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

HCV Infection

Monocyte-derived DC generated from hepatitis C patients have an impaired ability to stimulate allogeneic CD4 T cells [68, 69]. Functional impairment of DC diminished when HCV had been eradicated from patients, revealing the evidence of HCV-induced DC disability [68]. DC play a decisive role in shaping innate immunity by interacting with NK cells. In response to IFN-α, DC are able to express MHC class-I related chain A/B (MICA/B) and activate NK cells following ligation of the NK receptor, NKG2D [70]. Interestingly, DC from HCV-infected patients are unresponsive to exogenous IFN-α to enhance MICA/B expression and fail to activate NK cells [70]. It is tempting to speculate that the impairment of DC in NK cell activation is responsible for the failure of HCV control in the early phase of primary HCV infection, where HCV continues to replicate in spite of high-level IFN-α expression in the liver. Alternatively, NK cells from HCV-infected patients down-regulate DC functions in the presence of hepatocytes by secreting suppressive cytokines, IL-10 and TGF-β1 [50]. Such functional alteration of NK cells in HCV infection was ascribed to the enhanced expression of inhibitory receptor NKG2A/CD94 compared to the healthy counterparts [50]. Further study is necessary to determine if the NK-mediated DC suppression is instrumental or not in acute HCV infection.

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

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

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

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

ADAPTIVE IMMUNITY

Chronic HBV Infection

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

Chronic HCV Infection

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

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

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

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

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

Several investigators have reported that the frequency of regulatory T cells (Tregs) are increased in chronic hepatitis B or C patients, either in liver or in the periphery [13, 14]. It is well acknowledged that Tregs consist of distinct populations, thymus-derived natural Tregs, adaptive/inducible Tregs or IL-10-producing $CD8^+$ Tregs. Either type of Tregs are endowed with suppressing capacity on NK cells, DC and HBV- or HCV-specific T cells, thus leading to alleviation of collateral liver damage or impeding virus elimination. It has been speculated that Tregs actively enhance persistence of hepatitis virus in the acute phase, however no direct correlation was found between the expansion of $FOXP3^+$ cells and attainment of chronic HCV infection [109]. A question is still unsolved that how Tregs are generated either in HBV or HCV infection. The presence of viral epitope has been postulated that is specifically capable of expanding Tregs, though it is still controversial [110, 111]. Alternatively, multifaceted mechanisms have been committed to the induction of Tregs, such as tolerogenic MDC or PDC [112], galectins [113] and certain TLRs [114] as reported elsewhere.

IMMUNE RESPONSE DURING ANTI-VIRAL THERAPY

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

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

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

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

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

Recently, the genome-wide association study (GWAS) revealed that the single nucleotide polymorphisms (SNPs) neighboring IFN-λ3/IL28B gene are strongly associated with successful or unsuccessful viral response in Peg-IFNα/ribavirin therapy for chronic hepatitis C [124, 125]. Of particular interest, it is reported that major alleles in such SNPs give a significant and positive impact on spontaneous HCV clearance [125]. Since DCs are main producer of IFN-λ, these results again confirm that DCs are critically important for the control of HCV infection, either spontaneously or therapeutically.

The questions remain unsolved are if impaired immune system in chronic HCV infection is restored or not by the successful HCV eradication after anti-viral therapy. Controversial results have been reported about the durability of treatment-induced recovery in HCV-specific immune response [126, 127], which seems to be clearly distinct from that observed in spontaneous HCV resolvers.

IMMUNOLOGICAL INTERVENTION AGAINST HBV OR HCV INFECTION

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

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

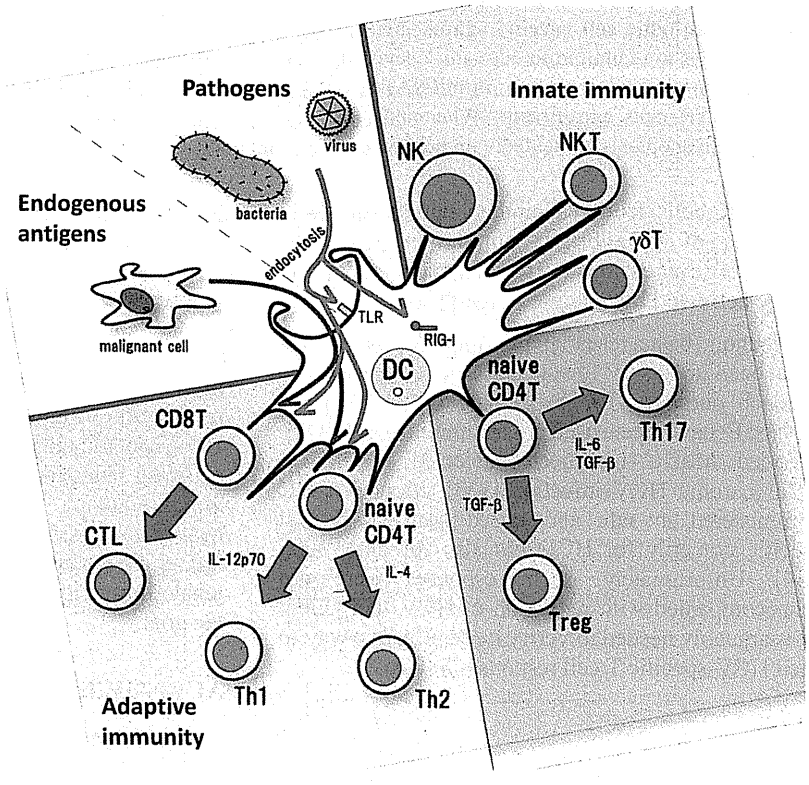

Fig. (2). Dendritic cell as a conductor of innate and adaptive immunity. Dendritic cells sense viral and endogenous antigens and evoke or regulate immune reactions by interacting with various lymphocytes. CTL, DC, NK, NKT, Th are as described in Fig. (**1**). γδ T cells, gamma delta T cells; Treg, regulatory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

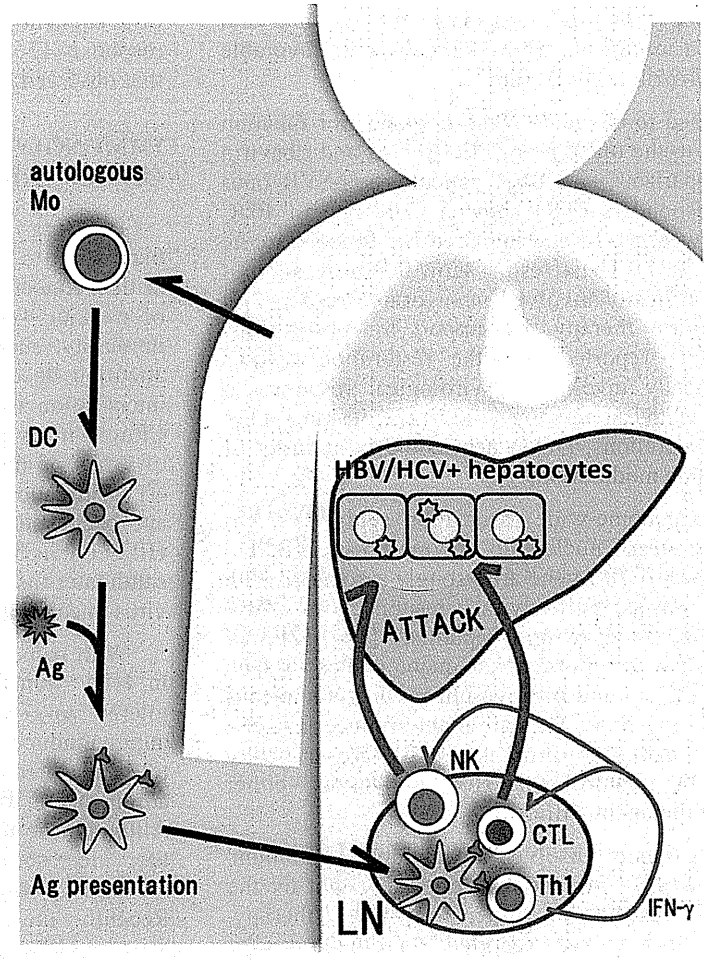

Fig. (3). Strategy of dendritic cell vaccine against hepatitis virus infection. Most of the clinical trials utilize autologous monocytes as source of DC. Monocytes are cultured *ex vivo* for a several days in the presence of cytokines, such as GM-CSF and IL-4. Mature DC are loaded with viral antigens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

points of view, NK cells from vaccinated patients showed higher percentages of CD56bright population and their CD244 and NKG2D expressions were enhanced, indicating that NK cells are activated by vaccine *in vivo* [130].

