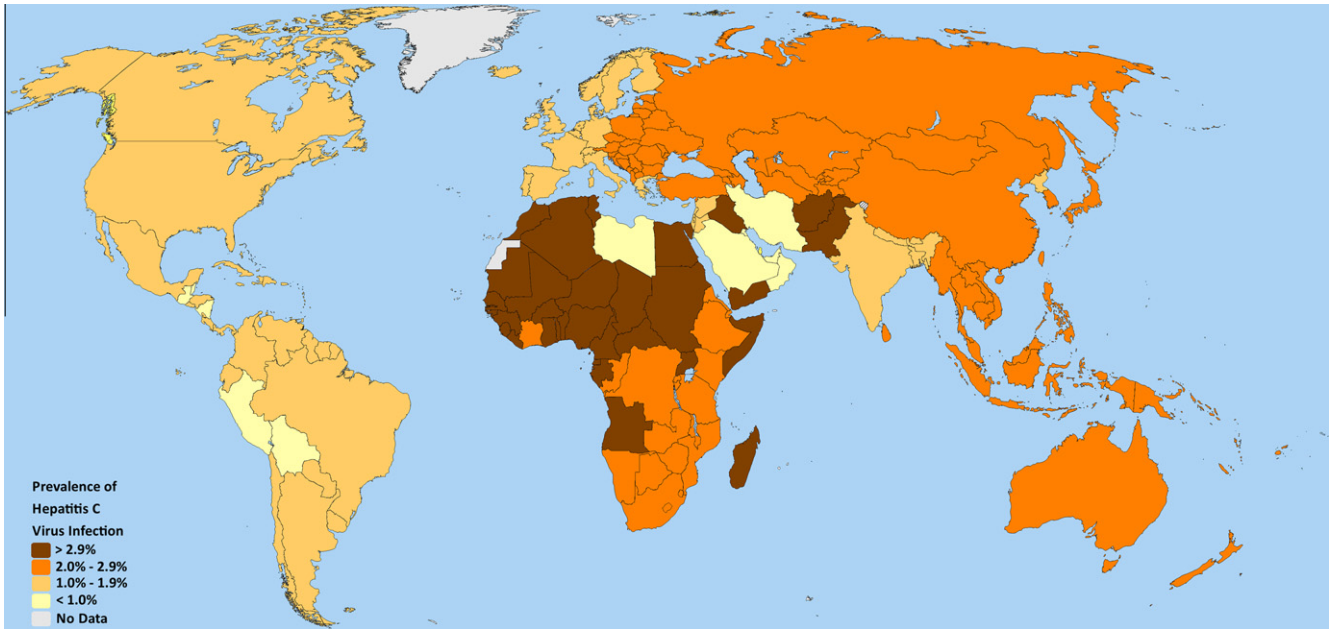

Fig. 1. Prevalence of hepatitis C virus infection. *Sources*: The US Centers for Disease Control and Prevention, Hepatitis C virus database project.

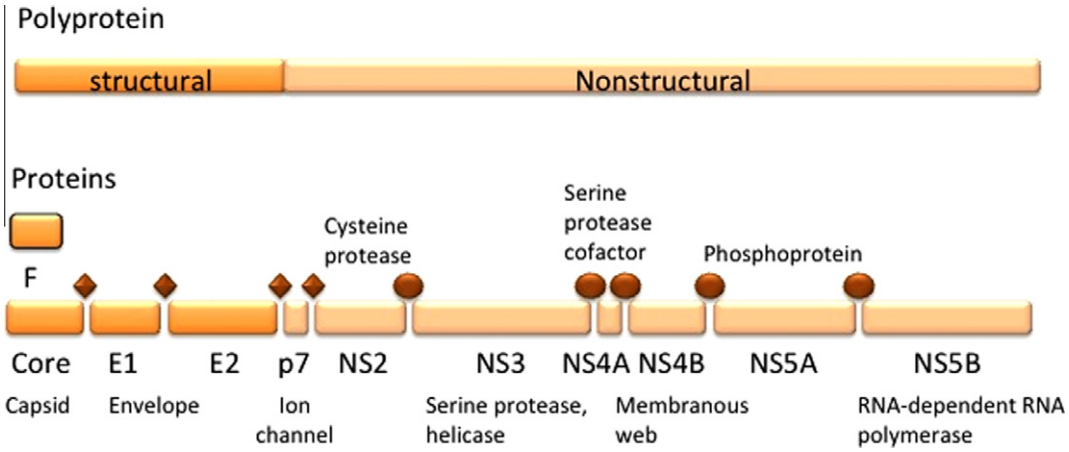

Fig. 2. Hepatitis C virus proteins. The HCV polyprotein is processed by cellular proteases (diamonds) or viral proteases (circles) into 10 viral proteins. The basic function of each protein is indicated and extensively discussed in reference Lindenbach and Rice (2005).

2.2. Pathogenesis

HCV represents a major cause of liver disease in humans and chronic HCV infection is also the primary cause for liver transplantation in the Western world. HCV is transmitted mainly by blood transfusion, re-use of medical devices and shared needles among injection-drug users. Both innate and adaptive immune responses are required for viral clearance and will be discussed in detail below. Inefficient immune responses and chronic HCV infection induce liver damage and inflammation which may lead to liver cirrhosis, liver failure and ultimately hepatocellular carcinoma. Hepatocarcinogenesis is caused by the interaction of several indirect mechanisms including chronic inflammation, steatosis, fibrosis and oxidative stress (Bartosch et al., 2009).

