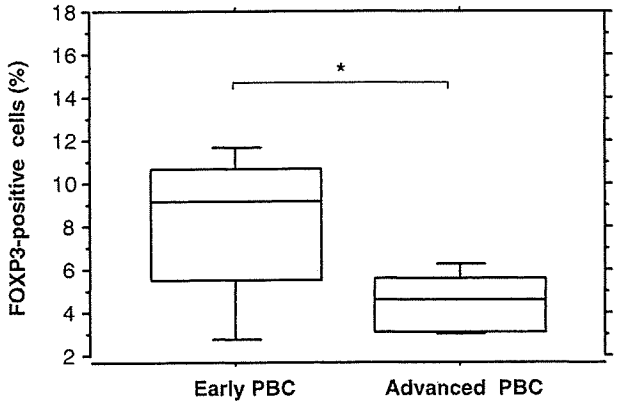


Figure 2 Frequency of regulatory cells (Tregs) in the liver of primary biliary cirrhosis (PBC) patients in terms of pathological stage of the disease advances. Intrahepatic forkhead box P3$^+$ (FOXP3$^+$) T cells in the PBC patients were divided into two groups as early stage (Scheuer's classification stage 1) and advanced stage (stages 2/3/4). Results are shown as the mean percentage of Tregs frequency ± standard deviation in each stage. $P < 0.05$.

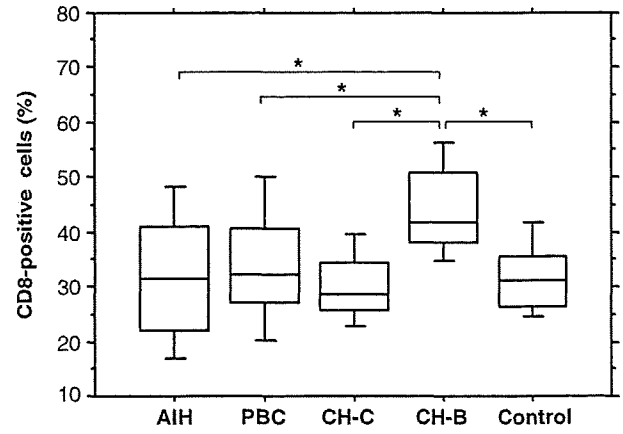


Figure 4 Intrahepatic CD8$^+$ T cells in autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), and controls. To compare intrahepatic CD8$^+$ cell frequency, intrahepatic CD8$^+$ cells in patients with AIH, PBC, CH-C, and CH-B were stained. CD8$^+$ cells were counted with the same procedure used for forkhead box P3$^+$ cells. Results are expressed as the median and range of all tested patents in each group. $P < 0.05$.

immune cells were involved in the immunopathogenesis of each liver disease. As shown in Figure 3, the frequency of CD4$^+$ T cells infiltrating the liver tissue was significantly higher in CH-B patients than in the controls. We found significantly less infiltrating CD4$^+$ T cells in the AIH patients than in the PBC patients ($P = 0.007$), CH-C patients ($P = 0.045$), and CH-B patients ($P < 0.001$). As shown in Figure 4, the frequency of CD8$^+$ T cells was significantly higher in the CH-B patients than in the controls. There were also significantly higher CD8$^+$ T cells in the liver tissues of the CH-B patients than in those of the AIH patients ($P = 0.003$), PBC patients ($P = 0.002$), and CH-C patients ($P < 0.001$).

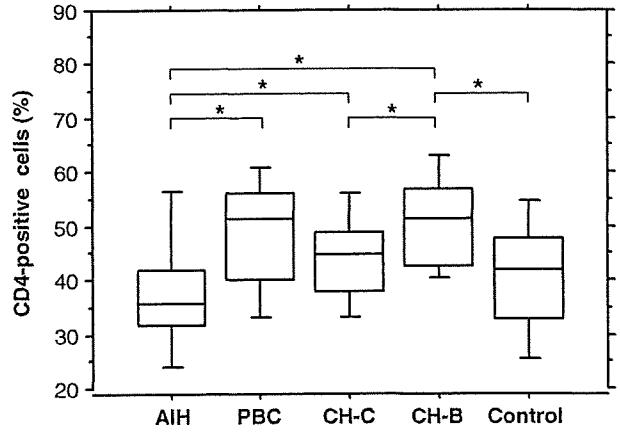


Figure 3 Intrahepatic CD4$^+$ T cells in autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), chronic hepatitis C (CH-C), chronic hepatitis B (CH-B), and controls. To compare intrahepatic CD4$^+$ cell frequency, intrahepatic CD4$^+$ cells in patients with AIH, PBC, CH-C, and CH-B were stained. CD4$^+$ cells were counted with the same procedure used for forkhead box P3$^+$ cells. Results are expressed as the median and range of all tested patients in each group. $P < 0.05$.

Furthermore, the CD4$^+$/CD8$^+$ ratio was lower in the CH-B patients than in the PBC patients (1.18 ± 0.26 *vs* 1.56 ± 0.66, $P = 0.037$) and CH-C patients (1.18 ± 0.26 *vs* 1.49 ± 0.39, $P = 0.007$). There was no difference in the total infiltration of mononuclear cells between patients with AIH, PBC, CH-C, and CH-B. Intrahepatic CD4$^+$ T cells and CD8$^+$ T cells in the control patients were significantly less than in the CH-B patients ($P = 0.013$ and $P < 0.001$, respectively), although we did not detect any differences between the control group and the other liver disease groups. There was no relationship between the biochemical data or histological activities and infiltration of the immune cells.

Since intrahepatic immune cells may directly affect inflammation in the liver, we compared the biochemical data, such as the serum ALT level, and histological activities with the intrahepatic frequencies of FOXP3$^+$, CD4$^+$, and CD8$^+$ cells. There was no relationship between the ALT, alkaline phosphatase, IgG, immuno-

globulin M level, or histological activities and the frequency of infiltrating immune cells other than described above (data not shown).

DISCUSSION

TREGS ARE THOUGHT to play roles in immune regulation, such as the suppression of severe inflammation and autoimmune diseases. The removal or reduction of Tregs can also enhance immune responses against infectious microbes, thus, Tregs affect the elimination of infectious microbes.[17–24] A higher proportion of $CD4^+$ and $CD25^+$ T cells in peripheral blood was found in patients with chronic HCV infection as compared to recovered patients and normal controls.[25] Tregs secrete transforming growth factor-β_1 and IL-10, and these cytokines may attenuate the function of macrophages. IL-10 also inhibits HCV-specific immunity when administered exogenously in patients with chronic HCV infection.[26] Thus, Tregs may disturb the eradication of HCV and lead to chronic infection. Chronic HBV patients harbor an increased frequency of Tregs in peripheral blood as compared to control patients, and Tregs have an immunosuppressive effect on HBV-specific T helper cells.[27] This may be one of the mechanisms that leads to chronic infection.

Several recent studies have focused on Tregs in patients with autoimmune liver diseases, such as AIH and PBC. Since Tregs prevent the proliferation and effector function of autoreactive T cells[16] and downregulate the production of IFN-γ by $CD8^+$ T cells in a murine model and in humans,[28,29] Tregs may be implicated in the pathogenesis of AIH and PBC. In fact, the relative frequencies of Tregs are decreased in peripheral blood samples of patients with PBC,[30] and Tregs are few in patients with AIH.[16] However, there are only a few reports regarding the status of intrahepatic Tregs.

Tregs maintain the ability to suppress IFN-γ production by $CD4^+$ and $CD25^-$ T cells in AIH, and circulating Tregs are significantly less in AIH patients than in controls.[16] However, few details regarding the roles of Tregs in the pathogenesis of AIH have been revealed.

In the present study, we demonstrated that intrahepatic Tregs were significantly more infiltrated in patients with liver diseases than in the controls. Indeed there are significantly fewer intrahepatic Tregs in AIH patients and CH-B patients than in PBC patients and CH-C patients, but as a whole, there is more infiltration of $FOXP3^+$ Tregs than in the controls, and there is not a great difference. In addition, we found significantly fewer infiltrating $CD4^+$ T cells in AIH patients than in the patients with other diseases, whereas $CD8^+$ T cells infiltrating liver tissue were detected with a significantly greater frequency in CH-B patients than in the other patients.

Although both AIH and PBC are representative autoimmune liver diseases, we identified differences in immune cell infiltration between these two autoimmune diseases in the present study. The results indicate that different mechanisms are involved in the pathogenesis of AIH and PBC. However, there are significantly more ratios of Tregs than control, and it seems that only a ratio of Tregs does not relate to the pathogenesis of these diseases.

We found that the frequency of Tregs decreased in the liver of PBC patients as the pathological stage of the disease advanced. A previous report demonstrated that there were few liver-infiltrating Tregs in PBC patients,[30] although it has not been confirmed by other researches. Sasaki *et al.* recently reported findings similar to ours. They found that the extent of $FOXP3^+$ Tregs in inflamed portal tracts with chronic non-suppurative destructive cholangitis in early stage (Scheuer's classification 1 and 2) of PBC was higher than that in late stage (Scheuer's classification 3, 4) of PBC.[31]

It is not clear whether this decrease of Tregs is a cause or a result of disease progression. Although we cannot explain the reason for these differences in Tregs' infiltration, the race of the study patients may be one of the factors. Functional investigations of intrahepatic Tregs in these autoimmune liver diseases may clarify this issue.

Since the frequency of intrahepatic Tregs in CH-C groups is diverse widely, we could not detect a significant difference in Tregs' accumulation between the CH-C and AIH groups. However, several CH-C patients had a large number of intrahepatic Tregs. When we divided the patients in each group into those with $FOXP3^+$ cells of less than 9% and those with $FOXP3^+$ cells of 9% or more, a significant difference was confirmed. In addition, patients who had a high frequency of intrahepatic Tregs were detected significantly more often in the CH-C group than in the CH-B group. In HCV infection, it has been suggested that HCV itself, especially in the NS3 region, induces Tregs in patients with HCV infection as well as in healthy donors,[32] and these Tregs are involved in the development of viral persistence, which occurs usually in acute HCV infection and rarely in acute HBV infection in adults. Thus, in chronic hepatitis, the pathogenesis of HCV should be different from that of HBV.

There were only a few Tregs in the pathologically normal tissue that surrounded metastatic liver tumors. The same phenomenon has been described in other reports.[30,33] The decreased frequency of Tregs was not likely to be the effect of metastatic tumors, because it has been reported that malignant tumors often induce Tregs.[34–39] In normal liver tissue, Treg infiltration may be suppressed because it is necessary to induce immunity against many pathogens flowing into the liver, rather than prevent inflammation or induction of autoimmunity.

Intrahepatic Tregs may be involved with immunopathogenesis and play a crucial direct role in the development of each liver disease. However, since immune systems in liver diseases are complicated, further investigations are needed to clarify the detailed relationship between Tregs and immunopathogenesis.

ACKNOWLEDGMENT

THIS STUDY IS supported in part by Grants-in-Aid for the Intractable Liver and Biliary Diseases from the Ministry of Health and Labor of Japan and for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1 Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002; 2: 389–400.
2 Sakaguchi S, Setoguchi R, Yagi H, Nomura T. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease. *Curr Top Microbiol Immunol* 2006; 305: 51–66.
3 Sugimoto N, Oida T, Hirota K *et al.* Foxp3-dependent and – independent molecules specific for CD25+CD4+ natural regulatory T cells revealed by DNA microarray analysis. *Int Immunol* 2006; 18: 1197–209.
4 Gregorio GV, Portmann B, Reid F *et al.* Autoimmune hepatitis in childhood: a 20-year experience. *Hepatology* 1997; 25: 541–7
5 Mieli-Vergani G, Vergani D. Progress in pediatric autoimmune hepatitis. *Semin Liver Dis* 1994; 14: 282–8.
6 Selmi C, Invernizzi P, Keeffe EB *et al.* Epidemiology and pathogenesis of primary biliary cirrhosis. *J Clin Gastroenterol* 2004; 38: 264–71.
7 Kaplan MM, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med* 2005; 353: 1261–73.
8 Ichiki Y, Shimoda S, Ishibashi H, Gershwin ME. Is primary biliary cirrhosis a model autoimmune disease? *Autoimmun Rev* 2004; 3: 331–6.
9 Kita H, Lian ZX, Van de Water J *et al.* Identification of HLA-A2-restricted CD8 (+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells. *J Exp Med* 2002; 195: 113–23.
10 Kita H, Imawari M, Gershwin ME. Cellular immune response in primary biliary cirrhosis. *Hepatol Res* 2004; 28: 12–17.
11 Shimoda S, Nakamura M, Ishibashi H, Hayashida K, Niho Y. HLA DRB4 0101-restricted immunodominant T cell autoepitope of pyruvate dehydrogenase complex in primary biliary cirrhosis: evidence of molecular mimicry in human autoimmune diseases. *J Exp Med* 1995; 181: 1835–45.
12 Matsumura S, Kita H, He XS *et al.* Comprehensive mapping of HLA-A0201-restricted CD8 T-cell epitopes on PDC-E2 in primary biliary cirrhosis. *Hepatology* 2002; 36: 1125–34.
13 Huang Z, Murray MG, Secrist JA 3rd. Recent development of therapeutics for chronic HCV infection. *Antiviral Res* 2006; 710: 351–62.
14 Bertoletti A, D'Elios MM, Boni C *et al.* Different cytokine profiles of intrahepatic T cells in chronic hepatitis B and hepatitis C virus infections. *Gastroenterology* 1997; 112: 193–9.
15 Bertoletti A, Naoumov NN. Translation of immunological knowledge into better treatments of chronic hepatitis B. *J Hepatol* 2003; 39: 115–24.
16 Longhi MS, Ma Y, Bogdanos DP, Cheeseman P, Mieli-Vergani G, Vergani D. Impairment of CD4 (+) CD25 (+) regulatory T-cells in autoimmune liver disease. *J Hepatol* 2004; 41: 31–7.
17 Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature* 2002; 420: 502–7.
18 Aseffa A, Gumy A, Launois P, MacDonald HR, Louis JA, Tacchini-Cottier F. The early IL-4 response to Leishmania major and the resulting Th2 cell maturation steering progressive disease in BALB/c mice are subject to the control of regulatory CD4+CD25+ T cells. *J Immunol* 2002; 169: 3232–41.
19 Xu D, Liu H, Komai-Koma M *et al.* CD4+CD25+ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, Leishmania major infection, and colitis in mice. *J Immunol* 2003; 170: 394–9.
20 Iwashiro M, Messer RJ, Peterson KE, Stromnes IM, Sugie T, Hasenkrug KJ. Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection. *Proc Natl Acad Sci USA* 2001; 98: 9226–30.
21 Montagnoli C, Bacci A, Bozza S *et al.* B7/CD28-dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to *Candida albicans*. *J Immunol* 2002; 169: 6298–308.
22 Raghavan S, Suri-Payer E, Holmgren J. Antigen-specific in vitro suppression of murine Helicobacter pylori-reactive

