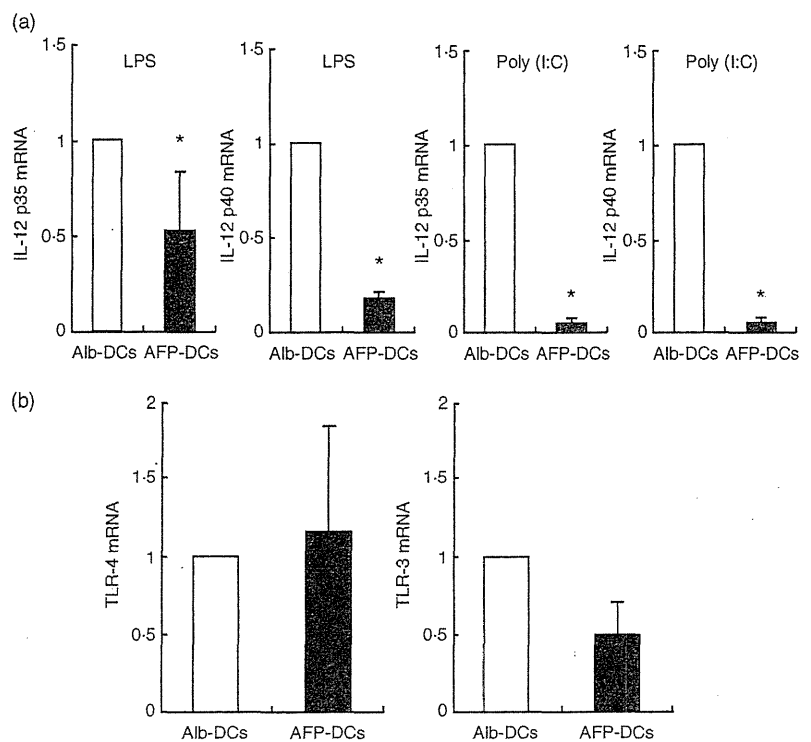


Fig. 5. The mRNAs of interleukin (IL)-12p35 and p40 from α -fetoprotein-dendritic cells (AFP-DCs) were lower than that from albumin (Alb)-DCs, but the mRNA of Toll-like receptor (TLR)-4 and TLR-3 were not. We cultured DCs for 7 days in RPMI-1640 with AFP (25 μ g/ml) or Alb (25 μ g/ml). On day 6, we added lipopolysaccharide (LPS) (10 μ g/ml) or Poly(I:C) (10 μ g/ml) to induce DC maturation. Twenty-four hours later, total RNA was isolated from LPS or Poly(I:C)-treated AFP-DCs or Alb-DCs and was subjected to real-time polymerase chain reaction (PCR) to detect mRNA of IL-12 (a: IL-12p35 mRNA, IL-12p40 mRNA) or mRNA of TLRs (b, TLR-4 mRNA or TLR-3 mRNA). Similar results were obtained from three independent experiments. We analysed statistically the mRNA levels of IL-12p35, IL-12p40, TLR-4 and TLR-3 between AFP-DCs and Alb-DCs. * $P < 0.05$.



cells co-cultured with AFP-DCs to the levels of that with Alb-DCs (Fig. 6b). These results demonstrated that NK activity was impaired in the co-culture with AFP-DCs possibly because of less IL-12 production from AFP-DCs.

Discussion

A variety of tumour-derived soluble factors have been reported to contribute to the emerging of complex local and regional immunosuppressive networks [15]. Recent study has demonstrated that innate immune system via NKG2D signals, expressed on NK cells, might play a critical role in tumour surveillance [16]. This led us to try to identify the immunosuppressive factors in innate immunity to develop a new strategy for cancer prevention. Elevation of serum AFP in cirrhosis patients is believed to be a high risk factor for HCC development [17]. AFP has already been reported to have immune regulatory function in T cells and B cells [9–11]. In this study, we hypothesized that AFP elevation might affect the immune-surveillance of innate immunity in HCC patients. We used a concentration of AFP (6.25–25 μ g/ml) that is in a range similar to that detected in the sera of cirrhosis or HCC patients. Our data show that AFP inhibited DC maturation and IL-12 production from DCs which might impair NK activity. This suggested that elevated AFP might affect HCC development by inhibiting NK activity in HCC patients.

The cytolytic activities of NK cells co-cultured with AFP-DCs against K562, NK-sensitive cells as well as Huh7

hepatoma cells were lower than those co-cultured with Alb-DCs. These results suggested that the presence of AFP-stimulated DCs could alter NK cytotoxicity. We have demonstrated previously that the expression of MICA/B on DCs, NK-activating molecules, plays a critical role in the pathogenesis of chronic hepatitis and HCC [14,18]. In this study, we examined these molecules on AFP-DCs and Alb-DCs. However, the expression of MICA/B on AFP-DCs were similar to those on Alb-DCs (Yamamoto *et al.* unpublished data), which suggested that the soluble factor from DCs was more important in the impairment of NK cytotoxicity. In NK activation by DCs, both direct contact with these cells and soluble factors such as IL-12 from activated DCs contribute to NK activation [19]. We demonstrated that the cytolytic activities of NK cells co-cultured with AFP-DCs were also lower than those with Alb-DCs in using Transwell culture, suggesting that the soluble factor contributed to the impairment of NK cytotoxicity. To examine the involvement of IL-12 from DCs in the activation of NK cells, we co-cultured NK cells with AFP-DCs or Alb-DCs with or without the presence of neutralizing antibody for IL-12. The cytolytic activity of NK cells co-cultured with Alb-DCs decreased to the level of that with AFP-DCs on addition of anti-IL-12 neutralizing antibody. Moreover, adding IL-12 to the co-culture of AFP-DCs and NK cells resulted in enhancement of the cytolytic activity of NK cells to the levels of Alb-DCs and NK cells. Taken together, these data demonstrated that IL-12 derived from AFP-DCs plays essential roles in the impairment of NK cytotoxicity, which is consistent

