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A novel combined vaccine candidate containing epitopes of HCV NS3, core and E1 proteins induces multi-specific immune responses in BALB/c mice

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ABSTRACT

Hepatitis C virus (HCV) has emerged as the major pathogen of liver disease worldwide. The mechanisms of HCV infection and interaction with a host are poorly understood. What exactly is required for efficient control of HCV infection is largely unknown. Standard treatment combining interferon-α (IFN-α) and ribavirine is effective in about 50% of the treated patients, however associated with significant toxicity and cost. Therefore, the development of new drugs or vaccines is urgently needed. An efficient vaccine against HCV infection requires induction of broad cellular and humoral immune responses against several viral proteins. We have engineered the combined vaccine candidate mT+mE1, an inclusion of multiple epitopes from HCV NS3, core (C) and E1 proteins. mT contains multiple conserved $CD4^+$ and $CD8^+$ T cell epitopes from HCV NS3 and C proteins. mE1 is based on eight dominant neutralizing epitopes of E1 protein from six HCV genotypes. In current study, we showed that immunization with mT+mE1 induced high titers of IgG, IgG1 and IgG2a antibodies to mE1, and high level of NS3- or C-specific CTLs. Furthermore, mT+mE1 elicited a Th1-biased immune response with secretion of high amounts of IFN-γ, compared with mT alone. Prophylactic as well as therapeutic administration of mT+mE1 in BALB/c mice led to protecting mice against SP2/0 tumor cells expressing HCV NS3 protein. These results suggested that mT+mE1 elicited strong humoral immune responses and multiple specific cellular immune responses. The vaccine candidate is now being tested in pre-clinical trials.

1. Introduction

HCV infection is a global health problem, being the second most common chronic viral infection in the world. According to the World Health Organisation [http://www.who.int/mediacentre/factsheets/fs164/en/index.html], more than 170 million people are infected and 3–4 million persons are newly infected each year by HCV worldwide. HCV infection is followed by persistence of the virus and chronic liver disease in 60–80%, cirrhosis in 10–20%, and hepatocellular carcinoma in 1–5% of infected individuals. The natural course of HCV infection and mechanisms of HCV interaction with a host are very complicated and still poorly understood. What exactly is required for efficient immunological control of HCV infection is largely unknown. Presently, the only available option for treatment of patients with chronic hepatitis C, combining interferon-α and ribavirine, is highly expensive, causes significant adverse side effects, and induces a sustained virological and biochemical response in 20–60% of treated patients, depending on the HCV genotype, the virus load and the age of the patients (Foster, 2004; Feld, 2005). Neither licensed vaccines nor selective drugs are available. In recent years, there has been a remarkable progress in the development of potent and selective inhibitors of HCV replication. Some well tolerated inhibitors are progressing through clinical development (Johan, 2006). Several studies in humans and chimpanzees with acute resolving HCV infection provide evidence that protective immunity exists, supporting the idea that viral persistence may be prevented by vaccination (Lanford et al., 2004; Houghton and Sergio, 2005).

The HCV genome encodes the structural protein C, envelope glycoproteins E1, E2 and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Schulzezur et al., 2003). Heterogeneity and the genetic variability of HCV may be a major obstacle for designing a HCV vaccine (Houghton and Sergio, 2005). The efficient HCV vaccine of the future would be based on two or several immunogens, one of which might contain multiple epitopes.

There is strong evidence to indicate that cooperative cellular immune responses mediated by $CD8^+$ and $CD4^+$ T lymphocytes

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Table 1
$CD4^+$ and $CD8^+$ T cell epitopes from HCV C and NS3 proteins for the construction of mT antigen.

HCV protein	Amino acid residues	Number of $CD4^+$ T cell epitopes	Number of $CD8^+$ T cell epitopes				
			HLA-A1	HLA-A2	HLA-A3	HLA-A24	HLA-B7
C	1–53	1 (Day et al., 2002)	–	1 (Wedemeyer et al., 2002)	1 (Chang et al., 1999)	–	1 (Lauer et al., 2004)
	111–119	–	–	–	–	–	1 (Lauer et al., 2004)
	169–177	–	–	–	–	–	1 (Chang et al., 1999)
NS3	1235–1321	2 (Day et al., 2002; Wertheimer et al., 2003)	3 (Hakamada et al., 2004; Nakamoto et al., 2003)	–	1 (Wertheimer et al., 2003)	1 (Hakamada et al., 2004)	–
	1371–1430	2 (Shoukry et al., 2004; Day et al., 2002)	–	2 (Chang et al., 1999)	1 (Hakamada et al., 2004)	–	–
	1031–1039	–	–	–	–	1 (Kurokohchi et al., 2001)	–

play a central role in the control and/or the resolution of HCV infection (Grakoui et al., 2003; Shoukry et al., 2003). Several studies reported that the presence of early, strong, and multi-specific T-cell responses targeting a large number of epitopes of HCV proteins associated with clearing viremia and avoiding viral escape (Chang et al., 2001; Rehermann and Nascimbeni, 2005). Nonstructural protein NS3 is the preferential target of natural or therapeutic viral clearance (Vertuani et al., 2002; Smyk-Pearson et al., 2006) and NS3 specific $CD4^+$ and $CD8^+$ T-cell responses were reported in self-limited patients (Rosen et al., 2002) and patient responders to therapy (Vertuani et al., 2002). C protein is among the most conserved genes in HCV genotypes that may elicit immune responses to a broad range of virus variants (Lechner et al., 2000). We have engineered a recombinant multi-epitope antigen mT based on multiple conserved $CD4^+$ T cell epitopes and HLA-A1, A2, A3, A24 or B7 restricted $CD8^+$ T cell epitopes from NS3 and C proteins, since these HLA-I molecules account for nearly 90% of the global population.

