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TOPIC HIGHLIGHT

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Immune mechanisms of vaccine induced protection against chronic hepatitis C virus infection in chimpanzees

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Abstract

Hepatitis C virus (HCV) infection is characterized by a high propensity for development of life-long viral persistence. An estimated 170 million people suffer from chronic hepatitis caused by HCV. Currently, there is no approved prophylactic HCV vaccine available. With the near disappearance of the most relevant animal model for HCV, the chimpanzee, we review the progression that has been made regarding prophylactic vaccine development against HCV. We describe the results of the individual vaccine evaluation experiments in chimpanzees, in relation to what has been observed in humans. The results of the different studies indicate that partial protection against infection can be achieved, but a clear correlate of protection has thus far not yet been defined.

Key words: Hepatitis C virus; Vaccines; Chimpanzees; Review; Prophylactic; Antibodies; T-cells

Core tip: With the near disappearance of the most relevant animal model for hepatitis C virus (HCV), the chimpanzee, we review the progression that has been made regarding vaccine development against this virus infection. An estimated 3 million people suffering from chronic hepatitis caused by HCV die each year. Currently, there is no approved vaccine available to prevent new infection.

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INTRODUCTION

Chronic hepatitis, caused by persistent infection with hepatitis C virus (HCV) is a major health threat worldwide[1]. The number of chronic HCV carriers is estimated

to be 170 million, about 1% to 2% of the population. HCV is a 9600 nucleotide, single-stranded positive-sense RNA virus belonging to the Flaviviridae. The open reading frame encodes for a large polyprotein with three structural proteins, Core (C), E1 and E2 that are linked *via* the presumed viroporin p7, to the nonstructural proteins NS1, NS2, NS3, NS5A and NS5B. The structural proteins form the viral particle, while the nonstructural proteins are involved in replication and maturation of the virus particle.

HCV infection is characterized by a high propensity for development of life-long viral persistence. Only one in five acute infections is spontaneously eradicated, normally within the first six months after infection.

During acute HCV infections, clinical symptoms are mild or absent. For that reason acute HCV infections are often not recognized. However, when acute HCV infection develops into a persistent infection, the majority of the patients develop chronic hepatitis and over decades the virus causes subtle but cumulative hepatic damage. Ultimately this may lead either to cirrhosis, decompensating liver congestion or hepatocellular carcinoma. To give a sense of the impact of HCV infection on the health care system, it has been calculated that worldwide, 27% of the cases of cirrhosis can be accounted for by HCV and population-based studies in the United States indicate that 40% of chronic liver disease is HCV related[2,3]. Overall, persistent HCV infection accounts for 3 million deaths each year[4].

TRANSMISSION

Transmission of HCV occurs *via* blood-blood contact. Nowadays in the western world, the majority of the new infections are associated with intravenous drug use, *via* sharing of contaminated needles[5]. There are several examples of drastically declined numbers of new HCV cases, after the introduction of surveillance programs and the distribution of fresh disposable needles amongst intravenous drug users[6,7].

In other geographical regions, the mode of transmission is different. The situation is especially worrying in Egypt, where an estimated 12% of the population is infected with HCV as a result of an unsafe treatment-procedure of an endemic schistosoma infection in rural areas during the years 60-80 s of the last century. Currently, the infrastructural organization of the Egyptian health care system is still seen as, at least partially, responsible for ongoing transmission in the region[8]. Recently, World Health Organization (WHO) has declared the large reservoir of chronic HCV carriers a serious risk, as tourism and migration contribute to spreading of the virus to places outside the region.

HIGH PROPENSITY FOR CHRONIC INFECTION

There are 7 major genotypes of HCV[9,10], each genotype consists of a cluster of different subtypes, and within each patient closely related quasi-species are present. The difference between two distantly related isolates can be as high as 30% at the nucleotide level[11]. Circulating quasi-species have the ability to mutate very quickly and can easily evade the immune system, and/or drugs that are used for treatment. In addition, the treatment protocol depends on the specific HCV genotype. Hence, it is difficult to develop a universal treatment regime for chronic HCV.

As indicated by the rapid upregulation of interferon-stimulated genes (ISGs) in the host's liver[12,13], HCV is present and recognized early after infection. However, differential HCV strains[14], the activation of distinct molecular pathways[15], kinetics of the ISG response[16] and even cellular composition of the microenvironment in the liver[17] may be responsible for inadequate mobilization of an effective immune response, ultimately leading to chronic infections. In this review we will focus on the role of the adaptive immune system in clearance of HCV infection, and place this in perspective of HCV vaccine evaluation studies in chimpanzees.

THERAPEUTIC DRUGS OR A VACCINE

For decades chronic HCV infection could only be treated with the broadly acting antiviral (pegylated) interferon, which was often accompanied by serious side effects and frequently not successful. Only in one out of five patients, a so-called sustained virological response was achieved, meaning that HCV RNA had declined to undetectable levels in peripheral blood after treatment. In 1998, the nucleoside analogue ribavirin (RBV) was added to standard therapy-protocols and this improved treatment efficacy to about 40%[18-20].

The year 2011 can be considered as a breakthrough in the treatment of chronic HCV infection. In that year, two direct-acting antiviral drugs (DAAs)-telaprevir and boceprevir-received regulatory approval and became available for patients. In combination with pegylated-interferon and RBV, these NS3/4A protease inhibitors have shown marked efficacy in patients infected with HCV genotype 1. However, this combination was found to be less effective against other genotypes, and patients still experienced the severe side-effects characteristic for treatment with interferon and RBV. In addition, the genetic background of the host can negatively affect treatment efficacy[21] and viral-resistance has been reported[22].

Regulatory approval of NS5B-targeting DAAs, like sofosbuvir has leads to further improvements in the treatment of chronic HCV infection. Not only do they have a better efficacy against genotypes other than genotype 1, also duration of the treatment is shorter[23,24]. In addition, these compounds can be administered orally and may possibly lead to interferon and ribavirin free treatment regimens.

More effective, more tolerable and safer treatment options however come with a price. Currently, oral DAA therapy is very expensive and therefore currently

not affordable in developing countries. Consequently, a prophylactic vaccine is imperative to contain HCV infection globally.

CHIMPANZEES IN BIOMEDICAL RESEARCH

Humans and chimpanzees (pan troglodytes) share a common ancestor who lived approximately 30 million years ago, before the hominoid lineage split. Chimpanzees are humans' closest living relatives with 98.9% identity at DNA level[25]. Since the 40 s of the last century, chimpanzees have been used in the United States space program and later also in biomedical research. The colonies of chimpanzees in research facilities were founded from animals that were imported from the wild in Western Africa. Soon, breeding programs assured enough offspring for experimental work and facilities became self-sustainable and no longer required import of chimpanzees from the wild.