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

PERSPECTIVE

Synthetic compounds that specifically suppress hepatitis virus replication are now ready to use in clinics [135]. Additionally, many others are still on the pipeline and are destined to clinical trials. They are quite promising as an alternative approach for hard-to-treat chronic hepatitis patients, such as those infected with drug-resistant HBV strain or non-responders in PEG-IFNα/ribavirin therapy. In addition to inhibitory effect on viral replication, such compounds are able to restore immunity either indirectly by reducing viral burden or directory by immune modulation. Therefore, extensive immunological studies are needed to elucidate if the therapeutic modulation of innate immunity could shape sustained HBV- or HCV-specific adaptive immunity or not. The next steps in evolving innovative approaches to establish virus-specific immunotherapy are to determine the means to, direct the magnitude, breadth, quality and duration of antigen-specific immune responses in a desired way. Active modulation of innate immunity may be one of the strategies to gain access to the goal.

ACKNOWLEDGEMENT

Declared none.

CONFLICT OF INTEREST

Declared none.

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Received: September 18, 2010 Revised: November 12, 2010 Accepted: February 10, 2011

Cancer Immunol Immunother (2011) 60:1565–1575
DOI 10.1007/s00262-011-1051-1

ORIGINAL ARTICLE

Comprehensive immunological analyses of colorectal cancer patients in the phase I/II study of quickly matured dendritic cell vaccine pulsed with carcinoembryonic antigen peptide

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Received: 8 March 2011 / Accepted: 24 May 2011 / Published online: 17 June 2011

Abstract Dendritic cell (DC) vaccine has been used to treat patients with advanced colorectal cancer (CRC). The results of vaccine-induced clinical responses have not always been satisfactory partially because of DC incompetence. In order to evaluate the feasibility of novel mature DCs for therapeutic adjuvants against CRC, we conducted clinical trials with carcinoembryonic antigen (CEA) peptide-loaded DC quickly generated with a combination of OK432 (Streptococcuspyogenes preparation), prostanoid, and interferon-α (OPA-DC). In the ten patients enrolled in this study, the OPA-DC vaccine was well tolerated and administered four times every 2 weeks except for two patients, who were switched to other treatments due to disease progression. Among the eight evaluable patients, one displayed stable disease (SD), while the remaining seven showed progressive disease (PD). In the SD patient, natural killer (NK) cell frequency and cytolytic activity were increased. In the same patient, the frequency of CEA-specific cytotoxic T cells (CTLs) increased stepwise with repetitive vaccinations; however, most of the CTLs exhibited central memory phenotype. In those with PD, NK cells proliferated well regardless of failure of response, whereas CTLs failed to do so. We concluded that the OPA-DC vaccine is well tolerated and has immune-stimulatory capacity in patients with CRC. Additional modulation is needed to attain significant clinical impact.

Keywords Dendritic cell · Vaccine · Cancer · Clinical study

Electronic supplementary material The online version of this article (doi:10.1007/s00262-011-1051-1) contains supplementary material, which is available to authorized users.

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Introduction

Colorectal cancer (CRC) is one of the most intractable malignant diseases causing the death of 600 thousand people annually around the world. Recently, the development of new chemotherapeutic regimens including molecular-targeted drugs has significantly improved the clinical outcomes of patients with CRC [1]. However, the severe adverse effects of chemotherapeutic agents often deteriorate their quality of life (QOL), thus limiting continuation of therapy at full dose. The development of better therapies against CRC is needed.

Dendritic cells (DCs) are the most potent antigen-presenting cells that enhance innate and adaptive immune reactions. Previous studies have demonstrated that antigen-loaded DC is one of the most promising candidates for a

therapeutic vaccine to induce tumor-specific immune reactions and preferable clinical responses in cancer patients with limited side effects. However, less than 10% of vaccinated patients showed favorable clinical responses [2, 3]. One of the primary reasons for such unsatisfactory results may be that the DCs have not been fully exploited to induce anti-tumor immunity.

Previous DC-vaccine studies have shown that mature DCs are better than immature ones for inducing anti-tumor responses in patients. The protocols of maturation stimuli are yet to be standardized. Although a combination stimulus using interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and prostaglandin (PG)-E2 is widely used as a monocyte-conditioned medium (MCM) mimic for monocyte-derived DCs (MoDCs), it lacks the ability to promote DCs to secrete IL-12p70 [4], a well-known enhancer of cytotoxic activity of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) [5, 6]. From the mechanistic point of view, DCs loaded with antigens migrate into draining lymph nodes, where they produce IL-12p70 and activate NK cells, prime type-1 helper-T (Th1) cells and CTLs [7, 8]. We have previously reported that novel mature DCs (OPA-DCs) more effectively exert such functions than MCM-mimic DCs, which can be generated even from monocytes of refractory cancer patients within 3 days by using a combination of OK432 (Streptococcus pyogenes preparation), low-dose prostanoid, and interferon (IFN)-α [9] (OPA). In order to evaluate the tolerability and the clinical or immunological responses of OPA-DC vaccine, we conducted a phase I/II clinical study of OPA-DC vaccine in patients with CRC, which shed light on the importance of NK cells and the limitations of CTL differentiation even with fully matured DC.

Subjects and methods

Subjects

Patients with advanced CRC (stage IV) who had been followed in or referred to Osaka University Hospital were enrolled as candidates in this clinical trial. The eligibility and exclusion criteria are summarized in Table 1. Among them, ten patients were selected. Their clinical backgrounds are given in Table 2.

Study design

The current clinical trial was performed to evaluate tolerability as well as immune and clinical responses; it has been registered with the University-hospital Medical Information Network Clinical Trials Registry (UMIN-CTR), under the code of UMIN000000743. The protocol was approved by the ethical committee of the Osaka University Graduate School of Medicine and also reviewed by the Translational Research Review Board, Osaka University Hospital. Standard operating procedures for manufacturing OPA-DCs were reviewed by the Medical center for Translational Research (MTR). Every detailed procedure described in the Manufacturing Instructions and Records was programmed to automatic process-control software to avoid operational errors. All patients gave written informed consent prior to the treatment.