immunopathological T cells by CD4CD25 regulatory T cells. *Scand J Immunol* 2004; 60: 82–8.
23 Hori S, Carvalho TL, Demengeot J. CD25+CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyper-inflammation driven by Pneumocystis carinii in immunodeficient mice. *Eur J Immunol* 2002; 32: 1282–91.
24 Singh B, Read S, Asseman C *et al.* Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 2001; 182: 190–200.
25 Cabrera R, Tu Z, Xu Y *et al.* An immunomodulatory role for CD4 (+) CD25 (+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* 2004; 40: 1062–71.
26 Nelson DR, Tu Z, Soldevila-Pico C *et al.* Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. *Hepatology* 2003; 38: 859–68.
27 Alvarez F, Berg PA, Bianchi FB *et al.* International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; 31: 929–38.
28 Piccirillo CA, Shevach EM. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol* 2001, 167: 1137–40.
29 Camara NO, Sebille F, Lechler RI. Human CD4+CD25+ regulatory cells have marked and sustained effects on CD8+ T cell activation. *Eur J Immunol* 2003; 33: 3473–83.
30 Lan RY, Cheng C, Lian ZX *et al.* Liver-targeted and peripheral blood alterations of regulatory T cells in primary biliary cirrhosis. *Hepatology* 2006; 43: 729–37.
31 Sasaki M, Ikeda H, Sawada S, Sato Y, Nakanuma Y. Naturally-occurring regulatory T cells are increased in inflamed portal tracts with cholangiopathy in primary biliary cirrhosis. *J Clin Pathol* 2006; 60: 1102–7.
32 Tajimi M, Ugajin T, Ota M, Hiroishi K, Nakamura I, Imawari M. Immune responses of liver-infiltrating lymphocytes and peripheral blood mononuclear cells to hepatitis C virus core and NS3 antigens. *Hepatol Res* 2006; 35: 250–5.
33 Xu D, Fu J, Jin L *et al.* Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. *J Immunol* 2006; 177: 739–47.
34 Yang XH, Yamagiwa S, Ichida T *et al.* Increase of CD4+ CD25+ regulatory T-cells in the liver of patients with hepatocellular carcinoma. *J Hepatol* 2006; 45: 254–62.
35 Woo EY, Chu CS, Goletz TJ *et al.* Regulatory CD4 (+) CD25 (+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 2001; 61: 4766–72.
36 Liyanage UK, Moore TT, Joo HG *et al.* Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 2002; 169: 2756–61.
37 Viguier M, Lemaitre F, Verola O *et al.* Foxp3 expressing CD4+CD25 (high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 2004; 173: 1444–53.
38 Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A. CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. *Cancer* 2003; 98: 1089–99.
39 Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 2003; 9: 4404–8.

doi:10.1111/j.1440-1746.2008.05475.x

MINI-SERIES OF REVIEW

Immune responses in hepatitis C virus infection and mechanisms of hepatitis C virus persistence

Kazumasa Hiroishi, Takayoshi Ito and Michio Imawari

Department of Gastroenterology, Showa University School of Medicine, Tokyo, Japan

Key words
cytotoxic T lymphocyte, dendritic cell, helper T cell, natural killer cell, regulatory T cell.

Accepted for publication 31 March 2008.

Correspondence
Dr Kazumasa Hiroishi, Department of Gastroenterology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan. Email: hiroishi@med.showa-u.ac.jp

Abstract

Immune responses against hepatitis C virus (HCV) play a crucial role in the pathogenesis of chronic hepatitis C. HCV infection often persists and leads to chronic hepatitis and eventually cirrhosis. Accumulated data suggest that HCV proteins suppress host immune responses through the suppression of functions of immune cells, such as cytotoxic T lymphocytes, natural killer cells, and dendritic cells. They also suppress the type 1 interferon signaling system. The resulting insufficient immune responses against HCV lead to the sustained infection. The appropriate control of immune responses would contribute to the eradication of HCV and the improvement of hepatitis, but there are still many issues to be clarified. This review describes the scientific evidence to support these emerging concepts, and will touch on the implications for improving antiviral therapy.

Introduction

Hepatitis C virus (HCV) infection often persists and causes chronic hepatitis that may progresses to cirrhosis, followed by the development of hepatocellular carcinoma. HCV infection is a worldwide health problem. HCV was first identified by Chiron's group in 1989,[1] following sustained attempts by many researchers to find by various methods to isolate the causative agent of the so-called non-A, non-B hepatitis. In 1989, one of our authors (MI) established cytotoxic T-lymphocyte (CTL) clones, which recognize hepatocytes from patients with non-A, non-B hepatitis, but not those of donors without non-A, non-B hepatitis.[2] Unfortunately, we were unable to clarify the target protein recognized by the clones. The discovery of HCV contributed much to the diagnosis of hepatitis C, and proved that HCV was the causative agent of most of chronic non-A, non-B hepatitis. It was also a breakthrough in investigations of the immune responses to HCV infection and the immunopathogenesis of chronic hepatitis C. Hepatitis viruses themselves are not cytopathic to hepatocytes directly. Rather, hepatitis is caused by host immune cells, such as natural killer (NK) cells and CTL, which attack and destroy virus-infected hepatocytes to eradicate viral infections. It has been reported that CTL, which recognize peptides derived from hepatitis viruses, exist in the liver and the peripheral blood.[3] CTL specific for particular hepatitis viruses are thought to be one of the major host defense mechanisms, and have been implicated in viral clearance and the immunopathogenesis of viral hepatitis.[4]

In this review, cellular immune responses against HCV, the immunopathogenesis of HCV infection, and the escape mechanisms of HCV from immunosurveillance are summarized.

Host immune responses in HCV infection and immunopathogenesis of hepatitis C

Non-specific immune response

As in other viral infections, non-specific immune responses are induced in the host infected by HCV (Fig. 1). In the early stages of HCV infection, type I interferons (IFN; α/β) are produced by HCV-infected hepatocytes and plasmacytoid dendritic cells (DC). Type I IFN induce the expression of 2′–5′ oligoadenylate synthetase which inhibits HCV replication. Type I IFN enhance the expression of human leukocyte antigen (HLA) class I on the surface of antigen-presenting cells, such as DC, and reinforce cellular immune responses. The latter include the activation of NK cells and CTL. NK cells activated by type I IFN injure HCV-infected hepatocytes, and this process initiates hepatitis. In turn, the destruction of hepatocytes stimulates myeloid DC. Subsequently, these DC promote the secretion of a high amount of IFN-γ through the activation of NK cells and NKT cells; the latter having characteristics of both NK cells and T cells, and exist mainly in the liver. IFN-γ then activates hepatic macrophages to enhance local inflammation.

HCV-specific immune response

When the host is unable to eradicate HCV despite the induction of non-specific immune responses, HCV-specific immune responses are induced to eliminate the residual HCV.

Figure 2 shows the process of inducing HCV-specific immunes. Type 1 helper T (Th1) cells play a crucial role in the induction and

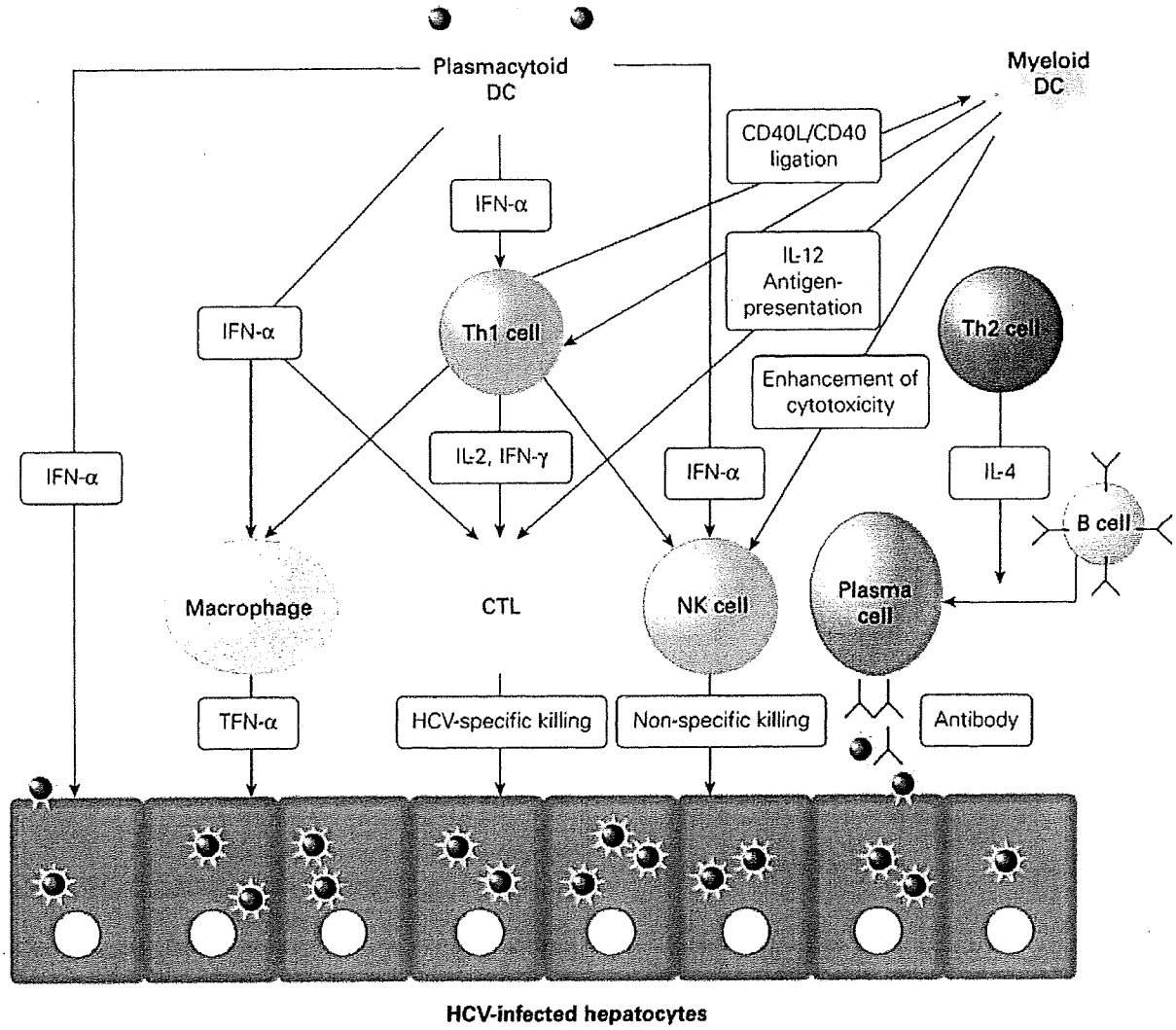


Figure 1 Immune responses in hepatitis C virus (HCV) infection. In the early stage of HCV infection, interferon (IFN)-α is produced by plasmacytoid dendritic cells (DC). IFN-α activates natural killer (NK) cells, helper T (Th) cells, macrophages, and cytotoxic T lymphocytes (CTL). NK cells activated by type I IFN injure the HCV-infected hepatocytes in a non-specific manner, whereas CTL do so in an antigen-specific manner. Destruction of hepatocytes stimulates myeloid DC. These DC secrete a high amount of interleukin (IL)-12, which promotes the activation of NK cells and type 1 helper T (Th1) cells. Activated Th1 cells in turn promote DC maturation by CD40/CD40 ligand (CD40L) interaction. Plasma cells secrete immunoglobulins to neutralize circulating HCV. Th2 cell, type 2 helper T cell.

activation of virus-specific cellular immune responses, such as CTL. After taking up HCV antigens in the liver, myeloid DC move to a draining lymph node. As they mature at the lymph node, the expression of HLA and costimulatory molecules is upregulated on their surface. These matured DC activate naïve helper T (Th) cells efficiently through stimulation with HLA class II and costimulatory molecules. In turn, the stimulated Th cells further activate DC by the expression of the CD40 ligand, as well as the secretion of cytokines, such as tumor necrosis factor (TNF)-α. Interleukin (IL)-12, produced mainly by myeloid DC, differentiates these stimulated Th cells towards Th1 cells. Activated Th1 cells secrete IL-2 and IFN-γ, which induce the activation and proliferation of CTL and NK cells. As a continuation of this process, naïve CTL recognize HCV antigens presented on the DC, and the stimulated HCV-specific CTL leave the lymph nodes for the liver. They recognize HCV antigens together with HLA class I on the surface of HCV-infected hepatocytes, and eradicate HCV by killing the infected hepatocytes.