Besides a multi-specific cellular immune response, induction of vigorous and cross-reactive antiviral antibodies is assumed to be necessary for an effective HCV vaccine (Bowen and Walker, 2005; Torresi et al., 2004). E1 and E2 are two envelope glycoproteins encoded by HCV, which play important roles in viral attachment and entry into target cells as well as virus–host immune interactions (Garcia et al., 2002; Triyatni et al., 2002). Vaccination with recombinant E1 and/or E2 proteins, has been shown to partially protect chimpanzees from a HCV infectious challenge or ameliorate the outcome of infection in chimpanzees (Choo et al., 1994; Forns et al., 2000). E1 shows a relatively high degree of conservation within the subtypes, compared with E2 (Maertens and Stuyver, 1997). To elicit broad cross-reactive E1-specific antibodies, we developed a recombinant antigen mE1 containing eight dominant neutralizing epitopes of N-terminal of E1 protein from six HCV genotypes and a pan-HLA-DR restricted $CD4^+$ T cell epitope.

Table 2
The neutralizing epitopes from HCV-E1 N-terminal for the construction of mE1 antigen.

HCV subtype	Neutralizing epitope sequence	GenBank accession no.
1a	YQVRNSSGLYHVTNDCPNSS	AF118605 (Ray et al., 1999)
1b	YEVRNVSGVYHVTNDCSNSS	AB008441 (Kato et al., 1998)
2a	VQVKNTSSSYMVTNDCSNDS	AB107935 (Higashi et al., 2005)
3a	LEWRNTSGLYVLTNDCSNSS	AF046866 (Unpublished)
4c	VNYRNASGVYHVTNDCPNSS	L16678 (Bukh et al., 1992)
4a	VHYRNASGVYHVTNDCPNTS	L16677 (Bukh et al., 1992)
1a	FTFSPRRHWTTQDCNCSIYP	AF118605 (Ray et al., 1999)
6a	LTYGNSSGLYHLTNDCPNSS	D88469 (Tokita et al., 1994)

The development of a vaccine against HCV capable of priming the multi-specific cytotoxic T lymphocyte (CTL) and helper T-cell (Th) responses, also inducing vigorous, broad and cross-reactive antiviral antibodies, represents the realistic way to control the infection caused by HCV worldwide. In current study, the humoral and cellular immune responses of immunization with the combined vaccine candidate mT + mE1 were evaluated, and the efficacy of NS3-specific cellular immune response in vivo was also evaluated in BALB/c mice carried tumor cells expressing HCV NS3 protein.

2. Materials and methods

2.1. Mice and cell lines

Female BALB/c mice, aged 6–9 weeks, were purchased from Hebei Laboratory Animal Center (Shijiazhuang, PR China), housed and manipulated according to the Care and Use of Laboratory Animals (PR China), and kept under specific pathogen-free conditions.

SP2/0-NS3 cell line steadily expressing HCV NS3 protein and SP2/0-C cell line steadily expressing HCV-core protein were constructed by Dr. Yan Jinqi in our laboratory (Yan et al., 2006). The cells were incubated in complete RPMI-1640 medium (10% fetal calf serum, 2 mM L-glutamine, 40 U/ml penicillin, 50 μg/ml streptomycin and 5×10^5 M2-mercaptoethanol).

2.2. Recombinant antigens

Gene fragments of several antigenic regions from HCV C and NS3 proteins containing multiple $CD4^+$ and $CD8^+$ T cell epitopes (Table 1) were synthesized, spliced by overlap extension PCR and ligated to pBV-IL-1 plasmid vector (containing human IL-1 gene) constructed by our laboratory (Song et al., 2001). The fusion protein mT was expressed in *E. coli* and purified by Q-Sepharose-FF and Sephadex-G50 chromatographies. Synchronously, eight dominant neutralizing epitopes were selected from E1 N-terminal variable region of six HCV subtypes (Table 2). A pan-HLA-DR restricted epitope (AKFVAAWTLKAAA) was used to induce $CD4^+$ T-cell response. The genes of these epitopes were synthesized and spliced by overlap extension PCR, and ligated to plasmid vector pBV-IL-1. The fusion protein mE1 was expressed in *E. coli* and purified by Q-Sepharose-FF and Sephadex-G50 chromatographies. Protein contents of pooled purified fractions were determined by the Lowry method (Lowry et al., 1951). The proteins were analyzed for purity and antigenicity by SDS-PAGE and Western-blot.