The patients were vaccinated four times every 2 weeks in conformity with the regulation in previous DC vaccine [10–12]. In order to collect peripheral blood mononuclear cells (PBMCs) as a DC source, they underwent leukocyte apheresis at the first day of each session. After OPA-DCs had been generated as described in the next paragraph, they were administered subcutaneously at bilateral groin sites. Blood samples were collected before and 2 weeks after DC injection for the purpose of immunological and biochemical tests. Serum carcinoembryonic antigen (CEA) levels were determined on the day of blood collection. Two

Table 1 Eligibility criteria for the enrollment of patients with colorectal cancer for receiving quickly matured dendritic cell vaccine

(1) Eligibility criteria	(a) Patients with colon cancer (stage IV)
	(b) Performance status: 0–2
	(c) Age: 20–75 years
	(d) Tolerability for apheresis
	(e) Stability in bone marrow, liver, and renal functions:
	2,000 < WBC (/μl) < 15,000, Plt (/μl) > 75,000, AST and ALT (IU/l) < 150, T-bil (mg/dl) < 2.0, Crn < 2.0
	(f) Acquired informed consent
	(g) Having metastatic lesions for the assessment of therapeutic efficacy
	(h) More than 4 weeks have passed from previous anti-cancer treatments
	(i) HLA-A*2402 positive
	(j) Increased serum CEA level: >5 ng/ml
	(k) Positivity for CEA in cancer tissues
(2) Exclusion criteria	(a) Pregnant or lactating woman
	(b) Severe bleeding tendency: PT < 50%, APTT > 60 s, fibrinogen < 100 mg/dl, FDP > 20 μg/ml
	(c) Patient with infectious diseases (HIV, HBV, HCV, HTLV, RPR)
	(e) Patient with autoimmune disorders
	(f) Patient who needs to take steroids or immunosuppressive drugs during treatment
	(g) Patient who has uncontrollable metastatic brain or intrathecal tumors
	(h) Patient whom the doctors define as inappropriate

Table 2 Clinical characteristics and outcomes of ten patients enrolled in this study

Case	Gender	Age	Metastases	CEA (ng/ml)[a]	Total tumor volume (cm^3)[a]	Administrated DCs (mean) ($\times 10^6$/injection)	DC injection time	CEA decline	RECIST
1	Female	62	Lung	111	4.7	106.7	8	+	SD
2	Female	64	Lung, liver	647	590.8	11.2	4	–	PD
3	Female	63	Lung, liver	987	603.3	19.2	4	–	PD
4	Male	60	Lung, liver	631	694.4	121.1	4	–	PD
5	Female	48	Lung, liver, peritoneal dissemination	482	Difficult to measure[b]	Withdrawn	2	Withdrawn	
6	Male	65	Liver	925	64.7	51.6	4	+	PD
7	Female	59	Liver, pancreas	692	543.7	115.0	4	–	PD
8	Female	42	Lung, liver	12	110.0	36.6	4	–	PD
9	Female	32	Lung, liver, abdominal wall, LN	402	163.7	Withdrawn	2	Withdrawn	
10	Male	49	Lung, abdominal LN	612	19.1	35.225	4	–	PD

[a] Before vaccination

[b] Peritoneal disseminations are too small to measure their diameter

weeks after the last vaccination, the clinical response was evaluated by the changes in tumor size measured on computerized tomography (CT) scans, based on the Response-Evaluation Criteria In Solid Tumors (RECIST) [13]. Toxicity was scored occasionally according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) v. 3.0. If severe toxicities of grade 3 or 4 developed, the patient was withdrawn from the study and switched to other appropriate treatment. A successive series of vaccinations could be applied to the patients evaluated as achieving a partial response (PR) or a stable disease (SD) condition based on the RECIST criteria.

Preparation of quickly inducible mature dendritic cells (OPA-DCs)

The OPA-DCs were generated as reported previously with some modifications [9]. At least 5×10^8 PBMCs were recovered via leukapheresis (Amicus) (Baxter, Deerfield, IL) from the patients. The PBMCs were incubated at a concentration of at least 5×10^6 cells/ml with serum-free AIM-V in 225 cm^2 culture flasks (Iwaki, Japan) for 2–3 h at 37°C in 5% CO_2. Subsequently, these flasks were washed with saline in order to separate monocytes by means of their preferential adherence to plastic [9]. Briefly, monocytes were cultured with 50 ng/ml GM-CSF and 20 ng/ml IL-4 for 3 days at 37°C in 5% CO_2. During the final 24 h, the cells were matured with 0.1 KE/ml OK432, 500 IU/ml IFN-α, and 50 ng/ml PG-E1. Concomitantly, 20 μg/ml CEA.652(9) was loaded to the cells. On day 3 of culture, non-adherent cells (OPA-DCs) were harvested. These procedures were performed in the cell-processing center affiliated with MTR under a clean condition according to the guidelines for translational research using human stem cells by Japanese Ministry of Health, Welfare and Labor.

Quality assessment of DC injections

During the DC-preparation process, samples of the culture supernatant were collected. No bacterial contamination was confirmed by means of their endotoxin measurement. Before handling the OPA-DC injections, we examined their viability and cell number using 0.3% trypan-blue staining. Viability was assessed as the percentage of viable cells among all countable cells. All the final products were confirmed as having at least 70% viability and containing at least 3.0×10^6 viable cells in conformity with the regulation in previous DC vaccine [10–12]. The purity of DCs was also examined. Briefly, the cells sampled from each final product were stained with fluorescent-material-conjugated anti-CD14 monoclonal antibody (mAb), anti-CD11c mAb, and anti-HLA-DR mAb. They were analyzed on a FACS Caliber (BD, Franklin Lakes, NJ). The live cells, except for lymphoid cells, were gated at forward scatter versus side scatter plot. Subsequently, the percentage of the CD14-/CD11c+/HLA-DR+ cells among the gated cells was analyzed as the purity. All final products were confirmed as having at least 70% purity.

Reagents

Recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 were purchased from Primmune Inc., Japan. OK432 (Picibanil®) was purchased from Chugai, Japan. The amount of OK432 is expressed in units designated as KE (Klinische Einheit

[clinical unit]). One KE OK432 is equivalent to 0.1 mg dry streptococci. Natural human IFN-α (OIF®) was purchased from Otsuka, Japan. PG-E1 (Prostandin®) was purchased from Ono, Japan. The 9-mer peptide (CEA.652(9): TYACFVSNL), reported to be a human leukocyte antigen (HLA)-A*2402 restricted CTL epitope in CEA [9], was purchased from TaKaRa Bio (Japan) or Mimotope-Genzyme Pharmaceuticals (Switzerland). Therapeutic grade medium (AIM-V) was purchased from Invitrogen (Carlsbad, CA).

Fluorescent antibodies and HLA/peptide-pentamer

Fluorescein-isothiocyanate (FITC)-conjugated Lineage-Cocktail 1, anti-CD14 mAb (M5E2), anti-CD3 mAb (UCHT1), anti-CD4 mAb (RPA-T4), anti-CD27 mAb (M-T271), and anti-CD45RO mAb (UCHL1) were purchased from Becton–Dickinson (BD), Franklin Lakes, NJ. FITC-conjugated anti-CCR7 mAb (150503) was purchased from R&D Systems, McKinley Place, NE. Phycoerythrin (PE)-conjugated anti-CCR4 mAb (1G1), anti-CD4 mAb (RPA-T4), anti-CD45RA mAb (HI100), and anti-CD69 mAb (FN50) were obtained from BD. PE-conjugated anti-FoxP3 mAb (PCH101) was obtained from eBiosciences, San Diego, CA. PE-conjugated anti-CXCR3 mAb (49801) was purchased from R&D Systems. Peridinin-chlorophyll-protein-complex (PerCP)-conjugated anti-CD4 mAb (SK3), anti-CD8 mAb (SK1), and anti-HLA-DR mAb were purchased from BD. Allophycocyanin (APC)-conjugated anti-CD11c mAb (B-ly6) and anti-CD56 mAb (B159) were obtained from BD. APC-conjugated anti-CD4 mAb (13B8.2) was purchased from Beckman Coulter Inc., Fullerton, CA. PE-conjugated HLA-A*2402/CEA.652(9)-pentamer (CEA(24)-pentamer) and HLA-A*2402/EBV peptide (HLA-A*2402 restricted peptide derived from Epstein-Bar virus (EBV) LMP2: TYGPVFMSL)-pentamer were purchased from ProImmune Ltd., Bradenton, FL.