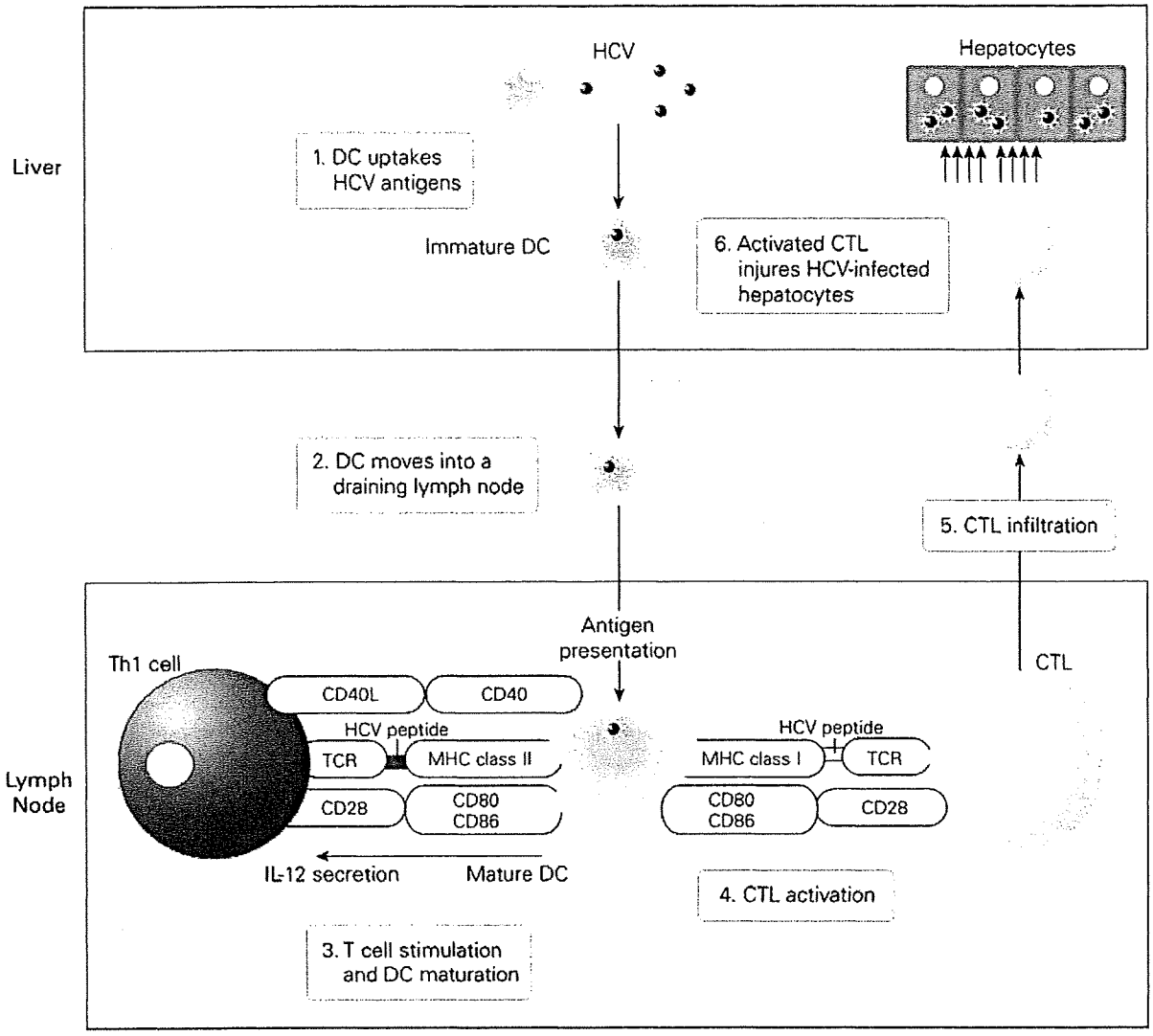


Figure 2 Induction of hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL). After taking up HCV antigens in the liver, myeloid dendritic cells (DC) move to a draining lymph node. Matured DC activate naïve helper T (Th) cells efficiently through stimulation with HLA class II and costimulatory molecules (CD80 and CD86). Stimulated Th cells in turn further activate DC by the expression of the CD40 ligand (CD40L) as well as secretion of tumor necrosis factor-α. Interleukin (IL)-12 produced by myeloid DC differentiates these stimulated Th cells towards Th1 cells. IL-2 and interferon-γ secreted by activated Th1 cells induce the activation and proliferation of CTL and natural killer NK cells. As a continuation of this process, naïve CTL recognize HCV antigens presented on the DC, and the stimulated HCV-specific CTL leave the lymph nodes for the liver. They recognize HCV antigens together with human leukocyte antigen (HLA) class I on the surface of HCV-infected hepatocytes, and eradicate HCV by killing the infected hepatocytes. MHC, major histocompatibility complex; TCR, T-cell receptor.

It has been reported that virus-specific CTL play an important role in viral eradication because CTL not only kill HCV-infected cells, but also inhibit viral replication. Since we first identified an HLA B44-restricted CTL epitope,[3,5] we have identified more than 20 CTL epitopes along the HCV polyprotein using synthetic peptides and enzyme-linked immunospot assay.[6,7] When peripheral blood lymphocytes of patients with HCV infection were stimulated with synthetic peptides corresponding to the CTL epitopes, we could frequently detect CTL responses in patients who had been infected with HCV within the past 3 years, but hardly ever in patients infected with HCV more than 10 years ago. It has been reported that patients who had spontaneously eradicated HCV infection up to 35 years earlier demonstrated persistent $CD4^+$ and $CD8^+$ T-cell responses specific to HCV peptides.[8]

At the time of HCV infection, if appropriate cellular immune responses are induced, viruses can be completely eradicated. In

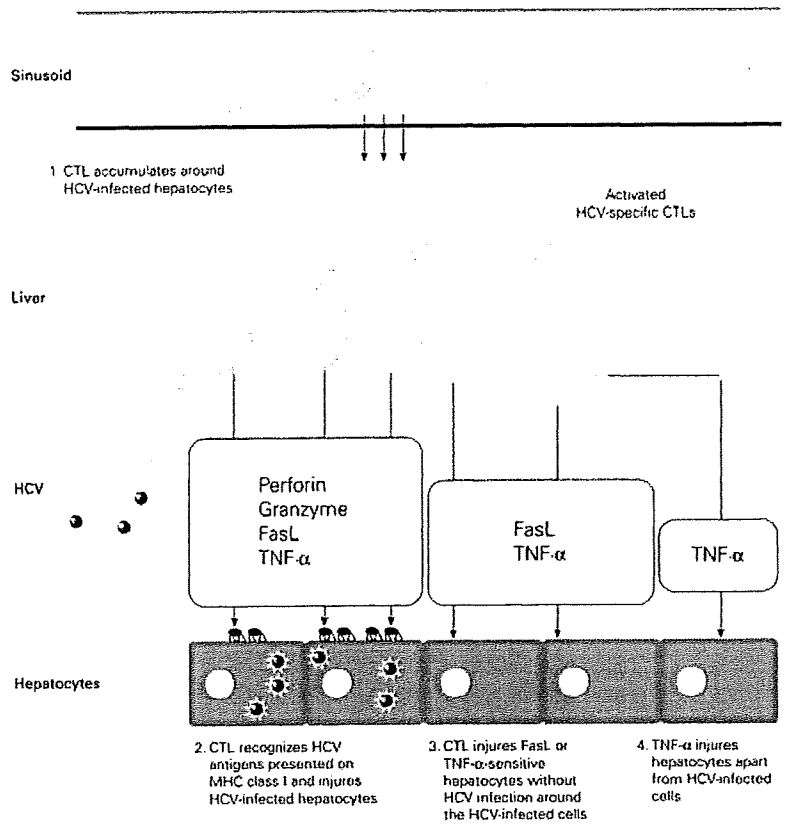


Figure 3 Killing mechanisms of hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTL) on hepatocytes. Activated HCV-specific CTL stimulated by matured dendritic cells (DC) infiltrate the liver tissue through a sinusoid. They recognize HCV antigen peptides and release perforin and granzyme. Activated CTL are also involved in killing hepatocytes through the expression of Fas ligands and the secretion of tumor necrosis-α (TNF-α). Hepatocytes in patients with severe hepatitis show increased susceptibility as a result of enhanced expression of Fas and/or TNF-α receptors. Activated CTL injure compromised hepatocytes, which have acquired sensitivity to the Fas ligand (FasL) and TNF-α, even if they are not infected with HCV. TNF-α released from activated CTL can injure compromised non-infected cells without cell-to-cell interaction.

HCV infection, however, immune responses are often not potent enough to eliminate the virus, and the weak immune responses result in relatively mild liver damage and lead to persistent infection. Conversely, when induced immune responses are too strong, severe liver injury, such as fulminant hepatitis, may occur, but this is rare in HCV infection. A moderate level of liver damage would occur through a balance between viral load and host immune responses.

It has also been reported that activated HCV-specific CTL accumulate in the liver of patients with chronic hepatitis C, and that they are involved in the inhibition of viral replication and liver damage. Given that CTL responses were frequently detectable in the peripheral blood of patients with a low titer of serum HCV-RNA, it was suggested that HCV-specific CTL may inhibit the replication of HCV, and that infection with high-titer HCV may suppress the CTL response.[9] Chronic hepatitis C patients with an HCV-specific CTL response had significantly high levels of serum alanine aminotransaminase, but there was no relationship between the CTL response, the clinical course, or the pathological severity.[10]

An extremely vigorous CTL response specific to a single epitope (HCV NS3 1073-1081), which cross-reacted with an influenza neuraminidase sequence, could be detected in patients with severe hepatitis, but not with mild hepatitis.[11] From this result, it was suggested that CTL cross-reactivity affects the severity of HCV-associated liver injury. A mutation of this CTL epitope at residues 1073–1081 of the NS3 protease may allow for escape from CTL recognition, but it would also diminish protease activity and RNA replication. Thus certain variants capable of immunological escape rarely appear as major viral species in HCV-infected patients.[12]

Killing mechanisms of HCV-infected hepatocytes by specific CTL

Activated HCV-specific CTL stimulated by matured DC infiltrate the liver tissue through a sinusoid. They recognize HCV antigen peptides, which consist of 8–11 amino acids, presented by HLA class I molecules through a T-cell receptor, and release perforin, which is inserted into the target cell's plasma membrane. This forms a pore and leads to the uptake of granzyme, which induces apoptosis of HCV-infected cells (Fig. 3). Activated CTL are also involved in killing hepatocytes through the expression of the Fas ligand and secretion of TNF-α. Perforin induces cell death in almost all cells, whereas the Fas ligand and TNF-α do so only in cells displaying their cognate receptors (Fas, TNF-R1). Normal hepatocytes show resistance against the Fas ligand and TNF-α while hepatocytes in patients with severe hepatitis show increased susceptibility as a result of the enhanced expression of Fas and/or TNF-α receptors. Although the cytotoxic activity of the Fas ligand and TNF-α is less potent than that of perforin, activated CTL

injure compromised hepatocytes, which have acquired sensitivity to the Fas ligand and TNF-α, even if they are not infected with HCV (Fig. 3). TNF-α released from activated CTL can injure compromised non-infected cells without cell-to-cell interaction.[13] This phenomenon may contribute to inhibiting the spread of HCV infection, although there is a possibility that it may further aggravate the hepatitis.

Mechanisms of persistence of HCV infection

Escape from immune surveillance by amino acid mutations

HCV exists in patients as various quasispecies induced by low-fidelity replication by RNA polymerase that attempt to escape from immune surveillance of the host. A mutation in an immunogenic region results in ignorance of HCV-specific CTL and antibodies, and the induction of tolerance towards CTL.[14,15] In addition, it is thought that quasispecies of HCV acquire the ability to infect various kinds of cells and become tolerant against multiple drugs.

Escape from humoral immune responses

A virus-specific neutralizing antibody eliminates viruses in the body fluid, but not in the infected cells. It takes a long time to generate a neutralizing antibody in HCV infection. It is therefore thought that an HCV-specific neutralizing antibody response contributes to the prevention of secondary infection rather than first infection. Amino acid mutations within the hypervariable region 1 (HVR1) of the HCV E2 region are highly diverse, and this may contribute to the persistence of infection by facilitating escape from neutralizing antibodies. However, it was reported that in chimpanzees, both with and without anti-HVR1 antibodies, the major HVR1 sequences were unchanged for a long time, and that sequences of HVR1 variants converged finally in each chimpanzee.[16] The data show that anti-HVR1 antibodies are unlikely to induce variation in HVR1. Recently, it was reported that the humoral response continuously failed to neutralize viruses, and that during chronic infection, selective pressures favor the continuous generation of escape variants from humoral and cellular immunity.[17]

Escape from cellular immune responses

It is possible that mutations in epitopes of HLA class I or class II-restricted T cells contribute to viral persistence by interfering with the recognition of HCV-infected cells. An amino acid mutation that interfered with $CD8^+$ CTL recognition was found through cloning the infecting HCV. A mutation of one amino acid within the epitope of the NS3 region recognized by Th1 cells resulted in a shift in cytokine secretion patterns from Th1 to type 2 helper T (Th2) cells.[18] Th2 dominance may lead to decreased antiviral responses.

Mutations within CTL epitopes were found to emerge in three of six patients with acute hepatitis C. CD8 cells could recognize the parental epitopes, but not variant sequences, and the variant sequences could not induce a CD8 response efficiently *in vitro*.[19] It was suggested that immunodominant CD8 responses are influenced by inhibitory mechanisms occurring early post-infection. However, CTL responses are diverse in the early stage of HCV infection, and thus a single amino acid mutation would not be able to explain persistent infection. Escape mutations may therefore be a result rather than a cause of persistent infection.

Immune suppression induced by HCV infection

Anti-HCV antibodies do not appear until 2–4 months after infection, and even if they are produced, HCV infection will persist and hepatitis will progress in most cases. Various T-cell responses can be detected in the acute phase, yet they are dramatically reduced in the chronic phase of HCV infection. Although a large number of HCV-specific $CD8^+$ T cells infiltrate the liver, they are unable to eradicate HCV. Recently, many reports have suggested that HCV itself may actively suppress host immune responses.

Inhibition of NK cells

As the major HCV envelope protein E2 has been shown to bind to CD81 with high affinity, the CD81 molecule is thought to be one of the receptors for HCV infection. It has been reported that CD81 cross-linking via the immobilized HCV E2 protein inhibits non-specific cytotoxicity mediated by NK cells, as well as IFN-γ production by NK cells.[20] Thus HCV may directly suppress the function of NK cells. NK cell infiltration can be found in the liver of patients 1 week after starting IFN therapy. Further, cytotoxicity of NK cells is thought to be an indicator of the efficacy of IFN therapy. It is therefore apparent that NK cells, representing innate immunity, are involved in the eradication of HCV, while the direct suppression of NK cells may be implicated in HCV persistence.

The HCV core protein enhances major histocompatibility complex (MHC) class I molecules by increasing the expression of transporter associated with antigen processing 1 in a p53-dependent manner.[21] As a result, the enhancement of MHC class I expression contributes to HCV persistence by suppressing the cytotoxicity of NK cells.

The expression of CD94/NKG2A, which is an inhibitory receptor on NK cells, has been reported to be enhanced in patients with chronic hepatitis C.[22] HCV peptide aa35–44, which is known as a well-characterized HLA-A2-restricted T-cell epitope, stabilizes the expression of HLA-E, which is a ligand for CD94/NKG2A, and thereby inhibits NK cell-mediated lysis.[23] It has been also reported that another inhibitory receptor of NK cells, KIR2DL3, is implicated in HCV eradication.[24]

Inhibition of humoral immunity

Since peripheral lymphocytes of patients with HCV infection show a high expression of CD81,[25] it is possible that HCV infects these cells and may affect antibody production. B-cell response is thought to be impaired, since the titer of the neutralizing antibody is too low to prevent reinfection with HCV.

