

Review Article

A New Insight into Hepatitis C Vaccine Development

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Chronic hepatitis C virus (HCV) infection remains a serious burden to public health worldwide. Currently, HCV-infected patients could undergo antiviral therapy by giving pegylated IFN- α with ribavirin. However, this therapy is only effective in around 50% of patients with HCV genotype 1, which accounts for more than 70% of all HCV infection, and it is not well tolerated for most patients. Moreover, there is no vaccine available. The efforts on identifying protective immunity against HCV have progressed recently. Neutralizing antibodies and robust T cell responses including both CD4⁺ and CD8⁺ have been shown to be related to the clearance of HCV, which have shed lights on the potential success of HCV vaccines. There are many vaccines developed and tested before entering clinical trials. Here, we would first discuss strategies of viral immune evasion and correlates of protective host immunity and finally review some prospective vaccine approaches against chronic HCV infection.

1. Introduction

More than 170 million people are currently affected by chronic hepatitis C virus (HCV) infection worldwide with the highest prevalence in Africa and Asia [1–3]. Since the adoption of the all volunteer blood donor system to screen blood donations in 1990s, the incidence of HCV infection has dropped dramatically. However, some populations remain highly susceptible including drug users sharing the same devices and patients that have received unsafe therapeutic injection or unsafe blood transfer [4]. Among all HCV infected individuals, 80% of them remain chronically infected [4, 5], 10%–20% of them develop cirrhosis, and 1%–5% of them acquire liver cancer over years [6]. Therefore, previous incidence as well as new incidence all account for future disease burdens. In developed countries, HCV infection has become the leading cause for the failure of liver transplants [1]. Up until now, there is no vaccine available for HCV infection. HCV-infected patients could receive anti-viral therapy by giving pegylated interferon- α (PEG-IFN) with ribavirin [7]. However, this therapy is long, expensive, toxic, and only effective in around 50% of patients for the most common genotype [8]. A regimen of 48-week therapy with PEG-IFN and ribavirin costing \$25,000 USD

is recommended for HCV genotype 1 and 24-week therapy for HCV genotype 2/3 [9]. There are many side effects associated with PEG-IFN, which have lead to early withdrawals or dose modification, including neutropenia, flu-like symptoms, neuropsychiatric disorders like depression, and autoimmune syndromes like autoimmune thyroiditis [8]. A sustained virological response (SVR) representing long lasting disappearance of viral RNA in the serum can be achieved in 80%–90% of genotype 2/3 but only around 40%–50% of genotype 1 [10, 11], which accounts for more than 70% of HCV infection in US [12, 13]. Therefore, the development of effective vaccines for HCV, especially therapeutic, is crucial in controlling chronic HCV infection.

HCV is an RNA virus with enveloped virion belonging to the family Flaviviridae. It contains a positive-sense single-stranded RNA genome that is 9,600 nucleotides in length. HCV genomic RNA is composed of one open-reading frame flanked by 5' and 3' noncoding region. The HCV polyprotein encoded by the only open-reading frame is approximately 3,000 amino acids in length and is cleaved into three structure proteins (core, E1, and E2), and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [14]. According to international standardization and coordination of the nomenclature of variants of hepatitis

TABLE 1: HCV genotypes, subtypes, and their geographical distributions.

Genotypes	Subtypes	Geographical Distribution
1	a, b, c	Central Africa, Europe, North America
2	a, b, c, k	Western Africa
3	a, b, k	Southeast Asia
4	a	Central Africa
5	a	
6	a, b, d, g, h, k	Southeast Asia

C virus, HCV is classified into 6 clades or genotypes with 31%–33% diversity in nucleotide based on partial sequences of core/E1 and NS5B, or complete sequences. Each genotype is further divided into different subtypes with 20%–25% differences (Table 1) [15]. The importance of HCV genotype lies in its geographical distribution and treatment response to PEG-IFN and ribavirin [8]. Due to the low-fidelity of RNA-dependent RNA polymerase, NS5A, in viral replication, there are many quasispecies within one infected individual [16]. Primarily HCV infects hepatocytes of humans and chimpanzees. Various molecules including CD81 [17], scavenger receptor class B type I [18], Claudin-1 [19], low density lipoprotein receptor [20], and glycosaminoglycan [21] have been shown to be the receptors for HCV. The recent discovered receptor, occludin, however, is the crucial factor allowing HCV replication in mice [22].

The studies on HCV evasion from host immunity and host immunity in HCV patients that have spontaneously recovered have allowed us to address important immunological parameters related to protective immunity. Spontaneous recovery has been linked to multifunctional CD4⁺ T cells, cross-genotype cytotoxic CD8⁺ T cells, as well as cross-genotype neutralizing antibodies. These studies have advanced our understanding on protective immunity against HCV and provide a blueprint for HCV vaccine developments. There were many vaccines developed and tested in preclinical setting in the past. Among them, several vaccines have now advanced to clinical trials. Herein, we would examine the immune evasion strategies used by HCV, discuss correlates of successful host immunity against HCV infection, and review some prospective therapeutic vaccines to chronic HCV infection.