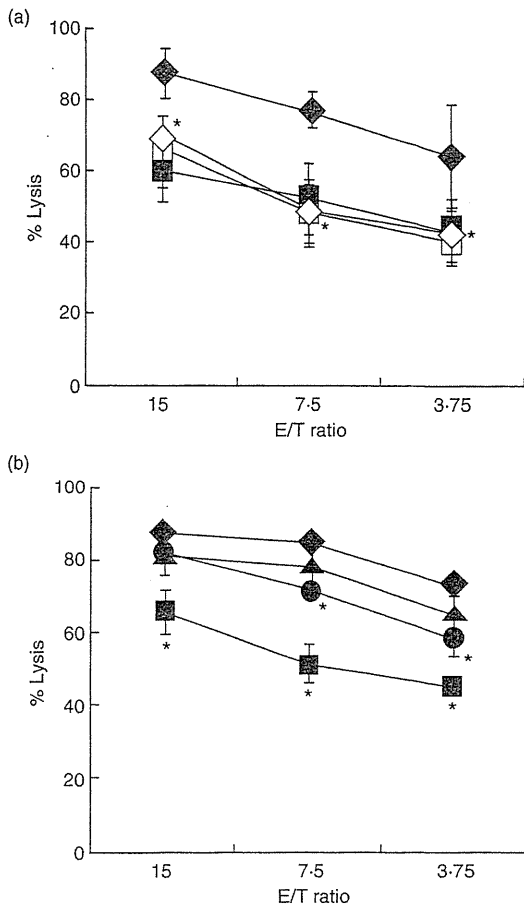


Fig. 6. Interleukin (IL)-12 derived from dendritic cells (DCs) played a critical role in natural killer (NK) cell activation. (a) Enriched NK cells were co-cultured with α -fetoprotein (AFP)-DCs or albumin (Alb)-DCs for 24 h with or without the presence of the neutralizing antibody of IL-12. The cytolytic activity of NK cells against K562 cells were evaluated by ^{51}Cr -releasing assay. The cytolytic activity against K562 cells of NK cells co-cultured with AFP-DCs (■) or Alb-DCs (◆) without neutralizing antibody of IL-12 or AFP-DCs (□) or Alb-DCs (◇) with neutralizing antibody of IL-12. We analysed statistically between the antibody-adding and not-adding groups in both AFP-DC and Alb-DC cultures, respectively. * $P < 0.05$ versus the cytolytic activity of NK cells cultured with Alb-DCs. Significant difference was observed between the antibody-adding group and not-adding group in the cytolytic activity of NK cells cultured with Alb-DCs. In contrast, no significant difference was observed between the groups in the cytolytic activity of NK cells cultured with AFP-DCs. (b) Enriched NK cells were co-cultured with AFP-DCs or Alb-DCs for 24 h with or without recombinant IL-12 protein (150 pg/ml, 300 pg/ml). NK cells were harvested and subjected to examine the cytolytic activity against K562 cells by ^{51}Cr releasing assay. The cytolytic activity co-cultured with AFP-DCs without IL-12 (■) or with IL-12 (150 pg/ml, ●; 300 pg/ml, ▲) or Alb-DCs (◆). * $P < 0.05$ versus the cytolytic activity of NK cells cultured with Alb-DCs. Representative results are shown. Similar results were obtained from three independent experiments in all experiments.

with the results of the production of IL-12 from AFP-DCs and Alb-DCs. Serum AFP is often high in patients with cirrhosis without HCC [8]. Oka *et al.* reported that the incidence of HCC development is significantly high in cirrhosis patients who had elevated serum levels of AFP [17], which suggests that high production of AFP in cirrhosis patients might also impair innate immunity, leading to HCC development. Our results might offer support for the hypothesis that elevation of AFP in cirrhosis patients impairs innate immunity which plays an essential role in the deletion of micro HCC, and thus results in promotion of HCC development.

Although the expression of antigen-presenting related molecules on AFP-DCs was not altered, the maturation of AFP-DCs was inhibited compared with Alb-DCs. This is consistent with a previous report [13] suggesting that the presence of AFP impairs DC maturation. DCs have been implicated in the activation of NK cells [19]. However, activated NK cells have been shown to recognize and lyse DCs *in vitro* and *in vivo*, but maturation of DCs results in resistance to NK lysis [19]. In HCC patients, it has been shown that impairment of DCs is associated with increased tumour progression [20] and that the levels of activated DCs are significantly low in HCC tissues [21]. High levels of AFP produced by HCC tissues may impair DC maturation, which would enhance HCC progression by removing DCs from HCC tumour areas.

IL-12 exhibits a number of immunologically important activities, including the ability to enhance NK cell and CTL functionality, to polarize CD4⁺ T cell responses by preferentially supporting the T helper type 1/cytotoxic T cell (Th1/Tc1)-type and to suppress Th2-type immunity [22]. We have demonstrated that the production of IL-12 protein from LPS-stimulated or Poly(I:C)-stimulated AFP-DCs was impaired significantly compared with that from Alb-DCs, which might affect immunosuppression in AFP-elevated patients. The expression of mRNA of both IL-12p35 and IL-12p40 were also inhibited significantly in AFP-DCs compared with Alb-DCs but not those of TLR-4, LPS receptor and TLR-3, Poly(I:C) receptor. The production of IL-12 is regulated strictly by positive and negative regulatory mechanisms and differential expression of TLRs affect the IL-12 production from DCs [23]. Our data revealed that adding AFP resulted in inhibition of IL-12 production at the transcriptional level, not by decreased expression of TLRs. Although the regulation of transcription of IL-12p40 and IL-12p35 has been elucidated in various studies [23,24], the detailed mechanism of inhibition of IL-12 transcription by AFP remains unclear. Further study is needed to clarify the detailed mechanism of inhibition of IL-12 by AFP. Taken together, IL-12 might play a mainly essential role in the impairment of NK activity by AFP. To evaluate the possibility of involvement of other immunosuppressive cytokines inhibiting NK activity, we examined the IL-6 and IL-10 levels in the supernatants of the co-cultures of NK cells and

AFP-DCs/Alb-DCs by specific ELISAs. IL-6 levels in the supernatants of AFP-DCs were similar to those of Alb-DCs, and IL-10 levels in the supernatants of AFP-DCs were significantly lower than those of Alb-DCs (M. Yamamoto, unpublished data). These results suggest that the addition of AFP might impair the ability of cytokine production of DCs.

In a previous report, Um *et al.* demonstrated that AFP impairs the function of dendritic cells and induces their apoptosis [13]. In their report, they used the commercially available human cord blood AFP. Thus, we used human cord blood AFP because this is the only commercially available AFP. The carbohydrates of AFP are heterogeneous, which is reflected by differences in the binding of individual AFP molecules to lectins. Therefore, we also added the supernatants of Huh7 cells, AFP-producing HCC cells or control medium on the DCs and evaluated IL-12 production after LPS stimulation by specific ELISA. The supernatants of Huh7 cells contained AFP (1.76 µg/ml) and control medium contained no AFP. The IL-12 production of DCs co-cultured with the supernatants of Huh7 cells was significantly lower than that with control medium (M. Yamamoto, unpublished data). These results were consistent with the results using human cord blood AFP. Although we cannot deny the possibility that unknown factors, except AFP, in the supernatants of Huh7 cell might affect the IL-12 production of DCs, these results suggest that another type of AFP might also have immunoregulatory ability on DCs.

In this study, we demonstrate that AFP might down-regulate IL-12 production from DCs which inhibit NK activity. Zhang *et al.* demonstrated that IL-12 improves the cytotoxicity of NK cells via up-regulated expression of NKG2D on NK cells [25]. We have demonstrated previously that NKG2D expression on NK cells was down-regulated in the progression of chronic liver disease, including HCC [18], which suggested that NK activities were impaired in HCC patients. The expression of NKG2D on NK cells in HCC patients with high serum AFP was significantly lower than those in HCC patients with low serum AFP (M. Yamamoto, unpublished data). This might be the result of long-term continuous suppression of NK cells by elevated AFP.

In conclusion, this study demonstrates that AFP impair the DC ability of activation of NK cells. These findings might provide new insight into understanding the mechanisms underlying the suppression of innate immune responses in chronic liver disease patients with high serum AFP levels.

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Disclosure

The authors have no conflicts of interest.