2.3. Standard-of-care therapy and novel drugs

The standard-of-care therapy of patients with chronic HCV infection consists of weekly injections of pegylated interferon-α and a twice-daily intake of ribavirin for 24–48 weeks. A sustained virological response defined as the absence of HCV RNA in serum for 6 months or longer after therapy, is achieved in about half of the treated patients. This treatment in most patients leads to side effects such as influenza-like symptoms and depression and about 20% of treated patients develop anemia (Fried et al., 2002). Two new direct-acting antiviral (DAAs) drugs, INCIVEK™/INCIVO® (telaprevir) and VICTRELIS™ (boceprevir), which both specifically inhibit the activity of the HCV NS3/4A protease, were licensed in 2011 by the US Food and Drug Administration to be used in combination with the standard-of-care therapy for patients infected with HCV genotype 1 (Jacobson et al., 2011; Poordad et al., 2011). New treatment schedule with either telaprevir or boceprevir is promising. In treatment-naïve populations response-guided therapy can be shortened to 24 weeks in approx. 55% of patients treated with the telaprevir-based regime and to 28 weeks in approx. 45% of patients treated with the boceprevir-based regime. (Dore et al., 2011). Side effects such as rash and anemia occur in around 40% of the treated patients (Jacobson et al., 2011; Poordad et al.,

2011). In two large phase 3 studies, the SPRINT-2 study (boceprevir, in combination with pegylated interferon-α and ribavirin) and the HCV RESPOND-2 study (Retreatment with HCV Serine Protease Inhibitor boceprevir and PegIntron/Rebetol 2), more than 40% of patients developed anemia and required erythropoietin administration for up to 150 days (Bacon et al., 2011; Poordad et al., 2011). Presently, many other drugs inhibiting HCV viral proteins or host proteins essential for viral replication such as cyclophilin A inhibitors, including alisporivir, are under investigation. However, due to the error-prone replication of HCV, combinations of antiviral drugs as a cocktail treatment will be required to prevent development of drug resistance. On the other hand, combination therapy may lead to side effects induced by drug–drug interaction of these new DAAs. Alternatively, therapeutic vaccines may, in combination with DAAs, not only reduce the duration of the standard-of-care therapy, but may also cure patients that do not respond to the standard-of-care therapy.

3. Immune response and vaccination against HCV infection

3.1. Immune responses in acute HCV-infected patients

Based on the level of viral load and alanine aminotransferase (ALT) in serum, HCV infection is divided into three stages, which are the incubation, the acute and the chronic phase. During the incubation phase, HCV is actively proliferating in the liver with a high level of HCV RNA detected in serum. However, this period is mostly asymptomatic and therefore difficult to be diagnosed. Upon infection, the viral dsRNA replication intermediates of the HCV RNA activate host innate immunity through Toll-like receptor 3 (TLR3) and the polyuridine motif at 3′ UTR activates the retinoic acid-inducible gene I (RIG-I) signaling in a viral 5′ triphosphate-dependent manner (Saito et al., 2008). This results in production of IFN-β by infected hepatocytes which is a first line of defense to control further infection by inducing antiviral IFN-α production in neighboring uninfected hepatocytes as described by Rehermann (Rehermann, 2009). Furthermore, HCV NS3/4A protein also reduces the production of IFN-α by infected hepatocytes by inhibiting TLR3 and RIG-I signaling through the cleavage of the adapter molecule TRIF (Toll-IL1 receptor domain-containing adaptor inducing IFN-β) and IPS1 (IFN-β promoter stimulator protein 1), respectively (Foy et al., 2003; Li et al., 2005). The incubation phase with the activation of innate response lasts for 8–12 weeks and HCV infection can be diagnosed only when the levels of ALT and HCV-specific T cells increase which characterize the acute phase of the disease (Rehermann, 2009).

During the acute phase of the disease, the level of HCV RNA fluctuates coincidentally with the level of ALT. HCV-specific T cells and HCV-specific antibodies are detectable at 5–9 and 8–20 weeks after primary HCV infection, respectively (Logvinoff et al., 2004; Thimme et al., 2002, 2001). HCV-specific adaptive immune responses are mild and delayed, which may be due to poor antigen presentation in the immune-tolerant environment of the liver (Crispe, 2003) and to direct inhibitory effects of HCV proteins. A protective role of anti-HCV antibodies is still controversial as it has been shown that antibody-deficient patients can recover from acute infection of HCV in the absence of anti-HCV antibodies (Semmo et al., 2006). Other studies demonstrate that neutralizing antibodies, induced in the early phase of disease, positively correlate with control of virus replication (Lavillette et al., 2005) and resolution (Pestka et al., 2007), reducing the chance to develop a chronic infection. Because of lack of an appropriate animal model for HCV infection, the potency of neutralizing antibodies was studied in a human liver-chimeric mouse model (Alb-uPA/SCID). These mice lack an adaptive immune system and are susceptible to HCV infection. Alb-uPA/SCID mice injected with neutralizing antibodies derived from patients with chronic HCV infection were protected from homologous HCV (Law et al., 2008; Vanwolleghem et al., 2008) or heterologous HCV challenges (Meuleman et al., 2011).

Cellular immune responses have been shown to play a crucial role in clearance of HCV as proliferation of HCV-specific T cells and increased levels of IFN-γ coincide with the first decline of HCV RNA level in blood. Virus clearance depends on the diversity and effector function of pathogen-specific $CD8^+$ T cells. HCV-specific $CD8^+$ T cells isolated from patients at early onset of the disease (1 week) exhibit a broad antigen specificity. Yet these cells often seem to have impaired effector functions, including proliferative capacity, IFN-γ production and cytotoxicity (Lechner et al., 2000; Urbani et al., 2002). The effector functions of HCV-specific $CD8^+$ T cells in patients who later recover from the acute disease improve over time. In patients who develop a chronic HCV disease this change does not occur (Urbani et al., 2002). HCV-specific $CD4^+$ T cells, perform differently between patients. Robust proliferation of HCV-specific $CD4^+$ T cells is detectable in patients who later recover from acute disease, but not in patients who progress to chronic infection. Only a minority of patients recovers from acute infection without treatment. However, these patients in general develop protective immunity against re-infection. (Grebely et al., 2006; Mehta et al., 2002; Osburn et al., 2010). Protective immunity against re-infection, which has been shown to be T cell-dependent (Mizukoshi et al., 2008), is characterized by a reduced duration of viremia and a lower amount of viral RNA in the serum of homologous or heterologous HCV re-infected patients (Osburn et al., 2010).