2. Immune Evasion by HCV

HCV can target many different effectors of the immune system, which enables its escape from host immune surveillance and ultimately leads to chronic infection. HCV can inhibit IFN- α production, inhibit NK activity, and produce escape mutants from antibody and CD8⁺ T cell recognition. All these have aided to the development of chronic HCV infection.

Double-stranded RNA expressed by many RNA viruses during replication could be recognized by host pathogen-

recognition receptors, such as TLR3 and RNA helicases (RIG-I and MDA-5), which lead to anti-viral responses. The recognition of dsRNA by TLR3 triggers its signalling pathways. In addition to the MyD88-dependent pathway, the MyD88-independent pathway leads to phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF-3) through adaptor protein TRIF [23–25]. The activation of transcription factor IRF-3 subsequently induces type I IFN production and other genes involved in host defence [26]. By comparison, RIG-I activates IRF-3 for type I IFN production through another pathway required a CARD-containing adaptor protein, Cardif. During replication, HCV NS3-4A protease recognizes and cleaves both TRIF and Cardif, which blocks the signalling pathways of TLR3 and RIG-I and ultimately inhibits the production of type I IFN [27–29].

The lack of type I IFN production in patients chronically infected with HCV may indirectly lead to a decrease in NK cell activity. Activated NK cells are important effectors in innate immunity against viral infection through the secretion of inflammatory cytokines like IFN- γ or the cytolytic ability like antibody-dependent cell-mediated cytotoxicity (ADCC) [30]. Type I IFN activates DCs which subsequently prime NK cells via the production and transpresentation of IL-15 [31, 32]. Thus, the lack of type I IFN production might have led to the lack of IL-15 production present in the serum, which eventually causes the decrease in total number of NK cells, especially cytotoxic CD16⁺CD56^{dim} NK cells, in chronic HCV- infected patients [33, 34].

Antibody responses may not be sufficient to protect individuals from HCV infection since neutralizing antibodies are rarely found in acute HCV patients but are found in the majority of chronically infected patients at relatively high titers (>320) [35, 36]. The failure of neutralizing antibodies in controlling HCV infection could be caused by several different factors. HCV can bind to very low-density lipoprotein (VLDL), which facilitates the uptake of HCV by hepatocytes via the interaction between ApoB and scavenger receptor class B type I, helping HCV avoid recognition by neutralizing antibodies [37]. E2 is highly glycosylated with 11 N-linked glycans located at the conserved region outside hypervariable region 1 (HVR1), which is targeted by most antibodies. These glycosylation sites are conserved across all HCV genotypes and subtypes. Three glycans located at the CD81-binding site of E2 decrease its immunogenicity and eventually protect viruses from antibody neutralization [38]. HCV can also infect surrounding cells through a direct cell-cell contact mediated by CD81 and Claudin-1, which can also avoid itself from the clearance of neutralizing antibodies [39]. Moreover, HCV can evolve into many quasispecies representing closely related but heterogenous RNA sequences within one individual during the course of infection. The number of quasispecies identified within a single sample ranges between 3–10 variants and the sequence variation occurs mainly in the HVR1 [40–42]. Studies following the evolution of HCV in one single patient well illustrated the development of quasispecies in chronic patients. Neutralizing antibodies to broad genotypes of HCV caused by continuous mounting immune response to evolving HCV

could be detected in this patient. However, these antibodies could not neutralize the dominant HCV isolate from this patient at the time of sample collection. Therefore, the antibody response failed to resolve HCV infection [41]. Furthermore, the presence of interference antibodies could diminish the function of true neutralizing antibodies. Two important epitopes located at E2 envelope glycoprotein have been identified (Table 2). Whereas epitope I located at residue 412–426 is an important neutralizing site and conserved between different genotypes, epitope II at 434–446 varies among different genotypes and generates antibodies interfering with the antibody to epitope I. When analysing the appearance of antibodies specific to these two epitopes, chronic HCV patients developed antibodies to nonprotective epitope II first. The appearance of antibodies to protective epitope I only appeared at very late time point together with equal abundant interference antibodies. Indeed, when antibodies specific to epitope II were depleted from patient plasma, this plasma, which contained antibodies to epitope I, could now provide better neutralizing capacity to a variety of genotypes [43]. In addition to the constant mutation occurred in HCV, the induction of interfering antibodies is yet another strategy of HCV to escape from immune response. Since these interference antibodies appear earlier than the protective antibodies, a vaccine effective in generating antibodies to epitope I would be critical against HCV infection.