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Dendritic Cells in Hepatitis Virus Infection: A Legatus Within

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Abstract: Hepatitis B or C virus (HBV or HCV) causes chronic liver diseases that eventually progress to liver cancer. Both viruses are armed with multiple machineries for modulating immune responses in infected hosts. Mild and pervasive immune cell dysfunction, but not fully compromised, is a hallmark of chronic HBV or HCV infection, of which fundamental mechanisms are yet to be clarified. Dendritic cells (DC) as immune sentinels sense virus via toll-like receptors (TLR) or retinoic acid inducible gene-I (RIG-I) and evoke a cascade of immune reactions by secreting cytokines or by interacting other lymphocytes. Reduced and disabled DC potentially give negative impact on adjacent cells, such as NK cells, NKT cells and T cells. However, lack of evidence for active viral replication in DC or blood cells imply the presence of undisclosed contrivances that are independent of infection. Successful treatment of chronically infected patients with anti-viral agents is accompanied with numerical and/or functional restoration of DC, suggesting that DC could serve as potential therapeutic targets. Further studies are warranted for the establishment of therapeutic DC vaccine in order to gain more vigorous and sustained virus-specific immune responses. Cross talk between DC and lymphocytes are thus critical in shaping innate and subsequent adaptive immune responses against hepatitis virus, either spontaneously or therapeutically.

Keywords: Dendritic cells, HBV, HCV, interferon, natural killer cells, natural killer T cells.

INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are two major causes of chronic liver disease worldwide. HBV is a small DNA virus that is a prototype of *Hepadnaviridae* family, while HCV is a single stranded RNA virus that belongs to *Flaviviridae*. Both viruses are hepatotropic, but not directly cytopathic and elicit progressive liver injuries resulting in end-stage liver disease unless effectively eradicated [1, 2]. Epidemiological studies revealed that the relative percentages of acutely infected patients developing chronic hepatitis are different when comparing HBV and HCV infection. Less than 10% of HBV-infected patients develop chronic hepatitis, while more than 80% of HCV-infected ones do so [1, 2]. Such difference may in part rely on the differences in the immunogenicity of viral proteins and the kinetics of viral replication during the early stages of infection [3]. One of the major determinants in the clinical course of viral hepatitis is the host immune response. It has been proposed that the ability of infected hosts to mount a vigorous and sustained cellular immune reactions to HBV and HCV is required for control in primary infection. Once HBV or HCV survives the initial interaction with the host immune system, it uses several means to nullify the selective immunological pressure during the later phases of infection. First, these viruses alter their antigenic epitopes recognized by T cells and neutralizing antibodies to escape immune surveillance [4, 5]. HBV and HCV also subvert immune functions, including those of NK cells, dendritic cells (DC) and T cells. Antiviral agents, pegylated interferon (IFN)- α ,

ribavirin and nucleot(s)ide analogues, widely used for the treatment of chronic HBV or HCV infection reduce the viral load or enhance immunity in order to prevent the subsequent development of liver cirrhosis or hepatocellular carcinoma (HCC) [1, 6].

Cumulative reports have shown that innate immune system dictates the direction and magnitude of subsequent adaptive immune response. It is generally accepted that HBV- or HCV-specific CD8⁺ T cells are responsible for virus elimination by inducing hepatocyte apoptosis [4, 5]. Innate immune cells, including NK cells and NKT cells, may contribute to HBV or HCV eradication after primary infection; however, their roles in chronically infected state remain elusive. Since dendritic cells (DC) orchestrate anti-viral immune response by linking innate and adaptive arms of immune system [7], functional impairment of DC leads to failure of NK cells, NKT cells, CD4⁺ and CD8⁺ T cells. Infiltration of disabled CD8⁺ T cells to the infected liver may result in weak liver inflammation that is not sufficient for HBV or HCV eradication [8].

In this paper, we discuss the current understandings of the roles of innate and adaptive immunity in the pathogenesis of hepatitis virus infection as well as efficacy of anti-viral therapy, especially focused on interaction of DC and other players.

KEY PLAYERS IN IMMUNE RESPONSES TO VIRAL HEPATITIS

After HBV or HCV infects the liver, viral replication continues and viral particles are continuously released into the circulation. Theoretically, the first lines of defense are provided by anti-viral type I IFN and subsequent IFN-inducible genes (ISGs). As for cellular components in innate immune system, NK and NKT cells play major roles in liver

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immunology, of which populations are relatively increased in the liver compared to the periphery. These cells are activated in the liver, where expression of IFN- α and ISGs are extremely high during the early phase of hepatitis virus infection [9]. Activated NK and NKT cells secrete IFN- γ , which inhibits replication of hepatitis virus mainly through a non-cytolytic mechanism (Fig. 1a) [10].

Dendritic cells (DC) or resident macrophages in the liver are capable of taking up viral antigens, and processing and presenting them to other immune cells (Fig. 1b) [7]. Since DC express distinct sets of toll-like receptors (TLRs) and cytosolic pathogen sensors [11], it is likely that some viral components stimulate DC through ligation of these receptors (Fig. 2). DC develop a mature phenotype and migrate to lymphoid tissues (Fig. 1c), where they stimulate effectors, including T cells and B cells (Fig. 1d). Following the encounter of DC with other cells, DC secrete various cytokines (IL-12, TNF- α , IFN- α and IL-10) instructing or regulating the functions of the adjacent cells [7]. In addition to these cytokines, DC express various co-stimulatory molecules and ligands to enhance or limit the functions of immune and infected cells. The existence of functionally and ontogenetically distinct DC subsets has been reported; i.e., myeloid DC (MDC) and plasmacytoid DC (PDC) [12]. MDC predominantly produce IL-12 or TNF- α following pro-inflammatory stimuli, while PDC release a considerable amount of IFN- α upon virus infection depending on the immune stimulus; both cytokines in actuality can be made by both cells. Helper T cells have an immunoregulatory function mediated by the secretion of cytokines that support either cytotoxic T lymphocyte (CTLs) generation (Th1 with secretion of IL-2, IFN- γ and TNF- α) or B cell function and antibody production (Th2 with secretion of IL-4, IL-5, IL-10 and IL-13) (Fig. 1e). In addition to Th1/Th2 paradigm, CD4⁺ T cells secreting IL-17 (Th17) are induced under distinct

cytokine conditioning and are involved in liver inflammation or autoimmunity. DC ontogeny and DC-derived cytokines are crucially associated with the differentiation or polarization of helper T cell subsets.

It is generally accepted that adaptive immunity performs a critical role during the clinical courses of hepatitis. The involvement of antigen-specific CD4⁺ T cells in HBV or HCV eradication has been well described during both acute or chronic infection [5]. However, there is little evidence that CD4⁺ T cells mediate direct liver cell injury in virus infection. Thus, it is likely that CD4⁺ T cells play a critical role in facilitating other antiviral immune mechanisms, such as enhancing CD8⁺ effector function. The antigen-primed CTLs recruit to the liver (Fig. 1f) and constitute the critical element in the eradication of virus-infected cells (Fig. 1g). The increment of specialized immune suppressors such as regulatory T cells (Tregs) has been shown in HBV or HCV infection [13, 14]. These cells are actively involved in the alleviation of Th1- or CTL-mediated liver inflammation, thus contributing to persistence of hepatitis virus (Fig. 1h).