3.2. Failure or immune responses in chronic HCV-infected patients

A majority of primary HCV-infected patients (60–80% of those infected with HCV genotype 1) develop chronic infection characterized by stable HCV serum RNA levels for decades. These HCV RNA levels are approximately 2–3 logs lower than the levels during the acute phase. The induction of virus-neutralizing antibodies is delayed but detectable during the entire period of chronic infection. Most importantly, HCV-specific $CD8^+$ T cells isolated from chronic patients are functionally exhausted which ultimately leads to a reduced breadth of the response (Kuyper et al., 2005; Wedemeyer et al., 2002). T cell exhaustion in general and also in chronic HCV infection is caused by a number of factors including:

- long-term antigen stimulation in the presence of a persisting substantial (HCV) viral load;
- upregulation of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF-β) and downregulation of pro-inflammatory cytokines such as IFN-γ, IL-2 and IL-21;
- reduced help from antigen-specific $CD4^+$ T cells;
- expression of inhibitory receptors including programmed cell death 1 (PD1), lymphocyte activation gene-3 (LAG3), CD244(2B4), CD160, T cell immunoglobulin and mucin domain-containing molecule (TIM3) and CTL4 on the surface of antigen-specific T cells (Crawford and Wherry, 2009);
- presence of immune suppressor cell populations such as regulatory T cells (Treg) (Wherry, 2011).

For instance, several groups reported on the presence of high frequencies of both Treg and HCV-specific $CD8^+$ T cells expressing PD1, CTLA4 and TIM3 in the periphery and liver of patients with chronic HCV infection (Chang, 2007; Golden-Mason et al., 2009; Thimme et al., 2008). Exhaustion occurs for both $CD4^+$ and $CD8^+$ T cells. Exhaustion of $CD8^+$ T cells is characterized by a gradual progression of dysfunction. It begins with a reduced, and later a complete lack of, production of tumor necrosis factor α (TNF-α)

and IL-2, followed by a reduced production of IFN-γ and loss of cytolytic function. At later time points, expression of inhibitory receptors such as PD1 and TIM3 is induced and apoptosis begins (Wherry, 2011). Exhaustion of $CD4^+$ T cells is not fully understood but it has been shown that HCV-specific $CD4^+$ T cells isolated from patients with chronic disease have a reduced production of IL-2 and IFN-γ (Urbani et al., 2006). Since dysfunction of HCV-specific $CD4^+$ T cells is not observed in patients who clear the infection, this may provide clues as to how to treat patients with a chronic infection. If the function of exhausted HCV-specific T cells can be restored and the diversity of HCV-specific T cells will be broadened, patients with a chronic infection may regain the ability to clear the virus. Fortunately, exhaustion of T cells is reversible; for example, it has been shown that HCV-specific cytotoxic T lymphocyte (CTL) activity can be restored by blockade of either PD1/CTLA4 or TIM3 (McMahan et al., 2010; Nakamoto et al., 2009). The function of exhausted T cells can also be restored by elimination of the original causes of dysfunction. For example, such as to increase help from $CD4^+$ T cells, to provide adequate amount of IFN-γ, IL-2 and IL-21 and to inhibit the function of Treg. All these strategies should be taken into account in the design of therapeutic vaccines against HCV infection in order to restore exhausted T cells and to increase the diversity of HCV-specific T cells.

Viral mutants or quasispecies, appearing under immune pressure (Salloum et al., 2008) may not be recognized by an intact immune system despite the fact that patients develop a spectrum of functionally active HCV-specific T cells (Cox et al., 2005). Mutations can be found in various regions of the viral genome including T cell epitopes, B cell epitopes or other regions. For example, viral mutants which have mutations within neutralizing epitopes have been described to infect hepatocytes more efficiently than their consensus virus in the transplanted liver of patients with chronic HCV infection (Fafi-Kremer et al., 2010). Mutations within T cell epitopes are positively correlated to the development of chronic disease in both chimpanzees (Weiner et al., 1995) and humans (Timm et al., 2004). Recent studies show mutations in protective HLA-B27-restricted epitopes in patients who developed chronic disease but not in those who recovered from acute infection (Dazert et al., 2009; Kim et al., 2011). Of note, the rate of mutation in CD8 T cell epitopes is comparable to the hypervariable region 1 (HVR-1) of the E2 gylcoprotein, the most variable region in the HCV genome (Cox et al., 2005). The lack of immune recognition of these mutants was demonstrated in studies which showed that HCV-specific T cells isolated from patients can be stimulated by consensus but not by mutated CD8 T cells epitopes (Cox et al., 2005; Neumann-Haefelin et al., 2008). Immune escape mutations may occur on the costs of viral fitness as the antiviral effect of T cells forces the virus to adopt a relatively unfavorable sequence (Uebelhoer et al., 2008; Salloum et al., 2008). In fact, half of the detectable CD8 T cell responses observed in patients with chronic disease were found associated with viral mutations (Neumann-Haefelin et al., 2008). Chronic HCV infection therefore results from the selection of immune escape mutants and the existence of functionally exhausted T cells.

3.3. Vaccination against HCV

3.3.1. Prophylactic vaccination

Conventional prophylactic vaccines against viral infections generally aim at induction of a humoral immune response resulting in the production of virus-neutralizing antibodies that eventually block receptor binding and cell entry of the viral pathogen involved. Although the neutralizing function of antibodies against the HCV envelope glycoproteins E1 and E2 in humans is still controversial (Bowen and Walker, 2005; Torresi et al., 2004), vaccination strategies which induce production of neutralizing antibodies against E1 and E2 do protect HCV-naïve chimpanzees from virus challenge (Choo et al., 1994; Forns et al., 2000). Several studies have now shown that polyclonal antibodies against HCV may prevent the attachment to and entry of virions into hepatocytes (Dreux and Cosset, 2007; Wintermeyer and Wands, 2007; Zeisel et al., 2007; Keck et al., 2004; Meunier et al., 2008). Although E1 gylcoprotein-specific antibodies can be identified in patients with chronic HCV infection (Meunier et al., 2008), the major antigenic determinants on the viral surface are located in the HVR-1 of the E2 glycoprotein (Prentoe et al., 2011) and this variability occurs not only between patients but also within a single patient making the design of prophylactic vaccines difficult. One possible solution is to delete the HVR-1 region from E2 glycoprotein allowing the presentation of other epitopes that may induce protective virus-neutralizing antibody responses. A recent study by Verstrepen and colleagues shows that virus-neutralizing antibodies against E1 glycoprotein can indeed clear HCV in chimpanzees in a T-cell-dependent manner (Verstrepen et al., 2011). Furthermore, a study in macaques has shown that antibodies against E1 and E2 glycoproteins possess neutralizing ability against both homologous and heterologous virus (Garrone et al., 2011). Clarification of the immunogenicity of E1 and E2 glycoproteins and the protective effect of neutralizing antibodies is essential for a more precise formulation of prophylactic and therapeutic HCV vaccines. To date, more neutralizing epitopes have been identified in patients, which may facilitate the development of prophylactic vaccines. Recently, in a phase I clinical study in healthy humans, E1- and E2-specific antibodies with *in vitro* virus-neutralizing activity were induced by vaccination with recombinant E1 and E2 protein and MF59 adjuvant (Ray et al., 2010).