2.3. Immunizations of mice

Mice were injected subcutaneously (s.c.) with mT (50 μg), mE1 (50 μg) or mT (50 μg) + mE1 (50 μg) diluted in 200 μl PBS containing 20% alhydrogel (v/v) (Danmark). The mice of control group were only immunized with 200 μl PBS containing 20% alhydrogel (v/v). Except where indicated, an interval of 3 weeks was invariably employed for the second and third immunizations.

2.4. ELISA assays

IgG, IgG1 or IgG2a antibody titers in sera from vaccinated mice were assessed by standard ELISA protocol (Depraetere and Leroux-Roels, 1999). Briefly, 96-well microtiter plates were coated overnight at 4 °C with mE1 protein in 100 μl of 50 mM sodium carbonate buffer (pH 9.6) per well. Twofold serial diluted serum samples were allowed to react with coated plates at 37 °C for 2 h, then incubated with 1:5000–10,000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG, IgG1 or IgG2a antibody (Southern Biotech) at 37 °C for 2 h. 100 μl of *O*-phenylenediamine (0.4 mg/ml in 0.1 M sodium citrate, pH 5.0, containing 0.03% (v/v) H_2O_2) was added to each well to allow color development at room temperature for 10 min. Reaction was stopped by adding 50 μl of 2 M H_2SO_4 and the OD was read at 490 nm. Antibody titers were expressed as the reciprocal of the last sample dilution giving an absorbance of at least twofold that of the pre-immune sample and with an OD ≥ 0.15.

2.5. Flow cytometry assay

Percentages of $CD4^+$ T and $CD8^+$ T cells in the spleen cells of mice were analyzed with flow cytometer. Briefly, 10 μl of FITC conjugated rat anti-mouse CD4 antibody and PE conjugated rat anti-mouse CD8 antibody (Invitrogen) were incubated with 1.0×10^6 spleen cells in a 100 μl volume for 20 min at room temperature. The spleen cells were washed twice with isotonic Na chloride and resuspended in 500 μl isotonic Na chloride for flow cytometry (Becton Dickinson), and FACS data were analyzed using Cellquest software.

2.6. Splenic CTL assays

Specific CTL activity was assessed using a Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (Cayman). Spleens from BALB/c mice immunized were removed 10 days after the last immunization. Splenocytes were suspended in complete RPMI-1640 and restimulated in vitro for 5 days with 20 μg of the antigen used for immunization to produce effectors. Target cells were SP2/0-NS3 and SP2/0-C cells. SP2/0 cells were used as a control. Effectors and targets (1×10^4 cells) were co-cultured for 6 h at 37 °C in 5% CO_2 with ratios of 50.0:1, 25.0:1, 12.5:1 and 6.25:1. Released LDH was measured according to the manufacturer's protocol. The percentage of specific killing was calculated as: % specific killing = (experimental release − spontaneous release)/(total release − spontaneous release). The data are represented as the mean percentages of the specific lysis values from six mice.

2.7. Enzyme-linked immunospot assay (ELISPOT)

IFN-γ and IL-4-secreting cells were quantified using an ELISPOT kit (eBioscience). Multi Screen 96-well filtration plates MAIP S4510 (Millipore, Bedford, MA) were coated with 100 μl of a 1:250 dilution anti-mouse capture antibody overnight at 4 °C, then washed with ELISPOT coating buffer and blocked for 1 h with 200 μl complete RPMI-1640. Splenocytes (1×10^6/well) were cultured for 24 h in complete RPMI-1640 alone (negative control), co-cultured with 20 μg/ml corresponding antigens or 4 μg/ml of Con A (as positive control) in triplicate wells. After removing cells and washing with wash buffer (PBS; 0.1% Tween 20 from SIGMA), 100 μl of a 1:250 dilution biotinylated anti-IFN-γ or anti-IL-4 antibodies were added and incubated for 2 h at room temperature. After washing, streptavidin-horseradish peroxidase was added at 1.2 μg/ml for 45 min at room temperature. After rewashing, the assay was developed by the addition of 100 μl/well freshly prepared 3-amino-9-ethyl carbazole substrate solution. Spots were then counted using an immunospot image analyzer (Software version: ImmunoSpot 4.0.16). Results are shown as the mean value obtained for triplicate wells.

2.8. In vivo efficacy of NS3-specific cellular immune responses

For prophylactic experiment, BALB/c mice were immunized with the same formulations as described above. Two weeks after the final immunization, the mice were challenged s.c. at the back with 1.0×10^6 SP2/0-NS3 cells and monitored daily for 2 months post-challenge. After 2 months, the mice were killed. The tumors were weighed. Tumor burden is reported as average tumor weight ± S.D. For therapy, 1.0×10^6 SP2/0-NS3 cells were implanted into the back of BALB/c mice. Then the mice were randomly divided into three groups. On 7 days after tumor implantation, mice were immunized three times with an interval of 2 weeks, and monitored daily for 2 months post-challenge. Length and breadth of tumors and survival rate were tested at several time points after the first immunization therapy.