NATURAL COURSE OF ACUTE HBV OR HCV INFECTION

Acute HBV Infection

During the early phase of primary HBV infection, HBV-DNA is not detectable in serum or the liver for 4-7 weeks following exposure. HBV infection of the liver directly induces type I IFN, which subsequently activates NK cells. Thus, even in the incubation phase, activated NK cells are thought to play crucial role in the control of HBV replication by producing IFN- γ . This is supported by the observation that circulating NK cells increases before the peak of HBV replication, which subsides following HBV reduction [15]. Activated NKT cells are involved in the inhibition of HBV replication, as evidenced by

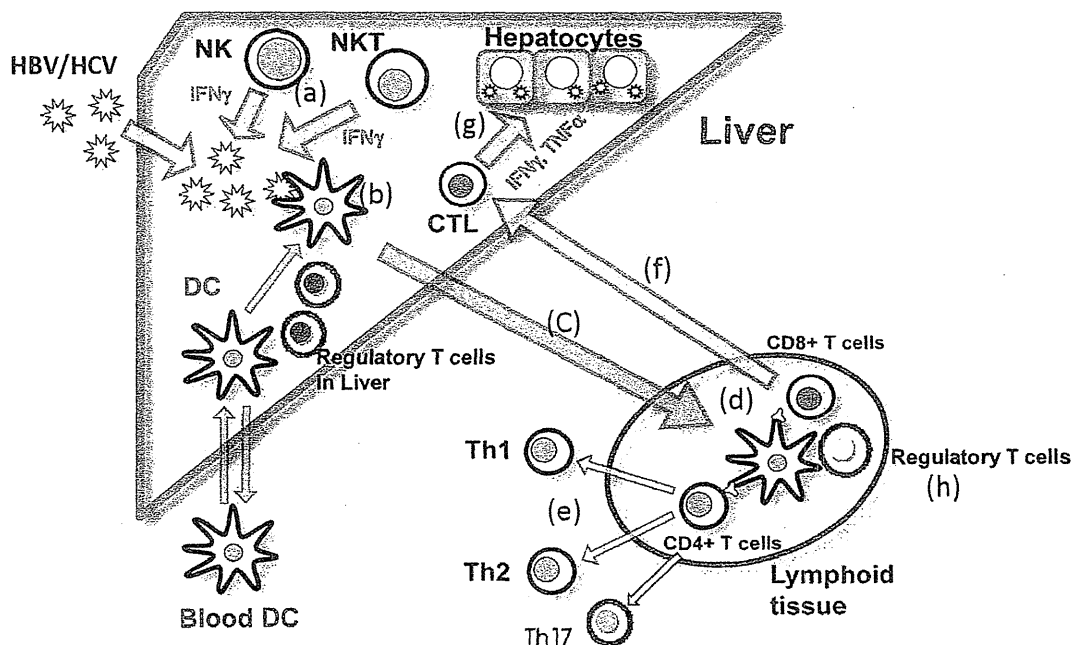


Fig. (1). Key players in immune reactions in viral hepatitis. CTL, cytotoxic T lymphocyte; DC, dendritic cell; HBV, hepatitis B virus; HCV, hepatitis C virus; NK, natural killer cell; NKT, natural killer T cell; Th, helper T cell. (a)-(h), see text.

HBV transgenic mouse model [16]. Increment of human NT cells, as defined as CD3⁺CD56⁺ cells, is observed in acute hepatitis B prior to the peak of T cell responses [17]. Subsequently a rapid increase in HBV replication occurs at 10-12 weeks of infection, which is accompanied by induction of adaptive immunity. HBV-specific CD4⁺ and CD8⁺ T cells are detectable in the blood even before the onset of overt hepatitis [15]. In chimpanzee inoculated with a single strain of HBV, the size of viral burden may be one of the determinants dictating the outcomes. The larger size of HBV is administered, the higher chance of immune-mediated HBV clearance is gained in chimpanzees [18]. Generally, strong and Th1-biased CD4⁺ T cell response and multi-specific CD8⁺ response are associated with HBV clearance [19]. HBV-specific CD8⁺ T cells continue to increase after a marked reduction in HBV-DNA and reach their highest number at the time of maximal ALT levels (0.1-1.3% of peripheral CD8⁺ T cells), then decline during the recovery phase, in parallel with a resolution of hepatitis [15, 19]. From a CD4⁺- or CD8⁺ T cell depletion study in chimpanzees, HBV-specific CD8⁺ T cells, but not CD4⁺, are the main effectors responsible for viral clearance [20]. In comparison with HCV-specific CD8⁺ T cells using tetramers, HBV-specific CD8⁺ T cells are highly activated and capable of proliferating and secreting much IFN- γ [21]. Since interactions among IFN, NK and T cells seem to be complicated in the acute phase, suppressive factors are involved as a compensatory mechanism. In this context, HBV-induced IL-10 at primary infection may be closely related with down-regulation of CD4⁺ and CD8⁺ T cell responses [22].

Acute HCV Infection

In clear contrast with HBV, HCV-RNA levels rapidly increase during the first few days of HCV infection and continue to be high during the incubation periods [23], which lasts for up to 10-12 weeks following infection. Although HCV triggers expression of type I IFN and ISGs in liver during this phase [9], the HCV viral load does not decrease. This suggests that HCV impedes the execution of anti-viral molecular mechanisms, including interferon regulatory factor (IRF)-3 [24], as well as NF- κ B and RNA-dependent protein kinase (PKR) [25]. In parallel with the onset of acute hepatitis, activated HCV-specific T cells enter the liver [26]. HCV-specific CD4⁺ and CD8⁺ T-cell responses and IFN- γ co-expression coincide with decreases in HCV quantity [26]. Vigorous, multi epitope-specific, Th1 type and sustained CD4⁺ T cell responses are detected in resolved cases [23]. By contrast, in cases that progress to chronic hepatitis, CD4⁺ T cell responses are weak, narrowly selected and short-lived [27]. The frequency of HCV-specific CD8⁺ T cells is high during the acute phase of infection (2-8% of peripheral CD8⁺ T cells), however, it decreases after HCV persistence develops (0.01-1.2%) [28]. Despite the high numbers of CTL, some of these cells are "stunned" in acute phase, as demonstrated by an inability to produce IFN- γ and to proliferate in response to HCV antigens [28, 29].

INNATE IMMUNITY

Interferon and Interferon-Stimulated Genes

Mammalian toll-like receptors (TLRs) sense some pathogen-associated molecular patterns (PAMP) embedded

in virus components and then induce inflammatory cytokines or type-I IFN, resulting in the augmentation of anti-virus immune reactions [11]. Retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen (MDA) -5 are cytosolic molecule that sense dsRNA as virus replicative intermediate, which subsequently activates IRF-3 and NF- κ B pathways [30]. Several lines of evidence have been presented that HBV or HCV impedes TLR- or RIG-I-dependent signal transduction, resulting in lesser magnitude of ISG responses.