3.3.2. Therapeutic vaccination

Therapeutic vaccines preferably activate both humoral and cellular immune responses, thereby generating antibodies and virus-specific $CD8^+$ cytotoxic T lymphocytes (CTL) that neutralize the circulating virus and eliminate virus-infected cells, respectively. The majority of the viral epitopes recognized by CTLs and $CD4^+$ T cells in patients with HCV infection are located in the NS3 region (Battegay et al., 1995; Cerny et al., 1995; Diepolder et al., 1997, 1995; He et al., 1999; Kurokohchi et al., 1996; Shirai et al., 1995; Thimme et al., 2001; Ward et al., 2002). Therefore, NS3 is considered to be a good cellular target candidate for a therapeutic vaccine. Yet as also CTL and $CD4^+$ T cells recognizing epitopes from other conserved HCV proteins such as core and NS5A/B, have been identified in self-limited HCV patients (Lauer et al., 2004), vaccines containing these epitope regions are currently being studied (Section 4).

The development of therapeutic vaccines against HCV is challenging, not only because such vaccines have to tackle the broad range of HCV types, but also because there is a lack of appropriate animal models and, most importantly, vaccination strategies have to deal with the ineffective host immune response against HCV, as discussed above. The high viral production rate and the presence of multiple HCV quasispecies impede the design of an effective universal therapeutic vaccine. The lack of immunocompetent small-animal models for HCV infection stands in the way of evaluation of the efficacy of vaccine candidates. Yet, recently an immunocompetent transgenic mouse has been generated expressing human CD81 and occludin thereby facilitating HCV cell entry (Dorner et al., 2011). This transgenic mouse could represent a crucial step in the development of an HCV-susceptible immunocompetent mouse model in the near future. And, as discussed in Section 3.2, co-administration of a therapeutic vaccine with immune-modulators and/or blockers of inhibitory molecules may potentially restore ineffective immune response.

4. Specific approaches in the development of a therapeutic vaccine against HCV infection

4.1. Peptide- or protein-based vaccines

Vaccines composed of viral peptides or recombinant viral proteins can be generated relatively easily and are being developed for infectious diseases and cancer. As peptide/protein vaccines in general are not very immunogenic, these vaccines are often combined with adjuvants. And to avoid the risk of immune escape, these vaccines often contain multiple epitopes for the induction of broad CTL and Th responses.

4.1.1. Preclinical studies

To induce an HCV-specific cellular immune response, already in 1999 Hiranuma et al. reported on a study on the vaccination with HCV peptides containing CTL and $CD4^+$ helper T (Th) epitopes (Hiranuma et al., 1999). To demonstrate the important role of Th epitopes in activation and maturation of CTL, mice were immunized with either CTL peptides only, a mixture of Th and CTL peptides or conjugated Th-CTL peptides. Th-mediated enhancement of HCV-specific CTL responses were only observed in the last group demonstrating the indispensable role of conjugated Th peptides for priming of a $CD8^+$ T cell response (Hiranuma et al., 1999).

Vaccination with recombinant HCV viral proteins has been reported to induce a Th2-biased response, which favors the production of IL-4 and antibodies rather than a Th1-biased response which favors the production of IFN-γ and activation of CTLs (Qiu et al., 2008). To induce a balanced immune response, Th1 adjuvants and/or immune modulators have been included in experimental vaccine formulations. Adjuvants such as Montanide ISA 720 or TLR agonists (TLR3, TLR9 or TLR4) shift the HCV-specific immune response in vaccinated mice from a Th2 to a Th1 phenotype (Mansilla et al., 2009; Qiu et al., 2008; Zabaleta et al., 2007). Currently, studies on the design of vaccines based on proteins, peptides, DNA and dendritic cells (DC) strongly focus on the selection and/or development of proper adjuvants and/or immune modulators.

4.1.2. Clinical studies

Peptide-based vaccination in clinical trials may either be predesigned (Klade et al., 2008; Schlaphoff et al., 2007) or personalized (Yutani et al., 2009, 2007). Predesigned peptides, which contain immunodominant regions of HCV in specific HLA genotypes, stimulate naïve and resting T cells in healthy individuals and HCV-infected patients. Personalized peptides are tailor-made and stimulate memory T cells and activated T cells that are already present in the patient. To identify effective peptides, peripheral blood mononuclear cells (PBMC) are isolated from the patient and cultured in the presence of different predicted HLA-restricted immunodominant peptides. Only peptides that can activate CTLs are administrated to the patients.

The efficacy of a personalized peptide vaccine derived from the HCV E1, E2, NS3 and NS5A regions has been evaluated in a phase I clinical study (Yutani et al., 2007). HLA-$A24^+$ patients with a chronic HCV infection, who did not respond to the standard-of-care therapy, were treated with 14 subcutaneous (s.c.) injections biweekly of a personalized peptide vaccine in Montanide ISA51 VG. The treatments were well-tolerated and there was no severe toxicity. These personalized vaccines stimulated peptide-specific IFN-γ production by CTLs in 50% of the vaccinees. However, a decrease of HCV RNA in serum was observed in only 3 out of 12 patients.

In a recent phase I clinical study with personalized peptides, HCV-infected patients were vaccinated with a peptide derived from the HCV core protein supplemented with Montanide ISA51 VG (Yutani et al., 2009). This peptide was tested in patients with different HLA genotypes as it contains a CTL epitope (Battegay et al., 1995; Cerny et al., 1995) which is conserved in various HCV genotypes and has binding activity to several HLA class I-A molecules (HLA-A2, HLA-A24, HLA-A26, HLA-A31 and HLA-A33). Peptide-specific CTL responses were enhanced after six vaccinations in 15 out of 25 patients and there was $a > 1$ log decline of HCV serum RNA level in two patients. The amount of peptide-specific IgG was significantly increased in more than 50% of the vaccinees in both studies using personalized peptides. This augmentation has been described to positively correlate with increased survival of advanced cancer patients (Mine et al., 2004; Noguchi et al., 2005; Yajima et al., 2005). Of note, vaccination with personalized peptides activates responses against specific epitopes but this strategy is relatively time-consuming and not applicable for all patients.

