Review Article

Immune Response of Cytotoxic T Lymphocytes and Possibility of Vaccine Development for Hepatitis C Virus Infection

Kazumasa Hiroishi, Junichi Eguchi, Shigeaki Ishii, Ayako Hiraide, Masashi Sakaki, Hiroyoshi Doi, Risa Omori, and Michio Imawari

Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan

Correspondence should be addressed to Kazumasa Hiroishi, hiroishi@med.showa-u.ac.jp

Immune responses of cytotoxic T lymphocytes (CTLs) are implicated in viral eradication and the pathogenesis of hepatitis C. Weak CTL response against hepatitis C virus (HCV) may lead to a persistent infection. HCV infection impairs the function of HCV-specific CTLs; HCV proteins are thought to actively suppress host immune responses, including CTLs. Induction of a strong HCV-specific CTL response in HCV-infected patients can facilitate complete HCV clearance. Thus, the development of a vaccine that can induce potent CTL response against HCV is strongly expected. We investigated HCV-specific CTL responses by enzyme-linked immuno-spot assay and/or synthetic peptides and identified over 40 novel CTL epitopes in the HCV protein. Our findings may contribute to the development of the HCV vaccine. In this paper, we describe the CTL responses in HCV infection and the attempts at vaccine development based on recent scientific articles.

1. Introduction

Hepatitis C virus (HCV) was first identified in 1989 [1]. The HCV is a member of the flavivirus family and is a type of positive-strand RNA virus. The discovery of HCV contributed to the diagnosis of hepatitis C; further, HCV has been implicated in many chronic non-A and non-B hepatitis infections. This virus spreads through needles used for vaccination or drug administration, and about 180 million people in the world are presumed to be infected with HCV. It has been clarified that HCV infection often persists, causing chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).

Cytotoxic T lymphocyte (CTL) plays a part in viral eradication [2]. These cells have been also implicated in the immunopathogenesis of viral infection [3], because HCV, by itself, does not produce cytopathic effects in hepatocytes directly. It has been thought that hepatitis is caused by the destruction of HCV-infected hepatocytes by immune cells such as natural killer (NK) cells and CTLs. Thus, the investigation of the roles of CTL in immunopathogenesis of HCV would contribute to the development of a new treatment strategy for HCV-induced hepatitis.

Interferon (IFN) therapy alone or with ribavirin and polymerase/protease inhibitor combination therapy has shown positive outcomes in more than 80% of patients with acute HCV infection and 50% of patients with chronic HCV infection. However, IFN causes severe adverse effects including flu-like symptoms, pancytopenia, hyperglycemia, depression, lung fibrosis, and cerebral bleeding. Therefore, there is an urgent need to establish an alternative therapy, which can afford a high rate of sustained virological response and performed with few adverse effects. Immunotherapy with HCV vaccine is one of the candidates of such therapies.

In this review, we have summarized the findings of recent investigations on CTL responses against HCV and the trials for the development of HCV vaccine.
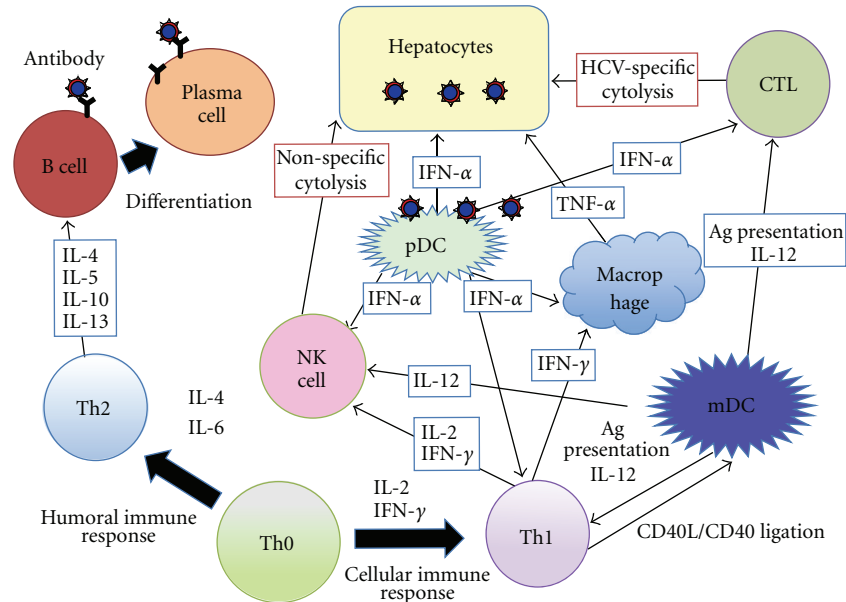
FIGURE 1: Cellular and humoral immune responses in HCV infection. Plasmacytoid dendritic cells (pDC) recognize HCV infection and produce IFN-α, which activates natural killer (NK) cells, helper T (Th) cells, macrophages, and cytotoxic T lymphocytes (CTLs). Activated NK cells destroy the HCV-infected hepatocytes in a nonspecific manner, whereas CTLs destroy the infected hepatocytes in an antigen-specific manner. Myeloid dendritic cells (mDC), which recognize dead hepatocytes, secrete IL-12, promoting the activation of NK cells, Th1 cells, and CTLs. Activated Th1 cells, in turn, promote DC maturation by interacting with the CD40/CD40 ligand. Macrophages stimulated by type 1 helper T (Th1) cells produce TNF-α, which accelerates local inflammation. In humoral immune responses, Th2 cells activate B cells. Plasma cells differentiated from B-cells secrete immunoglobulins to neutralize the circulating HCV. Abbreviated terms: CTL, cytotoxic T lymphocyte; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; Th1 cell, type 1 helper T cell; Th2 cell, type 2 helper T cell; NK cell, natural killer cell; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

2. CTL Responses in HCV Infection

2.1. Innate Immune Responses in HCV Infection.

HCV infection induces cellular and humoral immune responses (Figure 1). Similar to other viral infections, nonspecific immune responses are induced in the early stages of HCV infection for the eradication of HCV. Type I IFNs produced by HCV-infected hepatocytes and plasmacytoid dendritic cells (DCs) suppress viral replication by inducing enzymes such as 2′–5′ oligoadenylate synthetase (OAS) and RNA-dependent protein kinase (PKR) in hepatocytes [4]. The plasmacytoid DC recognizes HCV infection through toll-like receptor (TLR)-7, which interacts with single-stranded RNA [5]. The TLR-signaling upregulates PDC-TREM molecules on the cell surface, and PDC-TREM-dependent signal induces further production of IFN-α [6]. Activated OAS destroys viral RNAs, whereas PKR inhibits forming polysome of viral mRNA [4]. Moreover, type I IFNs activate innate immunity components such as natural killer (NK) cells [7]. The local inflammation further activates natural killer T-cells (NKT cells) and macrophages (Kupffer cells), thereby inducing the production of cytokines such as IFN-γ and tumor necrosis factor (TNF)-α. Hepatitis is thought to be initiated in this manner, and specific immune responses are generated if innate immune responses fail to eradicate HCV.