2.9. Statistical analysis

Statistical analyses were performed using the software SPSS 12.0. *p*-Values < 0.05 were considered as statistically significant.

3. Results

3.1. mT + mE1 induced humoral immune responses

There are evidences that ELISA titers of antibodies against E1 and/or E2 proteins appear to correlate with the protection of vaccination with recombinant E1 and/or E2 proteins (Choo et al., 1994; Forns et al., 2000). In current study, we tested the ELISA titers of mE1-specific antibody induced by co-immunization with mT + mE1. Mice were immunized three times with mT + mE1, mE1 or PBS. Serum samples were collected 3 weeks after the first and second immunization, and 10 days after the final immunization (20, 41 and 52 days after the first immunization). mE1-specific antibodies were determined by ELISA with mE1 as coating antigen. As shown in Fig. 1A, initial responses were first detected at 20 days after the first immunization, and continued to increase following subsequent immunization in mT + mE1 or mE1 group. Co-immunization with mT + mE1 or immunization with mE1 alone induced significantly higher mE1-specific IgG and IgG1 antibody titers compared with PBS groups ($p < 0.05$), and no statistic difference was observed between mT + mE1 and mE1 groups (Fig. 1A and B, $p > 0.05$). The production of IgG2a subclass is normally associated with, or a marker for, the development of a Th1-type immune response in mice (Hemmi et al., 2000). The data presented in Fig. 1C showed that both mT + mE1 and mE1 induced high titers of mE1-specific IgG2a in mice immunized, and IgG2a titers in mice immunized with mT + mE1 were significantly higher than those with mE1 ($p < 0.05$, Fig. 1C). These results indicated that co-immunization with mT + mE1 induced strong mE1-specific humoral responses, and mT in the vaccine formulation did not impair mE1-specific

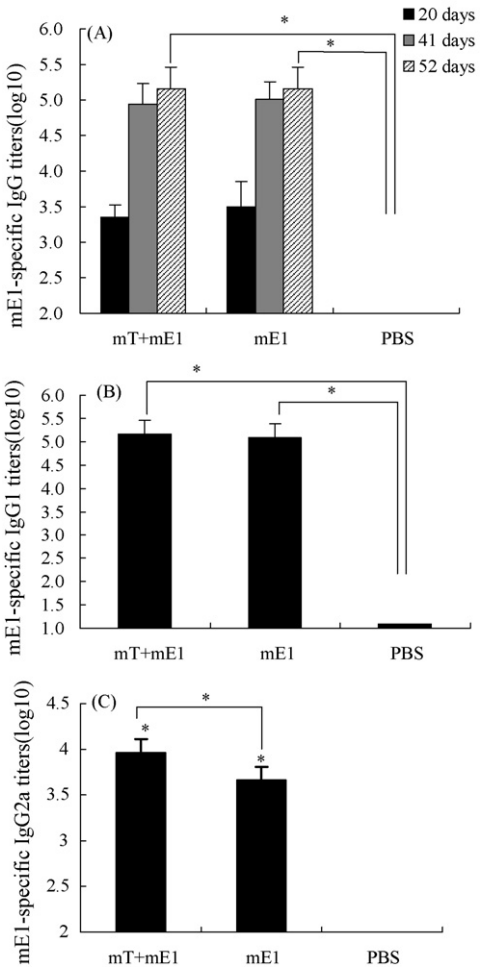

Fig. 1. mE1-specific antibody responses following immunization in BALB/c mice. Mice were immunized s.c. at 0, 21 and 42 days with mT + mE1, mE1 or PBS, 20% alhydrogel (v/v) was used as adjuvant for all immunizations as described in Section 2.3. Serum samples were collected at 20, 41 and 52 days after the first immunization. mE1-specific antibodies IgG (A), IgG1 (B) and IgG2a (C) were determined by ELISA. Results are expressed as geometric means of serum antibody titers. The single asterisk indicates $p < 0.05$.

humoral immune response. Moreover, it was implied that mT + mE1 elicited a mixed Th1/Th2 response, and mT could enhance Th1 response.

3.2. $CD4^+/CD8^+$ T cells in splenocytes

$CD8^+$ CTL and $CD4^+$ Th cells are all considered to be essential to protection against virus. Since mT contains multiple highly conserved $CD8^+$ and $CD4^+$ T cell epitopes, and mE1 contains also a strong $CD4^+$ T cell epitope, we speculated that mT + mE1 could activate $CD4^+$ and $CD8^+$ T cells. The hypothesis was tested in a flow cytometry assay. As shown in Fig. 2, percentages of $CD4^+$ and $CD8^+$ T cell subsets were significantly higher in mice co-immunized with mT + mE1 or immunized with mT alone than those in mice immunized with PBS ($p < 0.05$). The results suggested that mT antigen or complex of mT + mE1 could activate both $CD4^+$ and $CD8^+$ T cell subsets. Then we calculated the $CD4^+/CD8^+$ T cell ratios. While $CD4^+/CD8^+$ T cell ratio was 2.1 in PBS group, $CD4^+/CD8^+$ T cell ratio in mice immunized with mT + mE1 or mT dropped to 1.68 or 1.52, which indicated that mT + mE1- or mT-immunization activated more $CD8^+$ T cells than $CD4^+$ T cells.