Inhibition of T lymphocytes

The function of HCV-specific CTL as effectors is clearly impaired in chronic HCV infection. The expression of the CD3 ζ chain,

which is crucial for T cells to function properly, has been reported to be reduced on the surface of peripheral lymphocytes in patients with chronic hepatitis C.[26] The CTL responses are functionally defective with impaired IFN-γ production, low perforin content, and decreased capacity for proliferation and cytotoxicity.[27]

The HCV core protein, which is released from HCV-infected cells and exists in peripheral blood, interacts with gC1qR on T cells. This results in the inhibition of T-cell activation, proliferation, and IFN-γ production by T cells.[28,29] The HCV NS4A/B protein blocks the expression of HLA class I molecules on the cell surface by the inhibition of endoplasmic reticulum-to-Golgi traffic.[30] This may be one of the reasons why CTL tend to ignore HCV-infected hepatocytes. These functional defects may lead to persistent infection with HCV.

In acute hepatitis C, the cytotoxic activity of $CCR7^{-}CD8^{+}$ memory T cells is impaired, but it has been reported that IL-2 pushes semi-effector CTL to complete their effector function.[31] Therefore, IL-2 deficiency during T-cell activation may be responsible for incomplete effector differentiation of the memory CTL shown in patients with acute HCV infection. In addition, it is supposed that circulating the HCV core protein suppresses IL-2 and IL-2 receptor α gene transcription by the inhibition of phosphorylation of extracellular signal-regulated kinase (ERK) and the mitogen-activated ERK kinase.[32] Furthermore, in exhausted CD8 cells, programmed cell death 1, the ligation of which inhibits T-cell effector function, has been reported to be upregulated in acute hepatitis C.[33]

In the chronic phase, HCV-specific $CD4^{+}$ T cells can be detected, but their antigen-specific proliferation is impaired. As described later, the production of IL-10 and transforming growth factor-β (TGF-β) are detected in an antigen-specific manner. It is thought that these phenomena lead to persistent HCV infection by suppressing the proliferation and activation of Th cells and CTL.

In the liver tissue, immune cells, such as sinusoidal endothelial cells and Kupffer cells, can present viral antigens, but exhibit few costimulatory molecules, such as CD80 and CD86. Thus they are not able to stimulate T cells effectively, and may induce immune tolerance.[34]

Inhibition of DC

DC play a crucial role in inducing immunity. In chronic hepatitis C, it has been reported that the ability of DC to stimulate allogenic T cells is impaired, and that HCV core and E1 proteins inhibit DC maturation.[35] A decrease in the number of plasmacytoid DC and impairment of IFN-α production by them have been reported, while the ability of myeloid DC to stimulate allogenic lymphocytes is reduced despite no reduction in overall frequency of these cells.[36] HCV core and NS3 proteins impair the function of DC, which recognize those HCV proteins through Toll-like receptor (TLR) 2.[36] The available evidence suggests that HCV directly inhibits cellular immune responses in the host. It has also been reported that DC activated by TLR ligands derived from bacteria or viruses have a reduced cross-presentation ability, and this plays an important role in inducing immune responses.[37] HCV may possess the same mechanism to evade immune responses. In fact, the expression of TLR2 on immature DC is reduced in patients with chronic hepatitis C compared with healthy donors, and DC stimulated through TLR2 impair T-cell proliferation.[38]

Another study in acute hepatitis C suggested that the frequency and IFN-α-producing capacity of peripheral blood plasmacytoid DC are apparently reduced and inversely correlated with the severity of liver inflammation. In the chronic state, the recovery of plasmacytoid DC function is incomplete; this could also be due to the chronic inflammation.[39]

Concerning the relationship between DC and NK cells, it has been suggested that DC inhibit NK cell activation in HCV infection. DC activate NK cells by the expression of major histocompatibility complex class I-related chain A and B (MICA/B) on their surface after stimulation with IFN-α. However, in HCV-infected patients, the MICA/B expression is reduced. Impaired IL-15 production is one of the mechanisms of insufficient MICA/B expression on DC in response to type I IFN.[40]

HCV is thought to infect DC through the binding of the HCV E2 protein to DC-specific intercellular adhesion molcure-3 grabbing non-integrin on their cell surface.[41,42] Since the soluble E2 protein is also able to bind to DC, HCV may directly suppress DC function. Furthermore, it has been suggested that HCV core, NS3, NS5A, and NS5B proteins may induce apoptosis of mature DC.[43]

In an HCV-infected chimpanzee model, it has been shown that impairment of the above maturation and allostimulatory function are not necessary prerequisites, but rather consequences of chronic HCV infection.[44] Furthermore, there are some reports that neither plasmacytoid DC nor monocyte-derived DC are impaired in HCV-infected patients.[45] Human DC expressing HCV core and NS3 proteins showed normal phenotypic function, cytokine production, and normal T-cell stimulation capabilities.[46] It has also been reported that there are lower frequencies of myeloid DC and plasmacytoid DC in chronic hepatitis C patients than in healthy individuals. However, despite the decreased circulating myeloid DC, no phenotypic or functional defects were identified.[47] Although lower IFN-α production was responsible for the decreased numbers of plasmacytoid DC, these DC from HCV-infected patients produced almost the same level of IFN-α as DC from healthy individuals. Further investigations are needed to clarify DC function in chronic hepatitis C, the reason for its impairment, and the nature (if any) of functional defects.

Other effects of HCV proteins on immunity

It has been reported that IFN-induced signal transduction through the Jak–signal transducers and activators of transcription pathway is impaired in transgenic mice that express HCV proteins in their liver cells.[48] The HCV core protein induces apoptosis in Jurkat cells and immune cells via the Fas system.[49,50] The HCV core protein also drives liver injury by increasing Fas-mediated apoptosis and liver infiltration of peripheral T cells.[51] However, it has been also reported that although HCV-transgenic mice have an apparently normal T-cell response, their hepatocytes cannot eliminate an adenoviral infection, and that the defect in adenoviral clearance is responsible for resistance of the hepatocytes to apoptosis induced by Fas/APO1/CD95 death receptor stimulation.[52] *In vitro* data have also shown that the HCV core protein binds to TNF receptors. It has also been reported that the expression of the HCV core protein inhibits TNF-α-mediated apoptosis by the sustained upregulation of cellular Fas-associated via death domain-like IL-1β-converting enzyme-like inhibitory protein.[53] These data

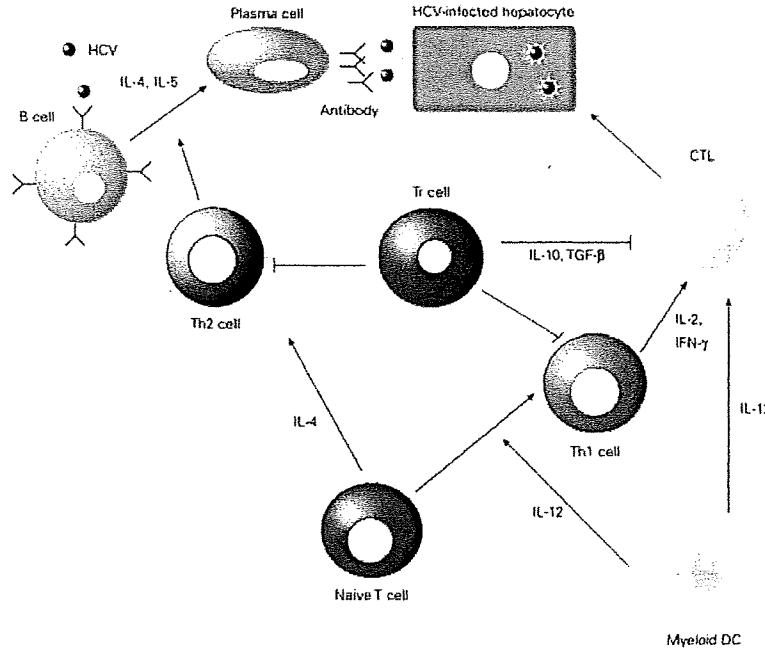


Figure 4 Induction of immune cells and inhibition of regulatory T (Tr) cells. IL-12 produced by myeloid dendritic cells (DC) differentiates T-helper (Th) cells towards type 1 helper (Th1) cells and activates CTL. Type 2 helper (Th2) cells stimulate humoral immune responses. Tr cells inhibit both cellular and humoral immune responses against hepatitis C virus (HCV). IL, interleukin; TGF, transforming growth factor.

emphasize that HCV can regulate apoptosis by direct effects of its own proteins, thereby escaping from host immune responses.

Involvement of regulatory T cells in persistent infection with HCV

Recently, the focus has been on regulatory T (Tr) cells, which secrete IL-10 and TGF-β, as potential regulators of immune responses.[54] In chronic hepatitis C, the frequency of $CD4^+$ $CD25^+$ T cells (considered Tr cells) is high, and these cells suppress T-cell function directly.[55] These cells can reduce HCV-specific cellular immune responses, thereby leading to persistent hepatitis (Fig. 4).[56] HCV core-specific Tr cells are induced from the peripheral blood of patients with chronic hepatitis C, and IL-10 produced by those cells is involved in the persistence of HCV infection.[57] Forkhead/winged helix transcription factor 3 Tr cells and IL-10 producing HCV-specific $CCR7^-$ $CD8^+$ Tr cells infiltrate the liver of patients with chronic HCV infection; IL-10 is identified as a soluble inhibitory factor mediating immune suppression.[58,59] These cells may play a crucial role in regulating intrahepatic T-cell responses. In addition, peripheral blood mononuclear cells from normal individuals secrete IL-10 in response to NS3 and NS4, suggesting that cells of the innate immune system, in addition to T cells, produce IL-10 in HCV-infected patients.[60,61]

While these data suggest the involvement of Tr cells in HCV-persistent infection, the involvement of Tr cells in the pathogenesis of chronic hepatitis C still remains to be clarified.

Other mechanisms of HCV persistence

The HCV NS5A protein binds to the IFN-induced, double-stranded, RNA-activated protein kinase, protein kinase R (PKR), which is a critical component of the cellular antiviral and antiproliferative responses, and inhibits PKR function.[62] This mechanism may also play a critical role in HCV persistence. The IFN sensitivity-determining region is a 40 amino-acid sequence located in the NS5A protein of hepatitis C virus genotype 1b. Mutations in this region abrogate the interaction of NS5A with PKR, and may result in increasing sensitivity of HCV to antiviral therapy.[63] HCV NS3/4A serine protease blocks the phosphorylation of interferon regulatory factor-3, which is a key cellular antiviral signaling molecule.[64] The NS3/4A serine proteases target Cardif, a caspase activation and recruitment-domain containing adaptor protein that interacts with retinoic acid-inducible gene I, and the interaction with Cardif leads to the inhibition of antiviral effects.[65] Immune suppression by HCV proteins is summarized in Figure 5.

Relevance of immune responses for HCV infection therapy

IFN-α is the mainstay for HCV antiviral treatment. Low doses of human IFN-α augment the cellular immune response by three to four-fold, whereas a further increase in IFN dosage suppresses the CTL response significantly.[66] A Th1 response is dominant in patients with sustained viral response to IFN-α-based therapies. Ribavirin, which is used in combination with IFN-α, has an antiviral mechanism that drives the Th2 response towards a Th1 response.[67] It was reported that intrahepatic and peripheral blood HCV-specific CTL activity could be detected in patients with a sustained response to IFN therapy more frequently than in patients who relapsed or had no treatment response.[68] The authors of that report stated that: "rather than the combination therapy acting to enhance the CTL response to achieve viral clearance, detectable CTL prior to treatment increases the likelihood of the host responding to the direct antiviral activity of IFN-α and ribavirin".

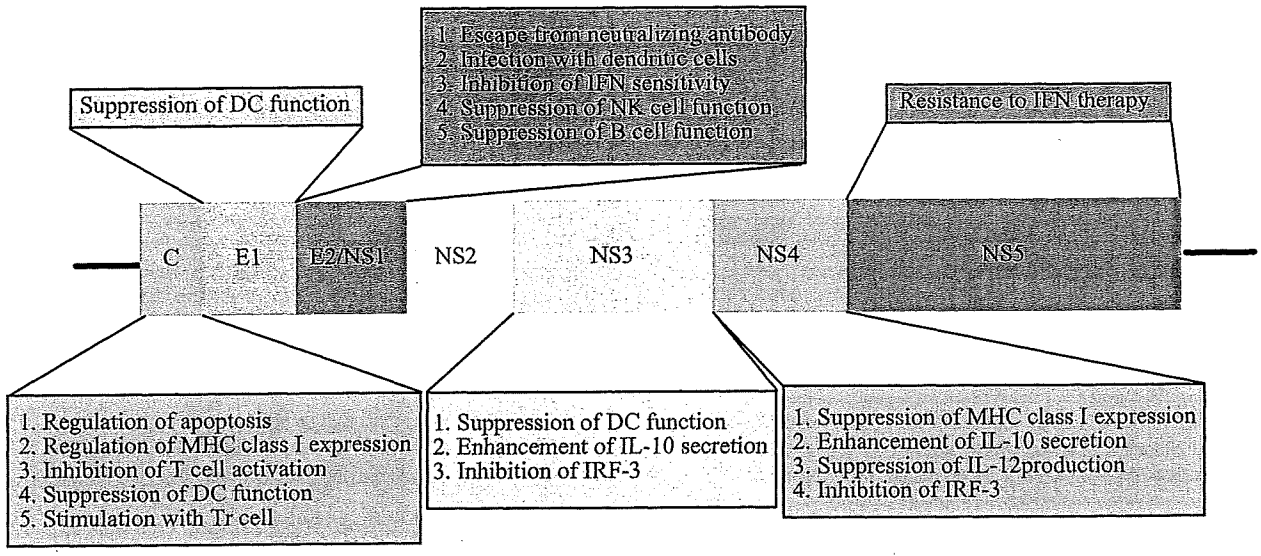


Figure 5 Immune suppression by hepatitis C virus (HCV) proteins. Suppressive effects of HCV proteins on host immune responses have been reported. Suppressive effects of HCV proteins at every HCV region are summarized. DC, dendritic cells; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; MHC, major histocompatibility complex; NK, natural killer; Tr cells, regulatory T cells.

In a mouse model, the adoptive transfer of HCV NS3 protein-pulsed DC matured with an oligodeoxynucleotide containing cytidine-phosphate-guanosine motifs *ex vivo* effectively promoted potent HCV-specific protective immune responses.[69] Thus DC-based therapy may be a candidate for immune therapy for chronic HCV infection in the future.