Klade and colleagues (2008) reported on a phase I clinical trial with a pre-designed peptide vaccine, IC41, containing five synthetic peptides derived from core, NS3 and NS4 of HCV with poly-L-arginine as an adjuvant. The peptides encompassed four HLA-A*0201-restricted CTL epitopes and three CD4 Th epitopes (Ward et al., 2002). The study indicates that both healthy volunteers and patients with a chronic HCV infection respond to IC41 vaccination. However, patients with a chronic HCV infection showed a lower induction of epitope-specific CTL activity, proliferation of $CD4^+$ Th cells and production of IFN-γ by epitope-specific $CD4^+$ and $CD8^+$ T cells compared to the healthy volunteers (Klade et al., 2008). Because of this suboptimal immune response, which is most likely the result of the low immunogenicity of synthetic peptides, the authors suggest to optimize T-cell responses by including more antigenic peptides or by combining IC41 treatment with the standard-of-care in the future.

An effective therapeutic vaccine also aims at maturation of memory $CD8^+$ T cells which are classified as either effector memory T cells (T_{EM}) or central memory T cells (T_{CM}) based on their location, functions and different expression of surface molecules (Bachmann et al., 2005). T_{EM} cells have been described to stably produce perforin and granzyme B, molecules required to lyse virus-infected cells (van Leeuwen et al., 2006). Of note, HCV-specific T_{EM} cells dominate in patients with an acute HCV infection, while T_{CM} cells dominate in patients with a chronic HCV infection. Further analysis of the phenotypes of HCV epitope-specific $CD8^+$ T cells in vaccinated patients showed a shift from T_{CM} cells to T_{EM} cells after IC41 vaccination (Schlaphoff et al., 2007). However, the T cell responses were too weak to induce reduction of HCV RNA in the serum of most of the patients, which may be due to the low immunogenicity of the peptides (Klade et al., 2008). Recently, to optimize immunogenicity, different vaccination routes and dosages of IC41 have been investigated in healthy subjects. Intradermal (i.d.) administration of IC41 induced more robust peptide-specific immune responses than s.c. administration. Although intradermal vaccination with IC41 proved to be safe, efficacy needs to be enhanced in order to obtain sufficient protective cellular immune responses in patients with chronic HCV infection (Firbas et al., 2010).

Vaccinations with peptides together with adjuvant in general are well-tolerated, even after repeated injections. And although most of the HCV peptide/protein vaccines clinically evaluated so far do induce cellular immune responses, these responses do not seem strong enough or do not encompass the appropriate phenotype of cells that would be required to induce viral clearance in patients with a chronic HCV infection.

4.2. DNA vaccines

In the early 1990s, it was shown that plasmid DNA can directly transfect mouse muscle cells *in vivo* (Wolff et al., 1990). Upon

intramuscular (i.m.) administration to mice, plasmid DNA expressing the influenza A virus nucleoprotein (NP) elicited both antigen-specific antibodies and CTLs (Ulmer et al., 1993). These studies initiated the development of DNA vaccines against infectious diseases and tumors (Donnelly et al., 1997).

4.2.1. Preclinical studies

Plasmid DNA encoding antigenic HCV protein(s) or peptide epitope(s) of varying sizes can induce both humoral and cellular immune responses *in vivo*. Antigen-specific memory responses have also been described in animal studies (Encke et al., 2006b; Shi et al., 2006). Yet, plasmid DNA injected i.m. or subcutaneously (s.c.) is poorly immunogenic and several studies have demonstrated that immune induction is lower in non-human primates than in mice (Park et al., 2008; Calarota et al., 1998; Coban et al., 2004). In order to stimulate the *in vivo* transfection rate, different immunization approaches such as the usage of a gene gun (Ahlen et al., 2005), micro-needles (Gill et al., 2010), gene-electrotransfer (Capone et al., 2006b) and *in vivo* local electroporation (Ahlen et al., 2007; Zhu et al., 2010) have been developed and tested in experimental animal models. All these approaches enhance both humoral and cellular immune responses against expressed HCV viral proteins when compared with conventional i.m. or s.c. injection.

Host immune responses against HCV viral proteins in experimental mice models can also be skewed and enhanced by co-administration of adjuvants such as CpG (Yu et al., 2004; Zhu et al., 2004), QuilA (Yu et al., 2004) and/or immune modulators such as Flt3-L and GM-CSF (Encke et al., 2006a), IL-2 (Encke et al., 2006b) or IFN-α (Gehring et al., 2005). Mice vaccinated with these adjuvants or immune modulators have increased HCV-specific humoral and cellular immunity. Interestingly, inclusion of sequences of the core protein of Hepatitis B virus, thus creating a priming environment by recruiting "healthy" heterologous T cells and by activating innate signaling, resulted in a restoration of HCV-specific responses in mice tolerant for HCV NS3/4 (Chen et al., 2011). Furthermore, co-administration of IFN-α, IL-2 and combinations of Flt-3L and GM-CSF with HCV DNA vaccines have been shown to protect mice from development of HCV-antigen-expressing tumors. Long-term memory responses are induced in mice vaccinated with HCV DNA vaccine together with a plasmid expressing IL-2 (Encke et al., 2006b). Some HCV viral proteins are known to directly suppress the host immune system (Rehermann and Nascimbeni, 2005). Modification of these proteins without removal of the antigenic epitopes could also enhance the immune responses against these antigens. Modification of antigens expressed by DNA vaccines, such as truncated core proteins (Zhu et al., 2010), truncated or secreted forms of the E2 protein (Li et al., 2006), and codon-optimized NS3/4A proteins (Ahlen et al., 2005) has been shown to improve the immunogenicity of these proteins when compared with that of native viral proteins. The main advantage of the use of DNA vaccines is the high flexibility of the approach thereby allowing combinations of strategies as mentioned above, to further improve the overall efficacy of DNA vaccines.