2.2. HCV-Specific Immune Responses and Immunopathogenesis of HCV-Specific CTLs.

The process of HCV-specific CTL induction and the destruction of HCV-infected hepatocytes by CTLs are shown in Figure 2. The destruction of HCV-infected hepatocytes releases HCV fragments; these fragments are taken up by myeloid DCs, consequently activating the DCs. These DCs migrate to the draining lymph nodes and express HCV antigens on human leukocyte antigen (HLA) class II molecules. Then, they enhance expression of costimulatory molecules (CD80, CD86) that interact with and activate antigen-specific helper T (Th) cells [8]. In turn, the activated Th cells promote the maturation of DCs by the expression of CD40 ligand and TNF-α. Subsequently, mature DCs stimulate specific CTLs by antigen presentation on HLA class I molecule and enhance the expression of costimulatory molecules [8]. Cytokines such as IL-2 and IL-12 produced by Th1 cells and DCs further promote CTL activation. These CTLs infiltrate the liver and recognize HCV antigens presented on the surface of HCV-infected hepatocytes together with HLA class I molecules. Then, the effector CTLs release perforin, granzyme, and TNF-α, or express Fas ligand, and initiate a direct attack on HCV-infected hepatocytes [9, 10]. In the previous study, we demonstrated that Fas ligand and TNF-α can also destroy noninfected hepatocytes in the vicinity of the HCV-infected cells [11].

When appropriate CTL responses are induced in hosts, HCV eradication is achieved. However, HCV-specific CTL responses are usually not strong enough to eradicate the virus, hence contributing to persistent infection. On the
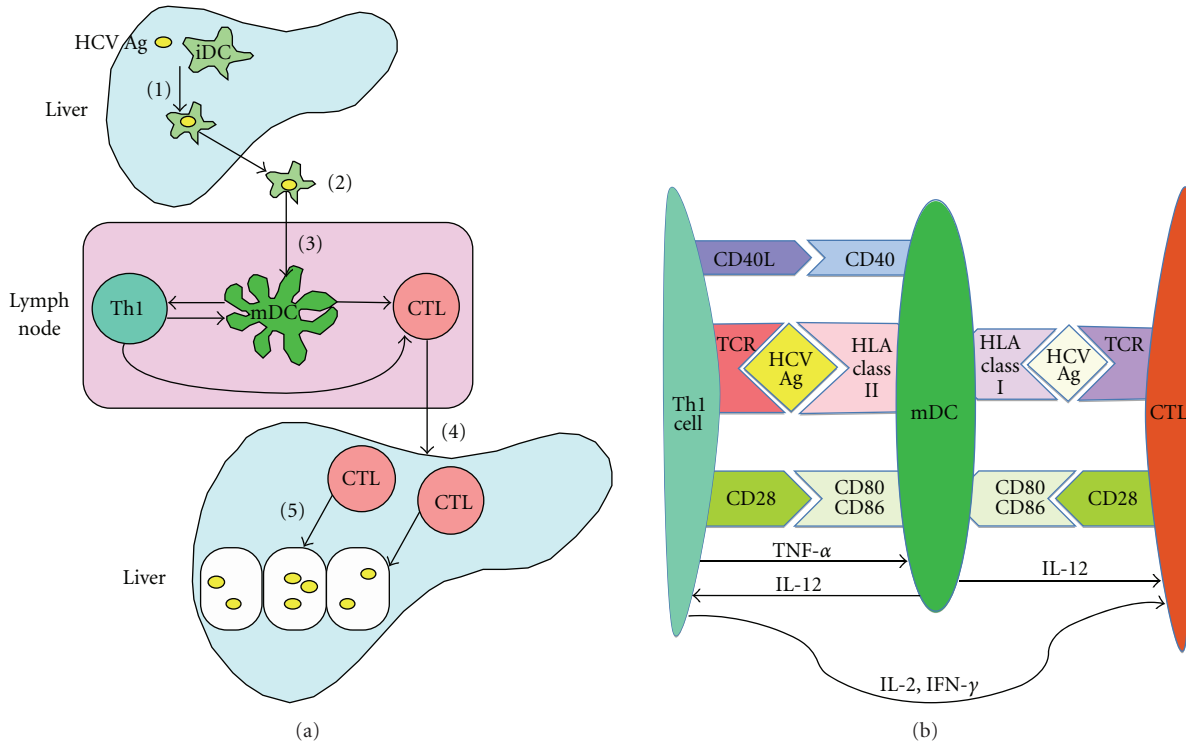
FIGURE 2: Destruction of HCV-infected hepatocytes by CTLs. (1) Immature myeloid dendritic cells (iDC) take up hepatitis C virus antigens (HCV Ag) in the liver. (2) The DCs move to a draining lymph node. (3) Matured DCs activate naïve helper T (Th) cells efficiently through stimulation with HLA class II, costimulatory molecules (CD80 and CD86), and cytokines such as IL-12. The stimulated Th cells, in turn, activate DCs by expressing CD40 ligand and secreting TNF-α. IL-12 produced by myeloid DCs differentiates these stimulated Th cells towards Th1 cells. Naïve cytotoxic T lymphocytes (CTLs) recognize the HCV Ag presented on the DCs. IL-2 and IFN-γ secreted by activated Th1 cells induce the activation and proliferation of the HCV-specific CTLs. (4) The stimulated HCV-specific CTLs leave the lymph nodes and move toward the liver. (5) They recognize HCV antigens together with HLA class I on the surface of HCV-infected hepatocytes, and try to eradicate HCV by killing the infected hepatocytes. Abbreviated terms: Th1 cell, type 1 helper T cell; mDC, myeloid dendritic cells; CTL, Cytotoxic T lymphocyte; CD, cluster of differentiation; CD40L, CD40 ligand; TCR, T-cell receptor; HLA, human leukocyte antigen; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon.

other hand, markedly potent immune responses would lead to severe hepatitis and fulminant hepatitis as proven in a hepatitis B virus (HBV) model [12], although this is a rare event in HCV infection.

We evaluated the relation between HCV-specific CTL responses and the clinical course of acute HCV infection and found that HCV eradication cannot be predicted on the basis of a strong CD8$^+$ T-cell response [13]. However, Lauer et al. reported that potent and broad CTL responses against HCV peptides were observed in patients with resolved infection but not in those with persistent infection [14]. Another report indicated that patients with complete resolution of HCV infection exhibited broader CTL responses with higher functional avidity and wider cross-recognition ability than patients with persistent HCV infection [15]. The opposite observations can be attributed to the differences in the monitoring methods of the CTL responses. Race and HCV genotype might also affect the contradiction of the results. Further investigation is needed to clarify this issue.

We analyzed the immune response of chronic HCV patients by studying their HLA-B44-restricted CTLs that recognized the HCV core amino acid residues 88–96; the CTL response and viral load were found to be inversely correlated [16]. The findings of this study suggested that HCV-specific CTLs may inhibit HCV replication. Otherwise, as many reports have suggested that HCV protein impairs the CTL responses by several mechanisms (see Section 3), HCV infection with a high titer of HCV RNA may suppress the HCV-specific CTLs by an excess of HCV antigens. No relation between other CTL responses recognizing other HCV epitopes and the HCV status was found in the study. From the data, it was supposed that the HLA-B44-restricted CTLs recognizing HCV core amino acid residues 88–96 were immunodominant.