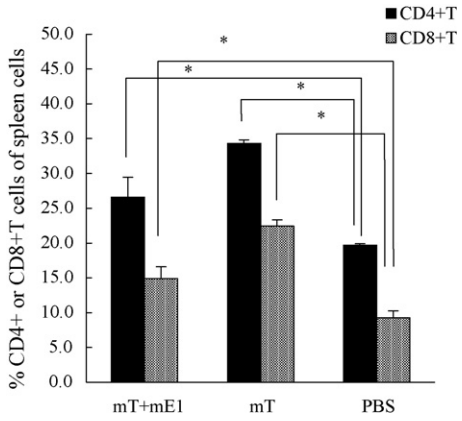

Fig. 2. $CD4^+$ T cells and $CD8^+$ T cells activated in BALB/c mice immunized. Mice were immunized s.c. with mT + mE1, mT or PBS three times. Spleens from mice immunized were removed 10 days after the last immunization. Splenocytes were incubated with FITC conjugated rat anti-mouse CD4 antibody and PE conjugated rat anti-mouse CD8 antibody, then detected by flow cytometry. Percentage of $CD4^+$ T cells or $CD8^+$ T cells in splenocytes of mice immunized. Results are shown as the mean value.

3.3. mT + mE1 induced NS3- and C-specific CTL responses

To determine the capacity of mT + mE1 containing multiple $CD8^+$ CTL epitopes from HCV NS3 and C proteins inducing specific CTL response, mice were immunized as described above and bled 10 days after the last immunization. Splenocytes from mice immunized were tested 5 days after restimulation with corresponding antigens in vitro. The data presented in Fig. 3 showed that immunization with mT + mE1 elicited high levels of CTL activity on SP2/0-NS3 and SP2/0-C target cells (Fig. 3A), compared with immunization with PBS (Fig. 3C) ($p < 0.05$). In comparison, less cytolytic activity was exhibited in mice immunized with mT alone (Fig. 3B, $p < 0.05$). The CTL activities were NS3 and C antigens specific, as little or no lysis was observed on non-transfected SP2/0 cells over a range of effector to target ratios (Fig. 3A and B). The above results suggested that the NS3 and C antigens specific CTL responses were enhanced dramatically by co-immunization with mT + mE1.

3.4. Frequency of IFN-γ or IL-4-secreting cells in splenocytes

Because IFN-γ is a representative Th1-biased cytokine that strongly contributes to the activation of cellular immune responses, and IL-4 is a representative Th2-biased cytokine that strongly contributes to the activation of humoral immune responses (Gajewski and Fitch, 1998), we compared the frequency of IFN-γ or IL-4-secreting cells in splenocytes of immunized mice by ELISPOT. Co-immunization with mT + mE1 induced significantly higher frequency of specific IFN-γ-secreting cells than immunization with mT or mE1 alone (Fig. 4, $p < 0.05$). Synchronously, frequency of specific IL-4-secreting cells in mice co-immunized with mT + mE1 was also dramatically higher, compared with those in mice immunized with mT or mE1 alone ($p < 0.05$). These results suggested that synergistic effect in frequencies of both specific IFN-γ-secreting cells and IL-4-secreting cells was observed in mice co-immunized with mT + mE1. In addition, in mT + mE1-immunized mice, frequency of specific IFN-γ-secreting cells was significantly higher than that of specific IL-4-secreting cells ($p < 0.05$), which indicated that mT + mE1 induced a Th1-biased immune response.

3.5. The effect of cellular immunity against NS3-expressing tumor cells in vivo

The above results presented in Fig. 3A indicated that mT + mE1 could restimulate specific cytotoxic effector activity against NS3-

expressing tumors in vitro. This suggested that the preventive and therapeutic effect in vivo might also be attainable. To assess this possibility, BALB/c mice immunized were injected s.c. with SP2/0-NS3 tumor cells. As expected, consistent with NS3-specific CTL detection, mT + mE1 completely prevented detectable tumor growth at the back of mice challenged with SP2/0-NS3 tumor cells, while tumorigenesis was observed in a few mice immunized with mT alone, and 100% of mice in PBS group generated tumors (Fig. 5A and B). Moreover, the survival rates of mice challenged with SP2/0-NS3 cells after immunization with mT + mE1 or mT alone were 100%, while that of PBS group was only 50% at the end of 2 months (Fig. 5C). These results suggested that NS3-specifc cellular immune response elicited by mT + mE1 could protect fully mice immunized from the challenge with SP2/0-NS3 cells, while that induced by mT alone partly protected mice against SP2/0-NS3 cells.