Conclusions and future directions

Immune responses against HCV have been investigated by analyses of clinical samples and animal models. It has been clarified that cellular immune responses play an important role in the pathogenesis of hepatitis. The mechanisms of liver damage and inhibition of immune responses have also been investigated. The appropriate control of immune responses would contribute to the eradication of HCV and improvement of hepatitis, but there are still many issues to be clarified. Therapies capable of complete HCV eradication could be developed by detailed exploration of immunological mechanisms as well as the virology of HCV.

Acknowledgment

We thank to Professor Geoff Farrell for his critical review of our manuscript.

References

1 Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-a, non-b viral hepatitis genome. *Science* 1989; **244**: 359–62.
2 Imawari M, Nomura M, Kaieda T *et al.* Establishment of a human T-cell clone cytotoxic for both autologous and allogeneic hepatocytes from chronic hepatitis patients with type non-A, non-B virus. *Proc. Natl. Acad. Sci. USA* 1989; **86**: 2883–7.
3 Kita H, Moriyama T, Kaneko T *et al.* HLA B44-restricted cytotoxic T lymphocytes recognizing an epitope on hepatitis C virus nucleocapsid protein. *Hepatology.* 1993; **18**: 1039–44.
4 Yap KL, Ada GL, McKenzie IF. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature* 1978; **273**: 238–9.
5 Kita H, Hiroishi K, Moriyama T *et al.* A minimal and optimal cytotoxic T cell epitope within hepatitis C virus nucleoprotein. *J. Gen. Virol.* 1995; **76** (Pt 12): 3189–93.
6 Kaneko T, Nakamura I, Kita H, Hiroishi K, Moriyama T, Imawari M. Three new cytotoxic T cell epitopes identified within the hepatitis C virus nucleoprotein. *J. Gen. Virol.* 1996; **77** (Pt 6): 1305–9.
7 Hakamada T, Funatsuki K, Morita H *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. *J. Gen. Virol.* 2004; **85**: 1521–31.
8 Wertheimer AM, Miner C, Lewinsohn DM, Sasaki AW, Kaufman E, Rosen HR. Novel CD4+ and CD8+ T-cell determinants within the NS3 protein in subjects with spontaneously resolved HCV infection. *Hepatology* 2003; **37**: 577–89.
9 Hiroishi K, Kita H, Kojima M *et al.* Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection. *Hepatology* 1997; **25**: 705–12.
10 Rothman AL, Morishima C, Bonkovsky HL *et al.* Associations among clinical, immunological, and viral quasispecies measurements in advanced chronic hepatitis C. *Hepatology* 2005; **41**: 617–25.
11 Urbani S, Amadei B, Fisicaro P *et al.* Heterologous T cell immunity in severe hepatitis C virus infection. *J. Exp. Med.* 2005; **201**: 675–80.
12 Soderholm J, Ahlen G, Kaul A *et al.* Relation between viral fitness and immune escape within the hepatitis C virus protease. *Gut* 2006; **55**: 266–74.
13 Ando K, Hiroishi K, Kaneko T *et al.* Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. *J. Immunol.* 1997; **158**: 5283–91.

14 Kaneko T, Moriyama T, Udaka K *et al.* Impaired induction of cytotoxic T lymphocytes by antagonism of a weak agonist borne by a variant hepatitis C virus epitope. *Eur. J. Immunol.* 1997; **27**: 1782–7.
15 Hiroishi K, Eguchi J, Ishii S, Okamoto H, Mitamura K, Imawari M. Differential effect of cytotoxic T lymphocyte variant epitopes on generation and cytotoxicity in chronic hepatitis C virus infection. *Hepatol. Res.* 2002; **24**: 91–4.
16 Zhou YH, Sugitani M, Esumi M. Sequences in the hypervariable region 1 of hepatitis C virus show only minimal variability in the presence of antibodies against hypervariable region 1 during acute infection in chimpanzees. *Arch. Virol.* 2002; **147**: 1955–62.
17 von Hahn T, Yoon JC, Alter H *et al.* Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection *in vivo*. *Gastroenterology* 2007; **132**: 667–78.
18 Wang JH, Layden TJ, Eckels DD. Modulation of the peripheral T-Cell response by CD4 mutants of hepatitis C virus: transition from a Th1 to a Th2 response. *Hum. Immunol.* 2003; **64**: 662–73.
19 Urbani S, Amadei B, Cariani E *et al.* The impairment of CD8 responses limits the selection of escape mutations in acute hepatitis C virus infection. *J. Immunol.* 2005; **175**: 7519–29.
20 Tseng CT, Klimpel GR. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *J. Exp. Med.* 2002; **195**: 43–9.
21 Herzer K, Falk CS, Encke J *et al.* Upregulation of major histocompatibility complex class I on liver cells by hepatitis C virus core protein via p53 and TAP1 impairs natural killer cell cytotoxicity. *J. Virol.* 2003; **77**: 8299–309.
22 Jinushi M, Takehara T, Tatsumi T *et al.* Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection. *J. Immunol.* 2004; **173**: 6072–81.
23 Nattermann J, Nischalke HD, Hofmeister V *et al.* The HLA-A2 restricted T cell epitope HCV core 35–44 stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells. *Am. J. Pathol.* 2005; **166**: 443–53.
24 Ahlenstiel G, Rehermann B. Hepatitis C virus and the threshold of natural killer cell inhibition. *Hepatology* 2005; **41**: 675–7.
25 Zuckerman E, Slobodin G, Kessel A *et al.* Peripheral B-cell CD5 expansion and CD81 overexpression and their association with disease severity and autoimmune markers in chronic hepatitis C virus infection. *Clin. Exp. Immunol.* 2002; **128**: 353–8.
26 Maki A, Matsuda M, Asakawa M, Kono H, Fujii H, Matsumoto Y. Decreased CD3 zeta molecules of T lymphocytes from patients with hepatocellular carcinoma associated with hepatitis C virus. *Hepatol. Res.* 2003; **27**: 272–8.
27 Missale G, Cariani E, Ferrari C. Role of viral and host factors in HCV persistence: which lesson for therapeutic and preventive strategies? *Dig. Liver Dis.* 2004; **36**: 703–11.
28 Kittlesen DJ, Chianese-Bullock KA, Yao ZQ, Braciale TJ, Hahn YS. Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. *J. Clin. Invest.* 2000; **106**: 1239–49.
29 Yao ZQ, Eisen-Vandervelde A, Ray S, Hahn YS. HCV core/gC1qR interaction arrests T cell cycle progression through stabilization of the cell cycle inhibitor p27Kip1. *Virology* 2003; **314**: 271–82.
30 Konan KV, Giddings TH Jr, Ikeda M, Li K, Lemon SM, Kirkegaard K. Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus. *J. Virol.* 2003; **77**: 7843–55.
31 Francavilla V, Accapezzato D, De Salvo M *et al.* Subversion of effector CD8+ T cell differentiation in acute hepatitis C virus infection: exploring the immunological mechanisms. *Eur. J. Immunol.* 2004; **34**: 427–37.
32 Yao ZQ, Nguyen DT, Hiotellis AI, Hahn YS. Hepatitis C virus core protein inhibits human T lymphocyte responses by a complement-dependent regulatory pathway. *J. Immunol.* 2001; **167**: 5264–72.
33 Urbani S, Amadei B, Tola D *et al.* PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J. Virol.* 2006; **80**: 11398–403.
34 Mehal WZ, Azzaroli F, Crispe IN. Immunology of the healthy liver: old questions and new insights. *Gastroenterology* 2001; **120**: 250–60.
35 Sarobe P, Lasarte JJ, Zabaleta A *et al.* Hepatitis C virus structural proteins impair dendritic cell maturation and inhibit in vivo induction of cellular immune responses. *J. Virol.* 2003; **77**: 10862–71.
36 Szabo G, Dolganiuc A. Subversion of plasmacytoid and myeloid dendritic cell functions in chronic HCV infection. *Immunobiology* 2005; **210**: 237–47.
37 Wilson NS, Behrens GM, Lundie RJ *et al.* Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat. Immunol.* 2006; **7**: 165–72.
38 Yakushijin T, Kanto T, Inoue M *et al.* Reduced expression and functional impairment of Toll-like receptor 2 on dendritic cells in chronic hepatitis C virus infection. *Hepatol. Res.* 2006; **34**: 156–62.
39 Ulsenheimer A, Gerlach JT, Jung MC *et al.* Plasmacytoid dendritic cells in acute and chronic hepatitis C virus infection. *Hepatology* 2005; **41**: 643–51.
40 Jinushi M, Takehara T, Tatsumi T *et al.* Autocrine/paracrine IL-15 that is required for type I IFN-mediated dendritic cell expression of MHC class I-related chain A and B is impaired in hepatitis C virus infection. *J. Immunol.* 2003; **171**: 5423–9.
41 Lozach PY, Lortat-Jacob H, de Lavalette A *et al.* DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J. Biol. Chem.* 2003; **278**: 20358–66.
42 Pohlmann S, Zhang J, Baribaud F *et al.* Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J. Virol.* 2003; **77**: 4070–80.
43 Siavoshian S, Abraham JD, Thumann C, Kieny MP, Schuster C. Hepatitis C virus core, NS3, NS5A, NS5B proteins induce apoptosis in mature dendritic cells. *J. Med. Virol.* 2005; **75**: 402–11.
44 Rollier C, Drexhage JA, Verstrepen BE *et al.* Chronic hepatitis C virus infection established and maintained in chimpanzees independent of dendritic cell impairment. *Hepatology* 2003; **38**: 851–8.
45 Piccioli D, Tavarini S, Nuti S *et al.* Comparable functions of plasmacytoid and monocyte-derived dendritic cells in chronic hepatitis C patients and healthy donors. *J. Hepatol.* 2005; **42**: 61–7.
46 Li W, Li J, Tyrrell DL, Agrawal B. Expression of hepatitis C virus-derived core or NS3 antigens in human dendritic cells leads to induction of pro-inflammatory cytokines and normal T-cell stimulation capabilities. *J. Gen. Virol.* 2006; **87**: 61–72.
47 Longman RS, Talal AH, Jacobson IM, Rice CM, Albert ML. Normal functional capacity in circulating myeloid and plasmacytoid dendritic cells in patients with chronic hepatitis C. *J. Infect. Dis.* 2005; **192**: 497–503.
48 Blindenbacher A, Duong FH, Hunziker L *et al.* Expression of hepatitis c virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. *Gastroenterology* 2003; **124**: 1465–75.
49 Moorman JP, Prayther D, McVay D, Hahn YS, Hahn CS. The C-terminal region of hepatitis C core protein is required for Fas-ligand independent apoptosis in Jurkat cells by facilitating Fas oligomerization. *Virology* 2003; **312**: 320–9.
50 Hahn YS. Subversion of immune responses by hepatitis C virus: immunomodulatory strategies beyond evasion?. *Curr. Opin. Immunol.* 2003; **15**: 443–9.

51 Soguero C, Joo M, Chianese-Bullock KA, Nguyen DT, Tung K, Hahn YS. Hepatitis C virus core protein leads to immune suppression and liver damage in a transgenic murine model. *J. Virol.* 2002; **76**: 9345–54.
52 Disson O, Haouzi D, Desagher S *et al.* Impaired clearance of virus-infected hepatocytes in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2004; **126**: 859–72.
53 Saito K, Meyer K, Warner R, Basu A, Ray RB, Ray R. Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. *J. Virol.* 2006; **80**: 4372–9.
54 van Driel IR, Ang DK. Role of regulatory T cells in gastrointestinal inflammatory disease. *J. Gastroenterol. Hepatol.* 2008; **23**: 171–7.
55 Boettler T, Spangenberg HC, Neumann-Haefelin C *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. *J. Virol.* 2005; **79**: 7860–7.
56 Sugimoto K, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* 2003; **38**: 1437–48.
57 MacDonald AJ, Duffy M, Brady MT *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. *J. Infect. Dis.* 2002; **185**: 720–7.
58 Accapezzato D, Francavilla V, Paroli M *et al.* Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J. Clin. Invest.* 2004; **113**: 963–72.
59 Sakaki M, Hiroishi K, Baba T *et al.* Intrahepatic status of regulatory T cells in autoimmune liver diseases and chronic viral hepatitis. *Hepatol. Res.* 2007.
60 Brady MT, MacDonald AJ, Rowan AG, Mills KH. Hepatitis C virus non-structural protein 4 suppresses Th1 responses by stimulating IL-10 production from monocytes. *Eur. J. Immunol.* 2003; **33**: 3448–57.
61 Tajimi M, Ugajin T, Ota M, Hiroishi K, Nakamura I, Imawari M. Immune responses of liver-infiltrating lymphocytes and peripheral blood mononuclear cells to hepatitis C virus core and NS3 antigens. *Hepatol. Res.* 2006; **35**: 250–5.
62 Gale MJ Jr, Korth MJ, Tang NM *et al.* Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 1997; **230**: 217–27.
63 Gale M Jr, Blakely CM, Kwieciszewski B *et al.* Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol. Cell Biol.* 1998; **18**: 5208–18.
64 Foy E, Li K, Wang C *et al.* Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 2003; **300**: 1145–8.
65 Meylan E, Curran J, Hofmann K *et al.* Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167–72.
66 Gehring S, Gregory SH, Kuzushita N, Wands JR. Type 1 interferon augments DNA-based vaccination against hepatitis C virus core protein. *J. Med. Virol.* 2005; **75**: 249–57.
67 Fang SH, Hwang LH, Chen DS, Chiang BL. Ribavirin enhancement of hepatitis C virus core antigen-specific type 1 T helper cell response correlates with the increased IL-12 level. *J. Hepatol.* 2000; **33**: 791–8.
68 Freeman AJ, Marinos G, Ffrench RA, Lloyd AR. Intrahepatic and peripheral blood virus-specific cytotoxic T lymphocyte activity is associated with a response to combination IFN-alpha and ribavirin treatment among patients with chronic hepatitis C virus infection. *J. Viral. Hepat.* 2005; **12**: 125–9.
69 Yu H, Huang H, Xiang J, Babiuk LA, Hurk S. Dendritic cells pulsed with hepatitis C virus NS3 protein induce immune responses and protection from infection with recombinant vaccinia virus expressing NS3. *J. Gen. Virol.* 2006; **87**: 1–10.