The emergence of escape mutations in CD8⁺ T cell epitopes requires a balance between virus infectivity and host immune response. The low-fidelity of RNA-dependent RNA polymerase, NS5A, is generally known to be the reason for the emerging of many quasispecies within HCV-infected individuals [16]. The rapid accumulation of mutated variants could be tracked back to the slow immune response generated against HCV, which has allowed many mutations to accumulate *in vivo* [40, 44]. The emerging of mutational variants in CD8⁺ T cell epitopes has been carefully described both in chimpanzees [44, 45] and in humans [46–48]. Firstly, when the mutation rate was analysed, amino acid substitutions within CD8⁺ T cell epitopes occurred more frequently compared to other regions [47]. Secondly, the lack of CD4⁺ T cell help in chronic phase has prevented the effectiveness of CD8⁺ T cells to clear the virus [49]. When chimpanzees with resolving HCV infection were rechallenged after CD4⁺ T cell depletion, they developed chronic HCV infection. The inability of HCV-specific CD8⁺ T cells to control HCV viremia correlated with the emerging of mutations in CD8⁺ T cell epitopes. Therefore, these ineffective CD8⁺ T cells have provided the selective pressure on shaping mutational variants of HCV. Recently, studies analysing different HCV variants within one chronically infected individual have shown that some HCV variants were actually emerging early during acute infection. Despite poor viral production while infecting hepatocytes, these variants survived due to poor recognition by host CD8⁺ T cells [50]. Therefore, a fine balance between virus infectivity and host immune response could shape HCV mutants present in chronically infected individuals.

3. Protective Immunity to HCV

Despite the ineffectiveness of the host immune system to eradicate HCV, studies on patients that have spontaneously recovered from HCV infection and vaccinated chimpanzees that have recovered from HCV challenge have allowed us to address the important immunological correlates related to HCV clearance. Some reports have shown that the clearance of HCV could be associated with certain host genetic background including host HLA types, cytokine and chemokine expression (e.g., IL-10, IL-28B, and CCR5) [51–56]. For example, HCV clearance is often linked to patients with HLA-B27 allele in their MHC class I locus [55, 57]. Since MHC class I is directly associated with antigen presentation to CD8⁺ T cells, this implies the importance of cytotoxic T cells in HCV eradication. Many studies published recently have provided critical immune parameters on protective immunity against HCV.

CD8⁺ T cells are the most important effectors in controlling HCV infection. It was first recognized in earlier studies analysing acute HCV infection in chimpanzees. Few chimpanzees have resolved HCV spontaneously while most of them became chronically infected. When comparing these two groups of chimpanzees for the development of neutralizing antibodies and cytotoxic CD8⁺ T cells, spontaneously recovered chimpanzees exhibited a strong CD8⁺ T cell response toward multiple viral epitopes and across multiple MHC class I restrictions (Table 2) [58]. When these spontaneously recovered chimpanzees were challenged with HCV for the second time, they recovered more rapidly, within 14 days compared to 40 days in primary infection. Specific CD8⁺ T cell number inversely correlated with HCV viral load in the blood. Additionally when CD8⁺ T cells were depleted before the third challenge, a prolonged HCV infection was observed [59]. This was further supported by similar findings in human studies. When patients with chronic HCV infection were investigated, a general lack of broad specificity and cytotoxicity in CD8⁺ T cells toward HCV epitopes was observed (Table 2) [60]. A more detailed study on HLA-matched individuals with chronic infection or spontaneously resolving infection was set to investigate the breadth, magnitude, phenotype, and function of HCV-specific CD8⁺ T cells. Individuals with resolved HCV infection contained stronger CD8⁺ T cell responses (17/20 versus 9/20; $P = .019$) and specific CD8⁺ T cells to broader range of epitopes (Table 2; mean 2.3 versus 1; $P = .039$) with higher frequency in circulation (mean 584 versus 95 per 1 million PBMC; $P = .027$) measured by IFN- γ secretion in response to HCV peptides [61]. These specific CD8⁺ T cells proliferated vigorously in response to antigens and expressed memory CCR7^{hi}CD45RA^{low}CD27⁺CD28⁺ phenotype [60, 61]. Notably a predominant expression of IL-7 receptor (CD127) on HCV-specific CD8⁺ T cells was identified in patients who recovered from infection [62]. Additionally cross-genotype CD8⁺ T cells could limit the escape of HCV and help the clearance of viruses [57]. Thus, a robust multispecific and cross-genotype CD8⁺ T cell response to different epitopes implies a successful response against HCV infection.

TABLE 2: Important epitopes of HCV recognized by T cells and B cells.