Public concerns about research with non-human primates, chimpanzees in particular, has eventually led to stop the use of apes for HCV research in Europe, and a significant reduction of the number of animals used in the United States[26]. With the near disappearance of the most relevant animal model for HCV, we review the progression that has been made regarding vaccine development against HCV describing the results in chimpanzees in relation to what has been observed in humans. To obtain a complete overview, a literature search was performed in PubMed combining the keywords chimpanzee(s) and hepatitis or HCV in combination with any of the following keywords; vaccine(s), vaccination, immunization or immunized. Furthermore, there are only a limited number of groups working on this subject and animals used can be identified by name or number and thereby tracked through the literature.

CHIMPANZEES AND HCV RESEARCH

No doubt, chimpanzees have been the most important animal model to study HCV[27]. In the late 80 s, after it became clear that the majority of blood borne chronic liver inflammations were not caused by hepatitis A or B virus, serum from a non-A-non-B hepatitis patient was inoculated into a chimpanzee[28]. From this chimpanzee, a cDNA bank was derived and in 1989 Michael Houghton and his coworkers at Chiron Inc. identified HCV as the main causative agent for non-A-non-B hepatitis[28].

Only chimpanzees and humans can be productively infected with HCV and this limited host range has seriously hampered HCV research. To date, the chimpanzee is the only validated animal model to study immunity associated with acute resolving infection, and protective immunity against HCV reinfection. Over the past 35 years, experimental infection of chimpanzees with HCV has provided groundbreaking information regarding identification, characterization, transmission, early responses after HCV infection and triggering of the innate as well as the adaptive immune system. Studies in chimpanzees have enabled us to identify immune mechanisms associated with viral clearance and chronic infection, critical for optimal prophylactic vaccine design. Subsequently, chimpanzees were used to evaluate the efficacy of vaccine candidates and vaccination strategies.

PRIMARY HCV INFECTION IN CHIMPANZEES

To be able to study the effect of a vaccine or vaccination strategy, it was necessary to identify the virological characteristics of HCV without any intervention. There are numerous reasons why it is difficult to study early events in HCV infection in humans. Firstly, in the vast majority of the cases acute HCV infection is asymptomatic and patients therefore rarely seek medical attention. Secondly, collecting serial blood samples (and occasional liver-biopsy material) from one individual during acute HCV infection is very difficult and, getting pre-exposed biospecimen from humans is complicated. Therefore, experimental inoculation of chimpanzees was pivotal to study early events of HCV infection.

In chimpanzees, similar to humans, intravenous exposure to HCV can either lead to a transient self-limiting infection or it may develop into a persistent infection[29]. In both humans and chimpanzees, viral RNA is detectable by reverse transcription polymerase chain reaction in plasma and liver tissue[30]. In addition, anti-HCV antibodies appear in peripheral blood of both species 6 to 8 wk after HCV exposure[31,32]. In the majority of human individuals, antibodies remain detectable in blood after viral clearance, while in chimpanzees sometimes a gradual loss of HCV specific antibodies after viral elimination has been reported[30,33]. However, in humans, HCV specific cellular immune responses have been found in seronegative individuals, implying also there the loss of HCV-specific antibodies after viral clearance[34-37].

Published data on cellular immune responses showed that HCV specific CD4 and CD8 T-cell responses in both humans and chimpanzees were weak after HCV infection. Spontaneous clearance was associated with somewhat stronger cellular responses compared to the individuals that became persistently infected[38-42]. Also in liver biopsies taken from HCV infected patients and chimpanzees CD4 and CD8 T-cells were observed[43-46] and relatively strong liver-associated T-cell responses were associated with viral clearance[46].

VIRAL PERSISTENCE IN HUMANS AND CHIMPANZEES

Based on antibody data, WHO estimates that 70% to 90% of the infections eventually develop into a persistent HCV infection. However, this percentage may be an overestimation as exposed seronegative individuals are

not included in these calculations[34-37]. The documented percentages of chimpanzees with persisting HCV infection varies between different laboratories from 39% to 70%[33,47-49]. This wide range reflects the heterogeneous nature of infection with HCV. Not only do virological differences, like genotype and dose of infection, play a role but also genetic factors of the host. In humans, the outcome of HCV infection is associated with protective human leukocyte antigen alleles HLA-B27, HLA-B57 and HLA-A3. And although the exact same major histocompatibility complex class I alleles are not present in chimpanzees, homologues with similar peptide-binding characteristics have been identified in these animals[50]. Genome wide association studies have also shown genetic variation linked to the *IL28B* gene, whose product directly interferes with the antiviral interferon (IFN)-pathways and determines the ability of patients to spontaneously resolve HCV infection[51,52]. In chimpanzees similar mechanisms may play a role[53].

Chimpanzee colonies in research facilities are not fully outbred. As a result higher frequencies of certain MHC class I molecules may be present in one facility compared to another facility. This so called "founder effect", in combination with the fact that the total number of human patients outnumbers the total number of chimpanzees used in experimental infection studies may affect the percentage of chronic infection per institute.

In conclusion, these contributing factors make it difficult to directly compare the percentage of persistent infection between humans and chimpanzees. Maybe even more relevant is the difference in "life-style" regarding diet and alcohol intake. Also differences in HCV inocula, route and dose of exposure may partly explain the difference. Similar factors may apply to distinct effects on changes in liver enzyme levels and progression to fibrosis. To our knowledge, it has never been documented that a chimpanzee developed liver fibrosis as a result of persistent HCV infection.

HCV REINFECTION IN CHIMPANZEES

Documented reinfection studies in humans are relatively sparce[54-57]. Longitudinal analysis of human intravenous drug users were performed, but results were inconclusive as to whether a previously cleared HCV infection induces functional immunological memory[55,56,58] that correlate with a shortened viremia and decreased HCV persistence. Important insights were obtained from chimpanzees in which experimental HCV re-exposure was studied in a controlled setting (genotype, dose and route of infection) and longitudinal follow up studies could be performed[59-66].

Reinfection studies in chimpanzees have demonstrated that all of the three possible outcomes: *i.e.*, protection from infection[63,64], protection from viral persistence[59,63-65] or persistent HCV infection[59], can occur.