HBV Infection

Limited information is available for which HBV components stimulate relevant PAMP receptors. In general, activation of TLR-dependent pathways suppresses HBV replication both *in vitro* and *in vivo* [31]. In chronic HBeAg-positive patients, it is reported that TLR2 expression is down-regulated in hepatocytes, Kupffer cells or monocytes, suggesting that TLR2-dependent pathway in these cells is impaired [32]. Acute-on-chronic hepatitis B is a critical liver disease that frequently becomes fatal. In such patients who failed to survive, the expression of TLR3 and IFN- β in monocyte-derived DC (MoDC) was significantly decreased compared to those who survived [33]. These results suggest that TLR3-dependent pathways are involved in either a cause or a consequence of liver inflammation. In mice, HBs, HBe antigens and HBV virions are capable of inhibiting TLR-dependent pathways [34]. Recently, it has been shown that HBV-X protein directly degrade adaptor molecule MAVS, resulting in the inhibition of RIG-I-dependent signaling pathways [35]. Alternatively, Yu *et al.* reported that, in the shared downstream of TLR and RIG-I, HBV polymerase is responsible for IRF3 inhibition at the levels of TBK1/IKK ϵ [36].

HCV Infection

Large-scale cohort study on US veterans revealed that the prevalence of various infectious diseases, including virus, bacteria and parasites, in HCV-infected individuals is significantly higher than those in uninfected controls [37]. These observations suggest that first-line defense against pathogens, of which system is initiated by TLR/RIG-I stimulation, is functionally impaired in HCV infection. Several mechanisms have been proposed regarding to HCV-induced suppression of innate immunity. By using HCV subgenomic replicon system, it has been demonstrated that HCV NS3/4A proteins influences on the functions of adaptor molecules mediating TLR-dependent and RIG-I-dependent pathways, resulting in an impairment of the induction of IFN- β as well as subsequent ISGs [38, 39]. However, it is yet to be proven whether the results obtained from HCV replicon are applicable or not for HCV-infected individuals. HCV core has been reported as one of the crucial immunogenic components. Several molecules have been regarded as targets of HCV core-mediated immune alteration, such as STAT1 [40] or SOCS3 [41], all of which eventually dampen IFN and ISG induction. Alternatively, in mouse macrophage cell line, HCV NS5A is shown to inhibit TLR-dependent IFN response by interacting adaptor molecule, MyD88 [42].

To investigate the roles of TLR/RIG-I in HCV infection, we compared their expressions and the functions in MDC and PDC between the patients and donors. In MDC from HCV-infected patients, TLR2, TLR4 and RIG-I expression were significantly higher than those in healthy counterparts [43]. Of particular interest, regardless of the higher expressions, specific agonists for these sensors stimulated patients MDC to induce lesser amount of IFN- β and TNF- α compared to donor MDC [43]. These results show that, in MDC, the signal transduction *via* these receptors is strongly impeded in HCV infection. Further investigation is needed to clarify which TLR or RIG-I is predominantly utilized by HCV to evoke immune reactions.

Natural Killer Cells

Within the liver, NK cells comprise 20-30% of mononuclear cells, as compared to that they consist of 10-15% in PBMC [44]. It thus needs to be stressed that such regional differences in the distribution of NK cells may raise conflicting analytical results of these cells. In HBV- or HCV-infected liver, NK cells are deemed to be activated by type-I IFN and have gained ability to produce substantial amount of IFN- γ . Recently, it is reported that such NK activation at this phase is not always correlated with the outcomes of infection, but they are capable of dictating further T cell responses [45, 46]. Cumulative data have shown that activated NK cells, not only T cells, could be effectors in liver injury in HBV or HCV infection, in which TRAIL-mediated apoptosis is involved.

Natural Killer cells express various functional receptors; the one group that transduces inhibitory signals (Killer Inhibitory Receptors/KIRs, CD94, NKG2A) and the other does activating signals (NKG2D). The function of NK cells is dynamically regulated *in vivo* by the balance between expressions of counteracting receptors and their association with relevant ligands [47]. Large scale cohort studies in HCV infection have disclosed that certain combinations of HLA-C and KIR haplotypes are closely associated with spontaneous HCV clearance [48, 49]. Such epidemiological observations raise a possibility that NK cells play an active role in HCV eradication.

We compared the expressions of NK cell receptor between HCV-infected patients and healthy donors. As for inhibitory receptors, KIR expressions are not different between the groups; however, CD94 and NKG2A expressions are higher in patients than controls [50]. In contrast, activating receptor NKG2D expression is comparable between the groups. It is yet to be determined how the expression of NK cell receptor is regulated. In our hands, HCV pseudo-virus did not enter purified NK cells, suggesting that NK cells are not susceptible to direct HCV infection (unpublished data). Thus, some soluble factors and/or direct binding of HCV particles to NK cells might be the cause of NK receptor dysregulation.

Natural Killer T Cells

Natural killer T (NKT) cells are a unique lymphocyte subset co-expressing T-cell receptor (TCR) and NK cell markers [51]. The NKT cell population is highly heterogeneous according to the differences in types and

tissue distribution; invariant (or classical) NKT (iNKT) cells express an invariant TCR, composed of V α 24-J α Q preferentially paired with V β 11 in humans [51], whereas non-invariant NKT cells express diverse TCR. Invariant NKT cells recognize glycolipid antigens presented on CD1d expressed by DCs [51]. Although endogenous ligands of iNKT cells are little known, α -galactosyl-ceramide (α GalCer) has been used as a surrogate for natural ligands. In contrast, non-invariant NKT cells are activated by CD1d-dependent manner but are not reactive to α GalCer. Baron *et al.* reported that, in HBV-transgenic mice, non-invariant NKT cells are critically involved in acute liver injury [52]. As for a human counterpart, Exley *et al.* observed that CD1d-restricted non-invariant NKT cells are infiltrated in HCV-infected liver, where they presumably exert their promoting role in liver inflammation [53]. Hepatic inflammatory cells or biliary cells up-regulate CD1d expression which subsequently supports NKT cell activation [54]. In addition, hepatic stellate cells are capable of activating NKT cells *via* surface CD1d and secretion of IL-15 [55].

For chronic HCV infection, some controversial reports have been published about the frequency of iNKT cells [56, 57], however, their functional roles in HCV-infected patients are largely unknown. We demonstrate that the number and functions of iNKT cells from HCV-infected patients are comparable with those from healthy subjects at the steady state [58]. By contrast, activated iNKT cells from patients released more Th2 cytokines, most significantly IL-13, than those from the controls [58]. Recently, other groups have reported that IL-4 and IL-13 from fresh iNKT cells were increased in liver cirrhosis caused by HBV or HCV, implying that these cells are pro-fibrogenic to the liver [59]. If this is the case, our findings suggest that iNKT cells in chronic HCV infection are pro-fibrogenic per se even in the pre-cirrhotic stage. The reason why iNKT cells in HCV infection are Th2-biased needs to be further investigated.

Dendritic Cells

HBV Infection

Several reports have been available for functional alterations of DC subsets in HBV infection [60]. Infection of HBV to DC is still controversial and the described functional defects are minimal when compared with HCV [61, 62]. It is reported that peripheral PDC are phenotypically and functionally intact [63]. In contrast, PDC ability of secreting IFN- α is reported to be impaired specifically in response to TLR9 but not to TLR7 agonists [64]. As for the mechanisms, HBs antigen is responsible for such PDC disability partly by up-regulating suppressor molecule SOCS-1 [64]. Similarly, other investigators reported that PDC frequency as well as their TLR9 expression are decreased [65]. In addition to PDC, MDC dysfunction is observed as well, which is presumably caused by HBV particles and HBs antigen [66].