Hence, there is a need to investigate HCV-specific CTL responses and clarify some issues. First, HCV exists as quasispecies in hosts and it has a high replicative ability and low fidelity RNA polymerase [17]. Thus, many HCVs with mutations in different amino acid sequences in the epitopes may be present in the host. Other issue is that most HCV-specific CTLs may infiltrate and compartmentalize in the host liver where inflammation occurs, and thus, only a few circulating HCV-specific CTLs can be detected. Although it is very crucial to investigate liver-infiltrating CTLs, the difficulty associated with obtaining liver specimen limits such study.
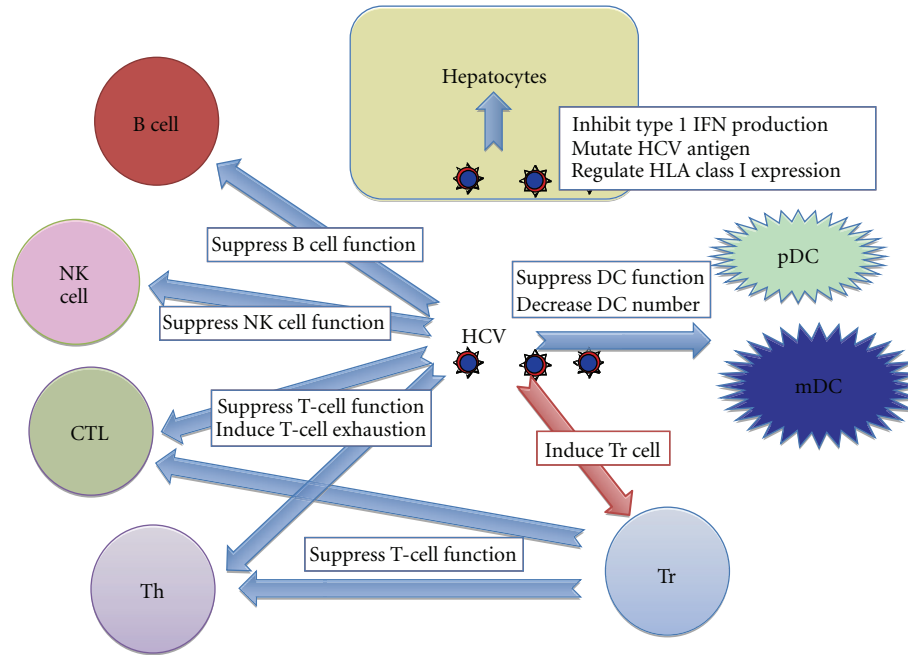
FIGURE 3: Immune suppressive mechanisms in HCV infection. HCV mutates its amino acid sequence to escape from immune surveillance, inhibits type 1 IFN production, and suppress NK cell function, T-cell function, and DC function. In addition, HCV induces Tr cells, which suppress T-cell function. Abbreviated terms: CTL, Cytotoxic T lymphocyte; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; Th cell, helper T cell; NK cell, natural killer cell; IFN, interferon.

3. Immunosuppression in HCV Infection

3.1. Escape from Immune Surveillance of Cellular Immune Responses. It was reported that amino acid mutations have been detected in the immunodominant regions of HCV in all patients with acute HCV infection, and mutations by which HCV escapes from CTL surveillance have been observed only in patients with viral persistence [18]. Hughes et al. investigated the variable intensity of purifying selection on CTL epitopes, and reported that the purifying selection of CTL epitopes on nonenvelop proteins was strong, particularly when the epitope was matched [19]. Since a variety of CTLs are induced in the early stage of HCV infection, a single amino acid mutation within a CTL epitope does not appear to contribute to persistent infection. It is supposed that escape mutation is a result rather than a cause of persistent HCV infection.

3.2. Impaired Function of CTL in HCV Infection. HCV inhibits cellular immune responses in the host by several ways; immune suppressive mechanisms in HCV infection are summarized in Figure 3.

In our study, the stimulation of peripheral blood lymphocytes of HCV-infected patients with synthetic peptides corresponding to CTL epitopes revealed that patients who were infected with HCV within the past 3 years exhibited CTL responses, while those infected with HCV more than 10 years ago did not exhibit this response. There are some reasons why HCV persistence is so common although a variety of HCV-specific CD8+ T-cells can be detected in the

liver and peripheral blood. The impaired function of HCV-specific CTLs as effector cells is due to the reduced expression of CD3 ζ chain [20], defective IFN-γ production, low perforin content, and decreased capacity for proliferation and cytotoxicity [21]. Incomplete differentiation of the memory CTLs to effector cells in patients with acute HCV infection may be due to IL-2 deficiency during T-cell activation [22]. Programmed cell death 1 (PD-1) receptor, the ligation of which inhibits the function of effector T-cells, is upregulated on exhausted CD8+ cells in patients with acute and chronic hepatitis C [23–25]. Another inhibitory receptor, namely, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), has also been reported to be upregulated on PD-1+ T-cells in the liver of HCV patients. The blockade of both these molecules is critical for the restoration of the function of HCV-specific effector cells [26].

Accumulated data have suggested that HCV itself actively suppresses host immune responses. Although spontaneous liver disease did not occur in mice expressing liver-targeted HCV NS5A transgene, both innate and adaptive immune responses were impaired [27]. HCV core protein inhibits IL-2 and IL-2 receptor α gene transcription [28], T-cell activation and proliferation, and IFN-γ production by T cells [29, 30]. HCV NS4A/B protein blocks the expression of HLA class I molecules [31].

Impaired function of DCs, which play the crucial role of antigen-presenting cells in inducing immunity, may be responsible for the impaired immune responses. It has been reported that the HCV core, E1, and NS3 proteins inhibit DC maturation [32, 33]. HCV is thought to infect DCs through the binding of HCV E2 protein and thereby suppress
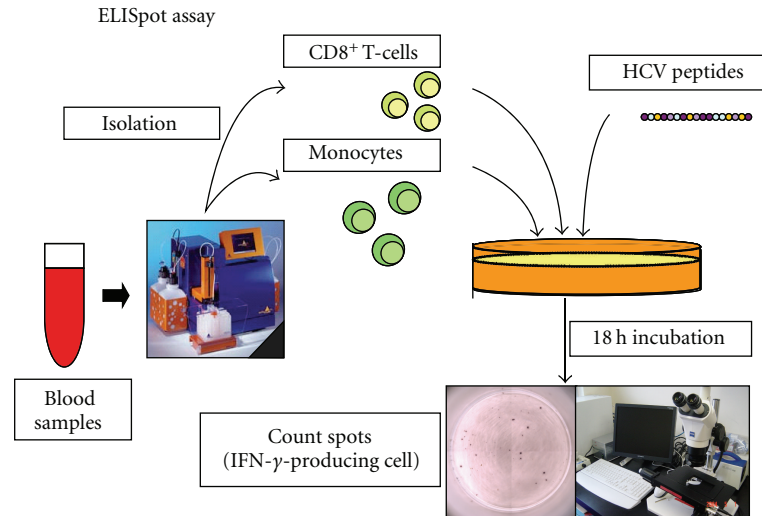
FIGURE 4: The procedure of enzyme-linked immuno-spot (ELISpot) assay. To detect CTL responses against HCV, we performed IFN-γ-based ELISpot assay. CD8$^+$ cells and monocytes were separated from peripheral blood samples by magnetic beads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and used as effector cells and antigen-presenting cells, respectively. These cells with synthetic HCV peptides were incubated for 18 h at 37°C in 5% CO_2 atmosphere. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted.