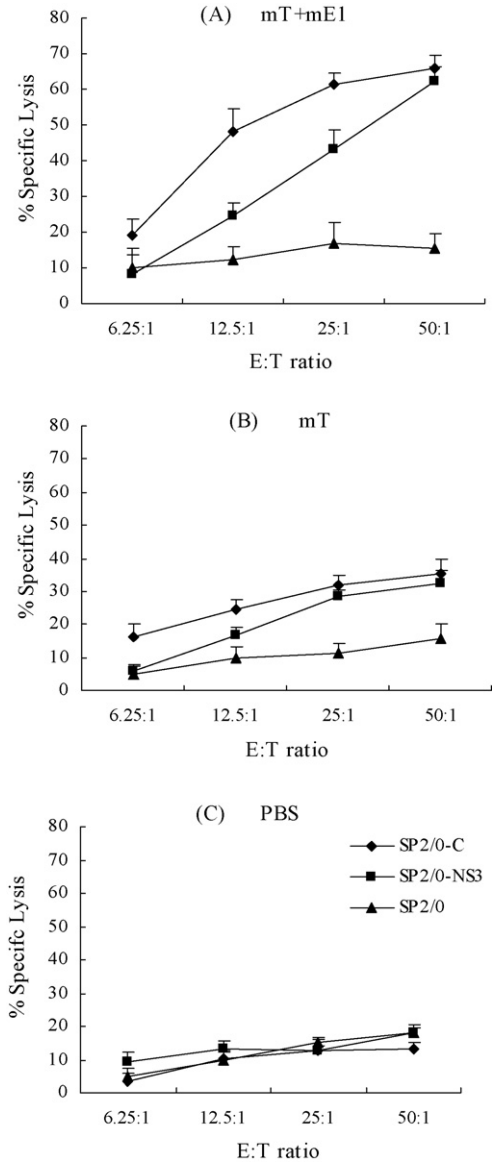

Fig. 3. Induction of NS3- and C-specific CTL responses following immunization in BALB/c mice. Spleens from mice immunized with mT + mE1 (A), mT (B) or PBS (C) were removed 10 days after the last immunization and restimulated in vitro for 5 days with 20 μg of the corresponding antigens to produce effectors. Target cells were SP2/0-NS3 and SP2/0-C cells. SP2/0 cells were used as a control. The data represent as the mean percentages of specific lysis from six mice at different effector to target (*E*/*T*) ratios.

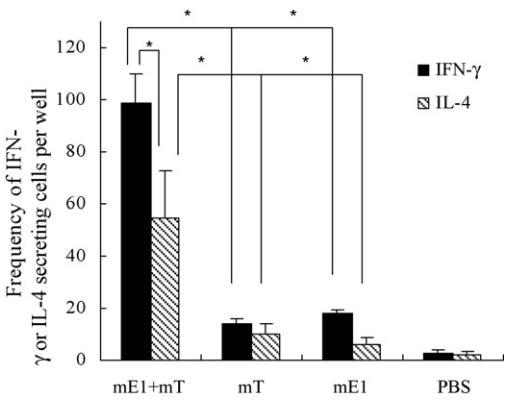

Fig. 4. Frequency of antigen-specific IFN-γ- or IL-4-secreting T cells in splenocytes of mice immunized. Mice were immunized s.c. three times with mT + mE1, mT or PBS. Spleens from mice immunized were removed 10 days after the last immunization. Splenocytes were suspended in complete RPMI-1640 and restimulated for 24 h with the corresponding antigens. Number of specific IFN-γ-secreting T cells and IL-4-secreting T cells were evaluated using an ELISPOT assay as described in Section 2. Results are shown as the mean value of the number of spots observed for 10^6 spleen cells, obtained from triplicate wells. The single asterisk indicates $p < 0.05$.

We next evaluated the therapeutic efficacy of NS3-specifc cellular immune response on NS3-expressing tumor in mice. Mice were injected s.c. 1.0×10^6 SP2/0-NS3 cells at the back. 7 days after injection, immunization therapy was carried out three times with an interval of 2 weeks. All SP2/0-NS3-bearing mice treated with PBS showed tumor rapid growth (Fig. 6A); 40% of them died before 35 days, and all mice died before 42 days (Fig. 6B). In contrast, immunization with mT + mE1 showed dramatic tumor inhibition, and the median survival in this group was increased significantly compared to that observed in PBS group (Fig. 6A and B, $p < 0.05$). Although tumor in mice immunized with mT was significantly smaller than that in control group, the therapeutic effect of mT was weaker than that of mT + mE1 ($p < 0.05$). These data indicated that NS3-specific cellular immune response induced by mT + mE1 showed potent therapeutic effect in SP2/0-NS3-bearing mice, and co-immunization with mE1 and mT enhanced the therapeutic effect, compared with immunization with mT alone.

4. Discussion

With the aim to develop a safe, poly-antigenic, prophylactic and/or therapeutic HCV vaccine, we have engineered and evaluated the combined vaccine candidate mT + mE1 containing multiple epitopes of HCV NS3, C and E1 proteins. It has been suggested that the humoral immune response to the E1 protein is largely impaired in patients with chronic hepatitis C, and that such response may be important for clearance of HCV (Depraetere and Leroux-Roels, 1999). In this study, co-immunization with mT + mE1 elicited high titers of mE1-specific IgG, IgG1 and IgG2a antibodies. mT in the combined vaccine did not weaken humoral immune response elicited by mE1, furthermore, enhanced the capacity of mE1 inducing IgG2a isotype that is known to be cytophilic in nature and to promote the clearance of infected cells.