Dendritic cells stimulated with cytidine-phosphate-guanosine oligodeoxynucleotides and interferon-α-expressing tumor cells effectively reduce outgrowth of established tumors *in vivo*

Ayako Hiraide, Kazumasa Hiroishi,[1] Junichi Eguchi, Shigeaki Ishii, Hiroyoshi Doi and Michio Imawari

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

(Received January 10, 2008/Revised April 8, 2008/Accepted April 22, 2008/Online publication July 29, 2008)

Dendritic cells (DC) are potent antigen-presenting cells that elicit immune responses to foreign antigens. We have previously demonstrated the synergistic effects of cytidine-phosphate-guanosine (CpG) oligodeoxynucleotides (ODN) and interferon (IFN)-α on DC maturation *in vitro*. In the present study, the antitumor effects of DC preincubated with IFN-α gene-overexpressing murine colorectal cancer MC38 cells (MC38-IFN-α) and CpG ODN were evaluated in a poorly immunogenic murine cancer system. When we injected DC preincubated with MC38-IFNα and CpG ODN subcutaneously to mice bearing MC38 wild-type tumors, the outgrowth of the established parental tumors was suppressed significantly compared with that following administration of DC with MC38-IFN-α ($P = 0.008$). All mice injected with DC preincubated with MC38-IFN-α and CpG ODN rejected a subsequent parental tumor challenge. Immunohistochemical and flow cytometric analyses showed that CD4$^+$, CD8$^+$, and NK1.1$^+$ cells markedly infiltrated the established tumors of mice treated with DC preincubated with MC38-IFN-α and CpG ODN. From the results in immune cell-depleted mice, CD4$^+$ and asialo-GM-1$^+$ cells seemed to contribute to the antitumor effects induced by the combination DC therapy. Furthermore, non-specific cytolysis was detected when splenocytes of mice inoculated with DC preincubated with MC38-IFNα and CpG ODN were used as effector cells. Using an interleukin (IL)-12-neutralizing antibody it was suggested that IL-12 stimulates natural killer cells and contributes in part to the antitumor effects induced by DC incubated with CpG ODN and IFN-α. As DC-based immunotherapy with CpG ODN and IFN-α-expressing tumor cells induces a potent antitumor immune response, it should be considered for clinical application. (*Cancer Sci* 2008; 99: 1663–1669)

Dendritic cells are known as professional APC, characterized by their potent ability to activate and stimulate naive T lymphocytes *in vivo*. It has been hypothesized that the colocalization of DC and tumor cells may generate APC capable of stimulating tumor-reactive T cells *in vivo* because the histological infiltration of DC into primary tumors is associated with prolonged patient survival and reduces the incidence of metastatic disease in patients with bladder, lung, esophageal, gastric, and nasopharyngeal carcinoma.[1–5] Therefore, DC are thought to be attractive adjuvant agents for cancer therapy and a number of clinical studies, as well as immunotherapy using DC in murine models, have been carried out.

In the immature state, DC are able to capture and process antigens. Then, as they mature, they express surface molecules, including CD40, CD80, and CD86, that appear to play crucial roles in costimulating the activation and expansion of antigen-specific CD8$^+$ CTL, and they migrate to draining lymph nodes where they encounter and prime CD4$^+$ and CD8$^+$ T cells. Concomitantly, their capacity to acquire antigens is decreased. Therefore, to establish DC-based clinical therapy, the changing phenotype should be characterized in detail.

Microbial molecules such as lipopolysaccharides, bacteria-derived RNA, and DNA are recognized by host cells through the TLR family, which belongs to the family of pattern-recognition receptors[6] and stimulates immune responses. It has been reported that synthetic oligodeoxynucleotides (ODN) containing the unmethylated CpG motif promote Th1-type immune responses.[7] CpG ODN bind to TLR9 and are then endocytosed by DC. The signal transduction cascade in such DC ultimately leads to their activation with subsequent maturation into professional APC,[8] which have enhanced expression of costimulatory molecules such as CD80 and CD86 and produce high amounts of cytokines such as IL-12 and TNF-α. Because matured DC may improve the effects of therapy on established tumors, many investigations have been carried out in murine models as well as human clinical studies. CpG-based immunotherapy has demonstrated enhanced antitumor responses in murine models[9–12] and, indeed, CpG ODN has been applied to clinical cancer therapy for melanoma patients.[13]

Interferon-α has many biological effects, including enhancement of IFN-α/β production,[14,15] antiviral function, inhibition of cell growth, and angiogenesis.[16] IFN-α upregulates the expression of MHC class I on the cell surface and enhances the proliferation of Th1,[17] and is important for the generation of CTL in specific antitumor immune responses.[18,19] In addition, we reported previously that IFN-α-expressing tumor cells promote the survival of tumor-specific CTL by preventing apoptosis.[20] Based on these immunomodulating effects of IFN-α, it has been used to treat patients with tumors such as melanoma, renal cell carcinoma, and leukemia.

It has been reported that DC maturation, such as upregulation of costimulatory molecules (CD80, CD86), MHC class II, and CD83 expression on human DC, follows stimulation with IFN-α,[21] and in the presence of IFN-α it has been reported that DC show a greater capability to stimulate the proliferation of allogenic lymphocytes.[21] Furthermore, we reported that IFN-α gene therapy

[1]To whom correspondence should be addressed. E-mail: hiroishi@med.showa-u.ac.jp

Abbreviations: APC, antigen-presenting cell; B6, C57BL/6; CM, complete medium; CpG, cytidine-phosphate-guanosine; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DC + a, DC preincubated with MC38-IFN-α; DC + a + C, DC preincubated with MC38-IFN-α and CpG ODN-1826; DC + WT, DC preincubated with MC38-WT; DC + WT + C, DC preincubated with MC38-WT and CpG ODN-1826; ELISA, enzyme-linked immunosorbent assay; E : T, effector to target ratio; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; MC38-IFN-α, IFN-α gene-overexpressing murine colorectal cancer MC38; MC38-WT, MC38 wild type; MHC, major histocompatibility complex; NK, natural killer; OCT, optimal clotting temperature; ODN, oligodeoxynucleotide; Th1, type I helper T cell; TLR, toll-like receptor; TNF, tumor necrosis factor; WT. wild type: YAC, yeast artificial chromosome.

doi: 10.1111/j.1349-7006.2008.00858.x

in combination with DC-based immunotherapy reduces the outgrowth of established tumors in a poorly immunogenic tumor model.[22] In that model, we demonstrated that $CD4^+$, $CD8^+$, and NK cells are involved in the antitumor effects induced by DC and IFN-α therapy. However, tumor regression was not observed in the parental tumor-bearing mice treated with DC and IFN-α, and it was considered that more effective treatment was required for clinical trials.

We previously demonstrated that coincubation with CpG ODN and IFN-α-overexpressing tumor cells, but not WT cells, effectively upregulated costimulatory molecules on the murine bone marrow-derived DC, and that CpG in combination with IFN-α effectively stimulated production by DC of cytokines such as IL-1β and TNF-α.[23] When DC that had been preincubated with CpG ODN and IFNα-overexpressing tumor cells were coincubated with allogenic splenocytes *in vitro*, proliferation of the splenocytes was enhanced significantly compared with those incubated with CpG ODN and WT cells. Because CpG ODN and IFN-α have synergistic effects on DC maturation, they are expected to induce potent antitumor immune responses, and should be considered for clinical application in combination therapy.

In the present study, a preliminary investigation of the combined therapy prior to clinical studies, the antitumor effects of DC preincubated with CpG and IFN-α-overexpressing murine colorectal cancer cells were evaluated in a poorly immunogenic murine cancer system. We showed that when parental tumor-bearing mice were injected with DC preincubated with CpG and IFN-α-overexpressing tumor cells outgrowth of the established parental tumor was suppressed significantly, and $CD4^+$, $CD8^+$, and $NK1.1^+$ cells markedly infiltrated the established tumors of mice treated with DC preincubated with CpG and IFN-α-overexpressing tumor cells. Combined therapy with DC showed a profound preventive antitumor effect on the parental tumor. Furthermore, we confirmed that IL-12 production contributes in part to the antitumor effects induced by DC preincubated with CpG and IFN-α-overexpressing tumor cells.

Materials and Methods

Mice. Female 6-week-old B6 mice were purchased from Sankyo Laboratory Service (Tokyo, Japan) for use in experiments from 8 to 12 weeks of age. Mice were maintained in an animal care facility at Showa University. The present study has been approved by the Ethical Committee for Animal Experiments of Showa University.

Cell lines, culture medium, and reagents. The MC38 poorly immunogenic murine colorectal adenocarcinoma cell line (B6 mouse origin) was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES buffer, 1 mmol/L Minimum Essential Medium sodium pyruvate, and 0.1 mmol/L Minimum Essential Medium non-essential amino acids (CM) in a humidified incubator with 5% CO_2 in air at 37°C. All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA).

The MC38 cell line was genetically modified to produce murine IFN-α (MC38-IFNα) as described previously.[24] Expression of IFN-α was confirmed by ELISA using a commercially available kit according to the manufacturer's instructions (mouse IFN-α ELISA; PBL Biomedical Laboratories, New Brunswick, NJ, USA). Gamma-irradiation (100 Gy for tumor cells and 30 Gy for DC) was carried out with Gammacell 3000 Elan (Nordion International, Kanata, Canada). As reported previously, 1×10^5 cells of MC38-IFNα produce approximately 20.8 ± 0.5 ng/48 h of IFN-α, and wild type MC38 (MC38-WT) cells do not produce any IFN-α. IFN-α gene transduction does not affect the growth of tumor cells *in vitro* or survival of γ-irradiated tumor cells.[22]

Cytidine-phosphate-guanosine (CpG) ODN-1826 has been reported to have maturation effects on DC.[25] CpG ODN-1826 (5′-TCC ATG ACG TTC CTC ACG TT-3′) and ODN-1911 (served as the control, 5′-TCC AGG ACT TTC CTC AGG TT-3′, non-CpG) were synthesized by Sigma-Aldrich Japan (Tokyo, Japan). In our previous study, we confirmed that ODN-1911 does not affect DC maturation[23] or antitumor activity (data not shown).

Preparation of DC and incubation with CpG ODN and tumor cells. DC were generated from bone marrow cells of B6 mice using murine granulocyte macrophage-colony stimulating factor (GM-CSF) (10 ng/mL) and IL-4 (10 ng/mL), obtained from Pepro Tech EC (London, UK) as reported previously.[22] DC were preincubated with MC38-WT (DC + WT), MC38-IFNα (DC + a), CpG ODN-1826 and MC38-WT (DC + WT + C), or CpG ODN-1826 and MC38-IFNα (DC + a + C). CpG ODN-1826 was added at a concentration of 6 μg/mL, and all tumor cells were γ-irradiated (100 Gy) before incubation with DC at a DC to tumor ratio of 10.[23]

Therapeutic effects of DC and genetically modified MC38 cells on established parental tumors *in vivo*. To evaluate the therapeutic effects of DC incubated with CpG ODN and IFN-α-overexpressing MC38 cells on established parental tumors, we measured the size of established MC38-WT tumors in mice before and after treatment with these DC, as described previously.[24] B6 mice were injected subcutaneously with 1×10^5 MC38-WT cells in the right flank. Seven, 10, and 14 days after the WT-inoculation, 1×10^6 DC prepared as above (DC + WT, DC + a, DC + C, DC + WT + C, and DC + a + C) were inoculated subcutaneously around the established parental tumors, which had reached 9–25 mm^2 in size. Tumor size was measured twice a week using vernier calipers. Each experiment involved six mice per group. Mice with ulcerated tumors or tumors larger than 20 mm in diameter were killed.

Preventive effects of inoculation with DC with CpG ODN and MC38-IFN-α cells on the development of parental tumors *in vivo* after immune-cell depletion. To determine the role of the immune system in reduction of *in vivo* tumor growth in the preventive model, $CD4^+$ T cells, $CD8^+$ T cells, or asialo-GM-1^+ cells were depleted by an antibody method as described previously.[26] Culture medium from hybridomas producing the following antibodies was used at appropriate concentrations: anti-CD4 (GK1.5, TIB207; American Type Culture Collection (ATCC) Manassas, VA, USA) and anti-CD8 (2.43, TIB210; ATCC). For depletion of asialo-GM-1^+ cells, antiasialo-GM-1 antibody was obtained from Wako (Osaka, Japan). All antibody doses and treatment regimens were determined in preliminary studies using the same antibodies used for the experiments. Treatment was confirmed by flow cytometric analysis to completely delete the desired cell population for the entire duration of the study (data not shown). After DC with MC38-IFNα cells (DC + a) or DC with MC38-IFNα cells and CpG ODN-1826 (DC + a + C) were inoculated twice intraperitoneally at 7-day intervals (days –14 and –7), we depleted $CD4^+$ T cells, $CD8^+$ T cells, or asialo-GM-1^+ cells (days –5, –4, and –3). Otherwise, rat IgG (obtained from Wako) was inoculated as a control on the same days. In another experiment, depletion of both $CD4^+$ and asialo-GM-1^+ cells was carried out to evaluate additional suppressive effects on antitumor immunity. One week after the vaccination (day 0), these mice were injected subcutaneously with 1×10^5 MC38-WT cells in the right flank. Tumor size was measured twice a week using vernier calipers. On day 35, tumor-free mice were reinjected subcutaneously with 3×10^5 MC38-WT cells, then tumor establishment was observed. Each experiment involved five or six mice per group. Mice with ulcerated tumors or tumors larger than 20 mm in diameter were killed.

Immunohistological analysis. B6 mice were injected subcutaneously in the right flank with 1×10^5 MC38-WT cells. On days 7, 10, and 14, 1×10^6 DC prepared as above (DC + WT, DC + a,

doi: 10.1111/j.1349-7006.2008.00858.x

DC + CC, DC + WT + C, DC + a +C) were inoculated around the established parental tumors. Tumor tissues were harvested 4 days after the last inoculation (18 days after WT inoculation), and were embedded immediately in optimal clotting temperature compound (Tissue Tek, Elkhart, IN) and frozen. Serial 5-μm sections were exposed to anti-CD4, CD8a, and CD11c antibodies (Nippon Becton Dickinson, Tokyo, Japan). Rat IgG2a (Nippon Becton Dickinson) was used as a control antibody. Immunostaining was completed with a Vectastain ABC kit (Vector, Burlingame, CA, USA). Immunoreactive cells were counted in 10 fields using light microscopy (×400) in a blinded fashion. Each experiment involved two mice per group.