Ref.	Antigen	Epitopes (aa sequence, restriction molecules)	Functional properties		
CD8 epitopes					
Cooper et al. [58]	E1	306–315 (CSIYPGHITG, Patr-A*0402); 366–375 (GNWAKVLVVL, Patr-C*0601/C*0602)	cytotoxic epitopes identified in recovered chimpanzees		
	E2	621–629 (TINYTIFKI, Patr-B*2001); 651–665 (RCDLEDRDRSELSPL, Patr-A*0601)			
	P7	781–791 (KWVPGAVYTFY, Patr-A*0601)			
	NS2	997–1008 (INGLPVSARRGR, Patr-A*0402)			
	NS3	1629–1637 (GAVQNEITL, Patr-B*1701)			
	NS5A	2055–2065 (MWSGTFPINAY, Patr-A*0601)			
Dazert et al. [57]	NS5B	2841–2849 (ARMILMTHF, HLA-B27)	IFN- γ secreting and protective epitope		
Lauer et al. [61]	core	41–49 (GPRLGVRAT, HLA-B7); 88–96 (NEGCGWMGW, HLA-B44); 111–119 (DPRRRSRNL, HLA-B7)	IFN- γ secreting epitopes identified in recovered individuals		
	E1	207–214 (CPNSSIVY, HLA-B35); 322–330 (MMMNWSPTT)			
	E2	541–551 (NTRPPLGNWFG, HLA-B57); 610–619 (YRLWHYPCTI, HLA-Cw7)			
	NS2	831–840 (LSPYYKRYIS, HLA-A25); 941–960 (LGALTGTYYVNHLPDRDWA); 957–964 (RDWAHNGL, HLA-B37)			
	NS3	1070–1089 (ATCINGVCWTVYHGAGTRTI); 1073–1081 (CINGVCWTV, HLA-A2); 1175–1183 HAVGLFRAA, HLA-A68); 1359–1367 (HPNIEEVAL, HLA-B35); 1395–1403 (HSKKKCEDEL, HLA-B8); 1406–1415 (KLVALGINAV, HLA-A2); 1435–1443 (ATDALMTGY, HLA-A1); 1610–1627 (CLIRLKPTLHGPTPLLYR)			
	NS4	1695–1702 (IPDREVLV, HLA-B35); 1745–1754 (VIAPAVQTNW, HLA-A24); 1751–1770 (VFTGLTHIDAHFLSQTQKQSG); 1758–1766 (ETFWAKHMMW, HLA-A25); 1771–1790 (GIQYLAGLSTLPGNPAIASL); 1801–1809 (LTTSQTLF, HLA-B57); 1966–1976 (SECCTPCSGSW, HLA-B37); 1987–1995 (VLDSFKTWL, HLA-A2)			
		2162–2170 (EPEPDVAVL, HLA-B35); 2225–2233 (ELIEANLLW, HLA-A25); 2461–2480 (TSRSACQRQKKVTFDRLQVL); 2594–2602 (ALYDVVTKL, HLA-A2); 2629–2637 (KSKKTPMGE, HLA-B57); 2819–2828 (TARHTPVNSW, HLA-A25); 2912–2921 (LGVPPRAWR, HLA-B57)			
		NS5		35–44 (YLLPRRGPR, HLA-A2); 132–140 (DLMGYIPLV, HLA-A2)	
	Wedemeyer et al. [60]	NS3		1073–1081 (CVNGVCWTV, HLA-A2); 1406–1415 (KLVALGINAV, HLA-A2)	cytotoxic and IFN- γ secreting epitopes identified in chronic & recovered individuals
	Urbani et al. [62]	NS3		1073–1081 (CVNGVCWTV, HLA-A2); 1406–1415 (KLVALGINAV, HLA-A2)	Differential expression of CD127 on IFN- γ secreting CD8 ⁺ T cells
CD4 epitopes					
Day et al. [67]	NS3	1248–1262 (GYKVLVLNPSVAATL, HLA-DRB1*0401); 1579–1597 (SGENLPYLVAQATVCARA, HLA-DRB1*0401)	CCR7 ⁺ CD45RA ⁻ CD27 ⁺ tetramer-positive T cells identified in recovered individuals		
	NS4	1770–1790 (SGIQYLAGLSTLPGNPAIASL, HLA-DRB1*0401)			

TABLE 2: Continued.

Ref.	Antigen	Epitopes (aa sequence, restriction molecules)	Functional properties
CD4 epitopes			
Lasarte et al. [68]	core	99–112 (SPRGSRPSWGPTDP, HLA-DR); 146–159 (GAARALAHGVRVLE, HLA-DR)	Epitopes recognized by IL-2 secreting Th cells in IFN- α treatment responders
Schulze zur Wiesch et al. [66]	NS3	1209–1219 (VFTDNSSPPVV, HLA-DRB3*0201); 1251–1260 (VLVLNPSVAA, HLA-DRB1*0101/0401/1104 & DRB3*0101); 1542–1550 (YMNTPLPV; HLA-DRB1*0701); 1587–1598 (VAYQATVCARAQ; HLA-DRB1*1001)	Broad specificity to NS3/4/5 proteins identified by proliferation and IFN- γ secretion in recovered individuals
	NS4	1775–1785 (LAGLSTLPGNP, HLA-DRB1*0401/0404/0407/1104); 1913–1922 (VQWMNRLIAF, HLA-DRB1*1104); 1915–1924 (WMNRLIAFAS, HLA-DRB1*1001)	
	NS5	2273–2286 (EILRKSRRFAQALP, HLA-DRB1*1104); 2423–2436 (SYSWTGALVTPCAA; HLA-DRB1*0701); 2577–2588 (ARLIVFPDLGVR, HLA-DRB1*0404/0407); 2944–2954 (YLFNWAVRTKL, HLA-DRB1*1104)	
Antibody epitopes			
Law et al. [72]	E2	396-424/436–447/523–540 (conformational epitope)	Conserved cross-genotype, neutralizing antibody epitope
Meunier et al. [70]	E1	313–327 (ITGHRMAWDMMMNWS)	Conserved cross-genotype, neutralizing antibody epitope
Perotti et al. [71]	E2	412–423/528–535 (conformational epitope)	Conserved cross-genotype, neutralizing antibody epitope
Zhang et al. [43]	E2	412–426 (QLINTNGSWHINSTA)	Conserved cross-genotype, neutralizing antibody epitope
	E2	434–446	Unconserved interfering antibody epitope