Priming HCV-specific T-cell responses may be an efficient strategy to protect from HCV infection. $CD4^+$ T lymphocytes play a central role in orchestrating the beginning and maintenance of the adaptive immune response. $CD4^+$ T cells not only help antibody responses (Mitchison, 1971), but also help the activation and expansion of $CD8^+$ T cells (Keene and Forman, 1982). Only in the presence of an efficient help provided by the $CD4^+$ T-cell response, $CD8^+$ T cells can complete their maturation program, acquiring a full antiviral activity which is essential for final and persistent control of infection (Rehermann and Nascimbeni, 2005; Bertoletti and

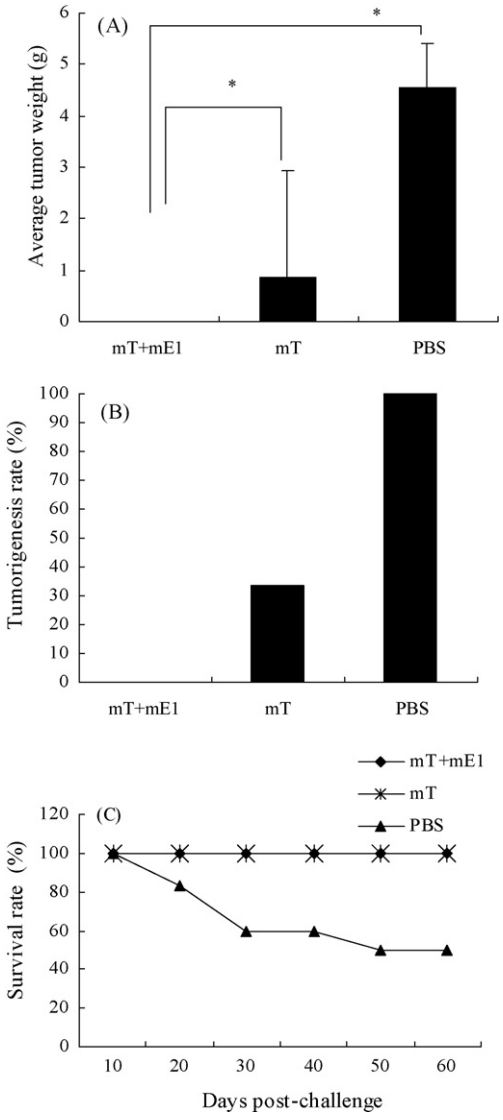

Fig. 5. Preventive efficacy against SP2/0-NS3 cells challenge in mice. Mice were immunized s.c. at 0, 21 and 42 days with mT + mE1, mT or PBS as described in Section 2.3. Two weeks after the final immunization, mice were challenged s.c. at the back with 1.0×10^6 SP2/0-NS3 cells. Mice were monitored for 2 months. Tumor weights were measured (A); tumorigenesis rate (B) and survival rate (C) of every group were compared. The single asterisk indicates $p < 0.05$.

Ferrari, 2003). Inducing both $CD4^+$ and $CD8^+$ T cellular immune responses may be essential for prophylactic vaccines to prevent the establishment of chronic infection, and for therapeutic vaccines to kill infected cells (Ward et al., 2002; Shoukry et al., 2004). Our results indicated that the immunization of BALB/c mice with mT + mE1 activated both $CD4^+$ and $CD8^+$ T cell subsets. In addition, $CD8^+$ T cells activated were more than $CD4^+$ T cells activated, which was consistent with more $CD8^+$ T cell epitopes (at least 14 epitopes) than $CD4^+$ T cell epitopes (at least 5 epitopes) in the vaccine candidate (Table 1). CTL, $CD8^+$ T lymphocytes with cytotoxic activity, is the most important effector cell in antivirus immunity. The CTLs derived from mice immunized with mT + mE1 were capable of killing effectively target cells expressing NS3 or C protein in a dose-dependent manner. In comparison, mT alone induced lower levels of NS3- or C-specific CTL activity. It is possible that the pan-HLA-DR T helper cell epitope in mE1 may contribute to the induction of CTL when mice were co-immunized with mT + mE1.

$CD4^+$ Th2-biased cytokines have been shown to be advantageous to the survival of the pathogen and/or lead to persistent infection with Mycobacterium and Respiratory Syncytial Virus (Sieling et al., 1993; Panuska et al., 1995). This is also the case in HCV infection (Zhu et al., 2005). $CD4^+$ Th1 cells produce cytokines such as IL-2 and IFN-γ that stimulate the proliferation and differentiation of CTLs. Moreover, they promote efficient antigen presentation, co-stimulation, and IL-12 production by dendritic cells (Hung et al., 1998). There is strong evidence that therapeutic as well as spontaneous HCV clearance is associated with vigorous specific Th1 cellular immune responses (Thimme et al., 2001; Ward et al., 2002). mT + mE1-immunization activated much more IFN-γ-secreting cells than mT- or mE1-immunization, and frequency of specific IFN-γ-secreting cells was significantly higher than that of specific IL-4-secreting cells in mT + mE1-immunized mice. These results suggested that mT + mE1 activated a Th1-biased immune response.