Analysis of the role of IL-12 and NK1.1⁺ cell infiltration in established parental tumors of mice treated with DC preincubated with CpG ODN and MC38-IFN-α. To explore the antitumor mechanism of DC preincubated with CpG ODN and MC38-IFN-α, we neutralized IL-12 using monoclonal antibody (C17.8; Nippon Becton Dickinson), and assessed the NK-cell infiltration of the tumors. B6 mice were injected subcutaneously in the right flank with 2×10^5 MC38-WT cells. On days 7, 10, and 14, 2×10^6 DC incubated with MC38-IFN-α and CpG ODN (DC + a + C) supplemented with 100 μg neutralizing rat antimouse IL-12 antibody[27] or control rat IgG (Nippon Becton Dickinson) were inoculated intraperitoneally. Tumor size was measured twice a week using vernier calipers. Each experiment involved five mice per group. Mice with ulcerated tumors or tumors larger than 20 mm in diameter were killed. Tumor tissues were harvested 4 days after the third inoculation (18 days after WT inoculation). Tumor-infiltrating mononuclear cells were separated from tumor tissue as described previously.[26] The cells were washed three times with CM. Flow cytometric analyses were carried out using FACScalibur (Nippon Becton Dickinson) to investigate the phenotype of the tumor-infiltrating mononuclear cells. The monoclonal antibodies used in this analysis were fluorescein isothiocyanate-conjugated anti-CD4, CD8, and NK1.1 antibodies (Nippon Becton Dickinson).

Evaluation of cytolytic activity of splenocytes from mice immunized with DC preincubated with CpG ODN and MC38-IFN-α cells by stimulation *in vitro*. Mice received injections of 1×10^6 DC preincubated with MC38-IFN-α (DC + a) or with MC38-IFN-α and CpG ODN (DC + a + C) on days 0 and 7. One week later, splenocytes were harvested and 2×10^6 splenocytes were incubated in the presence of 100 IU/mL recombinant mouse IL-2 (Nippon Becton Dickinson). Seven days later, cytolytic assays were carried out using the splenocytes as effector cells to assess non-specific cytolytic activity. For evaluation of cytolytic activity against MC38-WT cells, splenocytes from the immunized mice were stimulated three times weekly *in vitro* with 30 Gy γ-irradiated DC + a or DC + a + C, as described previously.[22] One week later, the stimulated cells were harvested and used as effector cells.

Cytolytic assays. Tumor-stimulated effector cells were assessed for cytolytic activity against MC38-WT and YAC-1 cells in triplicate in a 4-h ^{51}Cr-release assay. Target cells (1×10^6 cells/mL) were labeled with 3.7×10^6 Bq of $Na_2{}^{51}CrO_4$ (Amersham Pharmacia Biotech, Tokyo, Japan) for 1 h at 37°C. Labeled cells were washed and resuspended. Target cells (5×10^3) and various numbers of effector cells at the indicated E : T were plated in 200 μL CM in each well of the 96-well round-bottom plates. ^{51}Cr release was measured after a 4-h incubation at 37°C. Percentage lysis was determined using the formula:

(release in assay – spontaneous release) × 100/(maximum release – spontaneous release).

Maximum release was determined by lysis of labeled target cells with 1% Triton X-100. Spontaneous release was measured by incubating target cells in the absence of effector cells, and was less than 15% of maximum release.

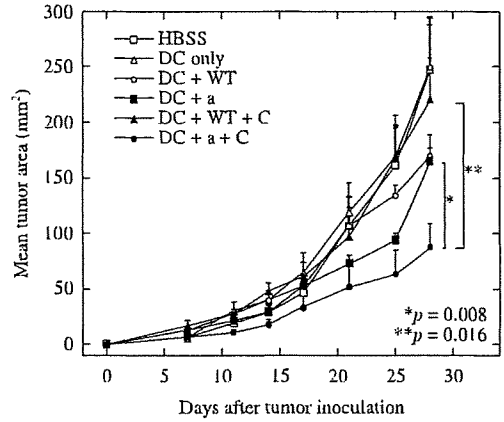


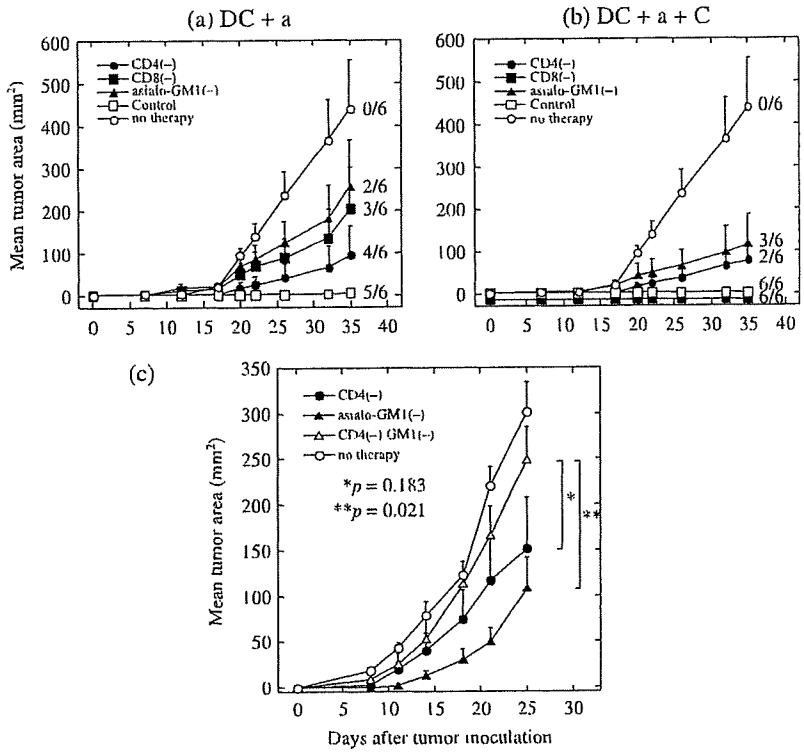
Fig. 1. Dendritic cells (DC) incubated with interferon (IFN)-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α) and cytidine-phosphate-guanosine (CpG) effectively suppressed established wild-type (WT) tumors. B6 mice were injected subcutaneously with 1×10^5 MC38-WT cells in the right flank. Seven, 10, and 14 days after the WT inoculation, 1×10^6 DC incubated with or without tumor cells and/or CpG oligodeoxynucleotides (ODN) were inoculated around the established parental tumors, which had reached 9–25 mm² in size. Tumor size was measured twice a week using vernier calipers. Each experiment involved six mice per group. The experiment was repeated twice and a representative result is shown. Results are shown as mean tumor area + SEM. DC + WT, DC incubated with MC38-WT; DC + a, DC incubated with MC38-IFNα; DC + WT + C, DC incubated with MC38-WT and CpG ODN; DC + a + C, DC incubated with MC38-IFN-α and CpG ODN; HBSS, Hank's balanced salt solutions.

Statistical analyses. Significance was assessed with Student's *t*-test or Wilcoxon's analysis. Differences between groups were considered significant when the *P*-value was lower than 0.05.

Results

Dendritic cells incubated with CpG ODN and IFN-α-overexpressing tumor cells effectively suppressed outgrowth of the established WT tumors. In our previous study, we demonstrated that DC maturation was effectively enhanced by coincubation with CpG ODN and IFN-α-overexpressing tumor cells *in vitro*.[23] To assess the antitumor effects of these DC *in vivo*, DC incubated with CpG ODN and MC38-IFN-α were injected into mice with established WT tumors. As shown in Figure 1, outgrowth of the established tumors was suppressed significantly by treatment with DC preincubated with CpG ODN and MC38-IFN-α (DC + a + C) compared with treatment with DC preincubated with MC38-IFN-α (DC + a) (DC + a + C vs DC + a, $P = 0.008$). In addition, we did not observe any therapeutic benefits of DC preincubated with MC38-WT and CpG ODN (DC + WT + C) in suppressing the outgrowth of parental tumors (Hank's balanced salt solutions (HBSS) vs DC + WT + C, $P = 0.663$; DC + a + C vs DC + WT + C, $P = 0.016$). Furthermore, of the 12 mice treated with DC + a + C, we observed regression of the parental tumor size in two mice.

CD4⁺ and asialo-GM-1⁺ cells were involved in the antitumor effects induced by DC incubated with CpG ODN and IFN-α-overexpressing tumor cells. DC in combination with IFN-α-overexpressing tumor cells have been proven to effectively suppress outgrowth of established WT tumors.[22] To clarify the different antitumor mechanisms in DC preincubated with MC38-IFN-α (DC + a) and DC preincubated with MC38-IFN-α and CpG (DC + a + C), we injected MC38-WT and observed tumor growth after treatment and depletion of immune cells. In mice treated with DC + a, when CD4⁺, CD8⁺, or asialo-GM-1⁺ cells were depleted by monoclonal antibodies, obvious tumor growth was observed in some



(d)

Therapy	CD4(–)	CD8(–)	Asialo-GM1(–)	Control	No therapy
DC + a	2/4	0/3	2/2	4/5	0/0
DC + a + C	2/2	0/6	3/3	6/6	0/0

Fig. 2. CD4⁺ and asialo-GM-1⁺ cells are involved in the antitumor effects induced by dendritic cells (DC) incubated with cytidine-phosphate-guanosine (CpG) oligonucleotides (ODN) and interferon (IFN)-α-overexpressing tumor cells. After (a) DC with IFN-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α) cells (DC + a) or (b) DC with MC38-IFN-α cells and CpG ODN-1826 (DC + a + C) were inoculated intraperitoneally twice at 7-day intervals (days –14 and –7), CD4⁺ T cells, CD8⁺ T cells, or asialo-GM-1⁺ cells were depleted on days –5, –4, and –3. Otherwise, rat IgG was inoculated as a control on the same days. (c) In another experiment, depletion of both CD4⁺ cells and asialo-GM-1⁺ cells was carried out to evaluate the additional suppressive effects on antitumor immunity. One week after the vaccination (day 0), these mice were injected with 1×10^5 MC38-WT cells in the right flank. Tumor size was measured twice a week using vernier calipers. The number of tumor-free mice on day 35 is also shown. (d) Numbers of tumor-free mice treated with DC + a or DC + a + C after the second parental tumor cell challenge (on day 70). On day 35, tumor-free mice were reinjected with 3×10^5 MC38-WT cells, and then tumor establishment was observed, and the numbers of tumor-free mice on day 70 are shown in the panel. Each experiment involved five or six mice per group.

Table 1. Immunohistochemical analyses of tumor-infiltrating immune cells after treatment with dendritic cell (DC), interferon (IFN)-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α), and cytidine-phosphate-guanosine (CpG)

Treatment	CD4⁺ cells	CD8⁺ cells	CD11c⁺ cells
DC only	4.2 ± 1.6	2.4 ± 1.1	6.0 ± 2.4
DC + WT	9.8 ± 3.5	0.6 ± 0.9	4.6 ± 3.4
DC + a	62.6 ± 31.9	44.2 ± 13.3	14.2 ± 11.1
DC + C	4.4 ± 2.9	0.0 ± 0.0	0.4 ± 0.55
DC + WT + C	6.4 ± 3.8	21.4 ± 4.1	0.0 ± 0.0
DC + a + C	94.6 ± 59.6	98.8 ± 79.6	44.8 ± 37.5
HBSS (control)	10.0 ± 7.1	3.6 ± 3.1	7.0 ± 14.6

Results are shown as mean counts ± SD of each cell in 10 fields using light microscopy (×400) in a blinded fashion. Each experiment involved two mice per group. DC + WT, DC incubated with MC38-WT; DC + a, DC incubated with MC38-IFNα; DC + C, DC incubated with CpG ODN; DC + WT + C, DC incubated with MC38-WT and CpG ODN; DC + a + C, DC incubated wit MC38-IFNα and CpG ODN. HBSS, Hank's balanced salt solutions.

mice of each group (Fig. 2a). In contrast, we did not detect any tumors in mice treated with DC + a + C when CD8⁺ cells were depleted (Fig. 2b). In other words, CD4⁺ and asialo-GM-1⁺ cells may be involved in the antitumor effects induced by DC + a + C, whereas CD8⁺ cells did not seem to be critical for the effects.

When both anti-CD4 antibody and anti-asialo GM-1 antibody were injected (Fig. 2c), the antitumor immunity was reduced significantly compared with administration with only anti-asialo GM⁻¹ antibody ($P = 0.021$). The mean parental tumor size of mice injected with anti-CD4 antibody was smaller than that of mice injected with both antibodies, although it did not reach a significant difference ($P = 0.183$). Thus, an additional suppressive effect of both antibodies on antitumor immune responses induced by the combined DC therapy was observed.

To evaluate the prolonged antitumor activity induced by the combined therapy, tumor-free mice were reinjected subcutaneously with 3×10^5 MC38-WT cells, and then tumor establishment was observed. As shown in Figure 2d, all six mice treated with DC + a + C rejected the subsequent parental tumor cell challenge, as did four of five mice treated with DC + a. In addition, all six mice treated with DC + a + C following injection with anti-CD8 antibody rejected the first parental tumor cell challenge, but obvious tumors were observed in all of these mice after rechallenge of the parental tumor cells. In three of six mice that were vaccinated with DC + a + C following depletion of asialo-GM-1⁺ cells, no obvious tumors were observed, and all three tumor-free mice rejected a subsequent challenge of the parental tumor cells. These results suggest that CD8⁺ cells are concerned with maintaining long-lasting antitumor immunity.

CD4⁺, CD8⁺, and asialo-GM-1⁺ cells markedly infiltrated the established parental tumors of mice treated with DC preincubated with CpG ODN and IFN-α-overexpressing tumor cells. To analyze the antitumor mechanisms induced by DC preincubated with MC38-IFN-α and CpG (DC + a + C), we carried out immunohistochemical staining using WT tumor tissue of mice treated with the DC therapy. The results showed that CD4⁺, CD8⁺, and CD11c⁺ cells clearly infiltrated the WT tumors of mice treated with DC + a (Table 1). Furthermore, extreme infiltration of these

doi: 10.1111/j.1349-7006.2008.00858.x

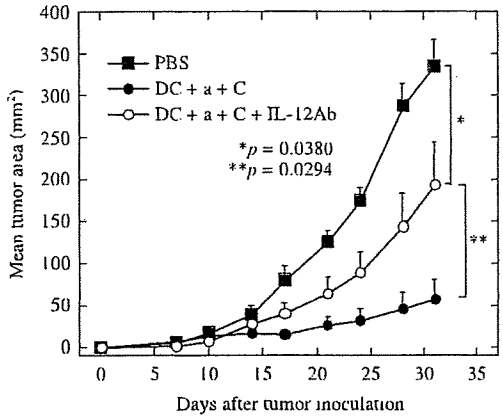


Fig. 3. Decreased antitumor effects of mice treated with dendritic cells (DC) incubated with cytidine-phosphate-guanosine (CpG) oligonucleotides (ODN) and interferon (IFN)-α-overexpressing tumor cells after neutralization of interleukin (IL)-12. B6 mice were injected in the right flank with 2×10^5 MC38-WT cells. On days 7, 10, and 14, 2×10^6 DC incubated with MC38-IFN-α and CpG ODN together with 100 μg IL-12-neutralizing antibody or control IgG were inoculated intraperitoneally. Tumor size was then measured twice a week using vernier calipers. Each experiment involved five mice per group. PBS, phosphate-buffered saline.