DC function [34, 35]. In addition, long-term ethanol consumption impairs CTL responses to HCV protein and subsequently alters DC function [36].

Regulatory T- (Tr) cells are also involved in HCV persistence. It has been shown that Tr cells (CD4$^+$ CD25$^+$ T cells) directly suppress T-cell function in chronic hepatitis C patients [37]. Forkhead box P3 (FOXP3)-positive Tr cells and IL-10 producing HCV-specific Tr cells infiltrate the liver of chronic HCV patients, and IL-10 mediates immune suppression in these patients [38, 39]. HCV core-specific Tr cells can be induced from the peripheral blood of patients with chronic hepatitis C [40].

4. Immunotherapy for Hepatitis C

4.1. IFN Therapy and Immune Response. Currently, chronic HCV infection can be resolved only with IFN-α-based therapy. IFN-α has been reported to have biologic effects on the immune system [41]. IFN-α upregulates HLA class I molecules on the cell surface. This cytokine appears to favor the proliferation of type 1 Th cells and activate CTLs. Ribavirin, which is used in combination with IFN-α, exerts an antiviral effect that drives the Th2 response towards a Th1 response [42]. During the primary immune response, IFN-α promotes both clonal expansion and survival of antigen-specific CTLs in vivo [43]. We also demonstrated that IFN-α prevents activation-induced cell death of CTLs [44]. A low dose of IFN-α augments cellular immune response, whereas a high dose suppresses CTL response [45]. Recently, it has been reported that although IFN-α upregulates MHC class I expression on hepatocytes, it reduces their sensitivity to CTL cytotoxicity, which may be due to the enhancement of granzyme-B inhibitor-proteinase inhibitor 9 (PI-9) expression [46]. Although it has been

reported that intrahepatic and peripheral HCV-specific CTL activity was detected more often in patients with a sustained response to IFN therapy than in patients who relapsed or did not respond to the treatment [47], further study is needed to clarify the effect of IFN therapy on host immune responses in vivo.

4.2. Identification of Novel Epitopes Recognized by HCV-Specific CTLs. As described above, we first identified an HLA B44-restricted CTL epitope [48, 49]. Then, we tried to identify more novel CTL epitopes in the HCV polyprotein, and performed IFN-γ-based enzyme-linked immuno-spot (ELISpot) assay [50, 51]. The procedure of this assay is presented in Figure 4. We synthesized 297 20-mer peptides overlapping by 10 residues and spanning the entire HCV sequence based on the amino acid sequence of HCV [13]. After separation with magnetic beads, we used CD8$^+$ T-cells as effector cells and monocytes as antigen-presenting cells. After the CD8$^+$ T-cells were incubated with the monocytes and the synthetic HCV peptides for 18 hours, IFN-γ-producing cells were counted. This procedure enabled to minimize the IFN-γ production for nonspecific response. Then, we identified more than 20 CTL epitopes in the HCV protein by using the synthetic peptides (Table 1). Furthermore, our group has identified several epitopes of HCV-specific CTLs using synthetic peptides and recombinant vaccinia viruses [52].

The HLA-24 allele of HLA class I is more common among the Japanese population. Thus, CTL induction by synthetic peptides based on HLA-A24 binding motifs has been investigated mainly in Japan [53]. HCV NS5A 2132–2142 peptide corresponding to the HLA-A24 binding motif has been reported to be able to induce both cellular and humoral immune responses in most HCV-positive patients

TABLE 1: CTL epitopes identified by using different procedures.

(a) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV sequence of genotype 1b

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt1	A*0207,2601 B*3501,4601 CW*0102,0303	NS3	1527–1546	WYELTPAETTVRLRAYLNTP	B*3501? A*2601?
		NS5B	2591–2605	KMALYDVVSTLPQAV	A*0207?
Pt2	A*2402,3303 B*4403,5401 CW*0803,1403	E1	332–351	LVVSQLLRIPQAVVDMVAGA	B*5401?
		NS3	1638–1656	THPITKFVMACMSADLEVV	B*5401?
		NS5B	2591–2605	KMALYDVVSTLPQAV	n.d.
Pt3	A*2602,3101 B*5101,5102 Cw1402,1502	NS3	1373–1380	IPFYGKAI	B*5101? B*5102?
Pt4	A*2402 B*0702,5201 Cw*0702,1202	E2	611–618	YPYRLWHY	n.d.
Pt5	A*1101,3101 B*6701,5101 Cw*0702,1401	NS5A	2290–2298	RPDYNPPLL	B*6701? B*5101?
Pt6	A*2402,2601 B*4002 Cw*0304	NS2	957–964	RDWAHAGL	B37
		NS5A	2122–2130	FTELDGVRL	n.d.
Pt7	A*2402,3303 B*0702,3501 Cw*0303,0702	Core	91–110	LGWAGWLLSPRGSRPSWGPT	A*3303? B*3501?
Pt8	A*2402 B*4801,5201 Cw*0803,1202	NS3	1643–1656	KFVMACMSADLEVV	n.d.
Pt9	A*2402 B*5201 Cw*1202	NS4	1760–1768	FWAKHMWNF	A*2402
		NS5B	2556–2564	TIMAKNEVF	n.d.
		NS5B	2803–2811	LTRDPTTPL	n.d.
Pt10	A*0201,0301 B*4402,4601 Cw*0102,0501	NS4	1958–1977	KRLHQWINEDCSTPCSGSWL	n.d.
Pt11	A*1101,2601 B*1501,5201 Cw*0401,1202	NS4	1858–1867	GVAGALVAFK	A*1101?
Pt12	A*2402 B*3501,4002 Cw*0303,0304	NS3	1618–1626	LHGPTPLLY	A*2402?

(b) CTL epitopes identified by HCV-derived synthetic peptides with binding motif of HLA-A24 [51]

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt13	A*2402,1101 B*3902,5201 Cw*0702,1202	NS3	1375–1385	FYGKAIPIEAI	n.d.
Pt14	A*2402,2601 B*4006,5401 Cw*0801,0803	E1	284–293	VFLVSQLFTF	n.d.
		E2	790–798	LYGVWPLLL	Cw*0801
		NS4	1759–1768	AFWAKHMWNF	n.d.
		NS5A	1990–1999	DFKTWLQSKL	n.d.
		NS5A	2280–2288	KFPPALPIW	A*2402
Pt15	A*2402,2601 B*3501,4002 Cw*0303,0304	NS2	910–919	PYFVRAQGLI	Cw*0303, 0304
		NS2	947–956	TYVYDHLTPL	B*4002
		NS3	1243–1252	AYAAQGYKVL	Cw*0303, 0304
Pt16	A*0206,2402 B*5201,5901 Cw*0102,1202	NS3	1443–1451	GFTGDFDSV	A*0206
Pt17	A*2402,3101 B*4801,5101 Cw*0304,0801	E2	790–798	LYGVWPLLL	Cw*0801
Pt18	A*2601,3101 B*3501,5101 Cw*0303,1402	NS5B	2456–2466	VYSTTSRSASL	n.d.