Pairwise comparison of virological parameters during primary infection in naïve chimpanzees *vs* animals that were rechallenged[47] showed that previous HCV clearance provided some protection, characterized by reduced duration, peak virus load and reduced frequency of development of persistent HCV infection[47]. Understanding the underlying mechanisms through which a cleared HCV infection can contribute to protection against infection, or virus persistence, and the involvement of the adaptive immune system has been an important research goal and pivotal for further HCV vaccine development. Since HCV-induced liver damage only leads to a fatal condition after decades of ongoing immunopathogenesis, a vaccine that could achieve a similar rate of protection from chronic infection as observed after a cleared infection, would already be of great value.

IMMUNE CORRELATES

Virus neutralizing antibodies

In 1994 it was already described that plasma components had an important role in protection against HCV infection[67]. In a hallmark experiment by Farci *et al*[67], *in vitro* neutralizing capacity was determined by mixing infectious virus with heat inactivated plasma from the same patient and subsequently testing it for residual infectivity by inoculating the mixture into a naïve chimpanzee. Patient plasma collected 2 years after infection was able to prevent infection, while plasma collected 13 years after infection could not. At that time there was no *in vitro* system to confirm the presence of neutralizing antibodies. However, simultaneous appearance of envelope HCV specific antibodies in circulation 7 to 8 wk after infection[32] and mutations in viral RNA in the hypervariable region of E2[61,68-70] substantiated the involvement of antibodies and demonstrated the flexibility of the virus to escape from immune pressure through mutation.

In vitro virus neutralization assays

Subsequently, several strategies were used to develop a technique to measure neutralizing capacity of antibodies in plasma of HCV infected individuals. However, it was not until 2003 that HCV envelope based neutralization could be adequately determined. The HCV pseudoparticle (HCVpp) system[71] is based on the expression of HCV envelope proteins on the surface of retroviral particles. After co-transfecting 293T cells with plasmids encoding for HCV envelope protein, a retroviral backbone and green fluorescent protein/luciferase, HCVpp are being secreted into the culture medium. Next, after mixing serum and HCVpp, residual infectivity can be determined in hepatocellular carcinoma cells. The system is very flexible with regard to envelope sequences expressed that can be expressed on the viral surface.

Because pseudoviruses may act different from HCV particles, a subgenomic replicon system was developed[72]. A robust cell culture-derived *in vitro* system was obtained when a replicon was constructed from a HCV genotype 2a clone named JFH-1, which was isolated from a Japanese patient with fulminant hepatitis. Transfection of Huh-7 cells with the *in vitro* transcribed full length JFH-1

resulted in the secretion cell-culture-derived infectious HCV particles (HCVcc)[73]. Similar to the HCVpp system, the HCVcc assay is based on the binding of antibodies to HCV envelope expressing particles before testing residual infectivity on hepatocellular carcinoma cells. Because of the high specificity of the neutralizing antibodies, this system did not suffice for measuring neutralization of genotype 2a based HCVcc and intergenotype clones were constructed[74]. Unfortunately, replacing the JFH-1 envelope proteins by envelopes from other genotypes resulted in less efficient production of viral particles.

Nevertheless, both HCVpp and HCVcc techniques have been shown to be very valuable in improving the understanding of viral entry and antibody neutralization[75].

Antibody correlates

HCV specific antibodies generated during the acute phase of the infection are mainly directed against linear epitopes within structural and non-structural viral proteins, while neutralizing antibodies have been mapped to conformational epitopes within the E1 and E2 envelope proteins[76-82]. While most neutralizing antibodies are rather strain specific[82-84], broadly neutralizing antibodies, antibodies that recognize epitopes that are highly conserved between genotypes, have also been described for E2[83,85,86].

Only for glycoprotein E2, specific targets for receptor binding have been identified: CD81 and scavenger receptor class B member 1 (SRB1) and coreceptors[87]. Neutralizing antibodies directed against domain I and Ⅲ of E2 interfere with its binding to CD81, while neutralizing antibodies directed against hypervariable region 1 (HVR-1) interfere with the binding of E2 to SRB1.

In humans, early induction of strain specific neutralizing antibodies was found to be associated with spontaneous recovery[88,89]. Unfortunately, in most cases these antibodies are only formed during the chronic phase of the infection, when viral clearance is more difficult to achieve. Nonetheless, these antibodies may exert immune pressure that could potentially lead to decreased viral fitness.

The paradigm that neutralizing antibodies play a less prominent role in chimpanzees compared to humans, is mostly based on data collected by Logvinoff *et al*[89]. In patient H, from which HCV clone H77 was derived, strain specific neutralizing capacity was observed 7 wk post infection[89], while in the majority of humans neutralizing antibodies are observed after 100 wk post infection[89]. In chimpanzees infected with H77, specific neutralization was detected only 15 to 20 wk post infection. This relatively late detection in chimpanzees may possibly be explained by the fact that the HCVpp were based on the exact same H77 sequence that was present in patient H. After inoculation of the RNA clone H77 in chimpanzees, it may however have rapidly adapted to its new host and therefore be slightly different from the original H77 clone, showing decreased HCVpp-H77 neutralizing capacity.

ROLE OF T-CELL RESPONSES

Since these early studies, it has been reported that hypogammaglobulinaemic patients have the ability to spontaneously clear HCV infection[90]. Hence, T-cell responses may have contributed to the protection against HCV challenge described above. Furthermore, antibody-mediated depletion experiments in chimpanzees showed that when CD8 T-cells were depleted, virus replication was prolonged despite the presence of memory CD4 T-cells and HCV was only cleared after recovery of HCV-specific CD8 T-cells in the liver[66]. But on the other hand, CD4 T-cells were required for a complete control of HCV replication despite the presence of functional intrahepatic CD8 T-cells[91]. Similarly, the association between HLA-class I molecules HLA-A*03, HLA-B27 and HLA-B57 and class Ⅱ molecules HLA-DRβ1*0101, HLA-DRB1*0401, HLA-DRB1*1101 and HLA-DRB1*0301, and HCV clearance, emphasizes the role of respectively, CD8 and CD4 cells (reviewed in[21]).