Robust CD4⁺ T cells with broad specificity and function predict a spontaneous recovery in individuals with acute HCV infection. CD4⁺ T helper cells are important in shaping adaptive immune effectors like B cells and CD8⁺ T cells. Even with the critical role of CD8⁺ T cells in controlling HCV infection, a broad specificity of CD8⁺ T cells to HCV could be found in some patients with chronic HCV infection [63]. The difference between spontaneous resolving and chronic persistence seems to lie on the quality of the CD4⁺ T cell response [62–65]. When studying the specificity of CD4⁺ T cells in acute HCV infected individuals, individuals with spontaneously resolving HCV infection have CD4⁺ T cells specific to many different HCV epitopes compared to chronically infected individuals (Table 2) [62, 66, 67]. A similar study was reported in patients responding to IFN- α treatment (Table 2) [68]. When the function of CD4⁺ T cells was analysed, multifunctional CD4⁺ T cells with the capacity to secrete IL-2 and IFN- γ seemed to correlate better with HCV clearance during acute HCV infection. In contrast, acute HCV- infected individuals became chronically infected when their specific CD4⁺ T cells secreted no IL-2 [62, 65]. Therefore, multi-specific CD4⁺ T cells capable of secreting IL-2 and IFN- γ are critical in the generation of quality CD8⁺ T cell responses necessary for HCV eradication.

In spite of the general lack of protection with neutralizing antibodies to HCV, cross-genotype neutralizing antibodies seem to render protection against HCV infection. HCV-infected patients can develop anti-HCV antibodies to HCV core, NS3, NS4, and NS5 proteins as measured by a third generation of anti-HCV assays [69]. Neutralizing antibodies to HCV, however, are detected using E1 and E2 expressing HCV pseudotype particles (HCVpp) [35]. Since E1 and E2 proteins are present on the surface of HCV virions and are critical for viral entry into hepatocytes, specific antibodies to certain E1 and E2 regions, mostly IgG isotype, have neutralizing capacity [35, 36, 70]. New findings using human monoclonal antibodies derived from HCV infected patients demonstrated that specific antibodies against certain E1 and E2 epitopes have in vitro cross-genotype neutralizing capacity to HCVpp (Table 2) [70–72]. In addition, when these cross-genotype neutralizing antibodies were given to humanized mice following intravenous HCV challenge, these antibodies could offer passive protection and prevent HCV replication in vivo [72]. Furthermore, a study on intravenous drug users with resolved HCV infection demonstrated the contribution of broad neutralizing antibodies in HCV clearance. When these individuals with resolving infection experienced a secondary HCV infection, the majority of

them (83%) would clear the virus spontaneously compared to 25% of them in primary infection. When the specificity of neutralizing antibodies was analysed, neutralizing antibodies reacting to a broad range of genotypes were found in patients spontaneously recovered instead of those who became chronically infected [73].

Studies on patients responding to IFN- α -based therapy have revealed the importance of innate immunity in HCV clearance. Several genome-wide association studies on chronic HCV-infected patients have identified a strong genetic association of IL28B gene, which encoded for IFN- λ 3, on the responsiveness to standard IFN- α and ribavirin therapy. Three different studies analysing patient populations at Australian [74], Japan [75], and United States [56, 76] have demonstrated that the polymorphism at the upstream of IL28B is associated strongly with sustained virological response. Patients with genotype expressing more IL28 mRNA respond better to standard IFN therapy. This genetic variation of IL28B has also been shown to be associated with individuals who were infected by HCV and experienced spontaneously viral clearance [56]. When different geographic populations are compared, C allele (rs 12979860) occurs most often in individuals from Asia, then Europe, and least common from Africa origin. Since 36.4% of non-Africa individuals and only 9.3% of Africa individuals spontaneously clear HCV, it further confirms the association of C allele to HCV clearance [76]. IFN- λ 3 together with IFN- λ 1 (IL-29) and IFN- λ 2 (IL-28B), which act through the receptor complex consisting of IL-28R α and IL-10R β and then signal through JAK/STAT pathway, has very similar anti-viral effects as type I interferon (IFN- α and IFN- β). Although the importance of IFN- λ 3 on the immune system to combat HCV infection remains mostly unknown, IFN- λ can inhibit HCV replication in hepatoma cells (Huh-7.5) [77]. In addition, IFN- λ can enhance antiviral activities of IFN- α and vice versa, which suggest the possible mechanism of IL-28B polymorphism in the responsiveness of PEG-IFN and ribavirin therapy [77, 78]. A pilot study on chronic HCV patients received pegylated IFN- λ alone or with ribavirin has showed some promising results in HCV RNA reduction after 4 weekly subcutaneous injections [79]. Overall, these data suggest the benefit of IFN- λ in controlling HCV infection.