(c) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV sequence [13]

	HLA class I alleles	Region	Amino acid residues	Sequence	HLA restriction
Pt19	A*2602,3101 B*5101,5102 C*1402,1502	NS3	1373–1380	IPFYGKAI	n.d.
Pt20	A*0402 B*0702,5201 C*0702,1202	E2	611–618	YPYRLWHY	n.d.
Pt21	A*1101,3101 B*6701,5101 C*0702,1402	NS5A	2290–2298	RPDYNPPLL	n.d.
Pt22	A*2402 B*5201 C*1202	NS4	1759–1768	AFWAKHMWNF	n.d.
		NS5B	2556–2564	TIMAKNEVF	n.d.
		NS5B	2803–2811	LTRDPTTPL	n.d.
Pt23	A*0201,0301 B*4402,4601 C*0102,0501	NS4	1958–1977	KRLHQWINEDCSTPCSGSWL	n.d.
Pt24	A*2402,4801 B*5201 C*0803,1202	NS3	1637–1656	LTHPITKFVMACMSADLEVV	n.d.

(d) CTL epitopes identified by peptides overlapping by 10 residues and spanning the entire HCV core sequence

Region	Amino acid residues	Sequence	HLA restriction	Reference
core	88–96	NEG(L,M,C)GWAGW	B*4403	[49]
core	28–36	GQIVGGVYL	B60	[50]

(e) CTL epitopes identified by HCV-derived synthetic peptides with binding motif of HLA-B*4403

Region	Amino acid residues	Sequence	HLA restriction	Reference
NS5a	2095–2103	AEVTQHGSY	B*4403	[16]

(f) CTL epitopes identified by comprehensive CTL induction from PBMC of HCV patients

Region	Amino acid residues	Sequence	HLA restriction	Reference
NS3	1373–1380	IPFYGKAI	B*5603	[52]

with HLA-A24 [54]. Three novel vaccine candidate peptides capable of CTL induction and antibody production have also been identified [55]. In the study, the HCV core 30–39 peptide was shown to induce peptide-specific CTLs from peripheral blood mononuclear cells (PBMCs) of patients with HLA-A11, -A31, or -A33.

Yerly et al. [56] developed a novel "epitome" approach and analyzed its in vitro performance. This approach compresses the common immune targets of HCV-specific cellular immune response into a short immunogen sequence and may be applied to induce cellular immune responses against highly variable antigens.

The most important concern in peptide vaccine development is the selection of peptides from among the CTL epitopes because some peptides may rather induce tolerance of effector cells [57] or Tr cells, which will result in immune suppression. Hence, it is necessary to develop tailor-made therapy using appropriate peptides according to the HLA haplotypes of the patients.

4.3. Trials for the Development of HCV Vaccine. Many attempts for inducing immune responses against HCV by vaccination have been performed using animal models. Splenocytes isolated from mice pretreated with Fms-like tyrosine kinase receptor 3 ligand exhibited NS5-specific cellular immune responses after vaccination with DCs containing magnetic beads coated with HCV NS5, lipopolysaccharide, and anti-CD40 antibody [58, 59]. It has been reported that the adoptive transfer of HCV NS3 protein-pulsed mature DCs could effectively promote potent HCV-specific protective immune responses in a mouse model [60]. From the data, DC-based therapy appears to be one of the candidates for immune therapy against HCV infection.

Since HCV envelope glycoproteins are heavily glycosylated, such modification would affect immune responses in hosts. The engineering of N-glycosylation of HCV E2 protein enhances HCV-specific cellular immune responses [61], whereas the deletion of N-glycosylation sites of HCV E1 protein augmented HCV-specific cellular and humoral immune responses [62].

Gene therapy has been tried to elicit strong immune responses in vivo. It has been reported that vector-based minigene encompassing 4 domains of HCV NS3, NS4, and NS5B proteins effectively induced CTL induction in HLA-A2 transgenic mice [63]. Using replication-incompetent adenoviruses expressing HCV core and NS3 proteins, HCV-specific CTLs could be induced from PBMCs of HCV-infected patients [64]. Administration of recombinant yeast cells producing HCV NS3-core fusion protein, namely, GI-5005, induced potent antigen-specific proliferative and CTL responses in mice [65]. As described above, gene therapy would be a candidate for HCV vaccine. However, a careful survey for adverse effects induced by the therapy must be performed before clinical application.

Adjuvants may help the induction of HCV-specific CTLs, and it is important to investigate what adjuvant we should use for HCV vaccination. Protein immunization using CpG and montanide ISA 720 have been reported to enhance HCV-specific Th-1 type immune responses [66]. Cytokines such as granulocyte-monocyte colony stimulating factor and IL-23 have been also used for argument of immune responses induced by HCV core vaccination [67]. In a mouse model, HBV precore protein enhanced HCV-specific CTL responses induced by the genetic immunization of DNA encoding truncated HCV core proteins [68]. In another model, HBs antigen enhanced the induction of HCV-specific CTLs by DNA vaccine harboring HCV CTL epitopes [69].

Not only animal experiments, but also several human trials have been proceeding. Yutani has reported a phase I study of HCV vaccine in Japanese patients who were either nonresponders to IFN therapy ($n = 23$) or had refused treatment ($n = 3$). A peptide derived from the HCV core region amino acid residues 35–44 is capable of inducing cellular immune responses in many patients with different HLA class I-A alleles [70]. This peptide was used to develop a series of 6 vaccine injections that enhanced the peptide-specific peripheral CTL activity in 15 out of 25 patients and 12 vaccine injections that augmented peptide-specific IgG production [71]. Improvement in serum alanine aminotransferase (ALT) level (>30% decrease) was also observed in 7 out of 24 patients in the study. The results revealed that the selection of candidate peptides is crucial for developing a successful HCV vaccine.

In another clinical trial of a synthetic peptide vaccine, IC41 containing the 7 relevant HCV-specific Th cell and CTL epitopes and the adjuvant poly-L-arginine were used. It has been reported that IC41 can induce HCV-specific responses in both Th1 cells and CTLs in patients not responding to or relapsing from IFN therapy [72, 73]. Although this vaccination was tolerated and induced serious adverse events, HCV RNA reduction was rarely observed in the study [73]. In the phase II trial of pegylated interferon plus ribavirin therapy in combination with this vaccine, an enhanced HCV-specific T-cell response was observed in 73% of patients, and the responses could be detected more frequently in patients with sustained virologic response than in those showing relapse [74].

A recent Phase I placebo-controlled study has revealed that a prototype vaccine, which consists of HCV core protein and the adjuvant ISCOMATRIX, induces cytokine production by T-cells, but CTL responses were detected in a few healthy individuals [75]. A tableted therapeutic bivalent vaccine, which consists of heat-inactivated HCV antigens derived from HBV- and HCV-infected donors, has been applied in the treatment of chronic hepatitis C patients. Oral administration of this vaccine showed no adverse effects, and the elevated liver enzyme levels observed before the study were reduced in all patients at the end of the study.