T-CELL RESPONSE PATTERNS

HCV specific T-cell responses have been reviewed in detail elsewhere[92-95]. As schematically depicted in Figure 1, four different scenarios can be used to describe HCV specific adaptive immune responses in relation to HCV clearance or viral persistence: (1) a spontaneous clearance of HCV infection, associated with early and effective T-cell responses (Figure 1A). The most important characteristics of this successful cellular immunity against HCV are relatively strongly expanding T-cells that are fully functional with respect to cytolytic capacity, reflected by granzyme and perforin secretion, or cytokine production[96-104]; (2) Transient immune control (Figure 1B) and ensuing viral escape that may be the result of either immune mediated viral selection or an exhausted immune response. Immune pressure may drive the generation of virus variants in which relevant T- or B-cell epitopes are mutated and therefore no longer recognized when they are presented on infected hepatocytes. During tolerance and/or exhaustion on the other hand, immune modulatory mechanisms result in dysfunctional T- or B-cells but with an intact and specific T- (or B-) cell receptor on its surface[105]; (3) Chronic or persistent HCV infection occurs when T-cells are not fully differentiated into functional effector cells (Figure 1C) or no neutralizing antibodies are produced; and (4) Protection of chronic HCV infection by vaccine-induced immune responses. Hypothetically, vaccine-induced neutralizing antibodies may prevent infection while functional HCV specific T-cells may protect from chronic infection (Figure 1D).

VACCINES

Several prophylactic vaccine efficacy experiments have been performed in chimpanzees[106-120]. Relevant information regarding vaccine components, strategy, adjuvants, genotype of the vaccine and the challenge virus and the

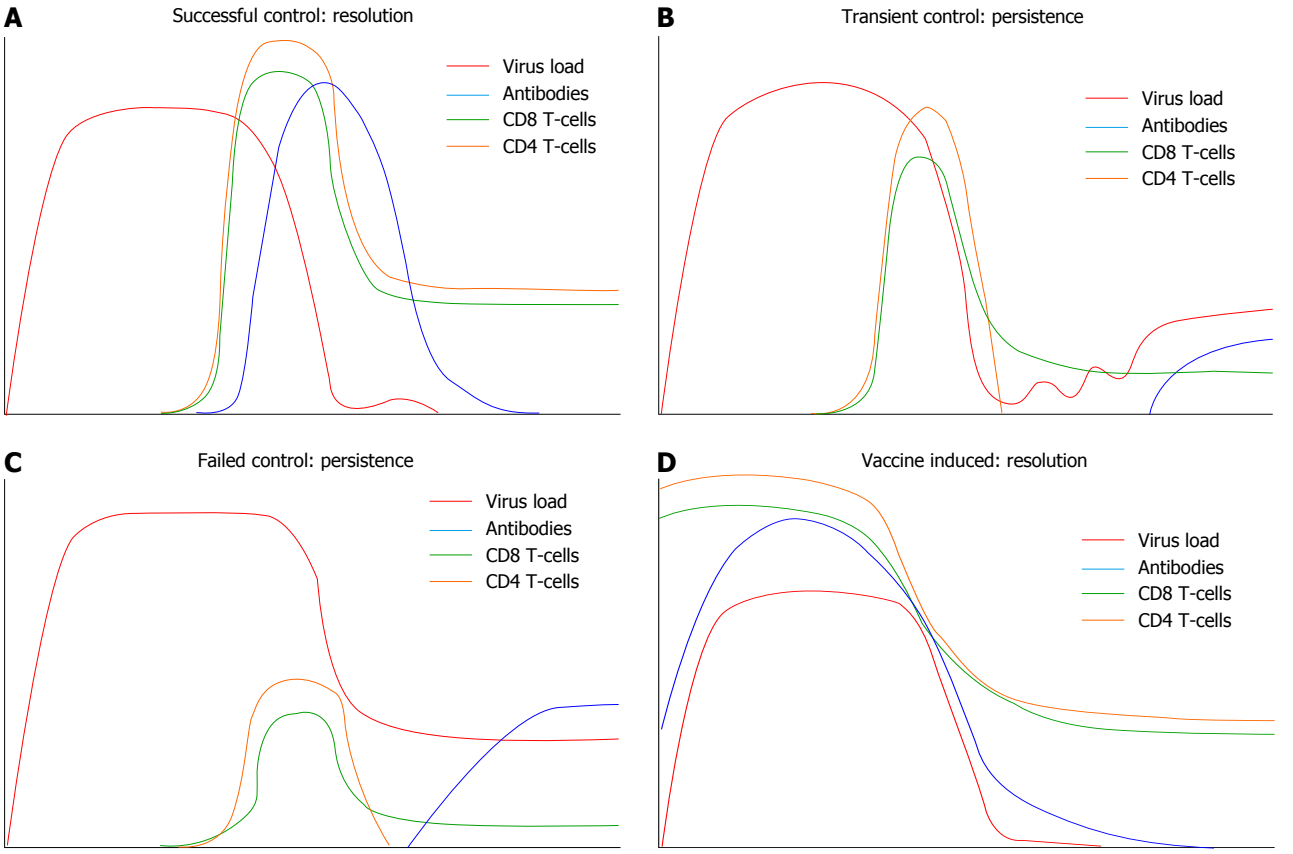

Figure 1 Schematic overview of the different causes of hepatitis C virus infection in relation to modulation of the adaptive immune response. A: Viral clearance. Viral RNA (red line) is normally detected in blood within 1-2 wk after exposure. The virus load will increase until the emergence of HCV specific CD4 (yellow line) and CD8 T-cell (green line) responses 4 to 8 wk after infection[98]. Ideally, strain specific neutralizing antibodies (blue line) are present around the same time[88,89]. After viral elimination, antibody responses can either remain present or decrease to undetectable levels. Memory T-cells remain usually present and can be detected by *in vitro* assays; B: Transient control. After the initial peak viremia (red line), T-cell responses emerge and virus load decreases but remains detectable in serum. CD8 T-cells (green line) remain detectable but CD4 T-cell (yellow line) responses decrease to low levels. There appears to be a constant battle between virus and the immune system. *De novo* escape variants are able to evade the T and B-cell responses but at the same time lose viral fitness. When effective T and B-cell responses contract because the correct epitopes are no longer present, the virus "mutates back" to a more fit variant and virus load may increase again. Thinner lines of the adaptive immune responses represent decreased functionality of CD4 (yellow), CD8 T-cell (green) and antibodies (blue); C: Failed control leading to persistent infection; After the initial peak viremia, T-cell responses emerge and virus load decrease to lower levels but virus remains detectable in serum. T and B cells are functionally impaired or present in too low numbers to efficiently eliminate the virus. The virus remains present at steady state levels. Thinner lines of the adaptive immune responses represent decreased functionality of CD4 (yellow), CD8 T-cell (green) and antibodies (blue); D: Vaccine induced protection model. Vaccine-induced broadly neutralizing antibodies are present at the time of exposure and prevent virus production by infected hepatocytes. The hepatocytes that are infected are successfully eliminated by cytolytic T-cells in the liver.

challenge outcome are summarized in Table 1. We will first focus on vaccine candidates that were developed for the induction of neutralizing antibody responses to protect against infection. Subsequently, vaccine strategies aiming to induce cellular immune responses to control viral infection are discussed.