4. Vaccine Approach

The development of vaccines against HCV has been hampered greatly by the availability of research tools. Due to the limited tissue tropism and host selection, HCV could be generated in vitro in tissue culture system only very recently. Without tissue culture techniques, there would not have been enough viruses for vaccine antigens and immunological bioassay. The development of HCV pseudotype particles by genetically expressing E1&E2 in retrovirus vector [80] has successfully filled the gap before the discovery of cell culture HCV (HCVcc) [17]. Moreover, it facilitates the identification of various receptors for HCV entry. Currently, various vaccines are primarily tested in chimpanzees and humans. Through the identification of different receptors

for HCV entry, it allows the construction of a humanized mouse model, which expresses HCV entry receptors like occludin [22]. Although it is still early in the development, the availability of small animal models could accelerate the preclinical screening for potential vaccine candidates.

After the diagnostic kits for HCV became available, the implementation of HCV screening during blood transfusions and organ transplants has dramatically decreased the numbers of new cases of HCV infection. However, chronic HCV infection still presents in many individuals. This emphasizes the importance of therapeutic vaccination against HCV infection. One of the challenges dealing with chronic infection is to rescue impaired T cells. Thus, the goals of therapeutic vaccines are to generate broad and multi-specific CD4⁺ T cells, to activate cytotoxic CD8⁺ T cells and finally to generate cross-genotype neutralizing antibodies. Due to the variability of HCV, a combination approach including vaccination, anti-viral therapy or immune modulation might be necessary. Many vaccines have been tested by both nonprofit and profit organizations. Most of them are still at preclinical stage with some advanced into phase I or II trials to determine safety and efficacy of the candidate vaccines in a small group of patients. Earlier vaccine approaches aiming to generate neutralizing antibodies against E1 failed to show efficacy in chronic HCV patients in spite of its effect on antibody production. Consequently, the recombinant E1 with alum adjuvanted vaccine has been discontinued after an unsatisfactory outcome in its phase II trial [88]. Therefore, most of HCV vaccines are focused on generating cytotoxic CD8⁺ T cells in addition to antibody responses. Different vaccines have been developed over years including epitope vaccines, vector vaccines, recombinant protein vaccines, and DNA vaccines. A review on the progress and efficacy of vaccines currently in clinical trials is summarized in Table 3.

4.1. Epitope Vaccines. HCV peptide-based vaccines with different adjuvants are among the earliest vaccines aiming to induce Th1 and cytotoxic T cell response in chronic HCV patients. One of these, IC41, contains 5 conservative peptides from core, NS3 and NS4 proteins, which are conserved within HCV genotype 1 and 2, and include 4 known HLA-A2 epitopes and 3 promiscuous CD4⁺ epitopes. In a randomized, dose escalating phase I trial, 128 HLA-A2⁺ healthy volunteers received 4 subcutaneous vaccinations every 4 weeks. IC41 adjuvanted with poly-L-arginine was well tolerated by these healthy volunteers [89]. When this vaccine was given to 60 chronic HCV nonresponders, there were 67% of patients with specific T cell proliferation, 33% with specific IFN- γ -secreting CD4⁺ T cells, and 25% with specific IFN- γ -secreting CD8⁺ T cells. Three responders with the strongest IFN- γ -secreting T cells had a transient decline in serum HCV RNA (>1 log) [81]. Since the response was not efficient in controlling HCV viral load, IC41 would require further modifications by using more intense regimens and stronger adjuvants or could be incorporated into the combination therapy with PEG-IFN and ribavirin [90]. Other two peptide vaccines composed of peptides derived from conservative region of HCV with ISA51, an

TABLE 3: Main vaccines in clinical trials for HCV.