A therapeutic DNA vaccine developed using the mixture of plasmid expressing HCV structural antigens and a recombinant HCV core protein, namely, CIGB-230, has also been used to treat chronic hepatitis C patients who did not respond to previous IFN therapy in a Phase I study [76]. This vaccination induced specific T-cell responses in 73% of the participants. Interestingly, 40% of the vaccinated patients showed reduction in liver fibrosis.

5. Conclusions and Future Directions

Since HCV was first identified, many investigations have been performed to resolve and prevent HCV infection. It has been demonstrated that HCV-specific CTLs are implicated in not only viral eradication but also the immunopathogenesis of hepatitis C. Development of IFN-based therapy in combination with ribavirin and protease/polymerase inhibitor has improved the sustained viral response rate of patients. However, there are still many nonresponders who suffer from chronic hepatitis C, cirrhosis, and hepatocellular carcinoma. Moreover, the HCV infection mechanism in many patients is still unknown. For these patients, a novel immune therapy and vaccination should be urgently established. For this purpose, we have to continue further investigation of immune responses in HCV infection.

References

[1] Q.-L. Choo, G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton, "Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome," *Science*, vol. 244, no. 4902, pp. 359–362, 1989.

[2] K. L. Yap, G. L. Ada, and I. F. C. McKenzie, "Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus," *Nature*, vol. 273, no. 5659, pp. 238–239, 1978.

[3] R. M. Zinkernagel, E. Haenseler, T. Leist, A. Cerny, H. Hengartner, and A. Althage, "T cell-mediated hepatitis in mice infected with lymphocytic choriomeningitis virus. Liver cell destruction by H-2 class I-restricted virus-specific cytotoxic T cells as a physiological correlate of the 51Cr-release assay?" *Journal of Experimental Medicine*, vol. 164, no. 4, pp. 1075–1092, 1986.

[4] C. E. Samuel, "Antiviral actions of interferons," *Clinical Microbiology Reviews*, vol. 14, no. 4, pp. 778–809, 2001.

[5] Y.-J. Liu, H. Kanzler, V. Soumelis, and M. Gilliet, "Dendritic cell lineage, plasticity and cross-regulation," *Nature Immunology*, vol. 2, no. 7, pp. 585–589, 2001.

[6] H. Watarai, E. Sekine, S. Inoue, R. Nakagawa, T. Kaisho, and M. Taniguchi, "PDC-TREM, a plasmacytoid dendritic cell-specific receptor, is responsible for augmented production of type I interferon," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 8, pp. 2993–2998, 2008.

[7] A. Ahmad and F. Alvarez, "Role of NK and NKT cells in the immunopathogenesis of HCV-induced hepatitis," *Journal of Leukocyte Biology*, vol. 76, no. 4, pp. 743–759, 2004.

[8] J. Banchereau and R. M. Steinman, "Dendritic cells and the control of immunity," *Nature*, vol. 392, no. 6673, pp. 245–252, 1998.

[9] D. Kägi, F. Vignaux, B. Ledermann, et al., "Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity," *Science*, vol. 265, no. 5171, pp. 528–530, 1994.

[10] H. Kojima, N. Shinohara, S. Hanaoka, et al., "Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes," *Immunity*, vol. 1, no. 5, pp. 357–364, 1994.

[11] K. Ando, K. Hiroishi, T. Kaneko, et al., "Perforin, Fas/Fas ligand, and TNF-α pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL," *Journal of Immunology*, vol. 158, no. 11, pp. 5283–5291, 1997.

[12] K. Ando, T. Moriyama, L. G. Guidotti, et al., "Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis," *Journal of Experimental Medicine*, vol. 178, no. 5, pp. 1541–1554, 1993.

[13] H. Doi, K. Hiroishi, T. Shimazaki, et al., "Magnitude of CD8+ T-cell responses against hepatitis C virus and severity of hepatitis do not necessarily determine outcomes in acute hepatitis C virus infection," *Hepatology Research*, vol. 39, no. 3, pp. 256–265, 2009.

[14] G. M. Lauer, E. Barnes, M. Lucas, et al., "High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection," *Gastroenterology*, vol. 127, no. 3, pp. 924–936, 2004.

[15] D. Yerly, D. Heckerman, T. M. Allen, et al., "Increased cytotoxic T-lymphocyte epitope variant cross-recognition and functional avidity are associated with hepatitis C virus clearance," *Journal of Virology*, vol. 82, no. 6, pp. 3147–3153, 2008.

[16] K. Hiroishi, H. Kita, M. Kojima, et al., "Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection," *Hepatology*, vol. 25, no. 3, pp. 705–712, 1997.

[17] S. Zeuzem, "Hepatitis C virus: kinetics and quasispecies evolution during anti-viral therapy," *Forum*, vol. 10, no. 1, pp. 32–42, 2000.

[18] S. Guglietta, A. R. Garbuglia, L. Salichos, et al., "Impact of viral selected mutations on T cell mediated immunity in chronically evolving and self limiting acute HCV infection," *Virology*, vol. 386, no. 2, pp. 398–406, 2009.

[19] A. L. Hughes, M. A. K. Hughes, and R. Friedman, "Variable intensity of purifying selection on cytotoxic T-lymphocyte epitopes in hepatitis C virus," *Virus Research*, vol. 123, no. 2, pp. 147–153, 2007.

[20] A. Maki, M. Matsuda, M. Asakawa, H. Kono, H. Fujii, and Y. Matsumoto, "Decreased CD3 ζ molecules of T lymphocytes from patients with hepatocellular carcinoma associated with hepatitis C virus," *Hepatology Research*, vol. 27, no. 4, pp. 272–278, 2003.

[21] G. Missale, E. Cariani, and C. Ferrari, "Role of viral and host factors in HCV persistence: which lesson for therapeutic and preventive strategies?" *Digestive and Liver Disease*, vol. 36, no. 11, pp. 703–711, 2004.

[22] V. Francavilla, D. Accapezzato, M. De Salvo, et al., "Subversion of effector CD8$^+$ T cell differentiation in acute hepatitis C virus infection: exploring the immunological mechanisms," *European Journal of Immunology*, vol. 34, no. 2, pp. 427–437, 2004.

[23] S. Urbani, B. Amadei, D. Tola, et al., "PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion," *Journal of Virology*, vol. 80, no. 22, pp. 11398–11403, 2006.

[24] L. Golden-Mason, B. Palmer, J. Klarquist, J. A. Mengshol, N. Castelblanco, and H. R. Rosen, "Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8$^+$ T cells associated with reversible immune dysfunction," *Journal of Virology*, vol. 81, no. 17, pp. 9249–9258, 2007.

[25] L. Golden-Mason, J. Klarquist, A. S. Wahed, and H. R. Rosen, "Cutting edge: programmed death-1 expression is increased on immunocytes in chronic hepatitis C virus and predicts failure of response to antiviral therapy: race-dependent differences," *Journal of Immunology*, vol. 180, no. 6, pp. 3637–3641, 2008.