The envelope glycoproteins as vaccine antigens

Structure and function of envelope glycoproteins: As stated above, HCV envelope glycoproteins E1 and E2 are key determinants for HCV entry. They mediate receptor binding, and the ensuing fusion process between the viral envelope and an endosomal host cell membrane[121,122]. E1 and E2 are heavily glycosylated proteins with a C-terminal transmembrane domain anchored in the lipid envelope of the virus particle. On the surfaces of HCV particles, the envelope glycoproteins are present as large disulphide-linked oligomers[123].

Little is known about the structure of the E1E2 heterodimer, but a proposed model of the E2 ectodomain[124] is comprised of three separate domains (D Ⅰ; described to be a discontinuous region containing the CD81 binding site, D Ⅱ; predicted to possess the fusion peptide and D Ⅲ; described to contain antigenic neutralization epitopes and to be involved in heterodimerization with E1[125]), and three immunogenic HVR1 (384-411), HVR2 (473-480) and HVR3; (431-466).

E1 is even less well characterized, and may be important for the correct folding of E2[126] and the E2 mediated fusion process[127]. E1 may also be involved in controlling virus assembly[87]. The structure of the E1E2 heterodimer is still largely unresolved. Both the functional characteristics of E1E2 and the detection of neutralizing antibody responses against these proteins make them obvious candidates as vaccine-antigen. Long before the presence of HCV neutralizing antibodies was actually confirmed, the

Table 1 Summary of vaccine experiments in chimpanzees

	Vaccine				Challenge		Outcome					
	Components	Adjuvant	Route (prime-boost)	GT	Strain	Dose (CID50)	# Sterile	# Chronic	# Resolved	#Total	%Chronic	Ref.
Recombinant protein												
	E1E2	MF59/MF57	i.m.	1a	HCV-1	10	5		2	7	0	[106]
	E1E2			1a	HCV-1			2	12	14	14	[107]
	E1	ALUM	i.m.	1b	HCV 1b J4	100			2	2	0	[109]
	E2deltaHVR-1	ALUM	i.m.	1b	HCV 1b J4	100		2		2	0	[109]
Recombinant protein-peptides												
	E1, E2, HVRpeptides	(In)complete Freund's	s.c.	4	HCV#6	10			1	1	0	[108]
DNA												
	E2	None		1a	1a	100			2	2	0	[110]
Virus like particle												
	Core, E1, E2	AS01B	i.m.	1b	HCV CG 1b	100			4	4	0	[111]
DNA protein												
	Core, E1, E2 and NS3	ALUM	i.m./i.d.-i.m.	1a/1b	HCV 1b J4	25		1	1	2	50	[112]
DNA-peptide protein												
	E1/E2 + HVR peptides	ALUM/RIBI	i.m.	1a	H7	100		1		1	100	[113]
DNA prime-vaccinia boost												
	NS3, NS5A, NS5B	CpG, rVV B7.1; ICAM-1; LFA-3	i.m./s.c.	1a	H77	100		1		1	100	[114]
DNA prime-MVA boost												
	Core, E1, E2 and NS3	None	i.m./i.d.-i.m/i.d.	1b	HCV 1b J4	25		3	1	4	75	[115]
Replicating rVV												
	Core, E1, E2, p7, NS2 and NS3	None	i.d.	1b	HCV 1b BK	2.5 and 24			4	4	0	[116]
DNA prime-adeno boost												
	Core, E1, E2, NS3-NS5	With/without IL-12	i.m.-i.m.	1b	HCV 1b BK	100	1	4	1	6	67	[117]
	NS3-NS5B	None	i.m./i.m.	1b	H77	100		1	4	5	20	[118]
	NS3, NS4, NS5A, NS5B	Liposomes/pIL-12	*iv-iv*	1b	H77	100		2	2	4	50	[120,134]

i.m.: Intramuscular; *iv*: Intravenous; rVV: Recombinant vaccinia virus; HCV: Hepatitis C virus; IL-12: Interleukin 12; ICAM-1: Intercellular adhesion molecule 1; LFA-3: Lymphocyte function associated antigen 3; HVR: Hypervariable region; s.c.: Subcutaneously; MVA: Modified vaccinia virus Ankara; GT: Genotype.

first envelope based vaccine experiments were already performed. Unfortunately, the envelope glycoproteins also show the largest genetic variance (30%) within HCV[9]. This variance not only poses problems for vaccine development with respect to target antigen selection, but it may also facilitate the formation of variants that escape vaccine-induced immunity giving rise to HCV persistence.

E1/E1 protein immunizations in chimpanzees: The first prophylactic HCV vaccine aimed at the induction of neutralizing antibody responses and was evaluated in chimpanzees by Choo *et al*[106] in 1994. The HCV envelope heterodimer gpE1/E2 was produced in mammalian cells infected with recombinant vaccinia virus that expressed the HCV E1/E2-genes. The protein was formulated in an oil/water micro-emulsion[106], and used to immunize seven chimpanzees. All seven vaccinees developed strong E1E2 antibody responses after the second protein immunization. After intravenous HCV exposure, the challenge control animals developed an acute HCV infection that persisted into a chronic HCV infection. In contrast, five out of seven gpE1/gpE2 vaccinated animals were fully protected from homologous HCV exposure and protection from infection

correlated with vaccine induced antibody responses (Table 1). The other two vaccinees showed overall lower viremia compared to the control animals and only minimal transient elevation of the liver enzyme alanine aminotransferase levels in plasma. From this experiment it was concluded that protection from-chronic-HCV infection was achieved by gpE1/gpE2 vaccination and the level of protection correlated with the level of antibodies directed against gpE1/gpE2.

During this vaccine-study, the lack of an efficient *in vitro* culture system made it impossible to determine the neutralizing capacity of the vaccine-induced antibodies. Retrospective analysis performed by Meunier *et al*[128] demonstrated robust neutralization in four out of five of the protected animals. However, since one of the protected animals showed only minimal HCVpp neutralizing capacity, and another animal with high neutralizing titers was not protected, neutralizing antibody responses alone cannot fully explain the results. Furthermore, vaccine antigens were derived from the same HCV strain that was used for the challenge.

Dahari *et al*[47] reported the results from 21 animals immunized with gpE1/E2. Included in these numbers were the seven animals described by Choo *et al*[106]. From the 14 animals that received a similar recombinant protein vaccine 12 vaccinees resolved HCV infection while 2 animals developed persistent HCV infection[47,107,119].