Since mT + mE1 elicited strong $CD8^+$ CTL and $CD4^+$ Th1 immune responses, the prophylactic and therapeutic efficacies should also be evaluated. Chimpanzees are the only animals that can be experimentally infected by HCV (Bukh, 2004). Because of their status of protective specie, their extreme cost and the fact that HCV infection in this model is associated with weak immune responses and none or weak signs of pathology, chimpanzees do not represent a mandatory pre-clinical model (Perrine and Geneviève, 2006). Live organisms expressing HCV antigens are used to 'challenge' mice either following vaccination (preventive vaccination) or before vaccination (therapeutic vaccination). The vaccinia virus (vv) expressing HCV antigens and a recombinant *Listeria monocytogenes* expressing NS3 challenge are used in prophylactic challenge assays as vv or *Listeria* infections are short lasting (4–7 days) (Arribillaga et al., 2005; Simon et al.,

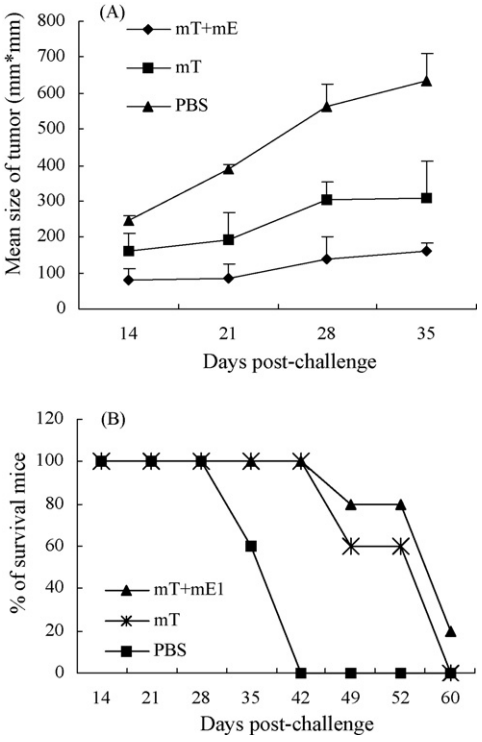

Fig. 6. Therapeutic efficacy against SP2/0-NS3 cells in mice. A surrogate was established by s.c. injecting 1.0×10^6 SP2/0-NS3 cells at the back of BALB/c mice. Then the mice were randomly divided into three groups. 7 days after injection, immunization therapy was carried out with mT + mE1, mT or PBS. The immunization was boosted two times at an interval of 2 weeks. Following observation for 60 days, the tumor size (length × breadth) and survival rate were tested at several time points after the first immunization. Results are shown as mean size of tumors (A) and percent of survival mice (B).

2003). Surrogate challenge with tumors expressing HCV proteins can be used to evaluate both prophylactic and therapeutic vaccines (Encke et al., 1998). In current study, SP2/0 cells expressing HCV NS3 protein constitutively were used in prophylactic and therapeutic studies. The cellular immune responses elicited by mT + mE1 achieved specific immune protection, which prevented completely tumor formation of the mice challenged with SP2/0-NS3 cells. As an immunotherapeutic, mT + mE1 showed potent therapeutic effect on SP2/0-NS3-bearing mice. Co-immunization with mT + mE1 inhibited effectively tumor growth in a high proportion of mice and increased survival. The above prophylactic and therapeutic efficacies were consistent with the vigorous CTL activity and Th1-biased immune response in mT + mE1-immunized mice. The vaccine candidate mT + mE1, an inclusion of multiple HLA-I restricted $CD8^+$ T cell epitopes, conserved $CD4^+$ T cell epitopes and dominant neutralizing epitopes from three HCV antigens, core, NS3 and E1, induced broad-based cellular and humoral immune responses. The use of mT + mE1 should therefore render effective immunoprophylaxis and immunotherapy even for the genetically diverse populations of HCV present in chronic infection. However, since mice are not permissive to HCV infection, it is important to be cautious in interpreting results from the model bearing HCV antigen-expressing tumors. More extensive studies will be required to assess the prophylactic and therapeutic efficacies of the vaccine candidate when more ideal animal model is available.

5. Conclusions

The study provided the important demonstration that mE1-specific humoral immune responses and NS3- or C-specific cellular immune responses could be successfully induced by the combined vaccine candidate mT + mE1. We provided evidence that synergistic effect in frequencies of both specific IFN-γ- and IL-4-secreting cells was observed in mice co-immunized with mT + mE1, and only when co-immunization with mT + mE1, a Th1-biased immune response with high amounts of IFN-γ was induced. Furthermore, prophylactic as well as therapeutic administration of mT + mE1 in mice led to rejection of SP2/0 tumor cells expressing HCV NS3 protein.

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