Table 2. Comparison of tumor-infiltrating immune cells after dendritic cell (DC) therapy with interferon (IFN)-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α), and cytidine-phosphate-guanosine (CpG) with or without anti-interleukin (IL)-12-neutralizing antibody

Treatment	$CD4^+$ cells	$CD8^+$ cells	$NK1.1^+$cells
Control IgG	38.02 ± 2.04	33.02 ± 6.73	19.90 ± 1.15
IL-12 antibody	38.91 ± 0.38	20.33 ± 0.12	11.23 ± 2.74

B6 mice were injected subcutaneously in the right flank with 2×10^5 MC38-WT cells. On days 7, 10, and 14, 1×10^6 DC incubated with MC38-IFN-α and CpG ODN, with 100 μg IL-12-neutralizing antibody or with control IgG were inoculated. Tumor tissues were harvested 4 days after this third inoculation (18 days after wild-type inoculation). Tumor-infiltrating mononuclear cells were separated from tumor tissue. Flow cytometric analyses were carried out using fluorescein isothiocyanate-conjugated anti-CD4, -CD8, or -NK1.1 antibodies to investigate the phenotype of tumor-infiltrating mononuclear cells. The results are shown as mean percentage positive cells ± SD.

cells was observed in the parental tumors of mice treated with DC + a + C (Table 1). These results suggest that the antitumor immune response would be activated effectively by DC preincubated with CpG and IFN-α-overexpressing tumor cells.

Decreased antitumor effects of mice treated with DC incubated with CpG ODN and IFN-α-overexpressing tumor cells after neutralization of IL-12. As shown previously, IL-12 production by DC was enhanced markedly by the addition of CpG ODN.[23] We carried out ELISA and detected 2.99 ± 0.15 ng IL-12 production by 1×10^6 mature DC incubated with CpG and IFN-α-overexpressing tumor cells. We evaluated the relationship between IL-12 and the antitumor effects of the DC therapy using neutralizing antibody against mouse IL-12. As shown in Figure 3, the outgrowth of WT tumors increased significantly in mice treated with DC + a + C together with injected IL-12 neutralizing antibody ($P = 0.029$) compared with control antibody, whereas the tumor size was still clearly smaller than the parental tumor of mice with no treatment (phosphate-buffered saline (PBS); $P = 0.038$). Because the antitumor effects of DC incubated with MC38-IFN-α and CpG ODN were partially abrogated by neutralization of IL-12, this cytokine contributes in part to the effects.

Decreased tumor-infiltrating $CD8^+$ and $NK1.1^+$ cells in the established parental tumors of mice treated with DC preincubated with CpG ODN and IFN-α-overexpressing tumor cells after neutralization of IL-12. We analyzed tumor-infiltrating NK cells of mice treated with the DC therapy because we had found previously that DC in combination with IFN-α-overexpressing tumor cells recruits many NK cells in tumors.[22] As shown in Table 2, we detected a high level of $CD8^+$ and $NK1.1^+$ cells in the parental tumors of mice treated with DC preincubated with MC38-IFN-α and CpG ODN. When anti-IL-12 neutralizing antibody was injected into mice treated with these DC, the levels decreased. However, the amount of intrahepatic $CD4^+$ cells in mice injected with anti-IL-12 neutralizing antibody was almost the same as that of mice injected with control antibody. These data suggest that IL-12 produced by the injected mature DC stimulates $CD8^+$ and NK cells *in vivo* and contributes to the antitumor effect of the combined DC therapy.

Cytolytic activity of splenocytes obtained from mice treated with DC incubated with CpG ODN and IFN-α-overexpressing tumor cells. To evaluate the cytolytic activity of mice treated with the DC therapy *in vivo*, splenocytes obtained from mice treated with DC incubated with MC38-IFN-α and CpG ODN were examined with a 4-h ^{51}Cr-release assay against YAC-1 cells, which are known to be sensitive to NK cells. As shown in Figure 4a, meaningful

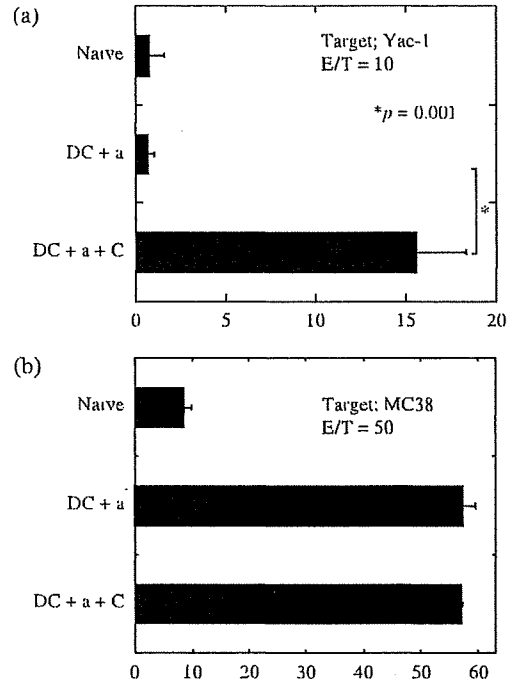


Fig. 4. Non-specific cytolytic activity was detected clearly when splenocytes obtained from mice treated with dendritic cells (DC) incubated with cytidine-phosphate-guanosine (CpG) oligonucleotides (ODN) and interferon (IFN)-α-overexpressing tumor cells were used as effector cells. Mice received injections of 1×10^6 DC preincubated with IFN-α gene-overexpressing murine colorectal cancer MC38 (MC38-IFN-α) (DC + a) or with MC38-IFN-α and CpG ODN (DC + a + C) on days 0 and 7. (a) To assess non-specific cytolytic activity, splenocytes were harvested and 2×10^6 splenocytes were incubated in the presence of 100 IU/mL recombinant mouse interleukin (IL)-2. Seven days later, a ^{51}Cr-release assay was carried out using the splenocytes as effector cells. The results are shown as mean percentage cytolysis against YAC-1 cells + SD. (b) For evaluation of cytolytic activity against MC38-WT cells, splenocytes from the immunized mice were stimulated three times weekly *in vitro* with 30 Gy γ-irradiated DC + a or DC + a + C. One week later, the stimulated cells were harvested and used as effector cells. The results are shown as mean percentage cytolysis against MC38-WT cells + SD.

cytolysis against YAC-1 cells was detected only with splenocytes from mice treated with DC preincubated when MC38-IFN-α and CpG ODN (% cytolysis = 15.5 ± 2.9, E : T = 10), and not with DC preincubated with MC38-IFN-α (% cytolysis = 0.7 ± 0.4, E : T = 10), were used as effector cells ($P = 0.001$). This result suggests that NK cells are activated *in vivo* by DC incubated with IFN-α-overexpressing tumor cells and CpG ODN.

We induced MC38-specific CTL using splenocytes of tumor-free mice treated with DC + a or DC + a + C by following repetitive *in vitro* stimulation with DC + a or DC + a + C, respectively, as reported previously.[22] Although we detected cytolytic activity against MC38 cells, there was no difference in tumor-specific cytotoxicity against MC38-WT cells between DC + a-treated mice (% cytolysis = 57.5 ± 2.1, E : T = 50) and DC + a + C-treated mice (% cytolysis = 57.1 ± 0.4, E : T = 50) (Fig. 4b). When splenocytes from mice treated with DC + a or DC + a + C twice weekly, but without any rechallenge of WT tumor cells *in vivo*, were stimulated with DC + a or DC + a + C *in vitro*, the cytotoxic activity against MC38 was 1.651% or 4.875%, respectively (E : T = 40). We could not detect any differences of CTL cytotoxicity against MC38-WT cells between them at any time points.

Discussion

We have demonstrated that DC preincubated with CpG and IFN-α-overexpressing tumor cells significantly suppress the outgrowth of an established parental tumor, and that the therapy recruits $CD4^+$ cells, $CD8^+$ cells, DC, and $NK1.1^+$ cells in the established tumors. The combined DC-based therapy showed a profound preventive antitumor effect on the parental tumor. IL-12 and NK cells contributed to the antitumor effects induced by DC preincubated with CpG and IFN-α-overexpressing tumor cells.

Dendritic cell therapy has been tried for patients with advanced tumors such as melanoma and renal cancer. However, the outcomes have been unsatisfactory; for example, the DC used in the clinical trials are not uniform, and it remains to be clarified which type of DC, mature or immature, is suitable for clinical cancer therapy. Mature DC would induce and activate antitumor immune cells more effectively than immature DC, but their phagocytosis and migration abilities diminish as they mature. DC that do not perceive an activating environment do not mature, and they induce tolerance rather than immunity.[28] As we revealed that mature DC stimulated by IFN-α-overexpressing tumor cells have potent antitumor activity *in vivo*,[22] we tried to induce more mature DC by CpG ODN, which is thought to stimulate DC through TLR9. Moreover, our previous study demonstrated that IFN-α-overexpressing tumor cells in combination with CpG ODN markedly induce DC maturation phenotypically as well as functionally, such as cytokine production and proliferative effects on allogenic lymphocytes.[23] Therefore, in anticipation of further antitumor effects of the DC therapy, we evaluated the therapeutic inoculation of more mature DC incubated with IFN-α-overexpressing tumor cells and CpG ODN.

In our previous study, IFN-α-overexpressing tumor cells, but not the parental tumor cells with exogenous IFN-α, promoted a tumor-specific immune response *in vivo*.[20,22] This suggested that a continuous source of IFN-α would be crucial for the induction and maintenance of immune responses. Therefore, IFN-α gene-transduced cells were used in our studies although establishment of the transduced cells, which produce a high amount of IFN-α, would be difficult in the clinical setting. We are now planning to use allogenic IFN-α-overexpressing tumor cell lines to treat hepatocellular carcinoma-bearing mice and evaluate the antitumor effects.

No tumors were observed in mice that were vaccinated with DC, CpG ODN, and MC38-IFN-α cells, and then depleted of $CD8^+$ cells following parental tumor cells, suggesting that $CD8^+$ cells do not contribute to the antitumor immune responses induced by the DC therapy with IFN-α-overexpressing tumor cells and CpG ODN. However, other results did not support this hypothesis. Cytolytic activity against MC38-WT cells was detected clearly in mice treated with DC, CpG ODN, and MC38-IFNα cells. All $CD8^+$ cell-depleted mice had the parental tumor after subsequent inoculation of MC38-WT. In addition, we did not detect any obvious tumors in three of six mice that had been vaccinated with DC, CpG ODN, and MC38-IFNα cells, and following depletion of asialo-GM-1^+ cells, and all three of these mice rejected a subsequent challenge of parental tumor cells. In these mice, a long-lasting antitumor immune response would be induced, and effector cells, which diminish tumor cells directly, would be $CD8^+$ cells. All $CD8^+$ cell-depleted mice were tumor-free because of potent activation of NK cells induced by the combined DC therapy, as NK cells exterminate the parental tumor cells without $CD8^+$ cells. In the previous study, we demonstrated the potent activation of tumor-specific $CD8^+$ cells as well as non-specific NK cells *in vivo* by DC with IFN-α therapy.[22] In addition, it was reported that peptide-coated DC generated memory $CD8^+$ T cells within 4–6 days, and that coinjection of CpG ODN with those DC prevented the rapid generation of memory T cells in an IFN-γ-dependent manner.[29] Thus, NK cells may mainly contribute to the antitumor effects induced by the DC-based therapy with CpG ODN in the early stage. From these phenomena, we speculate that potent activity of NK cells would reject the parental tumor cells in the $CD8^+$ cell-depleted mice, whereas stimulated $CD8^+$ cells could eliminate the tumor cells in some asialo-GM-1^+ cell-depleted mice.

The results of an IL-12 neutralization model showed that IL-12 plays a partial role in the antitumor activity induced by DC therapy with IFN-α-overexpressing tumor cells and CpG ODN. IL-12 was identified as a NK cell stimulatory factor, and was characterized as a heterodimeric cytokine with multiple biological effects on T and NK cells.[30,31] IL-12 is produced mainly by APC such as macrophages and DC following activation.[32] In the present study, not only the injected matured DC but also host DC stimulated with the therapy would produce IL-12, and then IL-12 might activate NK cells as well as $CD4^+$ and $CD8^+$ T cells. We observed partial inhibition of the antitumor effects induced by DC with IFN-α and CpG ODN by neutralization of IL-12. Further antitumor mechanisms other than IL-12 should exist in the combined DC therapy, although it is possible that the dose of IL-12 neutralizing antibody was insufficient because a high amount of IL-12 might be produced by the host immune cell stimulated with the treatment.

It has been reported that conventional CpG DNA, phosphorothioate-modified ODN called CpG-B, induce splenic B cell proliferation, DC maturation, and cytokine production from a variety of immune cells.[33,34] TLR9 is expressed essentially on human plasmacytoid DC, but not on human myeloid DC. However, it has been reported that TLR9 is expressed by all subsets of murine DC[35] and that CpG ODN can stimulate not only splenic but also bone marrow-derived myeloid DC through TLR9.[36] Our results support these previous observations that CpG ODN enhances murine bone marrow-derived DC maturation. However, as myeloid DC do not express TLR9 in humans, CpG ODN would not affect DC maturation effectively and might not induce antitumor effects as observed in this murine model. Therefore, further modifications such as OK-432, which is a potent stimulator to myeloid DC[37] and has indeed been used in clinical cancer therapy, are required for clinical application.

Here, we have demonstrated that DC preincubated with IFN-α-overexpressing tumor cells and CpG ODN have therapeutic benefits in suppressing parental tumor growth compared with DC preincubated with the parental tumor cells and CpG ODN, and with DC preincubated with IFN-α-overexpressing tumor cells. Thus, CpG ODN and IFN-α-overexpressing tumor cells have additive effects on DC-based immune therapy for tumors. To the

doi: 10.1111/j.1349-7006.2008.00858.x