In conclusion, while very promising results have been obtained with this vaccine candidate, there is some note of caution since these results could not be reproduced.

Induction of cross neutralizing antibodies: At the time of these experiments, heterogeneity in the envelope regions became evident, and it was assumed that multivalent vaccines were required to provide protection to heterologous virus stains. In order to broaden the immune response, and offer protection against a wider range of HCV isolates, Esumi *et al*[108] used truncated E1 and E2 glycoproteins produced in insect cells together with HVR-1 peptides from a different HCV isolate and immunized one chimpanzee. The vaccine, delivered in Freund's (in)complete adjuvant, induced E1 and E2 specific humoral responses, but only a low antibody titer against HVR-1. Upon challenge with HCV#6, the animal showed a transient peak of HCV RNA, which in view of the low propensity of this virus to cause chronic infection implies that the vaccine did not confer protection.

E1 neutralizing capacity: Because these HCV-envelope protein vaccines were based on the E1E2 heterodimer, the role of the individual glycoproteins could not be determined. Only recently, the gpE1 and a gpE2 lacking the HVR-1 were evaluated separately[109]. In two animals immunized with gpE1 HCV neutralizing antibodies were induced and after a heterologous HCV-1b challenge, both animals were able to resolve HCV infection shortly after challenge. In contrast, the two E2 delta HVR-1 immunized animals showed no HCVpp 1b neutralizing capacity, and despite the presence of E2 specific cellular responses both animals were not protected from chronic HCV infection. For the first time, this study showed that E1 neutralization can be achieved and has protecting potential. Possibly, epitopes within E1 are masked when administered as a heterodimer and may therefore have been missed until now. However, the exact role of E1 during the cell entry process needs to be further elucidated.

New insights in the role of E1 indicate that a better understanding of the interaction between E1 and E2 as well as the exact mechanisms of virus/receptor interaction and cell entry are needed.

Vaccine strategies for induction of protective T-cell responses

Although traditionally most vaccination strategies have relied on the induction of neutralizing antibody responses, the emergence of human immunodeficiency virus (HIV) and the realization that cellular immune responses are important in suppressing replication of this virus has boosted the development of new vaccine strategies for the induction of effective T-cell responses. The HCV vaccine research has greatly benefited from these developments and modeled their experimental vaccines on the knowledge gained in the HIV-field.

DNA vaccines encoding for HIV antigens have been proven efficient in the induction of HIV specific T-cell responses[129]. In the year 2000, Forns *et al*[110] performed a proof of principle experiment in two chimpanzees, using a DNA plasmid encoding for surface-expressed E2. One animal developed antibodies directed against E2 and HVR-1, while the other animal had only very low levels of E2 specific antibodies. However, no HCV specific T-cells could be detected. Nonetheless, upon challenge with the heterologous HCV, both vaccinees resolved HCV infection, while the control animal developed a persistent HCV infection. From this experiment it appears that DNA immunization can provide protection against infection, although the underlying mechanism is still unclear.

Virus-like particles: Delivery of antigens in the form of virus-like particles has been described as an efficient strategy to elicit T-cell responses[130]. This was evaluated in a study in chimpanzees, by giving four immunizations with HCV-like particles[111] consisting of the structural proteins Core, E1 and E2, in AS01B adjuvant. All four chimpanzees showed broad and strong T-cell responses, determined by IFN Enzyme-Linked ImmunoSpot (ELISPOT) and proliferation assay, in peripheral blood. In the liver antigen specific CD4 as well as CD8 T-cells were observed, comparable in magnitude to the blood. All four animals were able to control an intravenous challenge with HCV clone CG1b within 12 wk.

Multicomponent prime-boost vaccine strategies: Experience from the HIV vaccine field has shown that

the induction of cellular immune responses is greatly enhanced when two different vaccine modalities are given in a so-called "prime-boost" combination[131].

A multicomponent prime-boost vaccine strategy was evaluated by Rollier *et al*[112] using the relatively conserved regions, Core and NS3, in combination with the variable E1 and E2, as vaccine antigens to induce an immune response against a broad range of HCV variants. DNA plasmids expressing the individual antigens were used to prime the immune system and subsequently three recombinant protein immunization were given as boosts. Both immunized animals developed strong humoral as well as strong cellular responses. Animals were challenged with a heterologous HCV-1b strain and in contrast to the control animal, both vaccinees suppressed virus replication to below the detection limit early after exposure. However, in one vaccinee the virus kept reappearing in plasma at very low levels while no evidence for HCV replication could be observed in the other chimpanzee.

Puig *et al*[113] aimed to induce neutralizing antibodies by giving a prime with DNA encoding for E1E2 in combination with HVR peptides in ALUM adjuvant. The responses were boosted with recombinant E1E2 heterodimer in RIBI (squalene which is emulsified with saline containing Tween 80)[113]. Strong HVR-1 specific antibody responses were observed in peripheral blood and cellular proliferative responses and cytokine production were found in the liver. Despite these vaccine-induced responses, the animal became persistently infected after exposure to an homologous challenge strain. Compared to the naïve non-vaccinated control animal, a delay in the peak of virus replication was observed, but not a reduced viremia.

In another experiment performed by the same research group, priming with DNA plasmids encoding HCV-NS3, NS5A or NS5B, followed by a booster immunization with recombinant vaccinia constructs expressing the same HCV proteins, resulted in strong T-cell responses. After experimental HCV exposure, initially virus replication was controlled. However, the virus reemerged. Losing immune control coincided with emergence of new virus variants and the loss of CD4 T-cell recognition[114].

In a similar DNA prime modified vaccinia virus (MVA) boost strategy, but now directed against HCV core-E1-E2 and NS3, strong and broad T- and B-cell responses were reported[115]. However, despite strong humoral responses, no virus neutralizing capacity was found and after challenge with HCV-1b, all four animals showed acute viremia. Only one animal was able to control virus replication to undetectable levels. The other three animals became chronically infected. The vaccine induced vigorous T-cell responses as reflected by strong proliferation and HCV specific IFNγ, interleukin-2 (IL-2) and IL-4 cytokine responses. Retrospectively, vaccine induced T-cell responses were analyzed in more detail. It was found that, although the vaccine elicited NS3 specific cytokine producing CD4 and CD8 T-cells in all four vaccinees, only in the chimpanzee that cleared HCV infection, CD8 T-cells were found to have cytolytic capacity[132]. Interestingly, the animals that became chronically infected had higher mRNA expression levels of exhaustion markers programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Figure 2) and indoleamine 2,3-dioxygenase in the liver, suggesting the induction of T-cells with regulatory functions that might have prevented formation of a cytotoxic T-cell response[115].