4.2.2. Clinical studies

The first therapeutic HCV vaccine based on plasmid DNA evaluated in a phase I clinical study was CIGB-230. This vaccine is composed of a combination of recombinant HCV core protein and a plasmid DNA expressing HCV core, E1 and E2. The trial was performed in patients with a chronic HCV infection who did not respond to standard-of-care therapy (Alvarez-Lajonchere et al., 2009; Castellanos et al., 2010). Participants were immunized six times with CIGB-230 by i.m. injections with 4-week intervals. Moderate adverse effects such as headache were observed and no autoimmune responses, which could be possibly induced by DNA immunization (Klinman et al., 1999, 1997; Segal et al., 1997), such as anti-mitochondrial or anti-nuclear antibodies were generated. Almost half of the tested subjects had increased proliferation of $CD4^+$ and $CD8^+$ T cells upon *in vitro* antigen stimulation of PBMCs and increased titers of antibodies against E1 and E2 proteins in the serum. Although only 1 out of 15 vaccinated patients had a reduced level of serum HCV RNA, more than 40% of them had improved liver histology. The phase I trial showed CIGB-230 to be safe for humans. However further studies with optimized vaccination dose schedules and methods of vaccine delivery are required.

ChronVac-C® (ChronTech Pharma AB), which contains a plasmid expressing the NS3/4A proteins of HCV genotype 1 has passed a phase I clinical trial and is now under investigation in a phase II clinical trial. In the phase I clinical study, patients with a chronic HCV infection without previous treatment were immunized i.m. twice with a 4-weeks interval with ChronVac-C® in combination with *in vivo* electroporation using Inovio's Medpulser® DNA Delivery System. Standard-of-care therapy was given to all participants after the second administration of ChronVac-C®. Results showed that 83% of the participants (5 of 6 patients) achieved a sustained virological response. Standard-of-care normally results in a sustained virological response of 40–50% in this patient group. Yet, as the number of patients included in this ChronVac-C® trial was limited to six, no firm conclusions can be drawn so far. A phase II clinical trial with an increased number of patients is now underway to evaluate the safety, tolerability and efficacy of ChronVac-C® (ClinicalTrials.gov Identifier: NCT01335711).

4.3. Viral vector vaccines

Similar to DNA vaccines, viral vector vaccines encode target proteins or peptides. The major advantage being that in general viral vector vaccines are more immunogenic than DNA-based vaccines. The major disadvantage is that as these vaccines are derived from viruses, they require extensive evaluation by regulatory authorities, which may delay or even obstruct the progress of clinical application. The mostly tested viral vector vaccines against HCV are based on replication-defective adenovirus and modified non-replicative vaccinia virus Ankara (MVA).

4.3.1. Adenovirus vectors

Among the serotypes of Adenovirus (Ad) vectors investigated, Ad5 induces the strongest and longest lasting humoral and cellular immune response in mice (Tatsis and Ertl, 2004). However, a significant proportion of the world population has pre-existing humoral and cellular immunity against Ad5, with up to 50% in the United States and 88% in South Africa (Schulick et al., 1997; Sumida et al., 2004, 2005). To overcome pre-existing immunity, viral vectors based on low prevalence Ad serotypes such as Ad6 (Barnes et al., 2012), Ad24 or Ad35 (Fattori et al., 2006) or on adenovirus strains from chimpanzees (ChAd) (Barnes et al., 2012) with a prevalence of 12% (Colloca et al., 2012) are being developed. Ad6 induces strong humoral responses in Rhesus monkeys, although the effects on cellular immune responses are unknown (Capone et al., 2006a).

Apart from pre-existing Ad immunity, the efficacy of Ad-based vaccines and MVA-based vaccines, which will be discussed in the next subsection, is also diminished in homologous prime-boost immunization protocols due to the induction of neutralizing antibodies against the viral vector initiated by the prime immunization. Heterologous prime-boost immunizations may solve this problem as has been shown by combinations of different serotypes of Ad vectors (Fattori et al., 2006), plasmid DNA and Ad vector (Desjardins et al., 2009; Matsui et al., 2003; Rollier et al., 2005), peptides and Ad vector (Lin et al., 2008), liposomes and Ad vector

(Zubkova et al., 2009) or other viral vectors including Semliki Forest virus (SFV) (Rollier et al., 2005), ovine atadenovirus (Wuest et al., 2004), MVA (El-Gogo et al., 2008) and Ad vector.

Although an adjuvant is normally not required in viral vector-based vaccines, the polarity and breadth of the immune response can be further improved by giving different immune modulators before, during and/or after the immunization. Studies combining an Ad vector expressing HCV proteins and MHC class II chaperone protein invariant chain (Mikkelsen et al., 2011), IL-12 (Matsui et al., 2003; Zubkova et al., 2009), anti-CD137 antibodies (Arribillaga et al., 2005) or P60 (inhibitor of Foxp3) (Casares et al., 2010) have shown dramatic increases in both humoral and cellular responses in mice. The safety and efficacy of these immune modulators in humans is not yet known.

Recently, Barnes et al. developed an HCV vaccine in which Ad6 and ChAd3 vectors were being used to induce a broad HCV-specific T cells response (Barnes et al., 2012). A phase I study was performed in healthy human volunteers. Robust production of IFN-γ was induced 2 weeks after the prime immunization with an average of >1000 IFN-γ-producing cells per 1 million PBMCs at the optimal dose of recombinant virus. This vigorous production of IFN-γ by HCV-specific T cells has never been observed in other clinical trials in which on average 100–500 IFN-γ-producing cells per 1 million PBMCs have been observed. This HCV-specific T cell response is shown to be long lasting (at least 1 year) with wide diversity. Most importantly, the HCV-specific T cells have a mixed effector/central memory phenotype and have strong effector functions such as degranulation and production of IFN-γ, TNF-α. Of note, the vaccinations induced the production of neutralizing antibodies against the Ad vector which might affect the overall response of HCV-specific T cells. Robust HCV-specific immune responses can be induced in healthy volunteers but the efficacy of these vaccines is not known in patients with persistent HCV infection. Further studies on patients with chronic HCV infection are now underway aiming at the restoration of dysfunctional T cells and, at the same time, broadening of the HCV-specific T cell response (Barnes et al., 2012).