Cell lines

The NK-cell-sensitive cell line (K562) was obtained from ATCC (Manassas, VA). T2-A24 is a transporter associated with an antigen processing (TAP) deficient cell line (T2) transfected with HLA-A*2402 gene. This cell line expresses a high level of HLA-A24 protein and is used for targets in cytotoxicity assay. K562 and T2-A24 were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Calculation of total tumor volume

We calculated the total tumor volume of each patient before vaccination. Maximum diameters (D_1, D_2, ...) were measured for all visible tumors in CT images from the neck to the perineum, and total tumor volume was calculated as follows:

$$\text{Total Tumor Volume} = 3.14 \times \left(D_1^3 + D_2^3 + \ldots\right)/6.$$

Assessment of immune responses

(1) Frequency analyses of immune cells

PBMCs derived from blood samples were stained with fluorescent-material conjugated antibodies or a CEA(24)-pentamer. We analyzed the frequency of NK, Th1, Th2, FoxP3+ regulatory CD4+ T cells (Treg), and CEA.652(9)-specific CTLs on a FACS canto II (BD). For the analysis of Tregs, the cells were permeabilized with the Human FoxP3 Buffer Set (BD). Th1 and Th2 cells were detected as CD4+/CD45RO+/CXCR3+ and CD4+/CD45RO+/CCR4+ cells, respectively [14]. NK cells were characterized as CD3−/CD56+ cells and their active phenotypes were assessed by the expression of CD69. The ratios of CD69+ NK-cell frequency before and 2 weeks after every DC injection were calculated as the CD69+ NK variation rate (VR). We analyzed FoxP3+ Tregs as CD4+/CD45RO+/$CD25^{high}$/Foxp3+ cells. Highly avid CTLs for CEA.652(9) were identified as CD8+/CEA(24)-pentamer+ cells. Additionally, the CTLs were subdivided into CCR7+/CD45RA- central memory cells, CCR7−/CD4RA− effector memory cells, and CCR7−/CD45RA+ terminal differentiated effector memory cells [15].

(2) Analysis of NK cell activity

The cytotoxic ability of NK cells during vaccination was analyzed by flow-cytometric methods [16]. NK cells were separated from PBMCs using CD56 microbeads (Miltenyi Biotec). K562 cells were labeled with carboxyfluorescein succinimidyl ester (Invitrogen) and cultured with or without NK cells in 24-well culture plates (Falcon) for 12 h at 37°C in 5% CO_2 (E/T ratio: 24/1). At the end of the incubation, the samples were put on ice and 50 μg/ml propidium iodide (SIGMA) was added for DNA labeling of the dead cells. The samples were then incubated for 10 min on ice and analyzed within 60 min using FACS Canto II (BD). The percentage of specific-target-cell death (cytotoxicity) was calculated as:

$$\text{cytotoxicity}(\%) = \frac{\text{dead targets in the sample}(\%) - \text{spontaneously dead targets}(\%)}{100 - \text{spontaneously dead targets}(\%)} \times 100$$

The representative plots of flow-cytometric analyses are given in Online Resource 1.

Statistical analysis

For the vaccinated patients, a comparison was made between plasma cytokine level and total tumor volume

before vaccination using the Pearson's product-moment correlation coefficient (r), which was analyzed with Prism 5 software (Graph Pad Software, San Diego, CA). A P value is the probability that an r value is zero. Therefore, a P value of less than 0.05 indicates that the two variables are correlated.

Results

Quality of the OPA-DC vaccine

The mean DC number administered at each vaccination is shown in Table 2. The viability and purity of DC from all patients were 91.1 ± 2.6% (mean ± SD) and 88.8 ± 4.28%, respectively. All products met the quality assessment of the OPA-DC vaccine except for one form Case 3 due to its lesser number.

Clinical outcomes

Eight of the ten patients enrolled in this study were able to receive at least one series of vaccinations. Two patients (Case 5 and 9) were withdrawn from the study because of ileus, respiratory distress, or obstructive jaundice of grade 3 accompanied by cancer progression. One patient (Case 1) displayed a clinical response of SD lasting 2 months from the initiation of vaccination. Two weeks after the first session, the diameter of the patient's maximum lung metastasis increased by less than 20% and cavity formation was observed at the core of the lesion (Fig. 1a). Based on such clinical responses to the first session, this patient received the second series of vaccinations. Seven other patients had PD after the therapy.

Decline in the serum CEA level during the vaccination period was observed in two patients (Case 1 and 6) (Fig. 1b). They had clearly smaller total tumor volumes in the body before vaccination than the other patients (Fig. 2).

Immunological responses

We previously reported that OPA-DC has potent Th1 priming ability in vitro [9]. Therefore, we analyzed the frequency of peripheral Th1 and Th2 cells during the vaccination period (Fig. 3a). In Cases 1 and 8, the Th1/Th2 ratio remained high during vaccination compared with the pre-vaccination value. In Case 1, the patient with SD continued to show increment of the Th1/Th2 ratio after vaccination during the two series of sessions.

OPA-DCs have potent ability to activate NK cells and antigen-specific CTLs in vitro [9]. Therefore, we analyzed the frequency of NK cells before and during vaccination (Fig. 3b). In all except Cases 2 and 10, the mean frequency of NK cells during vaccination increased compared with the pre-vaccination value. Moreover, in Cases 1 and 6 demonstrating a CEA decline, the NK cells displayed strong lytic activity against K562 cells after vaccination (Fig. 3c).

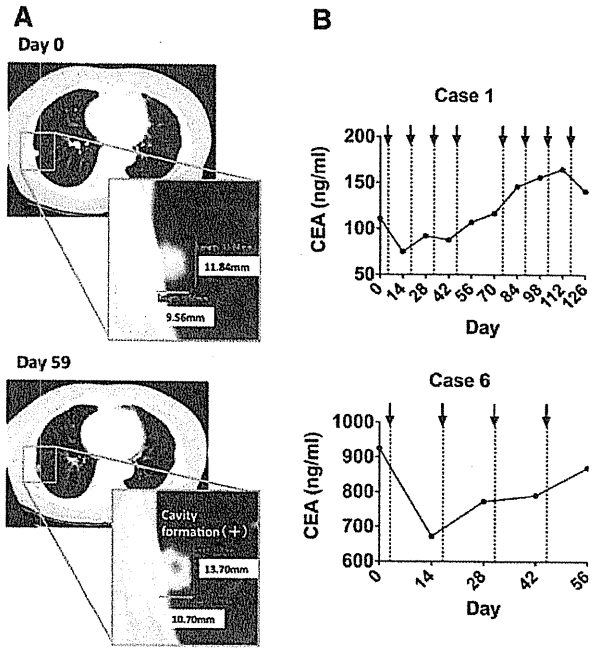


Fig. 1 Changes in CT images of metastatic lesion and serum CEA levels in patients after receiving the OPA-DC vaccines. **a** CT images of the patient with stable disease after OPA-DC vaccination (Case 1) are shown. The diameters of the maximum metastatic lesion in the lung are shown before (day 0) and after (day 59) the vaccination period. **b** The changes in serum CEA levels during vaccine treatment in the patients (Case 1 and 6) are shown. *Downward arrows* indicate the time points of OPA-DCs injections