In contrast to circulating PDC, liver-infiltrating counterparts are more vulnerable to HBV; liver PDC are significantly reduced and disabled in non-survivors of acute-on-chronic hepatitis B compared to those in survivors [67]. Blockade of PDC-derived IFN- α downgraded the amounts of IL-12 and TNF- α released from adjacent cells [67],

suggesting that liver PDC play a pivotal role in regulating immune responses *in situ*.

HCV Infection

Monocyte-derived DC generated from hepatitis C patients have an impaired ability to stimulate allogeneic CD4 T cells [68, 69]. Functional impairment of DC diminished when HCV had been eradicated from patients, revealing the evidence of HCV-induced DC disability [68]. DC play a decisive role in shaping innate immunity by interacting with NK cells. In response to IFN- α , DC are able to express MHC class-I related chain A/B (MICA/B) and activate NK cells following ligation of the NK receptor, NKG2D [70]. Interestingly, DC from HCV-infected patients are unresponsive to exogenous IFN- α to enhance MICA/B expression and fail to activate NK cells [70]. It is tempting to speculate that the impairment of DC in NK cell activation is responsible for the failure of HCV control in the early phase of primary HCV infection, where HCV continues to replicate in spite of high-level IFN- α expression in the liver. Alternatively, NK cells from HCV-infected patients down-regulate DC functions in the presence of hepatocytes by secreting suppressive cytokines, IL-10 and TGF- β 1 [50]. Such functional alteration of NK cells in HCV infection was ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts [50]. Further study is necessary to determine if the NK-mediated DC suppression is instrumental or not in acute HCV infection.

Limited works have been published on the roles of blood DC in acute HCV infection. Ulsenheimer *et al.* reported that, in acute hepatitis C, PDC are reduced and functionally impaired, however such decrease is not specific for HCV infection but due to liver inflammation [71]. In chronic HCV infection, MDC and PDC in HCV-infected patients were reduced in number and impaired in their ability to promote Th1 polarization and IFN- α production [72, 73]. One of the explanations for such reduction is enhanced apoptosis in MDC, which is partly due to diminished NF- κ B activity [74]. Of note is the finding that HCV is relatively a weak IFN- α inducer to PDC compared to other viruses such as HIV or herpes virus, thus contributing to HCV persistence [75]. Dysfunctional DC are involved in the exhaustion of CD8⁺ T cells, thus confirming a role of DC as a linker between innate and adaptive immunity [76].

Two plausible explanations exist to explain the mechanisms of DC dysfunction in chronic hepatitis C: 1] direct HCV infection of DC and 2] the presence of circulating HCV proteins which affect DC function and number. The HCV genome has been reported to be isolated from MoDC or blood DC [68, 77]. These results need to be interpreted carefully, since contamination with free virus in blood cannot be ruled out when amplifying PCR techniques are used. To exclude this possibility, HCV pseudo-virus has been developed to investigate the cell tropisms of HCV as well as to determine putative HCV entry receptors to cells. By using this, MDC, but not PDC, displayed susceptibility to HCV pseudo-virus possessing chimeric HCV E1/E2 proteins [78].

Recently, cell culture-derived HCV particles (HCVcc) have been established from replicon cells harboring full

genome JFH-1 clone [79]. Inoculation studies with HCVcc to various blood cells including DC, have been performed in order to reveal HCV tropism [80]. Consequently, no direct evidence was obtained for the replication and reproduction of HCVcc in these cells [81-83]. However, on PDC, HCVcc has given a negative impact on IFN- α production, of which was independent of HCV infection [84]. Of particular interest, Dolgunic *et al.* proposed a novel mechanism of third-party cells involving in PDC dysfunction. They have shown that HCV core and NS3 stimulate TLR2 on monocytes to release TNF- α , thereby inducing PDC apoptosis and dampening their IFN- α production [85].

Several criticisms have been raised about DC dysfunction in the setting of chronic HCV infection [86], failing to demonstrate any DC defects which may have to do with differences in the populations studied. Cohort studies on chimpanzees following HCV infection showed that functional impairment of DC was observed in some cases but was not a prerequisite of persistent infection [87]. Further study needs to be done to clarify whether DC are indeed disabled in the setting of human chronic hepatitis C and furthermore whether this contributes to the development of HCV persistence or it is simply a consequence of active HCV infection.

ADAPTIVE IMMUNITY

Chronic HBV Infection

Extensive work has been done on CD4⁺ and CD8⁺ T-cell dysfunction in chronic HBV infection. Patients with chronic hepatitis B are generally hyporesponsive to HBV proteins, and the level of T cell reactivity at this stage is significantly weaker than in acute self-limited hepatitis B. Even with tetramers, HBV-specific CD8⁺ T cells are barely detected in the circulation of HBe antigen-positive patients [88]. One of the proposed mechanisms of such CTL reduction is Bim-mediated CD8⁺ T cell apoptosis [89]. The importance of HBV-specific CD8⁺ T cells in the control of virus replication and the suppression of liver inflammation has been well established [88]. In HBe antigen-negative chronic hepatitis B patients without liver inflammation, tetramer positive CD8⁺ T cells were highly active and more frequently observed both in the liver and PBMC than HBe antigen-positive patients with active hepatitis [88]. It has been speculated that one of the mechanisms of T cell hyporeactivity in chronic HBV infection is the exhaustion of antigen-specific T cells due to the presence of large quantity of virus or viral proteins, such as HBe antigen [90]. In support of this, reduction of HBV-DNA in patients treated with lamivudine coincided with a significant increase in HBV-specific CD4⁺ and CD8⁺ T cell responses [91].

Chronic HCV Infection

The relevance of CD4⁺ and CD8⁺ T cells in chronic HCV infection is different from that observed with HBV. Many reports have been published on the importance of CD4⁺ T cell response in the clearance and control of HCV. In chronic hepatitis C patients, HCV-specific CD4⁺ T cells were functionally impaired and their activity was not sustained [92], which was in clear contrast with resolved cases.

Inoculation studies of infectious HCV to recovered chimpanzees demonstrated that CD4⁺ T cell help was indispensable for the development of effective CD8⁺ T cell response to protect from HCV persistence [93]. Since the liver is the major site where the hepatitis virus replicates, analyses of liver-infiltrated lymphocytes give quite distinct perspectives from that obtained with PBMC [94]. Compartmentalization of specific Th1 type CD4⁺ T cells occur in the chronic HCV-infected liver and can be distinguished from cells found in the periphery [95]. The immunological environment in the liver is potentially tolerogenic to infiltrating T cells [96]. Liver sinusoidal endothelial cells (LSEC), Kupffer cells, stellate cells and liver DC may mediate this tolerogenic effect [97].