4.3.2. Modified vaccinia virus Ankara vectors

Similar to Ad vectors, vaccines based on modified vaccinia virus Ankara (MVA) are safe in humans. However, pre-existing immunity and neutralizing antibodies against the vector backbone also reduces the efficiency of MVA-based vaccines. The levels of pre-existing immunity against vaccinia virus, as seen in persons previously vaccinated against smallpox virus, seem to be lower than those against Ad5 (Casimiro et al., 2003; Ramirez et al., 2000). And obviously this vaccine-induced pre-existing immunity will disappear as the population that received this vaccine grows older. Several preclinical studies have been performed and showed that MVA-based HCV therapeutic vaccines can induce HCV-specific cellular immune responses and protect mice from for example, NS3-expressing recombinant murine gammaherpesvirus 68 (El-Gogo et al., 2008) or recombinant *Listeria monocytogenes* challenge (Fournillier et al., 2007).

TG4040 (Transgene) is a viral vector-based therapeutic HCV vaccine that entered phase I and phase II clinical trials. The vaccine is based on MVA expressing NS3, NS4 and NS5B of HCV. Administration of TG4040 to patients with a chronic HCV infection induced no severe adverse effects in a phase I clinical study. Eight out of 15 patients had a reduction in HCV serum RNA and 4 out of the 8 responders had an increased number of HCV-specific IFN-γ-producing cells (Habersetzer et al., 2011). A randomized phase II study (HCVac study) involved 153 patients in three treatment groups: (A) standard-of-care alone, (B) combination of the standard-of-care treatment with TG4040 in the same treatment schedule or (C) pre-vaccination with TG4040 before the standard-of-care. Preliminary data, published on the website of Transgene shown that a reduction of viral load in treatment group C can be detected one week after the start of treatment which is faster than in both other groups (ClinicalTrials.gov Identifier: NCT01055821).

4.3.3. Alphavirus vectors

To overcome problems related to pre-existing immunity against Ad and MVA in humans, other viral vector vaccines such as alphaviruses including SFV, Venezuelan equine encephalitis virus and Sindbis virus, are currently being evaluated for the induction of HCV-specific immunity (Brinster et al., 2002; Frelin et al., 2004; Lin et al., 2008; Vidalin et al., 2000). In general, the immune and anti-tumor responses induced by alphavirus-based vaccines seem more robust than those induced by DNA-, peptide-, recombinant protein-, or adenovirus-based vaccines in animal studies (Frelin et al., 2004; Riezebos-Brilman et al., 2005, 2007). This can be ascribed to the high-level antigen expression by these vector systems and the low anti-vector responses induced by the viral vectors themselves (Lambeck et al., 2010; Walczak et al., 2011) and, possibly most importantly, the activation of multiple innate signaling pathways (Naslund et al., 2011). An additional advantage of alphavirus-based vaccines relates to the lack of pre-existing immunity in most humans against the native viruses.

4.4. Recombinant yeast-based vaccines

Recombinant *Saccharomyces cerevisiae* is one of the candidates for vaccine development because of its nonpathogenicity in humans and since this yeast can easily be engineered to express multiple proteins of varying size. Furthermore, recombinant yeast cells are able to activate both innate and adaptive immune responses by activating dendritic cells through direct yeast cells-dendritic cells interaction. Interaction of yeast cells with dendritic cells leads to increase antigen presentation and production of pro-inflammatory cytokines such as TNF-α, IFN-γ, GM-CSF, IL-2, IL-6 and IL-12 (Stubbs et al., 2001). Haller and colleagues showed that heat-inactivated *S. cerevisiae* yeast cells expressing HCV NS3 and core protein (GI-5005) induces effective NS3 and core-specific cellular immune responses in both C57BL/6 and BALB/c mice. Activation of cellular immune responses including induction of CTLs and helper T cells protected mice against a challenge with an HCV-expressing tumor (Haller et al., 2007).

GI-5005 (GlobeImmune) entered a phase I clinical trial in 2007 and results showed that it is well-tolerated and is able to induce significant HCV-specific immune response in patients with a chronic HCV infection (ClinicalTrials.gov Identifier: NCT00124215). The induction of HCV-specific cellular immune responses positively correlated to the reduction of serum viral RNA in vaccinated patients. A phase II trial aiming to investigate the treatment effect of combining GI-5005 and standard-of-care treatment is ongoing. Although not statistically significant, the preliminary results showed that 63% of the patients with combined treatment achieved sustained virological response as opposed to the 45% in patients receiving standard-of-care treatment alone. Additional patients were recruited in this trial in order to further evaluate the efficacy of GI-5005 in combination with standard-of-care (ClinicalTrials.gov Identifier: NCT00606086).

4.5. Vaccines based on dendritic cells

Vaccines composed of isolated DC, which are modified *ex vivo* to express foreign proteins, are considered very promising (Banchereau et al., 2001; Cerundolo et al., 2004). As natural antigen-presenting cells, DCs take up antigens and present these to both T cells and B cells. The phenotypes and immunological functions of antigen-loaded DCs can be extensively characterized before administration of the vaccine to the patient. However, tailor-made

treatment is expensive and time-consuming. In addition, the availability of functional DCs from patients depends on the severity of the disease, treatment history of the patients and/or other unknown factors (Barnes et al., 2008; Echeverria et al., 2008; Gelderblom et al., 2007).

4.5.1. Preclinical studies

Bone marrow-derived dendritic cells (BM-DC) from mice have been loaded with HCV antigen by transfection with either plasmid DNA or mRNA encoding HCV nsPs (Yu et al., 2007, 2008), infection with Ad vector expressing HCV nsPs (Diaz-Valdes et al., 2011; Echeverria et al., 2011; Racanelli et al., 2004; Xiang et al., 2006; Zabaleta et al., 2008), uptake of either HCV peptides (Diaz-Valdes et al., 2011), HCV lipopeptides (Chua et al., 2008) or HCV nsPs-coated magnetic microbeads (Gehring et al., 2009; Wintermeyer et al., 2010). Transfection of NS5A mRNA by electroporation into BM-DC resulted in the strongest immune response when compared with BM-DC loaded with plasmid DNA encoding NS5A or recombinant NS5A protein (Yu et al., 2008). A comparative study between BM-DC loaded with Ad vector encoding HCV NS3 DNA (AdNS3), NS3 peptides or recombinant NS3 protein showed that infection with AdNS3 resulted in the highest level of antigen loading and expression (Diaz-Valdes et al., 2011; Xiang et al., 2006; Yu et al., 2008). The infection rate of Ad vector could be further enhanced by co-administration of AdNS3 and an adaptor molecule CFm40L containing the Coxsackie adenovirus receptor and CD40L, the ligand for CD40 on the surface of DC, thus targeting AdNS3 directly to BM-DC *in vitro* (Echeverria et al., 2011).