Vaccine	Subject	Stage	Outcome	Ref.
Peptides (core, NS3, NS4)/poly-L-arginine (IC41)	60 HLA-A2 ⁺ chronic HCV nonresponders	II	67% responding to peptide plus adjuvant treatment versus 17% to peptide alone; 3 patients with transient decline of serum HCV RNA (>1 log)	[81]
Peptide (core)/emulsified with ISA51	26 chronic HCV patients	I	Well tolerated with no severe toxicity; 15/25 responder; 2/25 with 1 log decline on HCV RNA	[82]
Peptides (NS3)/Virosome	30 healthy volunteers	I	No result released	NCT00445419
MVA-HCV NS3/NS4/NS5B (TG4040)	15 chronic HCV patients	I	Well tolerated; 6/15 with decline on HCV RNA (0.5–1.4 log)	[83]
HCV gpE1/E2 glycoproteins/MF59	60 healthy volunteers	I	No result released	NCT00500747
Recombinant yeast transfect with HCV NS3-core fusion protein (GI5005)	Chronic HCV patients	II	Well tolerate and showed better virology response in chronic patients after triple therapy	[84]
HCV core protein/ISCOMATRIX	30 healthy volunteers	I	Well tolerated with mild local redness; all developed antibody response, 7/8 showed cytokine production & 2/8 showed cytotoxic T cell response in the group with highest antigen dose (50 µg)	[85]
NS3/4A DNA vaccine (ChronVac-C)	12 chronic HCV patients	I/IIa	Safe, immunogenic with transient effect on serum viral load	[86]
Recombinant core protein & core/E1/E2 DNA vaccine (CIGB-230)	15 chronic HCV patients	I	Safe, immunogenic, and stabilized liver function with persistence detection of HCV RNA	[87]

emulsified incomplete Freud adjuvant, were shown to be safe in HCV-infected patients [82, 91]. Preliminarily, 26 patients received subcutaneous injection of a conserved peptide derived from HCV core (C35-44, YLLPRRGPRLL) biweekly. 15 of 25 patients showed an increase in peptide-specific CD8⁺T cell response measured by IFN- γ production and 2 patients demonstrated 1 log decrease in HCV viral load after 12 vaccinations. The clinical efficacy would require further validation in phase II trial [82]. In addition, another phase I trial with virosome-based vaccine containing NS3 peptides derived from HCV is ongoing. It is a single-blinded placebo-controlled randomised trial with 30 healthy volunteers to evaluate dose-dependent safety and vaccine-induced immune response (ClinicalTrials.gov Identifier: NCT00445419). No data from this clinical study have been released at this time. Overall, the response with peptide-based vaccines shows good tolerability but their efficacy remains to be optimised.

4.2. Vector Vaccines. HCV vaccines delivered by attenuated virus vectors could induce effective CD4⁺ and CD8⁺ T cell responses. Modified Virus of Ankara (MVA), a highly attenuated poxvirus strain, is immunogenic and safe compared to other strains of poxvirus due to the lack of several genes coding for immunomodulatory proteins, such as the soluble receptors for IFN- γ , type I IFN, TNF- α , and CC-chemokines [92]. It has been used in several different vaccine

designs, such as HIV, tuberculosis, colorectal cancer, and melanoma [93–96]. Owing to its high immunogenicity and cross-reactivity, individuals immunized with vaccinia virus or MVA-based vaccines have a strong antivector response. However, this preexisting immunity would not affect the induction of immunity against vectored antigens despite lower amount of specific T cells and antibodies to vectored antigens were observed [97]. Vaccines based on MVA vector expressing HCV antigens including NS3, NS4, and NS5B have been shown to induce IFN- γ -secreting CD4⁺ T cells and specific CD8⁺ T cells capable of secreting IFN- γ and killing in vitro and in vivo when tested in HLA-A2.1 and HLA-B7.2 transgenic mice [98]. The phase I trial in 15 chronic HCV patients who received 3 weekly injections demonstrated that MVA-HCV (TG4040) was well tolerated, and 6 of 15 patients showed a decline in HCV viral load (0.5–1.4 log) associated with significant increase in IFN- γ -secreting T cells [83]. Currently, a phase II trial has been proposed to treat chronic HCV patients in combinational therapy with PEG-IFN and ribavirin.

4.3. Recombinant Protein Vaccines. Recombinant HCV proteins require a strong Th1 adjuvant in order to generate specific T cell response to HCV. HCV E1/E2 glycoproteins emulsified with MF59, a proprietary oil-in-water emulsion adjuvant, have shown to induce a strong CD4⁺ T cell response with significant production of neutralizing antibodies to E1

and E2 in nonhuman primates [99]. No information has been released from one double-blinded placebo-controlled randomized trial with 60 healthy volunteers to evaluate dose-dependent safety and vaccine-induced immune response (ClinicalTrials.gov Identifier: NCT00500747). The vaccine, GI-5005, designed to treat chronic HCV infection is containing heat-killed yeast cells expressing conserved NS3-core fusion protein. Because of its yeast components, this vaccine can induce robust CD4⁺ and cytotoxic CD8⁺ T cell responses. In preclinical studies, vaccinated mice exhibited strong Th1 with IL-2 and IFN- γ production and cytotoxic activity to NS3 and core proteins measured by *in vitro* killing assay and *in vivo* tumor challenge experiment. Biweekly repeated administration could effectively improve their specific immune response [100]. With this successful pre-clinical result, phase I trial has also demonstrated its safety and shown to induce immune response in chronic HCV patients [101]. Phase II trial was designed to compare a combined therapy with GI-5005 and PEG-IFN/ribavirin to PEG-IFN/ribavirin alone in chronic HCV patients. At the end of 48-week treatment, patients received GI-5005 and PEG-IFN/ribavirin had 74% of response rate determined by HCV RNA less than 25 IU/mL in contrast to 59% without GI-5005. In addition, clinical tests have suggested a better liver function by ALT normalization after the combined therapy [84]. Another vaccine based on conserved HCV core protein is adjuvanted with ISCOMATRIX, an adjuvant composed of saponin, cholesterol, and phospholipid to form sphere particles with 40 μ m in diameter. The first phase I trial in 30 healthy volunteers provided the evidence for its safety and tolerability. As to the efficacy measured by immune response, 8 volunteers who received the highest antigen dose, 50 μ g, all showed antibody response to core protein, 7 with cytokine-producing T cells, and 2 with CD8⁺ T cell responses measurable by intracellular IFN- γ staining [85]. A phase Ib trial is prepared to evaluate its safety and immune response in chronic HCV- infected patients.