[26] N. Nakamoto, H. Cho, A. Shaked, et al., "Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade," *PLoS Pathogens*, vol. 5, no. 2, Article ID e1000313, 2009.

[27] M. Kriegs, T. Bürckstümmer, K. Himmelsbach, et al., "The hepatitis C virus non-structural NS5A protein impairs both the innate and adaptive hepatic immune response in vivo," *Journal of Biological Chemistry*, vol. 284, no. 41, pp. 28343–28351, 2009.

[28] Z. Q. Yao, D. T. Nguyen, A. I. Hiotellis, and Y. S. Hahn, "Hepatitis C virus core protein inhibits human T lymphocyte responses by a complement-dependent regulatory pathway," *Journal of Immunology*, vol. 167, no. 9, pp. 5264–5272, 2001.

[29] D. J. Kittlesen, K. A. Chianese-Bullock, Z. Q. Yao, T. J. Braciale, and Y. S. Hahn, "Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation," *Journal of Clinical Investigation*, vol. 106, no. 10, pp. 1239–1249, 2000.

[30] Z. Q. Yao, A. Eisen-Vandervelde, S. Ray, and Y. S. Hahn, "HCV core/gC1qR interaction arrests T cell cycle progression through stabilization of the cell cycle inhibitor p27Kip1," *Virology*, vol. 314, no. 1, pp. 271–282, 2003.

[31] K. V. Konan, T. H. Giddings Jr., M. Ikeda, K. Li, S. M. Lemon, and K. Kirkegaard, "Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus," *Journal of Virology*, vol. 77, no. 14, pp. 7843–7855, 2003.

[32] P. Sarobe, J. J. Lasarte, A. Zabaleta, et al., "Hepatitis C virus structural proteins impair dendritic cell maturation and inhibit in vivo induction of cellular immune responses," *Journal of Virology*, vol. 77, no. 20, pp. 10862–10871, 2003.

[33] G. Szabo and A. Dolganiuc, "Subversion of plasmacytoid and myeloid dendritic cell functions in chronic HCV infection," *Immunobiology*, vol. 210, no. 2–4, pp. 237–247, 2005.

[34] P.-Y. Lozach, H. Lortat-Jacob, A. de Lacroix de Lavalette, et al., "DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2," *Journal of Biological Chemistry*, vol. 278, no. 22, pp. 20358–20366, 2003.

[35] S. Pöhlmann, J. Zhang, F. Baribaud, et al., "Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR," *Journal of Virology*, vol. 77, no. 7, pp. 4070–4080, 2003.

[36] C. Aloman, S. Gehring, P. Wintermeyer, N. Kuzushita, and J. R. Wands, "Chronic ethanol consumption impairs cellular immune responses against HCV NS5 protein due to dendritic cell dysfunction," *Gastroenterology*, vol. 132, no. 2, pp. 698–708, 2007.

[37] T. Boettler, H. C. Spangenberg, C. Neumann-Haefelin, et al., "T cells with a CD4$^+$CD25$^+$ regulatory phenotype suppress in vitro proliferation of virus-specific CD8$^+$ T cells during chronic hepatitis C virus infection," *Journal of Virology*, vol. 79, no. 12, pp. 7860–7867, 2005.

[38] D. Accapezzato, V. Francavilla, M. Paroli, et al., "Hepatic expansion of a virus-specific regulatory CD8$^+$ T cell population in chronic hepatitis C virus infection," *Journal of Clinical Investigation*, vol. 113, no. 7, pp. 963–972, 2004.

[39] M. Sakaki, K. Hiroishi, T. Baba, et al., "Intrahepatic status of regulatory T cells in autoimmune liver diseases and chronic viral hepatitis," *Hepatology Research*, vol. 38, no. 4, pp. 354–361, 2008.

[40] A. J. MacDonald, M. Duffy, M. T. Brady, et al., "CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons," *Journal of Infectious Diseases*, vol. 185, no. 6, pp. 720–727, 2002.

[41] F. Belardelli, "Role of interferons and other cytokines in the regulation of the immune response," *APMIS*, vol. 103, no. 3, pp. 161–179, 1995.

[42] S.-H. Fang, L.-H. Hwang, D.-S. Chen, and B.-L. Chiang, "Ribavirin enhancement of hepatitis C virus core antigen-specific type 1 T helper cell response correlates with the increased IL-12 level," *Journal of Hepatology*, vol. 33, no. 5, pp. 791–798, 2000.

[43] D. F. Tough, P. Borrow, and J. Sprent, "Induction of bystander T cell proliferation by viruses and type I interferon in vivo," *Science*, vol. 272, no. 5270, pp. 1947–1950, 1996.

[44] K. Hiroishi, T. Tüting, and M. T. Lotze, "IFN-α-expressing tumor cells enhance generation and promote survival of tumor-specific CTLs," *Journal of Immunology*, vol. 164, no. 2, pp. 567–572, 2000.

[45] S. Gehring, S. H. Gregory, N. Kuzushita, and J. R. Wands, "Type 1 interferon augments DNA-based vaccination against hepatitis C virus core protein," *Journal of Medical Virology*, vol. 75, no. 2, pp. 249–257, 2005.

[46] C. B. Willberg, S. M. Ward, R. F. Clayton, et al., "Protection of hepatocytes from cytotoxic T cell mediated killing by interferon-alpha," *PLoS One*, vol. 2, no. 8, article e791, 2007.

[47] A. J. Freeman, G. Marinos, R. A. Ffrench, and A. R. Lloyd, "Intrahepatic and peripheral blood virus-specific cytotoxic T lymphocyte activity is associated with a response to combination IFN-α and ribavirin treatment among patients with chronic hepatitis C virus infection," *Journal of Viral Hepatitis*, vol. 12, no. 2, pp. 125–129, 2005.

[48] H. Kita, T. Moriyama, T. Kaneko, et al., "HLA B44-restricted cytotoxic T lymphocytes recognizing an epitope on hepatitis C virus nucleocapsid protein," *Hepatology*, vol. 18, no. 5, pp. 1039–1044, 1993.

[49] H. Kita, K. Hiroishi, T. Moriyama, et al., "A minimal and optimal cytotoxic T cell epitope within hepatitis C virus nucleoprotein," *Journal of General Virology*, vol. 76, no. 12, pp. 3189–3193, 1995.

[50] T. Kaneko, I. Nakamura, H. Kita, K. Hiroishi, T. Moriyama, and M. Imawari, "Three new cytotoxic T cell epitopes identified within the hepatitis C virus nucleoprotein," *Journal of General Virology*, vol. 77, no. 6, pp. 1305–1309, 1996.

[51] T. Hakamada, K. Funatsuki, H. Morita, et al., "Identification of novel hepatitis C virus-specific cytotoxic T lymphocyte epitopes by ELISpot assay using peptides with human leukocyte antigen-A*2402-binding motifs," *Journal of General Virology*, vol. 85, no. 6, pp. 1521–1531, 2004.

[52] K. Ito, K. Shiraki, K. Funatsuki, et al., "Identification of novel hepatitis C virus-specific cytotoxic T lymphocyte epiotpe in NS3 region," *Hepatology Research*, vol. 36, no. 4, pp. 294–300, 2006.