To stimulate maturation and cytokine production of DC, BM-DC have been treated *in vitro* with immune modulators such as TLR2 antagonist, Pam_2Cys (Chua et al., 2008) or IL-10-inhibiting peptides resulting in an increased production of IL-12 (Diaz-Valdes et al., 2011). Enhancement of immune responses could also be achieved by repeated injection of antigen-loaded and unloaded BM-DC. Alternatively, splenocytes loaded with antigen-coated magnetic microbeads selected by magnetic field were used for *in vitro* immunization (Gehring et al., 2009).

The ratio of subpopulations of DC is one of the main concerns in the development of cell immunotherapy. $CD8\alpha^+$ DC have the highest antigen-presenting ability (Maldonado-Lopez et al., 1999). However, the expression of $CD8\alpha$ could not be increased by the *ex vivo* cultivation of BM-DC with GM-CSF, IL-4 and/or IL-10, which do increase the expression of CD11c (Maldonado-Lopez et al., 1999). In order to obtain balanced subpopulations of DC for *in vitro* loading of antigen, naïve mice were injected with plasmid DNA encoding Flt3-L to expand the populations of DC *in vivo* (Maraskovsky et al., 1996). DCs pretreated with Flt3-L have a low maturation level and retain their phagocytic properties that enable the *in vitro* uptake of antigen. These antigen-loaded DCs were able to induce robust $CD8^+$ and $CD4^+$ T cells and prevented tumor formation in mice (Gehring et al., 2009; Kuzushita et al., 2006; Wintermeyer et al., 2010).

4.5.2. Clinical studies

The application of DC therapy for patients with a chronic HCV infection progresses slowly due to contradictory results on the functionality of monocyte-derived dendritic cells (MoDC) from patients with a chronic HCV infection. Results from earlier studies have shown that MoDC from patients have an immature phenotype and deviating cytokine profile compared with those from healthy donors (Gelderblom et al., 2007). However, two studies have shown that the maturation status and cytokine-secreting ability of MoDC from patients with a chronic HCV infection are similar to those of MoDC from healthy donors (Barnes et al., 2008; Echeverria et al., 2008). These contradictory results could be explained by differences in the stage of disease and the pretreatment that the patients experienced. For example, ribavirin can suppress the functions of DC *in vivo* (Goutagny et al., 2004). MoDC obtained from patients who did not respond to the standard-of-care therapy showed reduced maturation markers and cytokine production (Gelderblom et al., 2007), while cells from patients with a chronic HCV infection with mild disease without the standard-of-care therapy have similar phenotypes and activity as those from healthy donors. Thus, treatment with DC can, at present, not be applied in all patients with a chronic HCV infection, since the disease stage and prior treatment affect DC function. Nonetheless, recently, Jirmo and colleagues showed that human monocytes, of which the functions are not affected by HCV infection, can be infected with a lentivirus vector resulting in the activation of both human $CD4^+$ and $CD8^+$ T cells *in vitro* (Jirmo et al., 2010). This study suggests that the use of monocytes as cell therapy warrants further investigation.

Recently, results of a clinical trial with DC treatment among patients with a chronic HCV infection were reported (Gowans et al., 2010). MoDC from patients were loaded *ex vivo* with six lipopeptides comprising HLA-A2.1 CTL epitopes (3 core, 1 NS3 and 2 NS4B) individually linked to one conventional Th epitope and a lipid moiety, Pam_2Cys. These antigen-loaded MoDCs were injected once i.d. and sequentially intravenously into patients. Patients had no severe adverse effects throughout the study. Furthermore, patients developed IFN-γ responses against the loaded epitopes but also against novel epitopes which were not present in the lipopeptides suggesting that killing of infected hepatocytes and cross presentation of HCV antigen occurred after vaccination. However, no significant changes in serum viral load, anti-core antibodies, level of cytokines and alanine transaminase were observed. Although the magnitude of the immune response was not robust, it can possibly be improved by increasing the dosage of antigen-loaded MoDCs.

5. Conclusions and future perspectives

Despite all efforts to develop a therapeutic vaccine against HCV over the last decades no therapeutic vaccine has yet reached a phase III clinical trial. Most clinical trials so far demonstrate some decline of HCV serum RNA level and antigen-specific immune responses, yet major therapeutic responses have not been observed. Peptide/protein-based vaccines, which are relatively easy to manufacture, require better adjuvants that enhance appropriate responses and at the same time do not elicit too severe adverse effects. DNA-based vaccines require better delivery methods and viral-vector based vaccines face problems associated with anti-vector responses. Similar problems are observed in the development of therapeutic cancer vaccines. In this research field more and more studies are now being conducted in which cancer vaccines are combined with other therapies. With an increasing number of HCV-specific antiviral drugs under investigation (Lemon et al., 2010), a combination of a therapeutic HCV vaccine and novel antiviral drugs may also become the trend of treatment in the future. Since outcome of any treatment is often affected by both viral and host factors, such as, HCV genotype, presence of quasispecies and HLA type of the patients, it would be worthwhile to study correlations between the effects of any treatment with both viral and host factor which may identify the most optimal therapy for each patient.

To enhance the efficacy of vaccination strategies, vaccines could possibly be combined with methods to direct HCV-specific immune effector cells to the liver and at the same time alleviate the immunosuppressive liver environment. Yet, it will be essential that therapeutic vaccines also restore the exhausted immune function of HCV-specific T cells and broaden their epitope-specific diversity,

while, also in a therapeutic setting, vaccines should induce neutralizing antibodies.

From preclinical studies in animal models, it appears that the choice of the antigen is an important factor affecting the quality of therapeutic vaccination against HCV. However, the results from these preclinical studies, performed in HCV non-susceptible animal models, are difficult to extrapolate to humans. Therefore, a prediction of the most effective approach and/or target antigens for humans based on these studies cannot be made. An immunocompetent small animal model, susceptible to HCV infection, would be very instrumental to evaluate therapeutic HCV vaccine candidates before their introduction into clinic. Such models will accelerate the development of therapeutic vaccines for HCV infection.

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