With regard to HCV-specific CD8⁺ T cells observed during the chronic stages of disease, conflicting results have been reported for their roles in HCV replication and liver inflammation. Several investigators have shown that the HCV-specific CTL response is inversely correlated with viral load, suggesting its inhibitory capacity on HCV replication [98]. However, others did not find a significant relationship between these parameters [99]. HCV-specific CD8⁺ T cells in chronic hepatitis C patients possess lesser capacity to proliferate and produce less IFN- γ in response to HCV antigens [100]. Since CD8⁺ T cells are reported to be involved in HCV-induced liver inflammation [101], inefficient CD8⁺ T cells may evoke only milder hepatocyte injury, which level is not sufficient for HCV eradication [8].

T cell exhaustion is a conceptual mechanism that is involved in persistency of hepatitis virus. Under continuous exposure to large amount of viral proteins, antigen-specific T cells become hypo-responsive to repetitive antigen stimulation in proliferation and cytokine production. With regard to an inducer of exhausted T cells, extensive studies have been done on PD-1 expression on HCV-specific CD8⁺ T cells [102]. In a transition from acute hepatitis to chronic phase, it is reported that CD8⁺ T cells expressing PD-1 are increased in patients who developed to chronic hepatitis [103]. However, inconsistent observation regarding to PD-1 has been reported by other investigators [104]. Of particular importance, HCV-specific CD8⁺ T cell response was restored *in vitro* in the presence of masking antibodies against PD-L1, suggesting that PD-1/PD-L1 pathway could be served as a therapeutic target [17, 105]. In addition to PD-1, multiple inhibitory receptors are co-expressed on HCV-specific CD8⁺ T cells, shaping characteristic aspects forcing exhausted phenotype [106].

Conflicting results have been published so far for the existence of epitope hierarchy in HCV-specific CTL. The prevalence of resolved cases was higher in those with HLA-B27 compared to those without, suggesting that HCV-specific CTL recognizing HLA-B27-binding peptides are somewhat involved in spontaneous HCV clearance [107, 108]. However, such responses are observed in limited patients with HCV genotype 1 infection, but not in those with genotypes 2 and 3 [108].

Several investigators have reported that the frequency of regulatory T cells (Tregs) are increased in chronic hepatitis B or C patients, either in liver or in the periphery [13, 14]. It is well acknowledged that Tregs consist of distinct populations, thymus-derived natural Tregs, adaptive/inducible Tregs or

IL-10-producing CD8⁺ Tregs. Either type of Tregs are endowed with suppressing capacity on NK cells, DC and HBV- or HCV-specific T cells, thus leading to alleviation of collateral liver damage or impeding virus elimination. It has been speculated that Tregs actively enhance persistence of hepatitis virus in the acute phase, however no direct correlation was found between the expansion of FOXP3⁺ cells and attainment of chronic HCV infection [109]. A question is still unsolved that how Tregs are generated either in HBV or HCV infection. The presence of viral epitope has been postulated that is specifically capable of expanding Tregs, though it is still controversial [110, 111]. Alternatively, multifaceted mechanisms have been committed to the induction of Tregs, such as tolerogenic MDC or PDC [112], galectins [113] and certain TLRs [114] as reported elsewhere.

IMMUNE RESPONSE DURING ANTI-VIRAL THERAPY

In chronic HBV-infected patients with active viral replication, nucleot(s)ide analogues such as lamivudine, adefovir or entecavir have been used to reduce viral burden [1]. The reduction of HBV DNA by lamivudine improves HBV-specific CD4⁺ as well as CD8⁺ T cell responses [91]. In similar with this observation, adefovir treatment reduces HBV load and subsequently improves number and function of MDC [115]. During IFN- α -based therapy, successful responses are associated with lasting and Th1-type CD4⁺ T cell responses [116].

Anti-viral agents, pegylated (PEG) IFN- α and ribavirin, have been widely used for the treatment of chronic HCV infection in order to prevent the development to liver cirrhosis and hepatocellular carcinoma [6]. In addition to providing direct inhibition of viral replication, these agents modulate antiviral immune responses, which greatly contribute to the successful therapeutic response. Earlier studies reported that HCV-specific CD8⁺ T cell response, as examined by CTL precursor frequency, was not enhanced after IFN- α monotherapy. Furthermore, analyses of MHC class-I tetramer-positive cells in patients who underwent IFN- α and ribavirin therapy revealed that CD8⁺ T cells did not increase following treatment and they were not associated with outcome [117]. Combination therapy of IFN- α and ribavirin increases antigen-specific CD4⁺ T cell proliferation and IFN- γ production by CD4⁺ T cells [118]. The "vigor" of the CD4⁺ T cell response to HCV eradication is reported to be variable, something which is considered quite controversial [117]. Recent report has shown that T cell responses as assessed by IFN- γ production and proliferation did not differ between sustained virological responders (SVR) and non-SVR groups [119].

Currently, no data is available for the involvement of innate immunity in the efficacy of IFN- α -based anti-HCV therapy. We thus examined whether IFN- α and ribavirin give a positive impact on DC capacity to induce CD4⁺ T cell (Th1) response. By using *in vitro* culture system, MoDC from chronic hepatitis C patients were impaired in the ability to drive Th1 in response to IFN- α . When we compared such DC capacity between patients who cleared HCV (SVR) by IFN- α /ribavirin therapy and those who failed to do so, impaired DC function was restored in response to IFN- α /ribavirin in SVR patients but not in non-SVR ones [120].

These results imply that DC responsiveness to anti-viral agents is restored in patients who potentially gain favorable outcomes in IFN- α /ribavirin therapy.

Next, we aimed to elucidate if the frequency or function of DC is related to the outcome of PEG-IFN- α and ribavirin therapy. In comparison with SVR patients, non-SVR ones and transient responders (TR) showed a decline of PDC frequency from weeks 1-12 and impaired DC function at the end of treatment [121]. These results show that restoration of DC function is critically involved in favorable response in PEG-IFN- α /ribavirin therapy. In support for our results, other investigators reported that the restoration of DC function is critically involved in virological response to PEG-IFN α and ribavirin therapy [122, 123]. Taking these results into considerations, DC system could be a target of therapeutic immune modulation.

Recently, the genome-wide association study (GWAS) revealed that the single nucleotide polymorphisms (SNPs) neighboring IFN- λ 3/IL28B gene are strongly associated with successful or unsuccessful viral response in Peg-IFN α /ribavirin therapy for chronic hepatitis C [124, 125]. Of particular interest, it is reported that major alleles in such SNPs give a significant and positive impact on spontaneous HCV clearance [125]. Since DCs are main producer of IFN- λ , these results again confirm that DCs are critically important for the control of HCV infection, either spontaneously or therapeutically.

The questions remain unsolved are if impaired immune system in chronic HCV infection is restored or not by the successful HCV eradication after anti-viral therapy. Controversial results have been reported about the durability

of treatment-induced recovery in HCV-specific immune response [126, 127], which seems to be clearly distinct from that observed in spontaneous HCV resolvers.