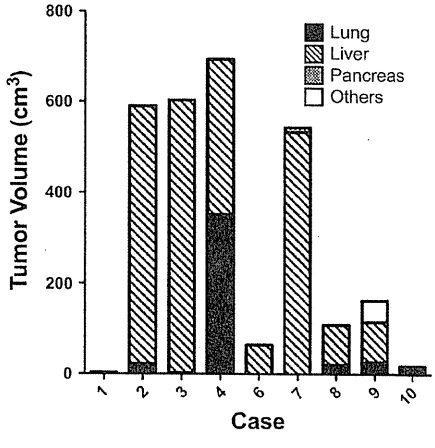
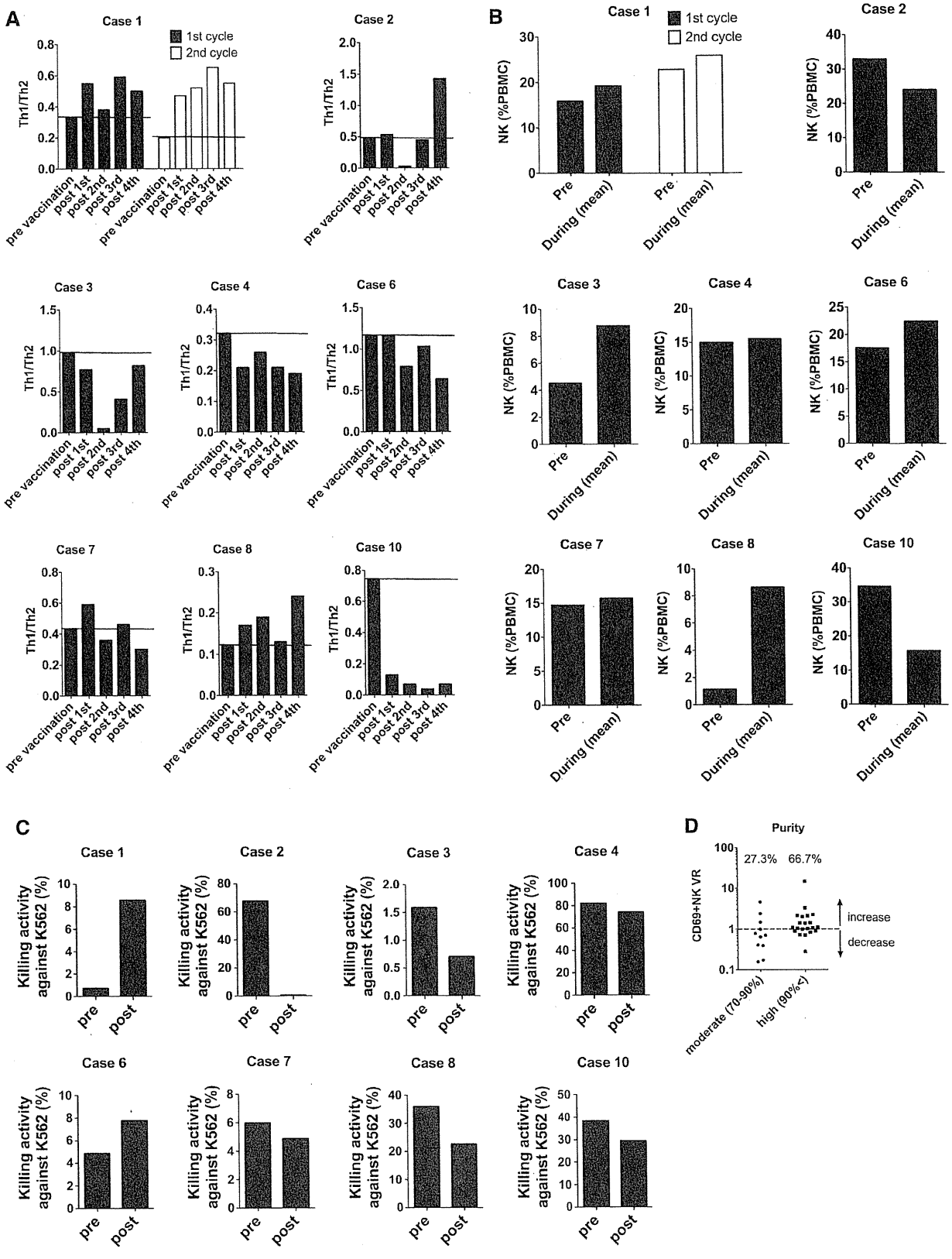


Fig. 2 Absence of correlation between total tumor volume and serum immunosuppressive cytokines in the vaccinated patients. Total tumor masses of each patient before vaccination were measured as described in "Subjects and methods". Localizations and volumes of metastatic tumors are shown



◀ **Fig. 3** Changes in Th1/Th2 ratio and NK cells before and during/after the OPA-DC vaccines. **a** Ratios between Th1 and Th2 frequency (Th1/Th2) were determined before and 2 weeks after every vaccination as described in "Subjects and methods". In Case 1, four additional injections of DCs were performed (*empty bars*). *Horizontal lines* in each graph indicate the Th1/Th2 value before vaccination. **b** NK cell frequency was examined before and during the vaccinations. The *values* are shown as the mean of the NK cell frequencies between the first and the last DC injections. **c** NK activities are evaluated by the killing of NK-sensitive K562 cells before and 2 weeks after the first DC injection. **d** CD69+ NK variation rates (VR) are shown for all DC injections of all patients in the first session. VR values above 1 mean that CD69+ NK cells increased with that single injection of DC. Percentages in the graphs depict the rate of CD69+ NK increment induced by DCs with quality

We and other investigators have found that DCs primed with OK432 can activate NK cells within 48 h [9, 17]. Such observations support the possibility of vaccine-dependent activation of NK cells with each DC injection. Therefore, we analyzed the increment of CD69 + NK cells after every DC injection with respect to its relationship with DC purity. In total, 32 injections were performed with eight patients in their first sessions. Among such vaccinations, 21 injections were done with highly pure DCs (purity: >90%) and the remaining 11 with moderately pure DCs (purity: 70–90%). As shown in Fig. 3d, regardless of the differences in clinical backgrounds of the patients, a post-vaccine increment of CD69 + NK cells was observed in 14 out of the 21 given highly pure DC injections (66.7%). In contrast, such an increment was detected only in 3 out of the 11 given moderately pure DC injections (27.3%). Therefore, highly pure DC injections resulted in a higher rate of NK activation compared with moderately pure DC injections.

Next, to assess the frequency of antigen-specific CTLs induced with OPA-DC vaccine, we analyzed PBMCs from vaccinated patients with CEA(24)-pentamer staining (Fig. 4a). In Case 1 with the SD response, the frequency of the pentamer-positive CTLs increased with vaccination. Of particular interest, during the second sessions, the frequency increased much more compared with those in the first sessions (Fig. 4a). In contrast, the frequency of specific CTLs against control peptide derived from Epstein-Bar virus (EBV) kept under 1% of all CD8+ T cells during all sessions (Online Resource 2). According to the differentiation stages, human CD8+ T cells have been subdivided into different populations based on their CD45RA and CCR7 expressions [15]. Therefore, we analyzed the frequencies of CEA.652(9)-specific central memory T (Tcm), effector memory T (Tem) and terminally differentiated Tem (Tem/td) cells during vaccination in Case 1 (Fig. 4b). The CEA.652(9)-specific Tcm cells increased gradually with OPA-DC vaccination; however, antigen-specific Tem or Tem/td cells were not induced. In other cases, including Case 6 with CEA decline, we did not observe such increment of antigen-specific CTLs after the vaccinations.