In 2008, a replicating recombinant vaccinia virus (rVV) vaccine; PolyVax (rVV-HBV-HCV) was evaluated in chimpanzees[116]. After immunization with PolyVax the animals were exposed to HBV and after resolution of the HBV infection, they were boosted with HCV-rVV, expressing HCV-1b based E1, E2, p7, NS2 and NS3. To assess the efficacy against HCV infection, animals were intravenously exposed to 2.5 CID50 of a homologous HCV strain. Unfortunately, this challenge was not successful and 17 wk later a second challenge was performed with the same inoculum with 24 CID50. After peak viremia, viral titers declined to non-detectable levels within 4 wk in all four vaccines while two controls became persistently infected. Eighteen months after the initial HCV clearance a multigenotype rechallenge was performed. Only one animal was able to clear infection while in three other animals, genotype HCV-1a remained detectable in plasma. PolyVax transiently induced HCV neutralizing antibodies. However, these were not present at the time of HCV exposure. On the other hand long lasting IFNγ secretion and proliferative responses were observed after PolyVax immunization and these cellular responses were boosted by HCV-rVV. To what extend these responses may have contributed to control of virus replication after the second challenge is difficult to establish due to possible contribution of the first 2.5 CID50 HCV exposure.

Adenoviruses are efficient vehicles for gene transfer and have a natural tropism for the liver[133], the site of HCV replication and therefore a good candidate for the delivery of HCV antigens. Youn *et al*[117] described a vaccine study with 6 chimpanzees, in which animals were primed with DNA encoding for HCV-Core, E1, E2, NS3-5 with three out of six animals receiving an additional plasmid encoding for IL-12 to promote the development of IFNγ producing Th1 cells. The prime was followed by an immunization with replication incompetent adenovirus expressing the same HCV antigens. Strong vaccine induced humoral as well as cellular responses were measured in proliferation assays, E2 specific enzyme-linked immunosorbent assay and neutralization assays. In the animal with the strongest responses at the day of challenge, no HCV RNA could be detected. All other animals had a delayed and lower peak virus load and four animals became persistently infected.

While viral vectors typically induce high cellular immune responses, they have the disadvantage that anti-vector responses are formed that limit their repeated application. To circumvent this problem, Folgori *et al*[118] used two different types of replication defective adenoviral

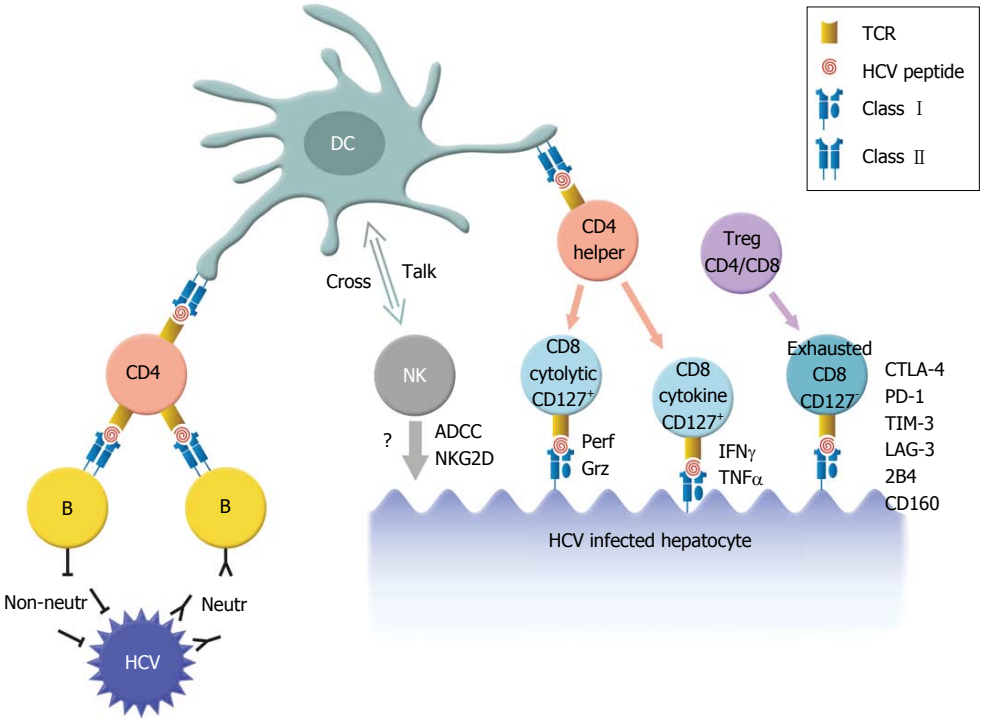

Figure 2 Summary of immune responses in hepatitis C virus immunized chimpanzees. Antigen presenting cells (DCs or Kupffer cells in the liver) present HCV peptides in the context of major histocompatibility complex class Ⅱ molecules to the T-cell receptor (TCR) on CD4 T-cells. CD4 cells may activate B-cells. Antibodies produced may be neutralizing and bind to circulating HCV particles and prevent the infection of hepatocytes. Or the antibodies may be non-neutralizing antibodies and potentially play a role in ADCC. CD4-helper T-cells can also stimulate cytolytic T-cells. CD8 T-cells may be directly responsible for lysis when they produce degranulation molecules like granzymes or perforin after the recognition of a peptide on the surface of an HCV infected hepatocyte. Or *via* indirect lysis, mediated by the secretion of cytokines. CD8 T-cells affected by Tregs and exhausted CD8 T-cells are functionally impaired and are incapable of lysing HCV infected hepatocytes. HCV: Hepatitis C virus; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; IFN: Interferon; TNFα: Tumor necrosis factor alpha; ADCC: Antibody dependent cellular cytotoxicity; DC: Dentritic cell; PD-1: Programmed cell death protein 1; TIM-3: T cell immunoglobulin and mucin protein 3; LAG-3: Lymphocyte-activation gene 3.

vectors for two subsequent booster immunizations[118]. For an optimal booster effect, the adenoviruses were selected based on low seroprevalence in humans and little or no immunological cross-reactivity between the two types. Both prime and booster immunizations were directed against the non structural proteins NS3 to NS5B. Upon HCV challenge, the immunized animals showed a reduction of acute viremia, which coincided with expansion of HCV specific CD8 T-cells in peripheral blood as well as the liver. In 4 out of 5 immunized animals, virus load was reduced to undetectable levels, while in the control group 3 out of 5 animals cleared the infection.