4.4. DNA Vaccines. DNA vaccines using naked DNA delivered by electroporation have been designed to treat chronic HCV patients. Due to the heterogeneity of HCV subtypes in most chronically infected patients, a DNA vaccine was designed to include the most conserved region including NS3 and NS4A. Through extensive codon modification, the DNA can be effectively expressed *in vivo* and elicit a Th1 response and cytotoxic response. These primed CD8⁺ T cells could effectively eliminate NS3/4A expressing hepatocytes and tumor cells in mouse model [102, 103]. This DNA vaccine (ChronVac-c) has been given to 12 patients with chronic HCV infection through intramuscular electroporation. Preliminary results suggested that the vaccine is safe and immunogenic after 4 monthly vaccinations. Two out of three patients received the highest dose of 1500 μ g showed a decrease in serum HCV RNA (1.2 & 2.4 log). Moreover, after completing the vaccination, three patients who received standard IFN- α -based therapy had an accelerated clearance in HCV viral load. Therefore, ChronVac-c has been proposed to treat chronic HCV patients in combination with standard IFN- α -based therapy [86]. Another DNA vaccine currently

in phase I clinical trial is CIGB-230, a mixture of recombinant HCV core protein and core/E1/E2-expressing plasmid DNA. Vaccination in 15 chronic HCV patients showed that this vaccine is safe, partially immunogenic, and able to stabilize liver histology despite persistent detection of HCV RNA [87].

The lessons from studying protective immunity against acute HCV infection have taught us the importance of multi-functional CD4⁺ T cells toward a broad spectrum of viral epitopes. With the help of these CD4⁺ T cells, the body can then generate functional cytotoxic T cells to eliminate virus-infected hepatocytes and produce neutralizing antibodies to prevent HCV from entering into uninfected cells [62–64]. The prototype of ideal vaccine would have to meet these requirements. HCV E1/E2 glycoprotein emulsified with MF59 can induce a strong CD4⁺ T cell response and neutralizing antibody to E1 and E2 [99]. Vaccination with defective alphaviral particles with RNA encoding for HIV gag has shown to generate a strong gag-specific CD8⁺ T cell response [104]. Through different adjuvant and antigen combinations, Lin et al. [105] have formulated a prime-boost regimen. Mice were first primed with E1/E2 glycoprotein, CpG, and MF59 to induce robust Th1 response, and followed by the boost vaccination with defective alphaviral particles with RNA encoding for HCV E1/E2/NS3-5 to generate a strong cytotoxic CD8⁺ T cell response. With this protocol, mice also generated neutralizing antibodies to E1/E2 capable of neutralizing heterogeneous HCV isolates. Although this vaccine approach remains to be evaluated in other pre-clinical trials, this would set a prototype for the next generation of HCV vaccines.

5. Conclusion

Various immunological parameters favoring HCV clearance have gradually been identified. Together with the knowledge on the strategies deployed by HCV, we now have a good picture on the war against chronic HCV infection. The issue would lie on how to use the information in various vaccine platforms the scientists worldwide have built for years. In addition to vaccine development, efforts on developing anti-viral drugs are underway. Several targets for drug development have been proposed including NS3-4A serine protease, NS5B RdRp, HCV 5'-NCR, HCV viral entry and fusion, and p7 ion channel. Among them, NS3-4A serine protease inhibitor has gone into clinical trial with edges on blocking viral replication and enhancing viral recognition by innate immunity. Since the nature of HCV in chronic infected patients is changeable, we would have to modify our strategy accordingly. A combination therapy including vaccination, anti-viral therapy like NS3-4A protease inhibitor, and immune modulation like IFN- α or IFN- λ would need to be tailored to meet individual requirements. With the help of NS3-4A protease inhibitor, antigen-presenting cells, especially DCs and Kupffer cells, and infected hepatocytes can now sense HCV infection by TLR3 and RIG-I pathways, which consequently activates innate and adaptive immune responses. Since the immune response to HCV is skewed in chronic HCV patients, it can be

redirected toward Th1 and cytotoxic T cell responses through the work of vaccines and immune modulators. Hence, the availability of multiple vaccines and treatment options is critical in treating chronic HCV patients.

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