We performed ELISPOT assay in order to enumerate IFN-γ-producing CD8 cells reacting to the CEA peptide. Also, we had intended to measure the antigen-specific lytic activity of CTLs against CEA.652(9) pulsed T2-A24 cells by ^{51}Cr-releasing assay, but in all cases, no such responses were observed (data not shown).

Regulatory T cells (Treg) play an active role in the suppression of anti-tumor immune responses. Many investigators reported that the accumulation of Tregs is observed in various types of cancers including CRC [18–21]. Therefore, we analyzed the frequency of Tregs during vaccination. In Cases 2, 3, 6, and 7, Tregs decreased after the vaccinations (Fig. 5). It is noteworthy that in Case 6, the reduction in Tregs was maintained throughout the vaccination period. Delayed-type hypersensitivity (DTH) against CEA.652(9) peptide was not observed in any of the cases (data not shown).

Toxicity

All patients experienced grade-1 fever after every vaccination, but this could be controlled with anti-inflammatory drugs. In addition, induration of the vaccinated groin sites was observed about 2 weeks after the first vaccination with all patients. They were followed without treatment, except for one patient (Case 8) who received temporal antibiotics for abscess formation of the induration. OPA-DC vaccine could be performed without severe toxicity for all patients.

Discussion

Dendritic cells pulsed with CEA peptide are one of the feasible vaccines to induce anti-tumor immunity in patients with CRC [22]. We have previously reported that novel mature DCs (OPA-DCs) can be generated from monocytes using OK-432, low-dose prostanoid, and IFN-α (OPA) by a short-term process. OPA-DCs possess potent migrating ability and stimulating activity for Th1, CTL, and NK cells, which are desirable for DC vaccines using peptide antigen against cancers [9]. In this phase I/II clinical study, we evaluated the safety and efficacy of vaccination with OPA-DCs pulsed with CEA.652(9) peptide in ten patients with metastatic CRC. We chose the peptide as a target antigen because it has been reported as a tumor-associated antigen achieving preferable responses in previous cancer vaccine studies [23, 24]. OPA-DCs offer several advantages in clinical settings [25]. First, even with serum-free media, OPA-DCs are likely to possess better functional abilities with large-scale yield. Second, they can avoid the possibility of contamination and can save costs with the

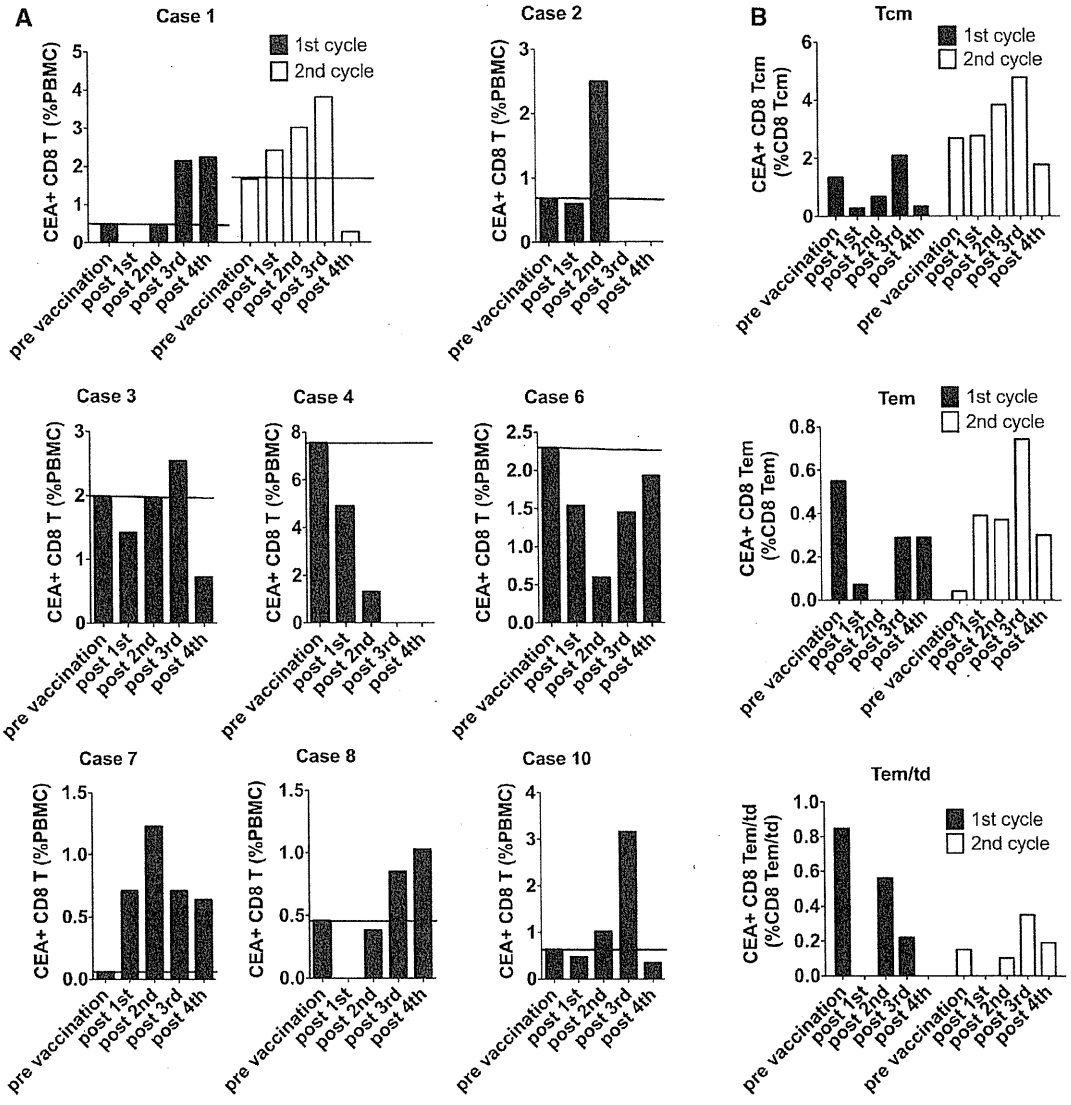


Fig. 4 Differentiation of CTLs was impaired regardless of the increased frequency of CEA-pentamer-positive CD8 T cells after DC vaccinations. **a** CEA-specific CTLs, as judged by CEA-pentamer-positive CD8+ T cells, were counted as described in "Subjects and methods". The *horizontal lines* in each graph indicate the frequency of CEA-pentamer-positive cells before vaccination. **b** In the patient who showed an SD response (Case 1), the frequency of CEA-pentamer-positive CD8+ central memory T cells (Tcm), effector memory T cells (Tem), and terminal differentiated effector memory T cells (Tem/td) was analyzed as described in "Subjects and methods"

generation of clinical-grade DCs. Alternate strategies using short-term cultured DCs have been reported elsewhere [26–28]; however, this is the first clinical study of anti-tumor vaccine using quickly generated DCs.

Regarding toxicity, the vaccination with OPA-DCs was well tolerated in patients with advanced CRC. The most common adverse events were grade-1 fever and indurations at the injection sites. Such toxicities were comparable to findings reported for previous DC-vaccine trials [29–32]. Two patients were withdrawn from the study because of grade-3 ileus, respiratory distress, or obstructive jaundice; however, these problems were caused by exacerbation of preexisting peritoneal disseminations, lymphangitic carcinomatosis, or liver metastases.