Similar results were reported in a study, where chimpanzees received a DNA prime followed by an adenovirus boost, both expressing NS3-5B + pIL-12[134]. One animal cleared the infection while the other became persistently infected. Evidence was found for the selection of escape mutants that evaded vaccine induced T-cells. Comparison of the nucleotide sequence of the circulating viruses in the two immunized animals and the control animal, showed a nonsynonymous/synonymous ratio indicative for positive selection. The exact same immunization strategy was then used to vaccinate two additional chimpanzees[120] and again one chimpanzee was able to control the infection early after challenge while in the other animal the virus persisted and the animal became chronically infected.

In conclusion, all immunization strategies that were evaluated in chimpanzees induced either humoral or cellular immune responses, or both. As nicely shown in the meta analysis performed by Dahari *et al*[47], compared to non-immunized animals, vaccinees generally showed reduced virus replication in the early phase of the infection, although complete protection from infection was rare. In addition, the analysis showed that the proportion of HCV persistence in vaccinees (28.3%) is much lower compared to 61.9% observed in the control animals that were included[47].

VACCINE INDUCED T-CELL RESPONSES IN BLOOD AND PREDICTION OF OUTCOME

A direct comparison between the individual chimpanzee experiments is not always possible because of disparity in experimental design, vaccine-antigens, vaccine regimen, heterologous or homologous challenge virus and challenge dose. Also, various methods have been used to assess the magnitude of vaccine induced T-cell responses. For instance for the quantification of cytokine production, real-time quantitative polymerase chain reaction, intracellular cytokine staining and ELISPOT assays have been used,

which do not necessarily yield the same answer. Despite these differences, important conclusions can be drawn on immune regulatory mechanisms that are potentially involved in HCV clearance.

Clearly, neither the magnitude of the vaccine induced immune responses nor the breadth of the responses could predict the protective effect of a vaccine within one experiment. There is a striking heterogeneity in vaccine-induced responses between individuals. This not only reflects the genetic variation of a population but also differences in pathways as well as regulatory mechanisms of T-cell responses, similar to the variation observed in human patients suffering from HCV infection[135]. Larger study groups would be needed to cover this diversity, but the special nature of the animals and the high costs involved, precludes larger experiments.

As an example, in the Folgori study, where a DNA-prime was followed by an adenovirus boost expressing the same antigens, it was not the animal with strong and broad vaccine-induced cytotoxic T-cell (CTL) responses that was protected from infection[118]. On the other hand the vaccinee with the lowest CTL response was the one animal that became persistently infected. Youn *et al*[117] described an association between E2-specific adaptive immunity and protection from (chronic) infection. However, in other experiments E2 was not identified as the key-antigen for protection against chronic infection.

ESCAPING VACCINE INDUCED IMMUNITY

HCV is notorious for its ability to mutate, resulting in development of different *de novo* variants that are generated under immune pressure and result in escape from T and B-cell responses. Data generated by Lavillette *et al*[127] describe two patterns of progressive emergence of neutralizing antibodies, which were correlated with a fluctuating decrease in virus load, leading to control of virus replication and ultimately viral clearance. These data strongly suggest escaping functional B-cell responses is at least one of the mechanisms for viral persistence. In addition, escape mutations have been described for both CD4[114] and CD8 T-cell[118] epitopes. Vaccine induced immune escape is therefore of great concern.

On the positive side, mutations induced by immune pressure can lead to a reduction in viral fitness that could potentially limit viral persistence. It was demonstrated that immune pressure induced changes of non-structural regions can be lethal to the virus[136], while specific changes in envelope glycoproteins may have serious implications in selective outgrowth[137], virus entry and sensitivity to neutralization[138].

OTHER MECHANISMS TO EVADE VACCINE INDUCED IMMUNITY

Apart from generation of escape mutants, HCV may evade immune pressure by modulating immune responses. HCV specific T-cells with an exhausted phenotype in terms of loss of CD127 expression, cytokine expression and increased levels of the inhibitory markers PD-1 and CTLA-4 have been described[139-141] (Figure 2). Moreover, the negative immune modulator Tim-3, LAG-3, CD160 and 2B4 have been associated with exhausted HCV specific T-cells[139,142,143].

Also active suppression of HCV-specific T-cell responses by regulatory T-cells or by the immunosuppressive cytokines IL-10 and transforming growth factor-β have been described[144]. The contribution of each of these immuno-regulatory mechanisms during HCV persistence varies between individual patients and also synergistic effects were found[135]. Restoration of dysfunctional HCV-specific T-cell responses by blocking inhibitory molecules temporarily restored anti-HCV T-cell responses resulting in a transient drop in virus load[143,145-147]. Combining the recovery of functional T-cells with a boost of T-cell responses will be of interest as a therapeutic vaccine strategy.

Natural killer (NK) cells play an important role during HCV infections[148] because of their potential to lyse infected hepatocytes *via* antibody dependent cellular cytotoxicity. However, because NK cell function has not been studied in the context of vaccine induced clearance of HCV in chimpanzees, this is not documented.

Some of the prophylactic vaccine candidates and regimen that were found beneficial in chimpanzees have been, or are currently, tested in humans. For two HCV-envelope vaccines, E1/E2[119] and E1[149], T-cell and antibody responses in healthy volunteers were comparable to the responses found in chimpanzees. Despite these promising results, the development of both candidates is currently on hold.

Both adenovirus and MVA were successful as vaccine delivery vehicles in chimpanzees and both platforms have advanced to human trails. To overcome vector specific immunity much effort was put into the design of even less immunogenic vectors or, when multiple immunizations are required, the design of immunization protocols with different serotypes of the vector. MVA and adenovirus based vaccines are currently incorporated in-mainly-therapeutic vaccination strategies in chronically infected patients.

CONCLUSION AND FUTURE VACCINE PERSPECTIVES

Studies in chimpanzees have provided important insights into the efficacy of different vaccine strategies and provided evidence for the central role of neutralizing antibodies in obtaining protection against infection. While most vaccine candidates that induce cellular immune responses, do not protect from infection they do lead to reduced viremia in the acute phase of the infection and reduce the risk for development of chronicity. The current challenge is to translate this newly acquired knowledge into an efficient prophylactic HCV vaccine that protects

from chronic HCV infection.

Due to further restrictions on the use of chimpanzees for biomedical research, future evaluation of a new vaccine candidates or strategies in these apes will be severely limited. We have summarized the work performed so far, discussing the different immunization strategies used and types of immune response induced. Although partial protection, defined as decreased chance to develop chronic HCV infection, can be achieved by immunization, a clear correlate of protection has not yet been established. Further studies are required and have to be based to a large extent on clinical trials.

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