IMMUNOLOGICAL INTERVENTION AGAINST HBV OR HCV INFECTION

Boosting virus-specific immune responses may be beneficial to HBV- or HCV-infected patients; however critical concerns remain unsolved regarding to potential risks of evoking severe type of hepatitis. In case of excessive immunity such as fulminant viral hepatitis, meticulous attention needs to be paid for the application of immune intervention in order to avoid progression of liver failure. Clinical trials have been underway in order to assess efficacy or safety of anti-HBV or anti-HCV vaccines. As reported in HIV infection, cell therapy is one of the innovative approaches for refractory chronic virus infection such as HBV or HCV. In the clinical settings, DC are attractive candidates as natural adjuvant that stimulate or regulate virus-specific immune responses (Fig. 3).

With respect to HBV, extensive studies have been done in HBV transgenic mice models. These mice are inbred tolerant to HBV antigens; however, DC pulsed with HBs antigen induced anti-HBs antibody, showing that DC act as a tolerance breaker [128]. For extension of the studies, they administered HBs antigen-pulsed DC to healthy volunteers. Although the size of the study is small, anti-HBs antibody titer increased in some recipients, showing that antigen-loaded DC vaccine is promising for controlling HBV [129]. Recently, the results of phase-I study using DNA vaccine encoding HBeAg have been published. From immunological

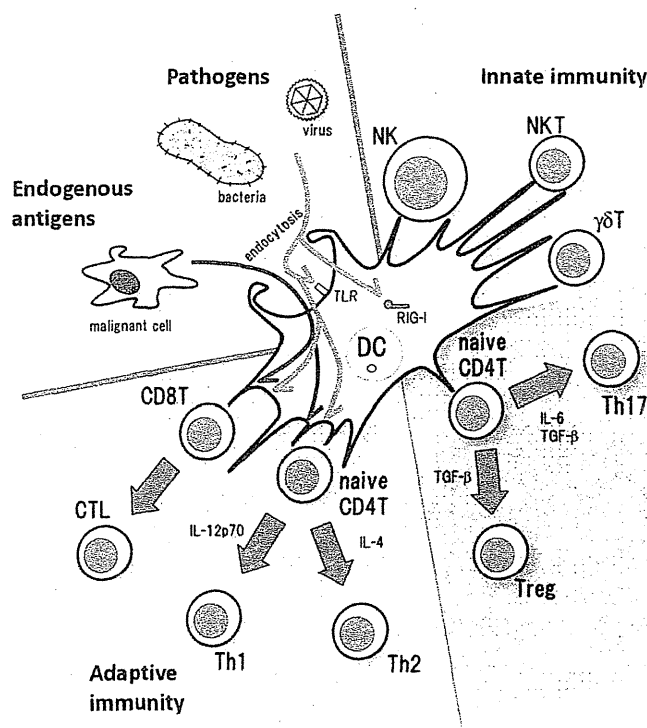


Fig. (2). Dendritic cell as a conductor of innate and adaptive immunity. Dendritic cells sense viral and endogenous antigens and evoke or regulate immune reactions by interacting with various lymphocytes. CTL, DC, NK, NKT, Th are as described in Fig. (1). $\gamma\delta$ T cells, gamma delta T cells; Treg, regulatory T cells

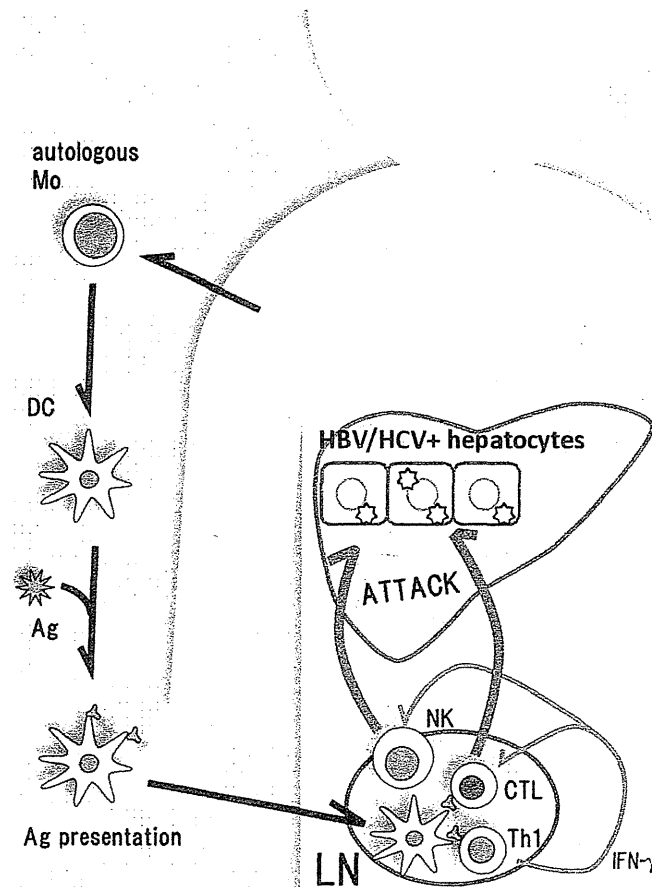


Fig. (3). Strategy of dendritic cell vaccine against hepatitis virus infection. Most of the clinical trials utilize autologous monocytes as source of DC. Monocytes are cultured *ex vivo* for several days in the presence of cytokines, such as GM-CSF and IL-4. Mature DC are loaded with viral antigens (peptides, proteins or mRNA) and subsequently administered to the patients. DC could migrate to lymphoid tissue where they stimulate NK cells and T cells. When induced, antigen-specific CTL migrate to the liver where they attack virus-infected hepatocytes, resulting in apoptosis coincided with hepatitis virus elimination.

points of view, NK cells from vaccinated patients showed higher percentages of CD56^{bright} population and their CD244 and NKG2D expressions were enhanced, indicating that NK cells are activated by vaccine *in vivo* [130].

In HCV infection, basic research regarding to therapeutic DC vaccine has been underway for the selection of appropriate antigens. For such purposes, DC expressing NS5A [131] or from NS2 to NS5B have been used as an immunogen [132]. Lipopeptide-loaded MoDC has been subjected to phase-I vaccine trial for chronic HCV infection [133]. Monocyte-DC has been administered in safe and raised transient T cell responses specific not only for HCV but also for other viral antigens [134]. Unfortunately, such responses were not sustained nor accompanied with reduction of HCV titer [134]. Further study is arguably needed to verify more effective way to prime sustained HCV-specific T cell responses *in vivo*.

PERSPECTIVE

Synthetic compounds that specifically suppress hepatitis virus replication are now ready to use in clinics [135]. Additionally, many others are still on the pipeline and are destined to clinical trials. They are quite promising as an

alternative approach for hard-to-treat chronic hepatitis patients, such as those infected with drug-resistant HBV strain or non-responders in PEG-IFN α /ribavirin therapy. In addition to inhibitory effect on viral replication, such compounds are able to restore immunity either indirectly by reducing viral burden or directly by immune modulation. Therefore, extensive immunological studies are needed to elucidate if the therapeutic modulation of innate immunity could shape sustained HBV- or HCV-specific adaptive immunity or not. The next steps in evolving innovative approaches to establish virus-specific immunotherapy are to determine the means to, direct the magnitude, breadth, quality and duration of antigen-specific immune responses in a desired way. Active modulation of innate immunity may be one of the strategies to gain access to the goal.

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CONFLICT OF INTEREST

Declared none.

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