In this clinical study, we observed an SD response in one patient (Case 1). Interestingly, several tumor lesions in this patient formed a central cavity, which was probably attributed to immunological tumor necrosis triggered by the OPA-DC vaccine. Histological analysis could have offered support for this, but we could not perform biopsies of the lesions for ethical reasons. Instead, some immunological events that could have contributed to her clinical outcomes were observed in the peripheral blood during vaccination. First, Th1 cells were dominant over Th2 cells

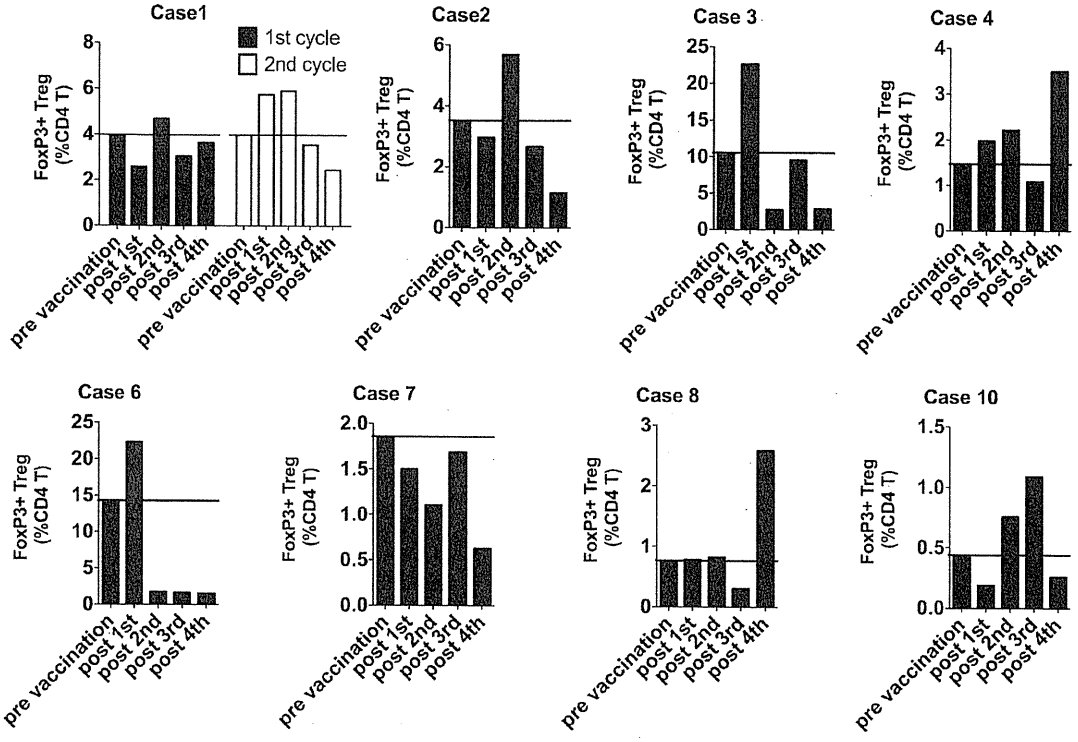


Fig. 5 Changes in FoxP3+ regulatory T cells varied during the OPA-DC vaccination period. Frequency of FoxP3+ CD4+ CD25+ T cells (depicted as Tregs) during the vaccination period is shown. *Horizontal lines* in each graph indicate the frequency of Tregs before vaccination

throughout her vaccination period. Second, NK cells had increased and were activated, and their in vitro lytic activity was enhanced after vaccination. In addition, specific CTLs that possess strong avidity to HLA-A*2402/CEA.652(9)-pentamer (CEA(24)-pentamer) were increased gradually with OPA-DC injections. These findings demonstrate that OPA-DCs can have significant immunological impact on patients even in refractory stages.

As for clinical outcomes, we observed decline in CEA in only 2 of 8 patients (Case 1 and 6) and stable disease in one (Case 1). Such results are comparable with those of previous reports regarding DC vaccine against CRC [31, 33, 34], which implies that additional modifications, other than highly active DC, are required to improve clinical responses with DC vaccine. In our study, the responders had lesser tumor volume than non-responders at the beginning of the vaccination period. In addition, regulatory T cells (Tregs) were reduced after OPA-DC vaccine in half of the treated patients. In a patient with CEA decline (Case 6), a sustained reduction in Tregs was observed throughout the vaccination period. It is not clear whether OPA-DCs directly reduce Tregs; however, Treg reduction may exert a favorable impact on the clinical outcome. Even if such inhibitory factors could be removed, a sizable number of cancer cells could not be completely eliminated by a limited number of effector cells educated by DC vaccine. Therefore, initiating vaccination at earlier stages of the disease could be a key to success for DC therapy.

Natural killer (NK) cells are potent anti-tumor effectors that reciprocally interact with DCs [35]. Many previous reports about DC vaccine have regarded CTLs as a principal effector providing anti-tumor immunity. However, there are few studies reporting NK cell activation in response to DC vaccine. Osada et al. [36] reported that NK number increased in 5 of 9 patients (55.6%) vaccinated with CEA-gene-transfected MoDCs, of which the clinical outcomes were correlated with NK activity rather than with T-cell responses. Our study showed that NK cell frequency was increased in 6 of 8 OPA-DC-vaccinated patients (75%). Such a high response rate shows that OPA-DCs possess potent ability to stimulate NK cells in vivo. In addition, our results showed that highly pure DC could activate NK cells compared with moderately pure one, which indicate that such NK activation is dependent on specific action of administrated DCs. Of interest, in two patients (Case 1 and 6), NK cells gained strong lytic activity after vaccination. Interestingly, only these two patients showed decline in serum CEA or a preferable clinical outcome as SD. In support of this possibility, Shimizu and Fujii [37] have reported that mice immunized with DC vaccine acquired "primed" NK cells, which could be quickly re-activated in response to tumor-cell challenge

even at 6–12 months after the vaccination. Such an observation suggests that NK cells primed with DC vaccine may contribute to long-term tumor rejection. Further exploration is needed to disclose how NK cells work in DC vaccine therapies.

In the patient displaying an SD response (Case 1), CEA(24)-pentamer-positive cells increased gradually with the injection of OPA-DCs. Interestingly, despite expansion of CTLs, we detected no IFN-γ-producing or antigen-specific-lytic ability of CTLs against antigen-loaded T2-A24 cells. One of the reasons for such a discrepancy may be the impaired differentiation of CTLs, exhibited by the predominance of Tcm phenotype as antigen-specific CTLs in this study. Sallusto et al. [15] identified different populations of memory CD8+ T cells in the peripheral blood of humans. The Tcm cells are reported to attain potent proliferative or IL-2-producing ability critical for maintenance of memory-T-cell pools, whereas Tem or Tem/td cells possess potent effector function such as IFN-γ/perforin production or cytotoxicity [15]. It is still uncertain which cells, Tcm or Tem, are more advantageous in providing protective immunity against cancer [38]. Mornarini et al. [39] reported that although most melanoma patients displayed antigen-specific CTLs belonging to Tcm subsets in tumor-invaded lymph nodes, few perforin-producing Tem or Tem/td cells infiltrated in the neoplastic tissues, and they found no evidence for tumor regression. Although the factors that regulate CTL differentiation are still unclear, further understanding of such regulators could provide clues to developing effective vaccines.

In summary, quickly inducible OPA-DC vaccine is tolerated and could induce preferable immunological responses in patients with advanced-stage CRC. OPA-DC vaccine exerted significant NK-activating ability, and such a response was partially linked to favorable clinical outcomes in the patients. Most antigen-specific CTLs induced with the OPA-DC vaccine belonged to a Tcm subset. In order to develop more practically effective DC vaccine against CRC, further investigation is necessary to explore the modality to induce coordinated and durable activation of both NK cells and CTLs.