[53] T. Mashiba, K. Udaka, Y. Hirachi, et al., "Identification of CTL epitopes in hepatitis C virus by a genome-wide computational scanning and a rational design of peptide vaccine," *Immunogenetics*, vol. 59, no. 3, pp. 197–209, 2007.

[54] Y. Takao, A. Yamada, S. Yutani, et al., "Identification of new immunogenic peptides in conserved regions of hepatitis C virus (HCV) 1b with the potentiality to generate cytotoxic T lymphocytes in HCV1b$^+$HLA-A24$^+$ patients," *Hepatology Research*, vol. 37, no. 3, pp. 186–195, 2007.

[55] S. Matsueda, A. Yamada, Y. Takao, et al., "A new epitope peptide derived from hepatitis C virus 1b possessing the capacity to induce cytotoxic T-lymphocytes in HCV1b-infected patients with HLA-A11, -A31, and -A33," *Cancer Immunology, Immunotherapy*, vol. 56, no. 9, pp. 1359–1366, 2007.

[56] D. Yerly, D. Heckerman, T. Allen, et al., "Design, expression, and processing of epitomized hepatitis C virus-encoded CTL epitopes," *Journal of Immunology*, vol. 181, no. 9, pp. 6361–6370, 2008.

[57] T. Kaneko, T. Moriyama, K. Udaka, et al., "Impaired induction of cytotoxic T lymphocytes by antagonism of a weak agonist borne by a variant hepatitis C virus epitope," *European Journal of Immunology*, vol. 27, no. 7, pp. 1782–1787, 1997.

[58] S. Gehring, S. H. Gregory, P. Wintermeyer, M. San Martin, C. Aloman, and J. R. Wands, "Generation and characterization of an immunogenic dendritic cell population," *Journal of Immunological Methods*, vol. 332, no. 1-2, pp. 18–30, 2008.

[59] S. Gehring, S. H. Gregory, P. Wintermeyer, C. Aloman, and J. R. Wands, "Generation of immune responses against hepatitis C virus by dendritic cells containing NS5 protein-coated microparticles," *Clinical and Vaccine Immunology*, vol. 16, no. 2, pp. 163–171, 2009.

[60] H. Yu, H. Huang, J. Xiang, L. A. Babiuk, and S. van Drunen Littel-van den Hurk, "Dendritic cells pulsed with hepatitis C virus NS3 protein induce immune responses and protection from infection with recombinant vaccinia virus expressing NS3," *Journal of General Virology*, vol. 87, no. 1, pp. 1–10, 2006.

[61] P. Li, Q. Wan, Y. Feng, et al., "Engineering of *N*-glycosylation of hepatitis C virus envelope protein E2 enhances T cell responses for DNA immunization," *Vaccine*, vol. 25, no. 8, pp. 1544–1551, 2007.

[62] M. Liu, H. Chen, F. Luo, et al., "Deletion of N-glycosylation sites of hepatitis C virus envelope protein E1 enhances specific cellular and humoral immune responses," *Vaccine*, vol. 25, no. 36, pp. 6572–6580, 2007.

[63] P. Martin, B. Simon, Y.-C. Lone, et al., "A vector-based minigene vaccine approach results in strong induction of T-cell responses specific of hepatitis C virus," *Vaccine*, vol. 26, no. 20, pp. 2471–2481, 2008.

[64] D. Thammanichanond, S. Moneer, P. Yotnda, et al., "Fiber-modified recombinant adenoviral constructs encoding hepatitis C virus proteins induce potent HCV-specific T cell response," *Clinical Immunology*, vol. 128, no. 3, pp. 329–339, 2008.

[65] A. A. Haller, G. M. Lauer, T. H. King, et al., "Whole recombinant yeast-based immunotherapy induces potent T cell responses targeting HCV NS3 and Core proteins," *Vaccine*, vol. 25, no. 8, pp. 1452–1463, 2007.

[66] Q. Qiu, R. Y.-H. Wang, X. Jiao, et al., "Induction of multi-specific Th-1 type immune response against HCV in mice by protein immunization using CpG and Montanide ISA 720 as adjuvants," *Vaccine*, vol. 26, no. 43, pp. 5527–5534, 2008.

[67] C. Hartoonian, M. Ebtekar, H. Soleimanjahi, et al., "Effect of immunological adjuvants: GM-CSF (granulocyte-monocyte colony stimulating factor) and IL-23 (interleukin-23) on immune responses generated against hepatitis C virus core DNA vaccine," *Cytokine*, vol. 46, no. 1, pp. 43–50, 2009.

[68] G. Liao, Y. Wang, J. Chang, et al., "Hepatitis B virus precore protein augments genetic immunizations of the truncated hepatitis C virus core in BALB/c mice," *Hepatology*, vol. 47, no. 1, pp. 25–34, 2008.

[69] A. Memarnejadian and F. Roohvand, "Fusion of HBsAg and prime/boosting augment Th1 and CTL responses to HCV polytope DNA vaccine," *Cellular Immunology*, vol. 261, no. 2, pp. 93–98, 2010.

[70] Y. Niu, N. Komatsu, Y. Komohara, et al., "A peptide derived from hepatitis C virus (HCV) core protein inducing cellular-responses in patients with HCV with various HLA class IA alleles," *Journal of Medical Virology*, vol. 81, no. 7, pp. 1232–1240, 2009.

[71] S. Yutani, N. Komatsu, S. Shichijo, et al., "Phase I clinical study of a peptide vaccination for hepatitis C virus-infected patients with different human leukocyte antigen-class I-A alleles," *Cancer Science*, vol. 100, no. 10, pp. 1935–1942, 2009.

[72] V. Schlaphoff, C. S. Klade, B. Jilma, et al., "Functional and phenotypic characterization of peptide-vaccine-induced HCV-specific CD8$^+$ T cells in healthy individuals and chronic hepatitis C patients," *Vaccine*, vol. 25, no. 37-38, pp. 6793–6806, 2007.

[73] C. S. Klade, H. Wedemeyer, T. Berg, et al., "Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41," *Gastroenterology*, vol. 134, no. 5, pp. 1385–1395.e1, 2008.

[74] H. Wedemeyer, E. Schuller, V. Schlaphoff, et al., "Therapeutic vaccine IC41 as late add-on to standard treatment in patients with chronic hepatitis C," *Vaccine*, vol. 27, no. 37, pp. 5142–5151, 2009.

[75] D. Drane, E. Maraskovsky, R. Gibson, et al., "Priming of CD4$^+$ and CD8$^+$ T cell responses using a HCV core ISCOMATRIX^{TM} vaccine: a phase I study in healthy volunteers," *Human Vaccines*, vol. 5, no. 3, pp. 151–157, 2009.

[76] L. Alvarez-Lajonchere, N. H. Shoukry, B. Grá, et al., "Immunogenicity of CIGB-230, a therapeutic DNA vaccine preparation, in HCV-chronically infected individuals in a Phase I clinical trial," *Journal of Viral Hepatitis*, vol. 16, no. 3, pp. 156–167, 2009.