Acknowledgments We are grateful to the members of MTR, especially to the following Drs. Toshiki Yoshimine (Director), Yoshiki Sawa (Director), Akira Myoui (Vice Director), ChunMan Lee, Junji Kawada, Haruki Ide, and Masao Umegaki (Project Managers).

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Hepatology Research

Hepatology Research 2011; 41: 30–38

doi: 10.1111/j.1872-034X.2010.00734.x

Original Article

Hepatitis C virus-specific CD8+ T cell frequencies are associated with the responses of pegylated interferon-α and ribavirin combination therapy in patients with chronic hepatitis C virus infection

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Aim: Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTLs) play critical roles in elimination of the HCV-infected hepatocytes. However, the mechanism of HCV elimination by pegylated interferon-α (peg-IFNα) plus ribavirin is not fully understood. We examined HCV-specific CTL responses during this combination therapy.

Methods: CD8+ T cells were isolated from 16 HCV infected patients treated by this combination therapy and were subjected to IFN-γ enzyme-linked immunospot (ELISPOT) assay.

Results: The numbers of IFN-γ spots against HCV Core or NS3 protein-derived peptides in HCV patients before treatment were similar to those in healthy donors, and those in HCV patients significantly increased 4 weeks after the initiation of combination therapy. All HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses in pre-treated patients were not associated with ALT levels and HCV viral loads of HCV patients before treatment. And those in pre-treated patients were similar between sustained virologic responder (SVR) patients and non-SVR patients. Significant increase of HCV Core or NS3 proteins-derived peptides specific CD8+ T cells responses between before and 4 weeks after this combination therapy were observed in SVR patients, but not in non-SVR patients.

Conclusions: These results demonstrated that significant increase of HCV-specific CD8+ T cells at 4 weeks after the initiation of IFN treatment might be associated with the elimination of HCV. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of the combination therapy of peg-IFNα and ribavirin.

Key words: chronic hepatitis C, HCV-specific CTL, IFN-γ ELISPOT, peg-IFNα, ribavirin

INTRODUCTION

CHRONIC INFECTION OF Hepatitis C virus (HCV) often leads to cirrhosis and hepatocellular carcinoma (HCC), which causes the poor prognosis of HCV-infected patients.[1,2] Combination therapy of pegylated interferon-α (Peg-IFNα) plus ribavirin is standard treatment for patients with chronic hepatitis C (CH-C), and sustained virologic response (SVR) in this combination therapy occurs in about 40–60% of genotype 1 patients,[1,2] which can improve the prognosis of HCV-infected patients. HCV-specific cytotoxic T lymphocytes (CTLs) is believed to play essential roles in determining the course of chronic infection,[3] and the insufficient activation, dysfunction, suppression of CTLs may cause persistent infection of HCV.[4–6] The elimination of HCV by HCV-specific CTLs is believed to consist of second slope of decay after viral decay during the first 24–48 h of IFN therapy.[7] However, the detail immune mechanism of HCV elimination by this combination therapy is not fully understood. In addition to direct antiviral

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Received 28 June 2010; revised 20 August 2010; accepted 24 August 2010.

property of Peg-IFNα and ribavirin against HCV infection, this combination therapy might have immunomodulatory activity. IFN-α enhances the maturation of antigen-presenting cells and CD4+ T cell function, but with little effect on CTLs. In contrast, ribavirin could induce a switch from Th2 to Th1 profile.[8] Although the base line immune responses of CTLs have been reported to be associated with the achievement of SVR in a few reports[7,8] , even now there are relatively little reports examining the detail of HCV-specific CTL responses during this combination therapy.

IFN-γ enzyme-linked immunospot (ELISPOT) assay allows detection of finally differentiated effector CTLs, which means the ELISPOT data reflect the in vivo situation.[9–11] In the current study, we evaluated the HCV Core and NS3 proteins-derived peptides specific CD8+ T cells responses of the HCV infected patients by IFN-γ ELISPOT assay and examined the relationship between CTL activity and the clinical outcome of the combination therapy of Peg-IFNα plus ribavirin. The frequencies of HCV-specific CD8+ T cells in pre-treated HCV patients were not associated with antiviral activity of this combination therapy in SVR. However, the significant increase of HCV-specific CD8+ T cells at 4 weeks after the starting of IFN treatment could be observed in SVR patients, but not in non-SVR patients. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

MATERIALS AND METHODS

Patients

SIXTEEN PATIENTS CHRONICALLY infected with HCV were examined for HCV specific CTL responses during the combination therapy of Peg-IFNα plus ribavirin. All patients enrolled in this study were infected with HCV genotype 1b with a high viral load and were HLA-A2 positive. The patients who were infected with other viruses (Hepatitis B virus, Human immunodeficiency virus) or had other forms of liver disease (alcohol liver disease, autoimmune hepatitis) were excluded from this study. Informed consent, under an Institutional Review Board-approved protocol, was obtained from each patient. All patients received Peg-IFNα-2b (PEGINTRON, Schering-Plough, Kenilworth, NJ) plus ribavirin (REBETOL, Schering-Plough) for the duration of the study of 48–72 weeks. In only one patient (Patients#11), treatment was stopped at 24 weeks because this patient remained HCV-RNA positive after 24 weeks and developed significant side effect. To evaluate the antiviral activity, serum HCV RNA levels were quantified during the combination treatment. Serum HCV RNA level was quantified using the COBAS AMPLICORE HCV MONITOR test (version 2.0; Roche Diagnostics, Branchburg, NJ). SVR was defined as the absence of detectable serum HCV RNA at 24 weeks after the end of the combination therapy. All treated patients were assessed the antiviral responses (SVR or non-SVR) as previously described.[12]The characteristics of patients with chronic HCV infection were summarized in Table 1.

CD8+ T cells isolation from peripheral blood mononuclear cells (PBMC)

PBMC was obtained from 16 treated HCV infected patients before IFN treatment (pre-IFN) and 4 weeks after starting of this combination therapy (IFN-4week) and six healthy donors. CD8+ T cells were isolated from PBMC by magnetic cell sorting using CD8 MicroBeads according to the manufacturer's instructions (Miltenyl Biotech, Auburn, CA). More than 95% of the cells were CD8+ lymphocytes.

IFN-γ ELISPOT assays for HCV Core and NS3 protein-derived peptide-specific CD8+ T cells responses

To evaluate the frequencies of CD8+ T cells recognizing peptide epitopes, IFN-γ ELISPOT assay were performed as previously described.[11] Briefly, 96-well multiscreen hemagglutinin antigen plates (Millipore, Billerica, MA) were coated with 10 μg/mL of anti-human IFN-γmAb (1-D1K; Mabtech, Stockholm) in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI 1640/10% human serum (1 h, 37°C), 1×10^5 CD8+ T cells were co-cultured with 2×10^4 T2.DR4 cells (HLA-A2 positive peptide-presenting cells generously provided from Dr Walter J. Storkus, University of Pittsburgh, School of Medicine, Pittsburgh, PA) pulsed with HCV Core and NS3 derived peptides (a final concentration of 10 μg/mL). HLA-A2-restricted HCV Core protein derived peptides ($Core_{35-44}$, YLLPRPGPRL, $Core_{131-140}$, ADLMGYIPLV) or NS3 protein derived peptides ($NS3_{1073-1081}$, CINGVCWTV, $NS3_{1406-1415}$, KLVALGINAV) were synthesized as previously described.[13] Negative control wells contained CD8+ T cells with T2.DR4 cells pulsed with HIV-$nef_{190-198}$ peptide (AFHHVAREL). After 24 h incubation of the plates, cells were removed from the ELISPOT well by washing and captured cytokine was detected at sites of their secretion